Characterization of the Nonvolatile Compounds Formed During the Thermal Oxidation of Triolein

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ABSTRACT

Triolein was subjected to thermal oxidation at 200 C for 24 hr with an air flow of 0.15 ml/min/gm. The oxidized material was saponified and converted to its corresponding methyl ester. Nonoxidized materials were separated from nonvolatile oxidation products using preparative thin layer chromatography. Further separation of these fractions was achieved using gas chromatography, gel filtration chromatography and solvent-solvent extraction. Several compounds were isolated in this manner and their structures determined with the aid of nuclear magnetic resonance and mass spectrometry, as well as by supplementary chemical methods and by comparison with known compounds. These compounds were: (1) 2-cyclohexyldodec-5-enoic acid and isomers; (2) a mixture of 2-, 8-, 9-, and 10-monohydroxystearic acid; (3) 9,10-dihydroxystearic acid; (4) 1-decyl-2-(dec-6-enyl)-cyclohexane; (5) a mixture of isomers corresponding to a cyclic monomer of oleic acid containing both exocyclic and endocyclic unsaturation.

INTRODUCTION

Recent reports have indicated that when fats are heated under normal deep frying conditions and fed to rats, little or no toxic effects are observed (1,2). However other reports have shown that fats heated under more severe conditions exhibit varying degrees of toxicity (3,4). It is probable that many of the same types of compounds in differing amounts are formed under both severe and mild treatments. Consequently it is essential to determine the types and amounts of compounds formed in such fats prior to individual toxicological studies on pure isolated compounds. The volatile decomposition products of heated fats have been well characterized (5,6); however the nonvolatile decomposition products have not been well defined. In the present study several of the nonvolatile oxidation products resulting from the oxidation of triolein, a simple triglyceride, were separated and identified.

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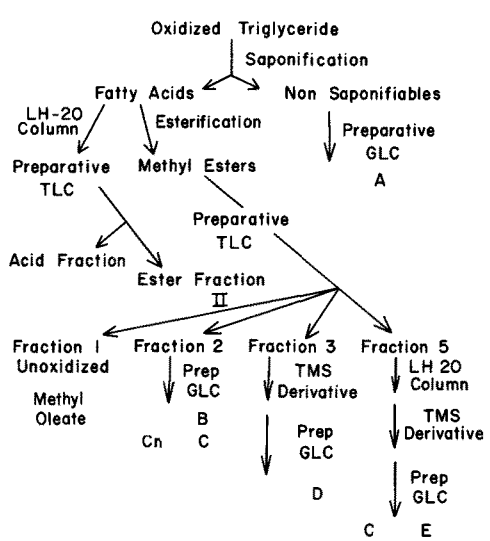


FIG. 1. Separation scheme for oxidized triolein.

Experimental Procedures

Emersol 233 LL oleic acid (Emery Industries, Inc., Cincinnati, Ohio) was converted to its methyl esters using 2% sulfuric acid-methanol and the resultant esters were subsequently distilled using a Podbielniak Semi-Cal 3650 fractional distillation unit. Purity of the distilled fractions (>98.5%) was monitored by gas liquid chromatography. Further purification of the methyl oleate was achieved by saponification of the esters and two crystallizations of the resultant acids from acetone at -60 C. The resultant product was at least 99.9% oleic acid containing less than 0.1% linoleic acid.

Oleyl chloride was prepared using oxalyl chloride following the method of Youngs et al. (7). Triolein was synthesized according to the method of Hartman (8). The product obtained was decolorized with activated carbon and recrystallized from acetone-Skellysolve F (4:1 v/v) to yield pure triolein. The triglyceride was oxidized in a three-necked pyrex flask equipped with a gas dispersion tube and thermometer by heating 170 g for 24 hr at 200 C while compressed air was dispersed through the hot oil. The air, 0.15 ml/min/gm oil, was first passed through two drying tubes and a gas dispersion tube before entering the oxidation flask. The oxidized oil was cooled and stirred at -20 C under nitrogen.

Esterification of samples was carried out using either diazomethane (9) or 2% sulfuric acid in anhydrous methanol.

Saponification was carried out using 1 M potassium hydroxide in methanol.

