Purification of Cyclic Fatty Acid Esters: a GC-MS Study[1]

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ABSTRACT

Gas chromatographic analysis of cyclic monomeric concentrates and fractions from argentation chromatography on packed columns containing SE-30, OV-25 and Apiezon L stationary phases yielded incompletely separated peaks representing the various isomers present in the mixture. Somewhat better separation was achieved using a 6 ft x 1/8 in. column packed with 15% EGS on Chromosorb W. This column, when coupled to a mass spectrometer, yielded information concerning the composition of each of the isomeric components. Comparable results were obtained using a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase and a 150 ft x 0.01 in. capillary column coated with Apiezon L. While argentation thin layer chromatography proved useful, an argentation column method using silicic acid coated with 10% $AgNO_3$ proved more efficient for larger scale preparations. Elution of the column with 2% diethyl ether in petroleum ether yielded material essentially free of conjugated linolenate. A comparison of the behavior upon argentation thin layer chromatography of conjugated methyl linolenate, methyl linoleate and cyclic monomer esters indicated that these esters migrated to the same relative position as methyl oleate.

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[3]Abstracted in part from the dissertation to be submitted to the University of Illinois Graduate College in partial fulfillment of the requirements for the Ph.D. degree.

INTRODUCTION

The cyclization of linolenic and linoleic acids has been studied extensively. Schofield and Cowan demonstrated that linolenic acid could be converted to a cyclized structure by heating in the presence of solvent and alkali (1). Subsequent papers have concerned themselves with methods for improving the preparation and yields of saturated cyclized product (2), its separation from straight chain fatty acids (3), determination by gas liquid chromatography (GLC) (4), and a structural study of these products as mixtures of the corresponding saturated and aromatic isomers (5). Extensive earlier structural work has been reviewed by Friedrich (5), including work on cyclic products from eleostearate.

Much of this interest in cyclic fatty acids stems from their potential as industrial chemicals (6). However of equal if not greater importance is the fact that cyclic monomers of fatty acids (primarily linoleic acid) have been found in fats heated under relatively mild conditions. For instance, cyclic monomers have been found as a component of heated cottonseed oil (7) and heated linoleic acid (8,9). They have been isolated from soybean oil (10) and corn oil (11) heated in deep fryers. In addition, they are formed during the thermal oxidation in the laboratory of pure triglycerides containing oleic (12) and linoleic (13) acids. Furthermore cyclic monomers have been isolated as by-products from the hydrogenation of fats and oils (14,15). The field of cyclic compounds in fats and oils has recently been reviewed comprehensively by Artman (16).

As part of our program to determine the metabolic properties of heated fat components, a mixture of cyclic fatty acids was prepared from pure linolenic acid. These experiments were to precede our objective of preparing a purified ^{14}C-labeled cyclic monomer for use in metabolic studies. It was therefore important to remove as much starting material as possible from the product, since the

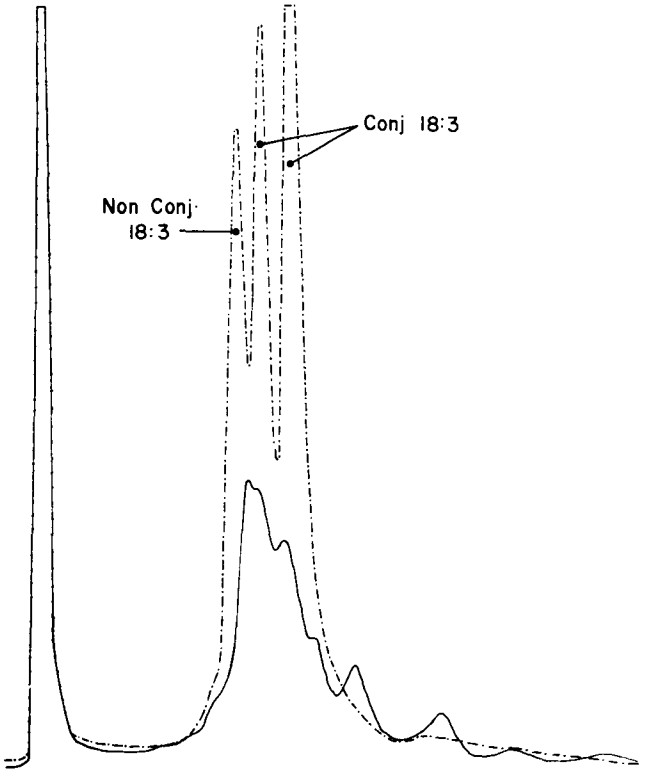


FIG. 1. Gas liquid chromatogram of cyclic monomer reaction product methyl esters with both conjugated and nonconjugated methyl linolenates (18:3) (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

