• TECHNICAL

Gas-Liquid Chromatographic Analysis of Monoglycerides as Their Trimethylsilyl Ether Derivatives[1, 2, 3]

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Abstract

A quantitative method is described for the rapid gas-liquid chromatographic analysis of monoglycerides of long chain fatty acids as their trimethylsilyl (TMS) ether derivatives. The TMS derivatives are formed rapidly and quantitatively at room temp without isomerization. The TMS derivatives of 1- and 2-monoglycerides may be separated on polyester columns, which can also be used for the analysis of methyl esters. A mixture of the 1- and 2-isomers of monopalmitin and monoolein was quantitatively analyzed. A mixture of monocaprin, monolaurin, monomyristin, monopalmitin and monostearin was also analyzed. A mixture of 1-monostearin, 1-monoolein and 1-monolinolein was also resolved. The analyses were carried out on packed and large bore capillary columns of diethylene glycol succinate polyester (DEGS) and Apiezon L. Capillary columns that required the use of a splitter were nonquantitative, resulting from what appeared to be fractionation at the splitter. Acid conditions, including those provoked by the use of phosphoric acid stabilized polyesters, cleaved the TMS derivatives to yield the original hydroxy compound.

Introduction

THE ANALYSIS of monoglycerides by gas-liquid chromatography (GLC) has lagged due to the lack of satisfactory derivatives to reduce their polarity and increase their volatility. McInnes et al. (1) reported the GLC analysis of monoglycerides as their allyl esters and isopropylidene derivatives. The retention times were long in both cases and the simultaneous determination of 1- and 2-monoglycerides was not possible. Acetylated monoglycerides were separated by Huebner (2) in which small peaks appearing before the major peaks were assumed to be the 2-isomer, but further support for this assumption has not been forthcoming.

The most common method used at present for the determination of 1- and 2-monoglycerides is essentially that of Martin (3), which involves periodic acid oxidation of the 1-isomer. The 2-isomer is then determined indirectly by difference. To determine the composition of a mixture of monoglycerides by this method, one must assume that the isomeric ratios are the same for all acids. On this basis, the composition can then be calculated from analysis of fatty acids.

The introduction of the TMS ethers has opened up new approaches for the gas chromatography of hydroxy compounds. TMS ether derivatives of several phenols have been successfully resolved by Langer et al. (4) and more recently by Shaw (5). Hedgley and Overend (6), and more successfully, Sweeley and colleagues (7) have reported the resolution of many isomeric carbohydrates as their TMS derivatives. Luukkainen and coworkers (8) and Wells and Makita (9, 10) prepared TMS derivatives of several steroids which made them volatile enough to analyze by GLC.

The present report describes the quantitative and qualitative analyses of mixtures of 1- and 2-monoglycerides of various fatty acids by this technique.

Experimental

Gas Chromatography. A Research Specialties Co. Model 600 Gas Chromatograph, equipped with a hydrogen flame ionization detector and an on-column injection system, was used in this study. A 100-ft large bore capillary (0.0625 in. OD, 0.030 in. ID) column was coated with a 15% carbontetrachloride solution of Apiezon L and conditionde according to the procedure described by Litchfield et al. (11). A high resolution 3 ft x ¼ in. U-type copper column was packed with 20% DEGS on 80–100 mesh Chromosorb "W." The stationary support was coated with the liquid phase by flash evaporation of the chloroform solvent. A 3 ft x ¼ in. straight piece of copper tubing, to serve as a column, was loosely packed with the packing material at the inlet and gradually tighter toward the exit. The column was then shaped to the desired form. It was observed that columns packed in this manner gave better resolution than uniformly packed columns. The polyester column was operated isothermally at 215C. These columns have been operated periodically over a two-month period without noticeable loss of resolution. However, the temp was reduced to 150C when not in use. An inlet pressure of 10 psi was used to maintain a helium flow of 60–70 ml/min through the columns. The hydrogen and air flow through the detector were 30 and 300 ml/min, respectively. The temp of the detector was maintained a few degrees above the maximum temp of the column.