Aromatization was carried out according to the procedure of Hutchinson and Alexander (10). Twenty milligrams of material was heated for 12 hr at 250 C with 1 mg of 10% palladium on charcoal in a sealed tube. The reaction mixture was washed from the sealed tube with diethyl ether

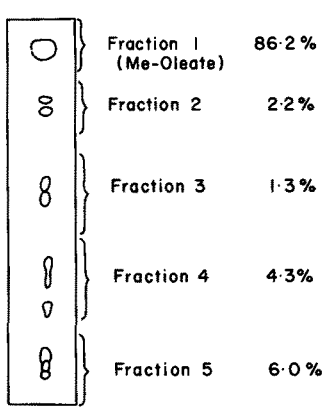


FIG. 2. Thin layer separation of oxidized triolein.

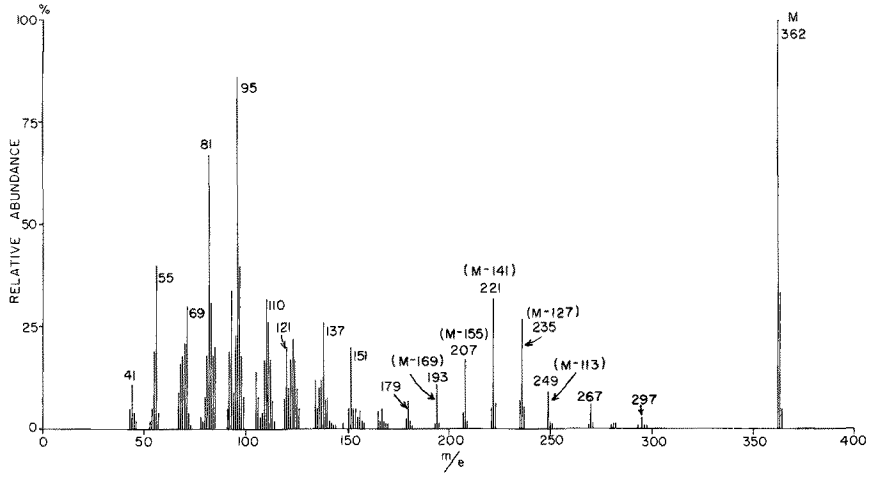


FIG. 3. Mass spectrum of A.

and filtered to remove the catalyst. The clear filtrate was then evaporated and the IR and UV absorption spectra of the residue determined.

Thin layer chromatography (TLC) was carried out according to the procedure of Stahl (11). Materials on the plates were visualized by spraying with 2,7-dichlorofluorescein, 0.2%/95% ethanol, and viewing under UV light. The separated material was recovered by extraction of the appropriate bands with moist ether.

Gas chromatographic separations were performed on a Barber-Colman Model 10 gas chromatograph, equipped with a programmable column bath and a flame ionization detector. Two types of glass columns were used: (1) a polar column 6 ft x 4 mm, packed with a 15% (w/w) ethylene glycol succinate polyester (EGS) coated on 100/120 mesh acid-washed Chromosorb W; (2) a nonpolar column (6 ft x 1/8 in. I.D.) packed with 15% Apiezon L on 60/80 mesh acid-washed Chromosorb W. Purified argon carried gas was employed at a flow rate of 80-90 ml/min. The columns were used either isothermally in the range of 175-250 C or programmed from 140-280 C. Methyl esters of known compounds were used as standards. The preparative column was equipped with a programmable column bath and a thermal conductivity detector. The material to be separated was injected onto a 12 ft x 3/8 in. I.D. glass column packed with approximately 250 g of 15% Apiezon L on 68/80 mesh acid-washed Chromosorb W. A flow rate of 350-400 ml/min of purified helium was maintained on the column.

A Cary Model 11M recording spectrophotometer was used for the determination of UV spectra. Samples with concentrations of 1 mg/ml of 95% ethanol were scanned from 200-400 mμ using 10 mm path quartz cells.

Samples were analyzed on a Beckman IR-7 infrared spectrophotometer. A beam condenser employed in conjunction with an ultramicro liquid cell was used to analyze small liquid samples.

Mass spectral analyses were performed using a Hitachi-Perkin-Elmer RMU-6E single focusing mass spectrometer. Spectra were produced at a standard ionization potential of 70 ev and then at 6-15 ev.

Nuclear magnetic resonance spectra were obtained on a Varian Associates Model A-60 high resolution nuclear magnetic resonance spectrometer.

Neutralization equivalents were determined according to Cheronis and Ma (12).