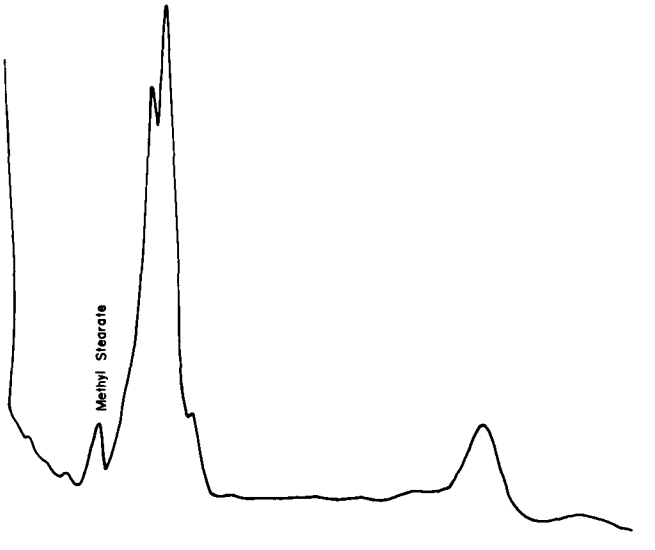


FIG. 2. Gas liquid chromatogram of hydrogenated cyclic monomer reaction product methyl esters (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

presence of such material, especially in the labeled conjugated form, would yield ambiguous results. The present paper is concerned with the preparation and partial characterization of a purified monomer fraction.

EXPERIMENTAL PROCEDURES

Pure linolenic acid was obtained from the Nu Chek Prep Co. and was analyzed as >99% by both GLC and thin layer chromatography (TLC). Pure aromatized cyclic monomer was obtained through J.C. Cowan, Northern Regional Research Lab., Peoria, Ill.

Methyl esters were prepared by reaction with diazomethane in diethyl ether followed by evaporation of solvent (17) and by reaction with methanol containing 2% H_2SO_4 (17) followed by the usual workup (17).

Samples were hydrogenated at atmospheric pressure with stirring by dissolving the sample (~5 mg) in ethyl acetate (10 ml) with 1-2 mg PtO_2 in an all glass system (18).

The cyclic monomers of pure linolenic acid were prepared according to a modification of the method of Schofield and Cowan (1) as follows: A typical preparation of the cyclic monomer of linolenic acid utilized 10 g pure trienoic acid dissolved in 100 g technical grade diethylene glycol and placed in a 250 ml three-necked pyrex flask. To this mixture was added 80-100% M excess of sodium hydroxide pellets (Reagent) and the flask and contents heated under an atmosphere of nitrogen gas for 1 hr at 240-245 C. During this time the level of solvent was kept constant by addition of diethylene glycol. The mixture was cooled, adjusted to pH 2 with 10% aqueous H_2SO_4 and the fatty acids extracted with petroleum ether. The organic extract was washed, dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was stored at 0 under nitrogen.

TLC was carried out according to Stahl (19). Glass plates (20 x 20 cm) were coated with a slurry of Silica Gel G (EM) containing 12% $AgNO_3$ in NH_4OH in layers 1 mm thick. Samples (80-100 mg) were applied as a narrow streak at 2 cm from the bottom of a plate. The plate was placed in a developing tank and developed twice with a solvent composed of petroleum ether-diethyl ether-glacial acetic acid 95:5:1. After drying, the plates were sprayed lightly with 1% of 2,7'-dichlorofluorescein in ethanol and viewed under UV light. The separated bands were removed by scraping into petroleum ether. The slurry was filtered and the fraction recovered by evaporation under N_2 to remove solvent. Analytical TLC was carried out under identical conditions as for preparative chromatography but employed a thinner silica gel layer (750 μ).

Purification of the crude cyclic monomer methyl ester was also carried out using column chromatography as follows: Mallinckrodt silicic acid (100-200 mesh) was washed with 50% aqueous HCl followed by distilled water rinses until succeeding rinses were Cl^- ion free. The washed silicic acid was heated in an oven at 150 C until dry. Silver nitrate (12.5% wt) was dissolved in distilled water and 100 g of the washed silicic acid added to form a slurry. This slurry was redried at 150 C and packed as a slurry in petroleum ether into a 2 cm diameter column. The average size column employed was 40 cm x 2 cm and contained ca. 80 g of packing. Samples of crude cyclic monomer methyl ester (2 g) were applied to the column and eluted with varying percentages of diethyl ether in petroleum ether. The elution was monitored by TLC and hydrogenation followed by GLC of the products.