Thin Layer Chromatography. Commercially available silica gel G, prepared according to Stahl (12,13), was applied in 1-mm thickness to 8 x 8 in. glass plates. These preparative thin layer plates were used to separate up to 250 mg of monoglycerides/plate. The chromatograms were developed with hexane-diethylether-acetic acid 70:30:1 v/v/v, in a saturated chamber. The monoglyceride region of the heavily loaded plates was located visually, or more distinctly with the aid of an UV light. The desired region was scraped from the plate and the monoglycerides extracted with diethyl ether.

Micro Balance. A Mettler Model M5 Micro Balance was used to prepare the standard mixture. The monoglycerides were weighed out accurately to the fifth decimal place.

Materials. The purified fatty acids used in the preparation of the acid chlorides and monoglycerides were obtained from commercial sources. The acid chlorides

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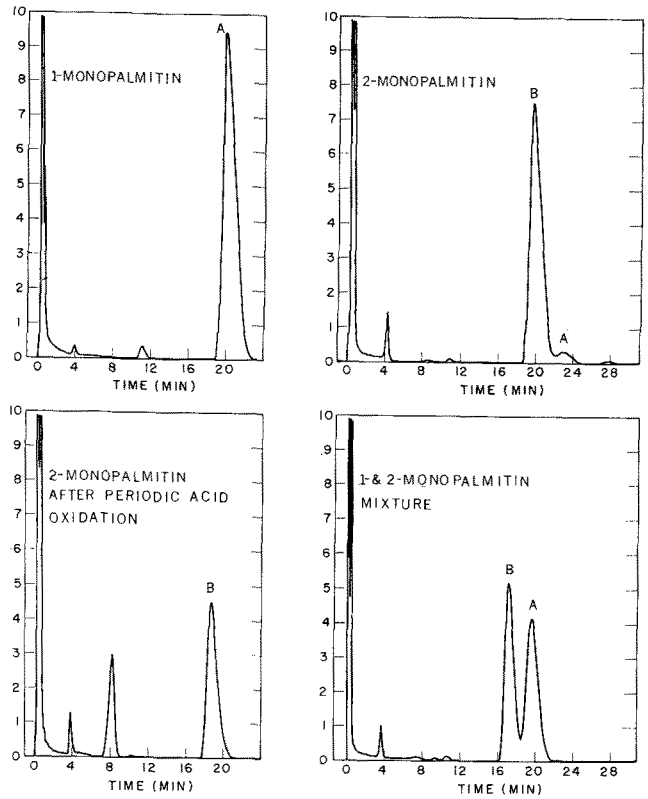


FIG. 1. Separation of 1- and 2-monopalmitin as TMS derivatives on a DEGS column at 215C. The major peaks are (A) 1-monopalmitin and (B) 2-monopalmitin.

were prepared according to the procedure of Youngs et al. (14). One and 2-monoglycerides were prepared from 1,2-isopropylidene glycerol and 1,3-benzylidene glycerol, respectively, by reacting with the acid chloride, as described by Mattson and Volpenhein (15).

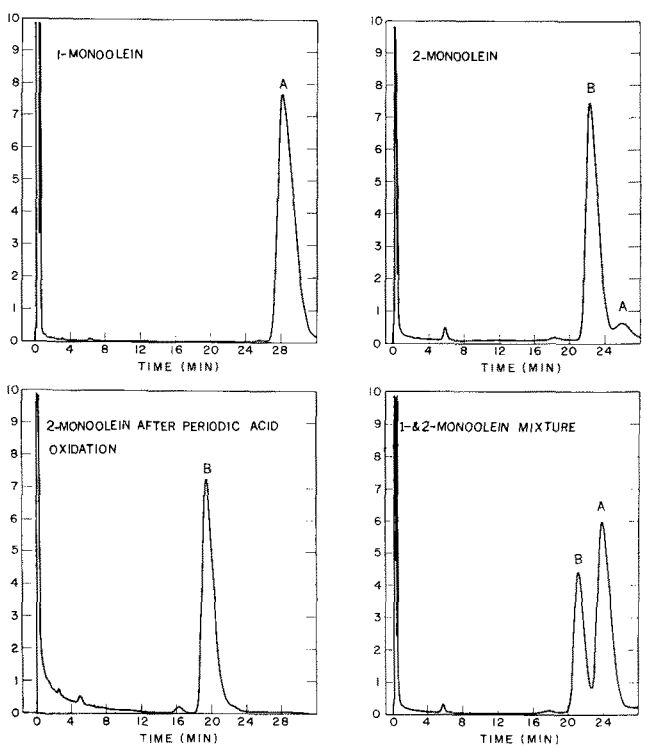


FIG. 2. Separation of 1- and 2- monoolein as TMS derivatives on a DEGS column at 215C. The major peaks are (A) 1-monoolein and (B) 2-monoolein.