The position of double bonds was determined by oxidative ozonolysis. The sample (2.25 mg) was dissolved in 1 ml of methyl acetate with the addition of a small droplet of Sudan Red. Ozone was bubbled through the cooled mixture (-20 C) until the red color faded. Excess ozone was expelled with nitrogen. Acetic acid (1 ml) and 0.75 ml of 30% hydrogen peroxide were added; the reaction mixture was sealed in a 5 ml ampule and incubated at 60 C for 12 hr. Solvent was evaporated and the residue dissolved in 2 ml of water-methanol (1:1). The monocarboxylic acids were separated from the dicarboxylic acids by ether extraction and solvent was evaporated under reduced pressure. The dicarboxylic acids were removed by evaporation of the aqueous methanol at 35 C under vacuum.

Side chain oxidations were carried out with potassium permanganate using 0.1 g of oxidant to 20 mg of sample (13).

Determination of C-methyl groups was performed by the Clark Microanalytical Laboratory, Urbana, Illinois.

A microhydrogenation apparatus described by Mead and Howton (14) was used to determine the extent of unsaturation of unknown compounds.

Trimethylsilyl ether derivatives were prepared from hydroxyl containing esters according to the procedure of Wood et al. (15).

Synthesis of Methyl 2-cyclohexyldodec-5-enoate

Commercially available 1-hydroxy-3-decyne (3 M) was reacted with phosphorus tribromide (1 M) in the presence of toluene at room temperature. The resultant product, 1-bromo-3-decyne, after isolation and purification by distillation under reduced pressure, was obtained in an 80% yield, of 99% purity as determined by gas liquid chromatography (GLC).

The condensation of cyclohexyl bromide with diethyl malonate to form diethyl 2-cyclohexylmalonate was performed according to the procedure of Adams and Kam (16), yielding upon distillation 85% of product (bp 125 C) with a purity of 99% by GLC.

The procedure of Adams and Kam (16) was again employed to condense the previously prepared alkynyl bromide and 2-cyclohexylmalonic ester to form diethyl 2-(3'-decynyl)-2-cyclohexylmalonate. The product was obtained in 64% yield, then saponified and subsequently decarboxylated to 2-cyclohexyl-5-dodecynoic acid by heating at 170 C for 4 hr at reduced pressure (1 mm Hg). The resultant alkynoic acid was esterified as previously described and the methyl ester purified by distillation under reduced pressure. This product had bp 188-189 C, 2.4 mm. The acetylenic ester was partially hydrogenated using Lindlar catalyst to form the corresponding *cis* olefin (17). The hydrogenated material was subjected to preparative GLC to yield pure methyl 2-cyclohexyl-5-dodecenoate. Anal: Calc. for $C_{19}H_{34}O_2$: C, 77.15; H, 10.87. Found: C, 77.31; H, 11.27.

Isolation of A: Gas chromatographic analysis of the ether extract of the nonsaponifiable fraction (1.2% of the

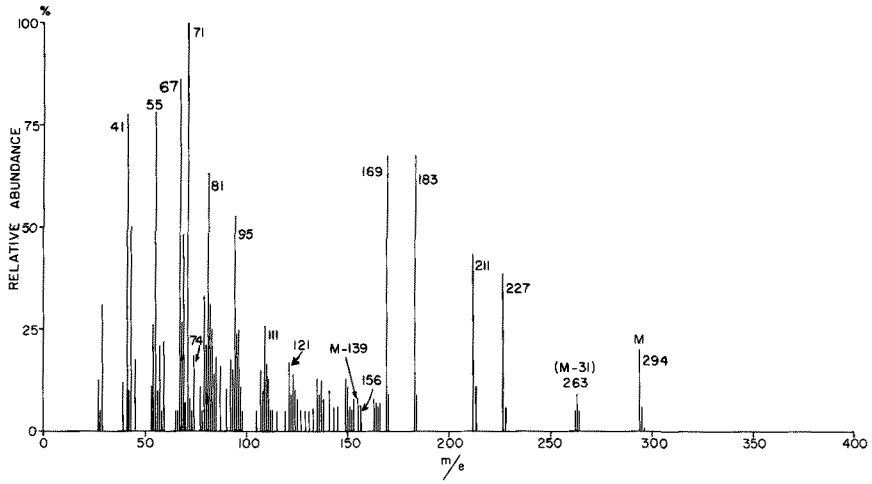


FIG. 4. Mass spectrum of B.

total oxidized material) of the oxidized triglyceride on an Apiezon L column revealed the presence of a major component (11% of the nonsaponifiable fraction) (A) with a relative retention time of 5.52 (methyl oleate = 1.00). Component A was the only one which had a relative retention time greater than that of methyl oleate and was subsequently isolated by preparative GLC.