Gas chromatography was accomplished using several columns. For ordinary analysis a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh Chromosorb W (Supelco, Inc., Bellefonte, Pa.) was employed. Other columns were: a 6 ft x 1/8 in. S.S. column packed with 3% SE-30 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 6 ft x 1/8 in. S.S. column packed with 3% OV-25 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase (Perkin-Elmer Corp., Norwalk, Conn.), and a 150 ft x 0.01 in. capillary column coated with Apiezon L (Perkin-Elmer Corp.). These columns were utilized at a column temperature of 170-185 C, at optimum flow rates for each column, and coupled with a flame ionization detector. The instruments employed were a Beckman GC-5 and a Varian Aerograph A-60A.

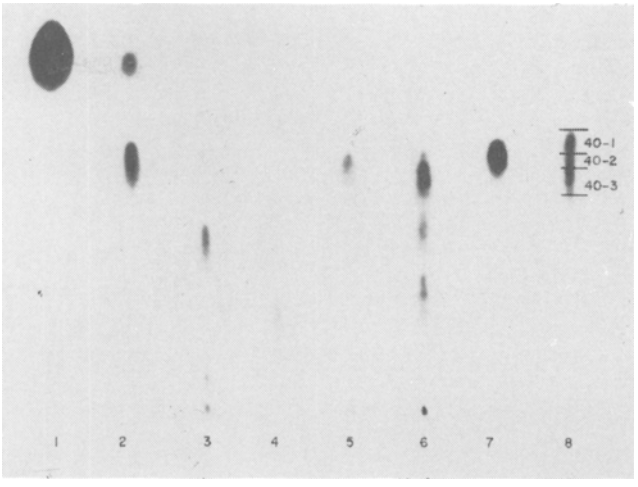


FIG. 3. Argentation TLC of cyclic, conjugated and nonconjugated fatty acid methyl esters: 1. methyl stearate, 2. methyl oleate, 3. methyl linoleate, 4. methyl linolenate, 5. conjugated methyl linoleate isomer mixture, 6. conjugated methyl linolenate isomer mixture, 7. methyl esters of aromatized cyclic monomer from linolenate, 8. methyl esters of cyclic monomer reaction product (dimeric and polymeric materials removed).

Mass spectra were determined with a Perkin-Elmer Hitachi RMU6E double focusing mass spectrometer adjusted to a resolution of 1000 and coupled with a gas chromatographic inlet system. The helium separator was maintained at 250 C, as was the ion source and short heated transfer line from the GLC. The ionizing current was 55 μA and the voltage set at 70 volts. Spectra were recorded every 3 sec to m/e = 600 during the elution of a GLC peak (1-10 μg material) as determined by both the FID detector of the gas chromatograph (10% split) and the continuous record produced by the total ion monitor. The mass spectra so obtained were stored on magnetic tape for further processing. High resolution element maps were obtained on selected samples at a resolution of 10,000 with a Varian Mat SM-1B instrument. The Varian Aerograph series 1200 Gas Chromatograph coupled to the mass spectrometer employed a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh acid washed Chromosorb W. A helium flow rate of 20-22 ml/min was employed. Approximately 90% of the column effluent was diverted to the mass spectrometer. Elution of individual components was accomplished by isothermal operation of the column at 175 C. The mass spectra collected (30-40 per sample) were processed using an on-line data system. The background contribution of the EGS column and that due to peak overlap were subtracted to yield spectra representative of the eluted components. The spectra were also averaged for changing ion current to obtain a representative spectrum for any one component.

RESULTS AND DISCUSSION

The formation of cyclic monomers of fatty acids in oils, which have been heated under both simulated and actual deep fat frying conditions, represents a potential public health problem. Artman and Smith (7) have shown that