The periodic acid oxidation procedure by Martin (3), with minor modifications, was used to degrade the 1-isomer which contaminated the 2-monoglyceride. When individual isomeric monoglycerides were not of concern, 1-monoglycerides were synthesized according to the procedure of Hartman (16), which contained approx 10% of the 2-isomer. Hexamethyldisilazane was obtained from Peninsular Chemresearch, Gainesville, Fla.: trimethylchlorosilane was obtained from K & K Laboratories, Plainview, N.Y. Solvents and other reagents were reagent grade and used without further purification.

Procedure. One mg or more of a monoglyceride sample was placed in a glass stoppered 15-ml conical centrifuge tube. One ml of pyridine was added followed by 0.2 ml of hexamethyldisilazane and 0.1 of trimethylchorosilane. The mixture was shaken for 15–30 sec and allowed to stand for 5 min. Five ml of hexane was added, followed by an equal volume of distilled water; then the mixture was shaken until both layers became clear. The hexane layer was removed and the aqueous layer extracted twice more with 5-ml portions of hexane. The combined hexane extractions were dried over anhydrous calcium sulfate and evaporated to dryness under a stream of dry nitrogen. Samples were retaken in hexane and evaporated until the odor of pyridine was not detectable. The samples were then stored at −20C until analyzed by gas chromatography.

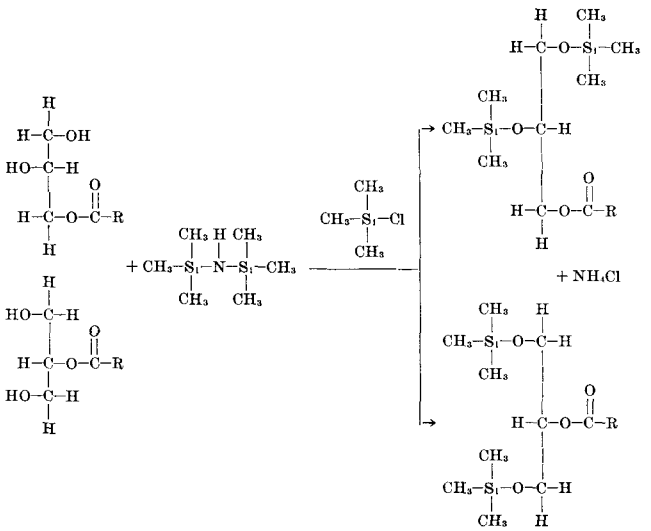
Results and Discussion

The separation of long chain isomeric monoglycerides as their TMS derivatives by GLC requires the columns used to be fast and efficient with respect to the separation of fatty acid methyl esters. A complete chromatogram of rat adipose tissue methyl esters through methyl linoleate, was obtained in 8–10 min on the columns used in this study with good resolutions of the monoenes from their corresponding saturated acids.

The packed DEGS column was superior to Apiezon L capillary column for the analyses of methyl esters, but each separated the isomeric monoglyceride TMS derivatives equally well. The large bore capillary column was used because the inside diameter allowed on-column injection and did not require a splitter. Quantitative results could not be obtained using small bore (0.010 in. ID) capillary columns which required the use of a splitter. This was attributed to the nonlinearity of the splitter. It appeared that fractionation did occur in the splitter since peak area deceased with an increase in mol wt, but no attempt was made to establish this. Thorough cleaning of the splitter and the addition of an auxiliary heater failed to improve the results.

Stabilized polyesters that give good resolution with short retention times of methyl esters appear initially to be ideally suited for the analysis of monoglyceride TMS derivative, but one should investigate the nature of the stabilizer before using. A sample of stabilized DEGS was obtained from Analabs (17) which proved satisfactory for the analysis of methyl esters but decomposed the TMS derivatives. It was later learned that the polyester had been stabilized with phosphoric acid. This explains the decomposition, since even weak acids cleave the TMS derivatives to yield the original hydroxy compound.