Isolation of B and C: Acids from the oxidized triglyceride were isolated in the usual way and converted to their methyl esters and chromatographed on Silica Gel G thin layer plates and developed in diethyl ether-Skelly F (70:30). Preparative TLC of the oxidized methyl esters yielded several fractions as shown in Figure 2. The fractions were extracted from the silica gel with moist diethyl ether. Fraction 1 was analyzed by gas chromatography and found to be 99% + methyl oleate, and comprised 86% of the total sample.

Gas chromatographic separation of Fraction 2 (Fig. 2) on an Apiezon L column revealed the presence of several components. This fraction, upon preparative gas chromatography yielded two fractions, B (0.53%) and C (0.55%), which had relative retention times of 1.27 and 1.60 respectively, compared to methyl oleate.

Isolation of D: Gas chromatographic separation of Fraction 3 (Fig. 2) on an SE-30 column revealed seven small peaks which exhibited tailing suggesting hydroxyl groups. Fraction 3 was therefore silylated according to the procedure of Wood et al. (15) yielding 14 peaks on gas chromatography. The material represented by the largest peak was isolated by preparative gas chromatography, using an Apiezon L column, was designated as D, and represented 0.21% of the total oxidized material.

Isolation of E: It appeared that Fraction 5 (Fig. 2) contained the most polar or the highest molecular weight products of oxidation, or both. Gas chromatographic analysis on a short column packed with 10% SE-30 at temperatures up to 300 C revealed several asymmetrical peaks. Further fractionation on the basis of molecular weight was accomplished by chromatography on Sephadex LH-20 using chloroform as the eluting solvent. A dark brown viscous liquid eluted, followed by fractions composed of a dark brown liquid which partly crystallized yielding white crystals. The latter fractions were combined and silylated, whereupon several peaks were observed on an SE-30 column. However only one component was present in sufficient quantity to isolate preparatively. This compound was designated as E and was present in the oxidized material to the extent of ~0.1%.

RESULTS

Structure A: The data obtained from elemental analysis (C, 86.9, H, 13.3) and mass spectrometry (MS) (M = 362) suggested the molecular formula of A as $C_{26}H_{50}$, suggesting two points of unsaturation (rings or double bonds). Quantitative hydrogenation substantiated the presence of one double bond which was confirmed by proton magnetic resonance spectroscopy (PNMR): a triplet at τ 4.60-4.75 attributed to olefinic protons and a doublet at τ 7.80-8.20 attributed to methylene protons adjacent to the double bond.

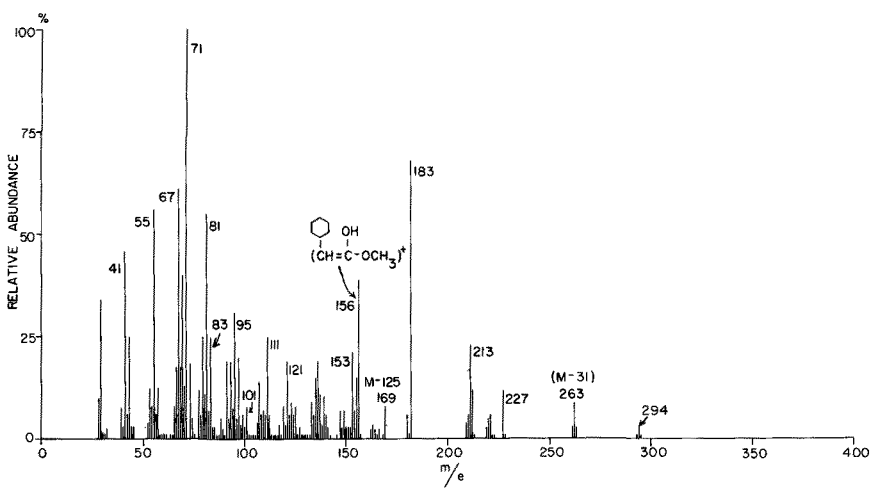


FIG. 5. Mass spectrum of methyl-2-cyclohexyl-dodec-5-enoate.