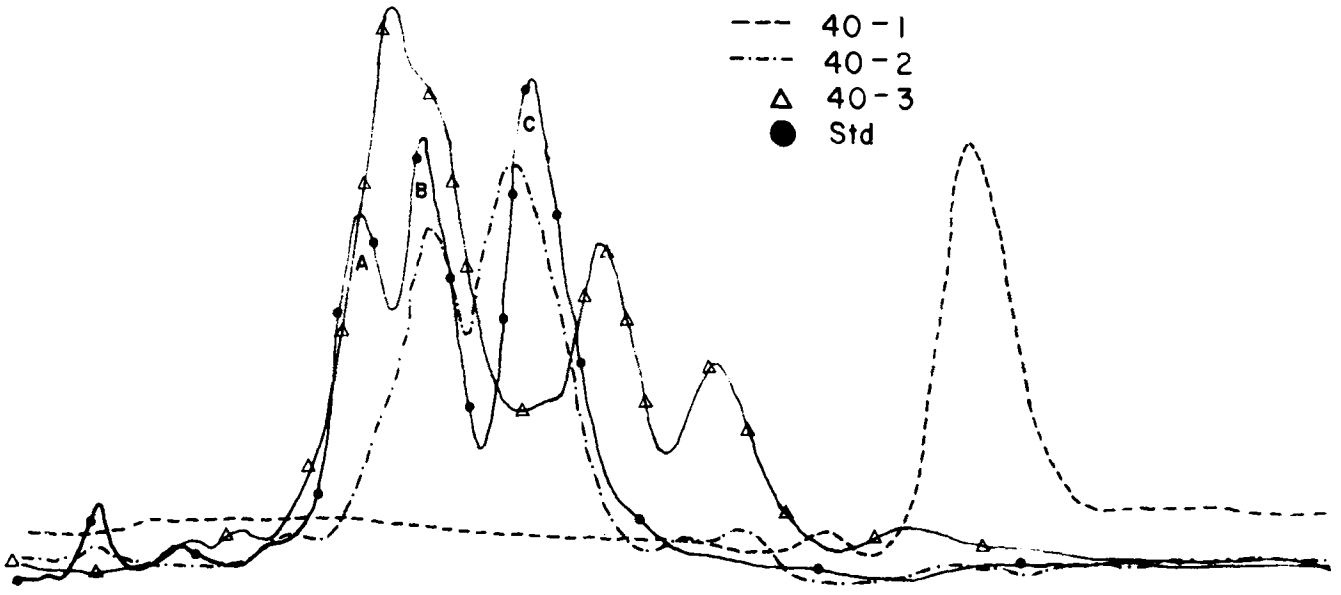


FIG. 4. Gas liquid chromatogram of fractions obtained by argentation thin layer chromatography of cyclic fatty acid methyl esters. Composition of standard: A. nonconjugated methyl linolenate, B. and C. conjugated isomers of linolenate (6 ft x 1/8 in. S.S., with 15% EGS in 60-80 mesh Chromosorb W [AW]).

cyclic monomer occurs in heated cottonseed oil as well as soybean oil heated in a deep fat fryer (10). Furthermore it appears that cyclic monomer may be the one component of heated oils with the most toxic potential that occurs in the largest quantities (7). For this reason it was essential to study the purification of cyclic monomer to remove as many other side reaction products as possible, with a view toward synthesis of approximately labeled compounds for future metabolic and toxicological studies.

The method of producing the cyclic monomer mixture used in the present study was a somewhat modified version of that reported previously (1). The formation of some small amounts of dimeric products appears to be a byproduct of the reaction at the high temperature and is probably due to a Diels Alder condensation between two molecules of linolenic acid. When the mechanism of cyclic acid formation (5) is considered, the side product of the reaction is, in addition to a small amount of dimeric material, primarily conjugated linolenic acid which is not readily separable from the reaction product.

Examination of the reaction products from a typical cyclization reaction using an ethylene glycol succinate (EGS) column (Fig. 1) indicated an ill-defined mixture of isomers similar to those reported for the cyclic monomer from linseed oil. There is considerable overlap indicated with the conjugated isomer mixture from methyl linolenate. The amount of conjugated linolenate isomers in the reaction mixture may be determined by hydrogenation and subsequent rechromatography of the hydrogenated crude reaction product (Fig. 2). The chromatogram of this sample indicated the presence of a small amount of methyl stearate (7-9%) and the presence of a component which did not change in retention time upon hydrogenation, probably due to an aromatic isomer.

Although the effect of conjugation on the Rf values of compounds separated via argentation TLC has not been fully investigated, it has been shown that the effect of silver complexing increased as the distance between double bonds increased (20). A comparison of the effects of argentation on the separation of conjugated and nonconjugated fatty acid methyl esters and cyclic monomer which demonstrates this effect is shown in Figure 3. The influence of increasing the number of double bonds in decreasing the Rf value in the series Rf 18:0>18:1>18:2>18:3, where the double bonds are all of the *cis* configuration, is readily apparent. However it is interesting to note that the complex mixture of geometrical and positional isomers representing conjugated 18:2 and 18:3 methyl esters exhibits the same Rf value as methyl oleate. The two lower spots in the conjugated 18:3 lane may be due to mixtures containing