The reaction involved in the formation of the TMS derivatives of monoglycerides is depicted below. Sweeley et al. (7) have shown that the reaction goes to



completion in one min for sugars. This also appeared to be the case with monoglycerides, but five min was allowed to assure completion. Most of the previous workers have injected an aliquot of the reaction mixture directly into the gas chromatograph or have previously purified it by distillation. The latter is unsatisfactory for small samples, whereas the insoluble ammonium chloride formed during the reaction makes the former unsatisfactory for capillary columns. Also, the pyridine used as a solvent for the reaction is objectionable since it does not elute quickly and sharply from the column and requires several min for the base line to return to its original position. This makes components eluting early somewhat difficult to quantitate. For the above reasons, the monoglyceride TMS derivatives were extracted from the reaction mixtures with hexane after the addition of water. Since pyridine is partially soluble in hexane, it was necessary to re-evaporate the TMS derivatives to dryness several times under a stream of dry nitrogen for its removal. Derivatives prepared in this manner and stored in the cold did not show measurable changes after four months.

Mattson and Volpenhein (15) have shown that 2-monoolein and, to a smaller extent, 2-monopalmitin quickly isomerize to an equilibrium mixture of the 1- and 2-isomer in a pH 8.0, 1.0M Tris buffer at 40C and have discussed other factors affecting stability. In order for the present method to be applicable to the quantitative analysis of isomeric monoglycerides, it was necessary to demonstrate that isomerization does not occur during the formation of the TMS derivatives. GLC analysis of the TMS derivatvies of the 1- and 2-isomers of monopalmitin and monoolein, both individually and in a mixture, are shown in Figures 1 and 2, respectively.

It should be noted that the retention time of any one component, shown in Figures 1 and 2, varied as much as four min. This is not unusual since most of the chromatograms were obtained on different days and the column oven temp was controlled by balancing heat input against radiation losses. This allowed the oven temp to change a few degrees depending on changes in room temp. Chromatograms obtained within one or two hr of each other showed no such variation. In both the case of monopalmitin and monoolein, the 2-isomer showed a shorter retention time than the 1-isomer. One-monopalmitin and 1-monoolein showed no isomerization during the formaiton of the TMS derivatives since no peak appeared with a retention time of that of the 2-isomer. However, the 2-isomer of the respective monoglycerides contained approx 10% of the 1-isomer. The 1-isomer could have risen from isomerization or contamination of the 2-monoglyceride. It was proven that the 2-isomer does not isomerize during the formation of the TMS derivatives, since GLC analysis showed that no peak was present with a retention time of that of the 1-isomer, after periodic acid oxidation of the contaminated 2-monoglycerides. GLC analysis of 2-monopalmitin after periodic acid oxidation showed a peak with a retention time of 8 min absent from the 2-monopalmitin before oxidation. This was assumed to be the methyl ester of palmitoyl glycolic acid formed from the oxidation of the 1-isomer. The peak was not observed if the sample was not methylated before the TMS derivatives were formed. The resolution of the isomeric mixtures of the respective monoglyceride TMS derivatives was almost complete in less than 30 min.

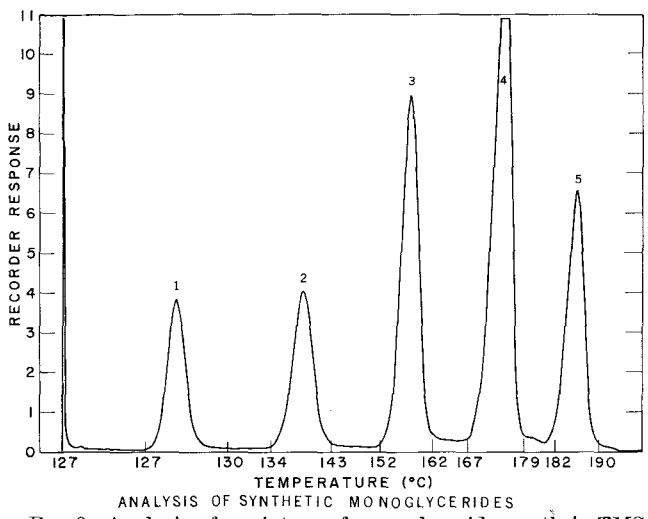


FIG. 3. Analysis of a mixture of monoglycerides as their TMS derivatives on an 18-in. 2.6% SE-30 column manually temp programmed from 127–190C. The major peaks are 1) monocaprin; 2) monolaurin; 3) monomyristin; 4) monopalmitin; and 5) monostearin. The 2-isomer was not resolved from the 1-monoglyceride.