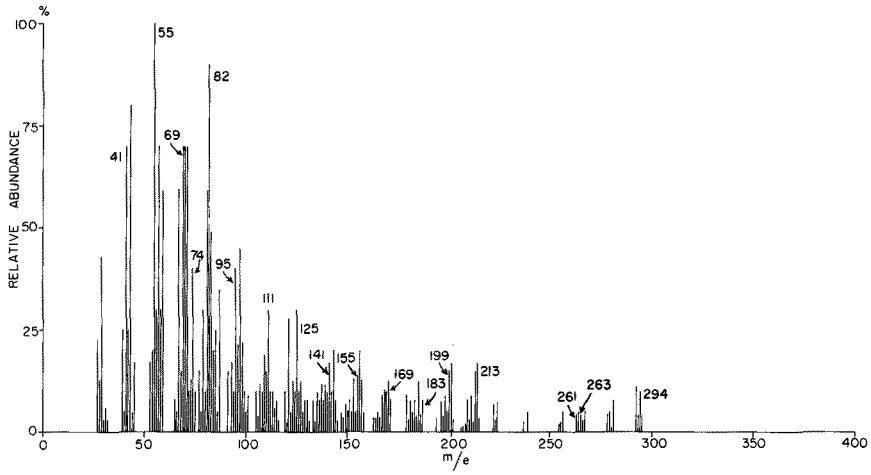
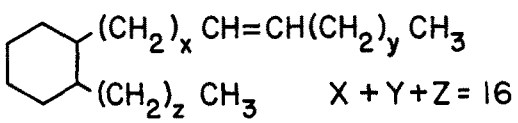


FIG. 6. Mass spectrum of C.

Catalytic dehydrogenation of A resulted in material with absorption maxima at 271, 267 and 264 mμ, representative of 1,2-disubstituted benzenes (18). In order to determine if ring formation and positional isomerization occurred during catalytic dehydrogenation, model compounds were subjected to conditions identical to those described earlier. Samples of methyl oleate, linoleate, and linolenate showed no absorption in the aromatic region after being subjected to dehydrogenation conditions. Only aliphatic conjugation was observed in the cases of linoleate and linolenate. Samples of isomerically pure 1,2-, 1,3- and 1,4-dimethylcyclohexanes were also dehydrogenated under the same conditions and the UV spectra of these materials were identical with those of the expected xylenes. There was no evidence obtained for isomerization of the methyl side chains during aromatization under these conditions.

The dehydrogenated material was then oxidized with alkaline permanganate (13) and the methyl ester prepared from the resultant acids. This ester was subsequently identified as dimethyl phthalate using IR and gas chromatographic data.

Oxidative ozonlysis of A afforded a 62% yield of hexanoic acid along with smaller amounts of pentanoic and propanoic acids. The mass spectrum of the mixture (Fig. 3) showed a series of homologous (14 amu) peaks beginning at m/e 81 and extending to m/e 249 indicating successive alkyl cleavage. A similar series indicating alkenyl cleavage was present in the ion clusters from m/e 93 to m/e 163. The presence of these series indicate that A may have alkyl chains of up to 17 carbons in length. However the presence of high intensity fragments at m/e 207, 221 and 235 indicate a large portion of chains of from 9-11 carbons in length. In addition, IR evidence indicated the presence of both *cis-* and *trans-* unsaturation. Kuhn-Roth oxidation demonstrated the presence of two terminal methyl groups (Calc.: 7.9% C-methyl; Found: 8.30%). These data indicated the structure of A as a series of materials of the general formula A where both *cis* and *trans* configurations are possible.



A

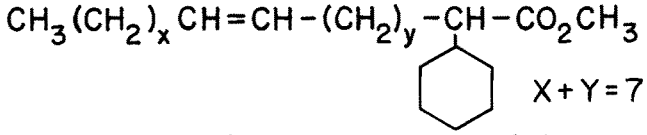
Structure B: Substance B (C, 77.2; H, 12.0) showed a mass spectrum (Fig. 4) indicating the presence of molecular ions at m/e 294, and an ion at M-31 corresponding to loss of a methoxyl ion (20) suggesting that the compound was a methyl ester. The PNMR spectrum showed a peak at τ = 6.32, also attributed to a methyl ester. These data support the molecular formula as $C_{19}H_{34}O_2$. On quantitative hydrogenation, A absorbed 1 mole of hydrogen. The PNMR spectrum of the unknown indicated that the ratio of methyl ester to olefinic protons was 3.0 - 2.1. Therefore the unknown contained one double bond and one ester group.