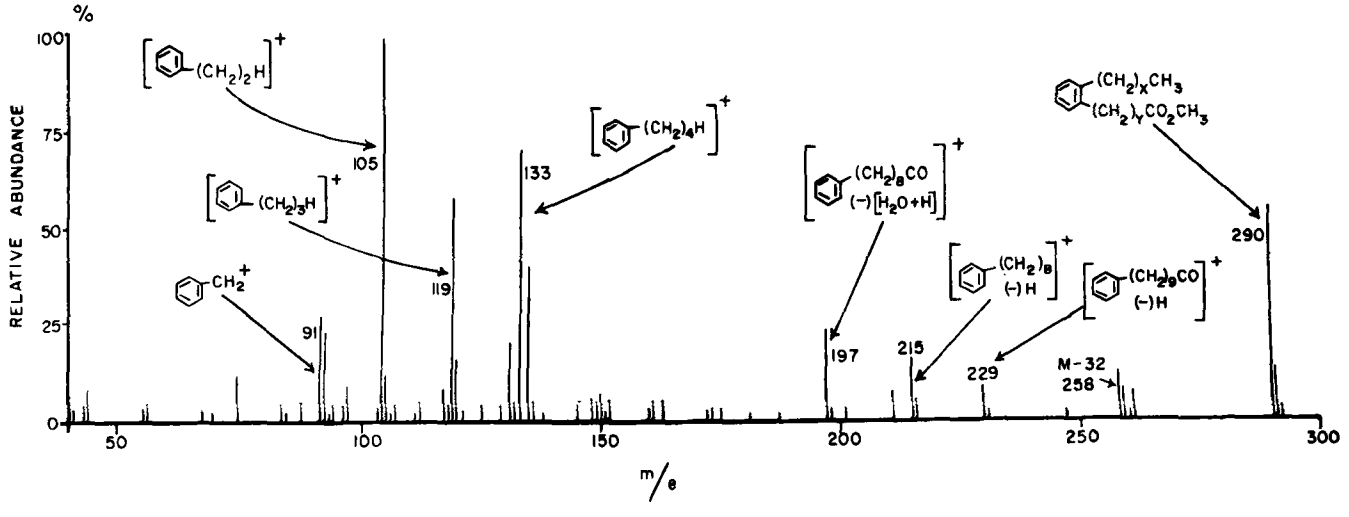


FIG. 5. Mass spectrum (70 eV) of isomeric aromatic cyclic monomer methyl esters (fraction 40-1).

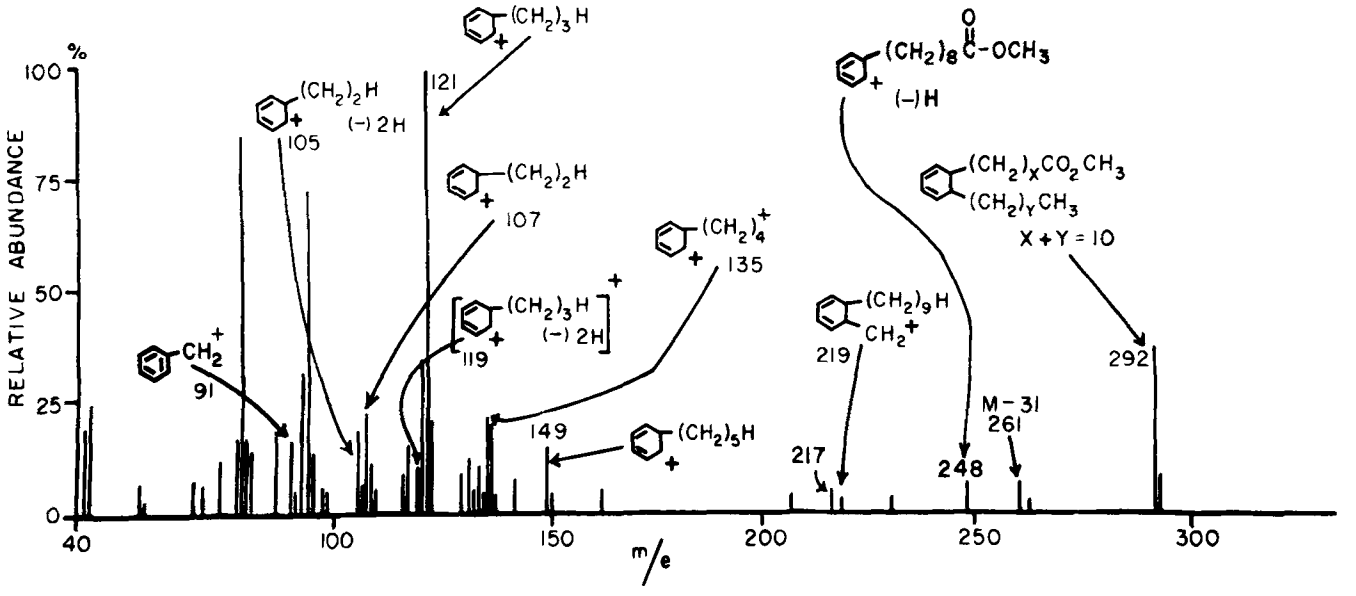
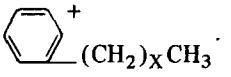
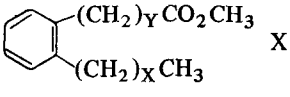