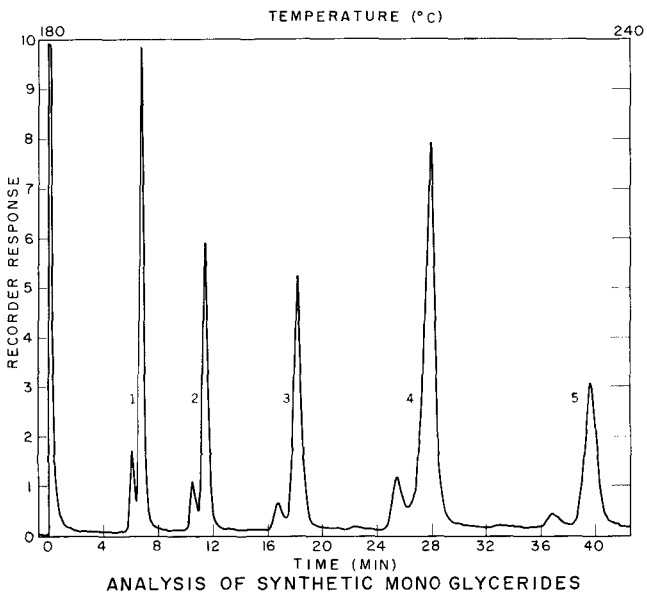


FIG. 4. Separation of a monoglyceride mixture on a 100-ft 15% Apiezon L large bore capillary column manually temp programmed from 180–240C. The major peaks are 1) monocaprin; 2) monolaurin; 3) monomyristin; 4) monopalmitin; and 5) monostearin. The small peaks appearing before the major peaks are the 2-isomer.

TABLE I

Quantitative Determination of 1- and 2-Monoglyceride Mixtures as Their TMS Derivatives on 3 ft x ¼ in. DEGS Column

Mixture	Components	Actual % [a]	% Found by TMS der.
Std. mixture no. 1	2-monopalmitin	18.2	18.0
	1-monopalmitin	18.7	18.5
	2-monoolein	24.3	24.1
	1-monoolein	38.8	39.5
Std. mixture no. 2	1- and 2-monolaurin	33.7	33.4
	1- and 2-monomyristin	31.3	33.3
	1- and 2-monopalmitin	35.0	33.3

[a] Prepared by weighing out purified monoglycerides.

Heating was sometimes employed to quickly dissolve long chain monoglycerides in pyridine. This practice can be used without isomerization since only 6–7% of 2-monopalmitin was isomerized to the 1-isomer and only 3–4% of the 1-monopalmiting was converted to the 2-isomer after being heated in pyridine at 100C for 24 hr.

The GLC analysis of monoglyceride TMS derivatives of different fatty acid chain lengths are shown in Figure 3. The instrument and the preparation of the column used to make the analysis have been described by Litchfield et al. (18). The 18 in. x ⅛ in. column packed 2.6% SE-30 was manually temp programmed from 127–190C. All components gave symmetrical peaks with no indication of the presence of 2-isomers. Figure 4 shows the GLC analysis of the same monoglyceride TMS derivative mixture shown in Figure 3, but on a 100-ft large bore capillary column coated with 15% Apiezon L manually programmed from 180–240C. Temp programming was necessary to permit the resolution of the two positional isomers of each component without peak asymmetry. It was not possible to analyze mixtures of such complexity of chain length on DEGS columns since DEGS does not lend itself to temp programming. However, it was possible to analyze any three successive homologues depending on the temp. Recently, a DEGS large bore capillary column was prepared by the authors which gave a resolution such as that shown in Figure 4 in 30 min under isothermal conditions.

Quantitative aspects of the method were also studied. Standard mixtures were prepared by accurately weighing out 97% + pure monoglycerides which had previously been purified by TLC. The percentage composition as determined from the triangulated peak area of the TMS derivatives was in close agreement with the actual values for both standard mixtures as shown in Table I.