The presence of rings was ascertained by aromatization (10). Absorption bands at 261, 264 and 267 mμ, characteristic of monosubstituted benzene rings (18) were observed. The aromatized unknown was oxidized to the corresponding acid, converted to the methyl ester and identified as methyl benzoate using IR and GLC. Additional data in support of the presence of a monosubstituted C_6 ring were obtained by MS. Major peaks were observed at m/e 67 and 81. Peaks from similar origin were noted at M-67 and M-83. The m/e 81 fragment is usually the base peak in cyclohexenyl branched compounds (19). A peak at m/e = 67 has been reported as a major ion in the spectrum of 2-cyclohexyloctane (21). The presence of ions of high abundance at m/e 82 and 83 usually indicate the presence of cyclohexyl substitution.

Oxidative ozonlysis and subsequent gas chromatographic separation of the methyl esters of the ozonolysis products suggested that the double bond was located at Δ^5, Δ^4 and Δ^3 positions since it was possible to isolate heptanoic (44.7%), octanoic (41.2%), and nonanoic acids (11.4%) as their methyl esters.

Mass spectrometric analysis (Fig. 4) suggested that the C_6 ring was attached to the aliphatic ester chain in the α-position. Thus the base peak in the mass spectrum of an unsubstituted straight chain methyl ester in the C_6-C_{26} range arises from cleavage with the transfer of a γ-hydrogen atom resulting in the formation of an ion at m/e 74 (22). B showed a relatively small m/e 74 peak suggesting that the α-carbon was probably substituted (Fig. 4). If the α substituent was either a cyclohexyl or cyclohexenyl group, peaks at m/e 156 and 154, respectively, would be expected for a β cleavage followed by the transfer of an α hydrogen.

The data obtained indicates that B is a mixture of positional isomers involving the position of a double bond and a C_6 cyclic, saturated and unsaturated, branch in an aliphatic ester molecule. One of the isomers is represented below.



Further support for the structure proposed above was obtained by comparison of a synthetic isomer (methyl 2-cyclohexyl-dodec-5 enoate) with the unknown. The mass

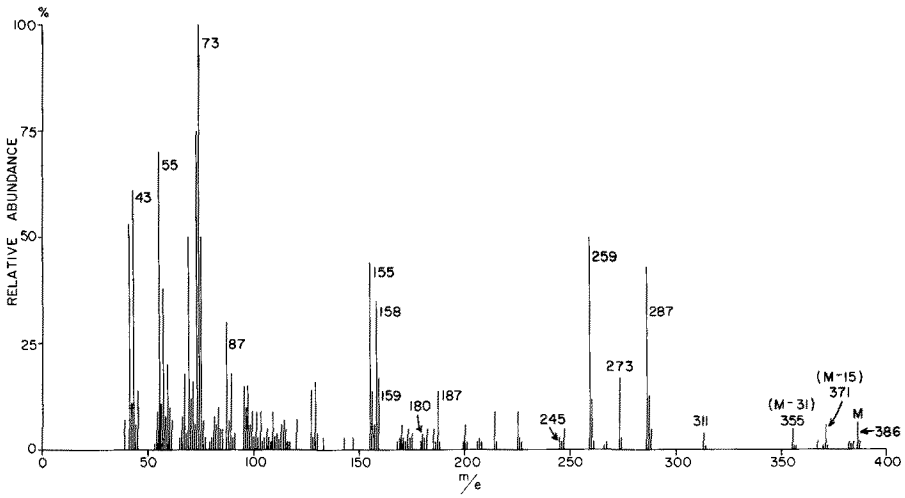


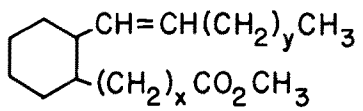
FIG. 7. Mass spectrum of D.

spectra were compared and, with the exception of the intensities, the spectra of B (Fig. 4) and methyl 2-cyclohexyl-5-dodecenoate (Fig. 5) are very similar. The major difference between the spectra is that an M-81 peak is found in spectra of the synthetic product, but an M-83 peak is found in the spectra of the unknown. The M-83 peak is a low intensity peak in the 70 ev spectra. At lower ionizing voltages the M-83 peak increased in intensity while the M-111 peak was observed to decrease in intensity. The presence of M-81 peak may indicate loss of a cyclohexenyl group and its presence in the molecule. Cleavage α and on the ester side of the double bond accounts for the presence of M-125 and M-111 peaks in the spectra of B. Other significant similarities in the spectra of the synthetic product and the unknown are the m/e 67 peak and the m/e 83, 81, 79 and 77 series.