FIG. 6. Mass spectrum (70 eV) of isomeric cyclic monomer methyl esters (fraction 40-2).

lower percentages of *trans* double bonds; all three components have identical mass spectra. The aromatized cyclic monomer and the present reaction product behave in the same fashion. The reaction product, methyl ester, separated into three poorly resolved bands upon preparative argentation TLC. Each of the fractions were collected and the organic material extracted and analyzed by GLC on an EGS column coupled directly to the mass spectrometer. The mass spectra of each peak was recorded. The gas chromatogram of each fraction is outlined in Figure 4, and the origin of the fractions is indicated on the photograph of the TLC plate (Fig. 3, lane 8). Fraction 1, the topmost spot, was composed of one major component, which did not change retention time upon hydrogenation and represented ca. 10% of the total cyclic material. The average mass spectrum of this peak indicated that it was a mixture of isomeric aromatic cyclic monomers with a molecular weight of 290 as the methyl ester (Fig. 5).

An examination of the mass spectrum indicates the presence of an intense molecular ion as well as ions at m/e 258 and 259, representing loss of methanol from the methyl ester. Cleavage of the alkylcarbonyl side chain leads to the formation of a series of ions at m/e 105 (C_8H_9, 105.0693, calculated 105.0704), 119 (C_9H_{11}, 119.0857, calculated 119.0861), and 133 ($C_{10}H_{13}$, 133.1015, calculated 133.1017) with the general structure  . The ions at m/e 119 and 133 contained a small percentage of ions (<2%) with the structure C_8H_7O (m/e 119.0490, calculated 119.0497) and C_9H_9O (m/e 133.0650, calculated 133.0653) due to a ketene ion. In addition, an intense ion at m/e 197 corresponding to $C_{15}H_7$ (m/e 197.1325, calculated 197,1330) may have originated from a corresponding ketene ion by loss of water and a proton. Less intense ions at m/e 215, $C_{15}H_{19}O$ (m/e 215.1436, calculated 215.1436) and m/e 229, $C_{16}H_{21}O_1$ (m/e 229.1592, calculated 229.1593) seem to be ketene type ions that have lost one proton. Ion structures that have been written in monosubstituted forms may also be written in the disubstituted form, as may be obtained by partial cleavage of one substituent. A fairly intense ion is present at m/e = 91 representing the tropilium ion.

The mass spectra of this fraction are quite similar to those published by Zeman et al. (21) for pure synthetic aromatic cyclic monomer, and by Scharmann et al. (8) for aromatic acids produced from linolenate and linoleate with the general structure:  X + Y = 10