TABLE II

Comparison of the Fatty Acid Composition of Several Representative Fats by GLC Analysis of Their Monoglyceride TMS Derivatives and Methyl Esters

Source	Type of derivative	Carbon chain length				
		10	12	14	16	18
Bayberry tallow	TMS derivative	trace [a]	1.09	57.74	41.18	.....
	Methyl ester	.....	.91	58.40	40.69	.....
Corn oil	TMS derivative [b]	.....	.....	trace	18.34	81.66
	Methyl ester	.....	.....	trace	17.46	82.54
Coconut oil	TMS derivative	4.26	53.23	21.19	10.24	11.08
	Methyl ester	3.00	49.33	23.46	10.87	13.36

[a] Represents less than 0.5% of the total.
[b] Values represent the total area of the 1- and 2-isomers of the saturated and corresponding unsaturated acid.

The percentage composition of standard mixture No. 2 was obtained by the summation of peak areas of the 1- and 2-isomers for each acid. The percentage composition of standard mixture No. 1 shows that the values obtained for the 1- and 2-monoglycerides of the saturated and unsaturated acids are in close agreement with the actual values. The small differences between actual and observed values could have been caused by lack of absolute purity of monoglycerides used in the standards. A typical chromatogram of the standard mixture No. 1 is shown in Figure 5. A comparison of the fatty acid composition of some natural fats as determined by GLC analysis on a 3 ft. x ¼ in. DEGS column of monoglyceride TMS derivatives and methyl esters is shown in Table II. Since we were unable to resolve the 2-monoglyceride isomers of the unsaturated acid from the 1-isomer of the corresponding saturated acid, the values for a particular chain length include the 1- and 2-isomers of the saturated and unsaturated acids. These values were compared to the sum of the methyl esters of that chain length. The close agreement of the values obtained by the two methods supports the results obtained from analysis of the standard monoglyceride mixtures. From these data one can safely conclude that 1- and 2-monoglycerides rapidly form TMS derivatives that give quantitative results by GLC analysis.

Although a mixture of 1-monostearin, 1-monoolein and 1-monolinolein TMS derivatives is resolvable on the DEGS column, mixtures of the 1- and 2-isomers are not.

The analysis of corn oil monoglycerides as their TMS derivative shown in Figure 6 demonstrates this.

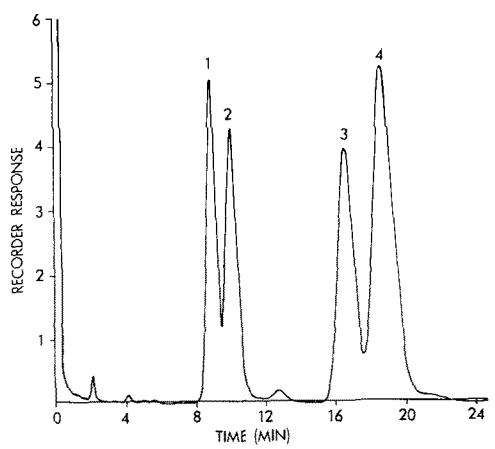


FIG. 5. Quantitative determination of 1- and 2-monopalmitin and monoolein standard mixture No. 1 shown in Table I. The analysis was carried out on a 3 ft x ¼ in. DEGS column. The major peaks are 1) 2-monopalmitin; 2) 1-monopalmitin; 3) 2-monoolein, and 4) 1-monoolein.

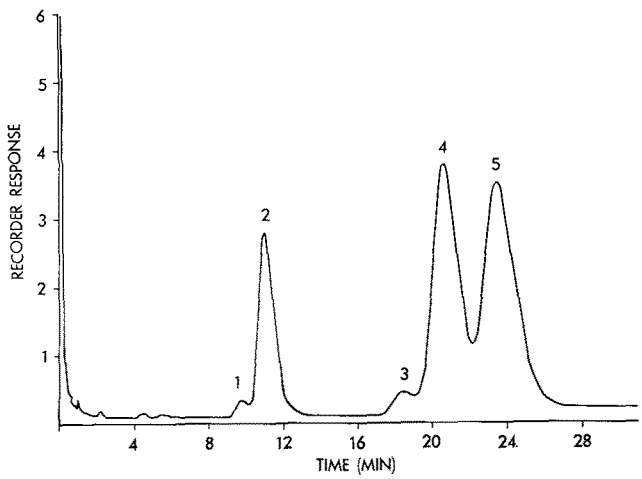


FIG. 6. Analysis of corn oil monoglycerides as their TMS derivatives on 3 ft x ¼ in. DEGS column. The major peaks are 1) 2-monopalmitin; 2) 1-monopalmitin; 3) 1-monostearin and 2-monoolein; 4) 2-monolinolein and 1-monoolein, and 5) 1-monolinolein.