Structure C: Elemental analysis of "C" (C, 76.8; H, 11.2) and the molecular weight from MS (294 with some 292) indicated the formula to be $C_{19}H_{34}O_2$. The PNMR spectra contained the following absorptions: a triplet at τ 4.58-4.78 (exocyclic olefinic protons); a singlet at τ 6.35-6.45 ($-OCH_3$); τ 7.30-8.00 (α-methylene protons); τ 8.25-8.85 (methylene protons); τ 8.90-9.30 (terminal methyl protons). Absorption bands were observed in the IR spectra of the unknown which indicated the presence of ester (5.74 and 8.00 μ) *cis* double bond (14.8 μ), and *trans* double bond (10.3 μ).

On the other hand quantitative hydrogenation indicated the molecule contained only one double bond. The ratio of methyl ester to olefinic protons (1:1) in the PNMR spectrum also indicated the presence of one double bond suggesting that a mixture of olefins was at hand. Aromatization of the unknown yielded a material which absorbed at 262, 266 and 271 mμ in the UV indicative of 1,2- and 1,3-disubstituted aromatic compounds (18). Oxidation of the aromatized material with potassium permanganate and conversion of the resulting acidic material into the corresponding ethyl esters yielded two major peaks upon GLC. These peaks were identified as diethyl phthalate and diethyl isophthalate, and they were present in a ratio of 3.54 parts of the 1,2-isomer to 1.00 parts of the 1,3-isomer.

The oxidative ozonolysis products were octanoic acid (1.2%), nonanoic acid (81.7%), and decanoic acid (4.4%), showing that C was also a mixture of positional olefin isomers. No dicarboxylic acids were found, indicating that the double bond was not present between the ester and the C_6 ring. The presence of a peak at m/e 292 also suggests the presence of a diunsaturated isomer, although this is in contrast to NMR and hydrogenation data. A possible structure for one of these isomers in accord with these facts is indicated below.



$Y = 6,7,8$

$X + Y = 8$

The mass spectrum (Fig. 6) supports the structure as shown.

Cleavage on both sides of the ring produces a cyclohexenl ion at m/e 82. This peak is found to be second in intensity to the m/e 55 peak which is prominent in the m/e 41, 55, 69 series associated with aliphatic olefins. The relatively low abundance of the m/e 74 ion indicates the

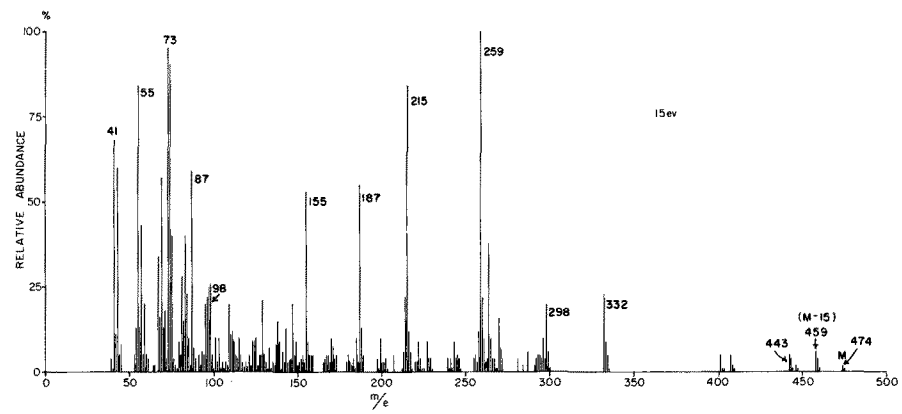


FIG. 8. Mass spectrum of E.

presence of substitution α to the ester function.

Structure D: The identification of D as a mixture of positional isomers of hydroxymethyl stearate is substantiated by comparison of IR spectra, GLC retention time, elemental analysis, and neutralization equivalent with authentic 12-hydroxy isomer.