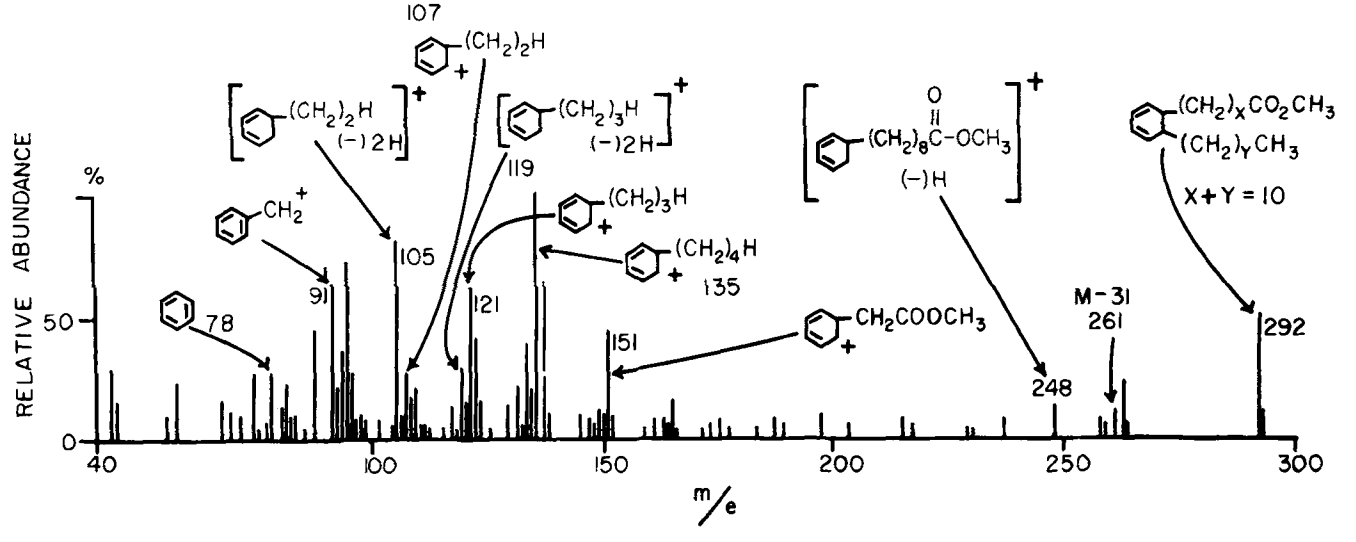


FIG. 7. Mass spectrum (70 eV) of isomeric cyclic monomer methyl esters (fraction 40-3).

The second fraction isolated was composed of two major components that were poorly resolved (Fig. 4). Peak number two coincided with the retention time of a conjugated 18:3 isomer mixture as shown by hydrogenation of this fraction. A small percentage (2.7%) of methyl stearate was observed, indicating overlap between conjugated 18:3 isomers and a major cyclic component. This overlap caused serious problems when evaluating the mass spectra of these components, since the mass spectra of conjugated methyl linolenate and its isomers were identical to those of the component cyclic esters. Several mass spectra were taken during elution of each of the major peaks. The mass spectra were all identical, varying only in intensity, indicating a series of closely related isomers. A typical spectrum, after spectrum averaging and background subtraction, is shown in Figure 6. The mass spectrum shown indicates a base peak at m/e = 121 (C_9H_{13}, 121.1008, calculated 121.1017) for an alkyl ion with a propyl substituent; other ions were present, representing alkyl substituents of up to 10 carbon atoms. Other ions representing a tropilium ion, formed by dehydrogenation, disubstituted ions, as well as a peak at m/e = 261 indicating loss of methanol are present. An ion at m/e = 248, which represented cleavage of the alkyl side chain from the molecular ion of one isomer, was also produced ($C_{16}H_{24}O_2$, 248.1764, calculated 248.1776). This spectrum and other obtained closely resembled those obtained by other workers (8,9,22) for cyclohexadienoid structured and aromatized monomers. This particular spectrum represented a mixture of isomers in which a short alkyl substituent group predominated. In the other major peak of the chromatogram (Fig. 4), the mass spectrum indicated predominance of compounds with other combinations of substituent groups varying in the number of carbon atoms in both the alkyl and carboxyl containing side chains.

Fraction 3, taken from the bottom portion of the band corresponding to the cyclic monomer reaction product as indicated in Figure 3, was subjected to GLC (Fig. 4). Another more complex series of poorly separated isomers was obtained consisting of a large doublet and two other fairly well resolved components. In this case also, there was considerable overlap with both linolenate and its conjugated isomers, and, upon hydrogenation of this fraction, 3.7% stearate was present. The mass spectra of each of the peaks were determined periodically during the elution of the peak, resulting in over 40 spectra. All spectra were identical with only intensity differences, as observed previously. This would be expected, since the gas chromatographic peaks represent a concentration of various isomers in terms of both substituent chain length differences as well as geometrical configuration. The average mass spectrum of the major component of the fraction is reproduced in Figure 7. The mass spectrum indicates the molecular weight as 292 with a peak at 261, representing loss of methoxy (methanol). Peaks are also present for alkyl-substituted cyclohexadiene and aromatic ions at m/e 105, 107, 119, 121, 135, 137, 151 and 165. Ions are present at m/e 78 and 91, formed by dehydrogenation and cleavage of the corresponding ion at m/e 93.

The intense ion at m/e 151 was resolved into three ions as follows. A major ion corresponding to $C_{10}H_{15}O$ (151.1111, calculated 151.1123) and minor ions corresponding to $C_{11}H_{19}$ (151.1463, calculated 151.1486) and $C_9H_{11}O_2$ (151.0748, calculated 151.0759). The second ion represents the alkyl substituted cyclohexadiene ion and the $C_9H_{11}O_2$ ion that fragment from which the alkyl group has been removed. The ion corresponding to $C_{10}H_{15}O$ probably represents a ketene ion. Other higher molecular weight ions consisted primarily of a homologous series of ketene ions.