The monoglycerides of each acid contains approximately 10% of the 2-isomer.

The 2-monoolein area includes the 1-monostearin peak while the 2-monolinolein peak is masked by the 1-monoolein peak. Monoglyceride TMS derivatives have been separated according to degree of unsaturation on thin layer plates. However, this technique is not perfected and is under further investigation. With the use of this additional step one can quantitatively determine the composition of any monoglyceride mixture, including the conen of the 1- and 2-isomers of each component.

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Helichrysum Seed Oil. I. Separation and Characterization of Individual Acids

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Abstract

Helichrysum bracteatum (family Compositae) seed oil contains a complex array of unusual fatty acids in addition to the usual palmitic, stearic, oleic, and linoleic acids. Its unusual constituents include 9.5% crepenynic acid; 14.0% epoxy acids, mainly coronaric; 4.4% *cis,trans*-hydroxy conjugated dienoic acids; and 7.2% of a previously unknown hydroxyacetylenic acid for which the name helenynolic acid is proposed. A method for determining helenynolic acid in the presence of hydroxy-conjugated dienes is described.

Introduction

Helichrysum bracteatum or strawflower is an annual originally from Australia. It is widely cultivated in the United States for its bloom, since the flower heads, when dried, hold their color for months.

The unusual nature of the oil was first noted in this laboratory during a routine gas-liquid chromatographic (GLC) analysis of the methyl esters. No linolenic acid was found although oil from a different accession of seed had been reported to contain 32.0% of this acid as determined by alkali isomerization (1). An unusual component appeared with equivalent chain lengths (ECL) of 18.1 on an Apiezon L column and 20.7 on a LAC-2-R446 column (2) and was tentatively identified as methyl crepenynate (3). A Durbetaki titration (4) of the oil gave a hydrogen bromide equivalent (HBE) (5) of 18.4, whereas GLC analysis of the methyl esters indicated a much lower quantity of epoxy acids to be present. An IR spectrum of the oil contained a noticeable hydroxyl peak, but no other peculiar feature that seemed significant. A UV absorption maximum at 229 mμ (6) indicated conjugated enyne. Thin-layer chromatography (TLC) of the methyl esters revealed the presence of three monohydroxy acids and at least one epoxy acid.

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This paper presents the isolation and purification of the individual component fatty acids of *Helichrysum* oil, and the characterization of some of the unusual acids present.

Experimental Procedures and Data

Methods

TLC was performed on glass plates coated with Silica Gel G (according to Stahl) with hexane, diethyl ether, acetic acid (70:30:1 or 80:20:1) as the solvent. Spots were detected by spraying with 50% sulfuric acid and heating at 120C.

GLC analyses were carried out as described by Miwa et al. (2).

Countercurrent distribution (CCD) studies utilized a 200-tube Craig-Post apparatus and the acetonitrile-hexane solvent system (7).

Partition chromatography of *Helichrysum* free acids was carried out essentially as described by Frankel et al. (8,9) using a 2.0% methanol in benzene as the eluting solvent. Our separations were not comparable to those of Frankel because of the large samples used; however, it was possible to obtain a hydroxy acid concentrate as described later in this paper.

Periodate-permanganate oxidations were as described by von Rudloff (10).

UV spectra were determined with a Beckman DK-2A spectrophotometer. IR spectra were determined on a Perkin-Elmer Model 137 instrument, using 1% solutions in carbon tetrachloride.

Oil Extraction. Oil was obtained from the ground seed of *Helichrysum bracteatum* var. *monstrosum* by Soxhlet extraction with petroleum ether (bp 30–60C). Solvent was removed *in vacuo* with a rotary evaporator. The yield of oil was 26.6%. It showed a rather broad maximum at 229.7 mμ, $E^{1\%}_{1\,cm}$ 80.5, in cyclohexane. In the IR, a hydroxyl peak (3450 cm^{-1}) was the only unusual feature. The oil had an HBE of 18.4 and contained 0.7% free fatty acid (calculated