The mass spectrum of "D" (Fig. 7), isolated as the trimethylsilyl ether derivative, resembled the corresponding derivative from a mixture of positional isomers of hydroxystearic acid (23). The presence of ions at m/e 245, 259, 273 and 287, formed by cleavage of the trimethylsilyl ether derivative, indicated that the unknown was primarily a mixture of 8, 9, 10 and 11-hydroxystearate. The presence of α-hydroxy stearate may not be discounted since the mass spectrum of the unknown ions at m/e 159-155 were present and may arise from the α isomer by cleavage and loss of hydrogen, or from other fragments, i.e., a m/e 157 ion could arise from cleavage of the 9- isomer.

Structure E: The presence of hydroxyl groups at 2.85 μ, and ester at 5.7 μ was shown by IR spectroscopy. The neutralization equivalent was 311 (Calc: 316). The trimethylsilyl ether derivative of the unknown was prepared to facilitate mass spectral and gas chromatographic analysis. The mass spectrum (Fig. 8) of the trimethylsilyl ether derivative at lower m/e values was typical of that of an aliphatic trimethylsilyl ether (24). At larger m/e values the spectrum suggested that the compound was methyl-9, 10-bis-trimethylsilyloxy stearate. A very small parent peak was observed at m/e 474. The major fragments at m/e 215 and 259 were formed by cleavage of the bond between the secondary carbons to which the substituent ether functions were attached.

A rearrangement ion at m/e 332 formed by combination of a trimethylsilyl radical (m/e 73) with the fragment with m/e 259 is also characteristic of bis-trimethylsilyloxy substituted dihydroxy fatty acids. To confirm this structure a periodate oxidation was performed on 9,10-dihydroxystearate, as on E. The products of oxidation were identical from both samples as shown by gas chromatography. The IR spectra of the standard and the unknown were then compared and found to be identical. The experimental values for the per cent carbon and hydrogen were in close agreement with those calculated for 9,10-dihydroxy methyl stearate. Calc: C, 69.2; H, 11.5. Found: C, 68.7; H, 11.3.

DISCUSSION

Component A, a mixture of isomers of 1-alk-2-(alk-6'-enyl)-cyclohexane, has been hard to rationalize. The possibility that this compound was an artifact introduced through the solvents used for solvent extraction during the isolation and purification of the compound was investigated, but no traces of materials were found which had a retention time similar to the isolated material designated as A. In view of their structures, we still consider it possible that these hydrocarbons may have been introduced from some other source of contamination.

B was found to be a mixture of isomers of α-cyclohexyl dodecenoic acid, as well as α-cyclohexenyldodecanoic acid. C was a mixture of isomers similar to those isolated by Artman and Alexander (25) from soybean oil which had been heated in a fryer. Similar structures have been isolated from heated linoleate by Michael (26). Fredrick (27) has isolated compounds of similar structure from linseed oil heated in the presence of alkali.

A mechanism proposed for the formation of cyclic monomeric materials isolated from heated linseed oil indicated a possible reaction sequence to involve free radical formation, free radical allylic proton extraction, double bond migration, and ring closure (26). The cyclic products isolated from heated linseed oil involved cyclization at the methyl end of the fatty chain while in the present case the carbon α to the ester group seemed to be also involved in cyclization.

D has been determined to be a mixture of the 8, 9, 10 and 11 isomers of hydroxystearic acid. The 9 and 10 isomers would result from oxygen attack directly on the carbons of the double bond and not on the methylene grouped to the double bond. The 8 and 11 isomers may have been formed either by direct attack on the double bond after migration to the 8 and 10 positions. Similar compounds, hydroxy octadecenoic acids, were identified by Artman and Alexander (25) from heated methyl oleate and soybean oil as their corresponding ethoxy derivatives. Evidence for the presence of the oxygen on the 8 and 11 position was presented. From the heated triglyceride these authors isolated a mixture of compounds having the double bond in various positions, comparable to the distribution of unsaturation in the original partially hydrogenated soybean oil.

E was primarily 9,10 dihydroxy stearic acid. This represents attack by oxygen directly on the double bond, or since saponification was used to simplify the separation of the oxidation products of triolein, it is probable that hydrolysis of an epoxide formed across the 9,10 position of oleic acid resulted in the formation of the dihydroxy acid. Dihydroxy stearic acid has previously been isolated from thermally oxidized corn oil (28). More recently it has been isolated from heated soybean oil as the ethyxy hydrosy derivative (25).

ACKNOWLEDGMENTS

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