This spectrum is also in accord with those reported earlier and is similar in fragmentation to that obtained for

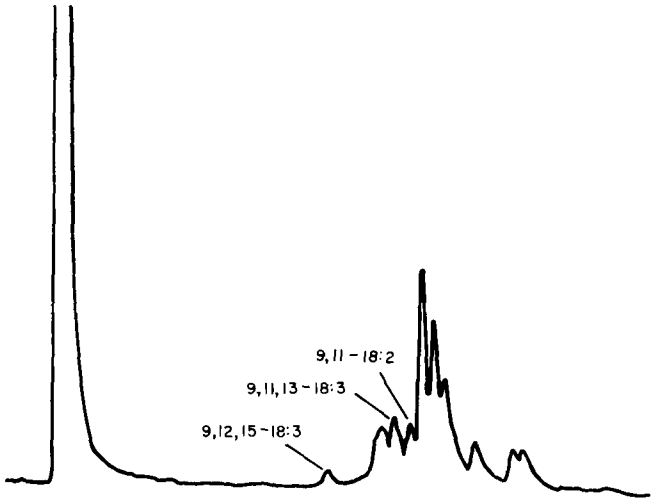
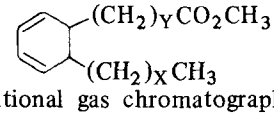


FIG. 8. Capillary gas liquid chromatogram of cyclic monomer ethyl ester reaction product with added 9,11-methyl linoleate, 9,11,13 and 9,12,15-methyl linolenate (150 ft x 0.01 in. capillary coated with Apiezon L).

aromatic isomers (8,9,21). The mass spectral studies have confirmed the structures of fractions 2 and 3 as mixtures of isomeric ω-(2-alkylcyclohexadienyl) carboxylic acids as shown:



X + Y = 10

Additional gas chromatographic studies using columns with OV-25 and SE-30 packings yielded inferior separations compared to that obtained with EGS. Results obtained with a 50 ft x 0.02 in. S.C.O.T. column coated with DEGS were not superior to those obtained from the EGS column. However the separation of cyclic monomer was improved considerably using a 150 ft x 0.01 in. capillary column coated with Apiezon L (Fig. 8). As indicated in the gas chromatogram, the separation of the cyclic monomer isomer, as well as octadecadienoate and trienoate samples was enhanced compared to that obtained on the 6 ft EGS column. However this separation is not of practical value for preparative use.

Argentation TLC has therefore allowed the partial removal of the undesirable side reaction products, such as conjugated linolenate and polymeric material, from crude cyclic fatty acid preparations. This method allowed partial fractionation of the isomeric monomers and produced one fraction very low in conjugated linolenate. The amount of material that may be fractionated by this method is low, although in the case of a ^{14}C-labeled substrate this would not be a major factor. Using a microgram scale (10-20 μg), separations are quite good and yielded complex chromatograms which appeared to be composed of separated positional and geometrical isomers of the cyclic monomer. However attempts to increase the plate loadings to preparative scale (20 mg) decreased the separation of the cyclic monomer and conjugated linolenate.

In order to facilitate the preparation of larger quantities of isomeric cyclic monomer mixtures free of conjugated linolenate isomers, argentation column chromatography was investigated. A column packed with acid-washed silicic acid coated with 10% $AgNO_3$, when eluted with 10% diethyl ether in petroleum ether, eluted large amounts of conjugated linolenate and cyclic material. When the ether content of the eluting solvent was decreased to 5% (v/v), less conjugated methyl linolenate isomers eluted. A fraction of pure cyclized methyl linolenate was eluted with 2% v/v ether in petroleum ether. With this solvent, yields of over 70% cyclized material were obtained by direct application of reaction mixture methyl esters to the column. Other reaction products, such as dimeric and higher polymeric colored products were retained on the column. With this

solvent system, the conjugated methyl linolenate was successfully removed from sample sizes of up to 10 g. The eluted material had on the average less than 1% conjugated linolenate isomers as determined by hydrogenation.

Although argentation TLC allowed partial separation of isomeric cyclic monomers and the aromatic isomer, column chromatography yielded a batch type separation of conjugated linolenate from the reaction product. The final purified cyclization product contained ca. 10% of the aromatic isomer, as well as those isomeric cyclohexadienyl products described previously.

ACKNOWLEDGMENTS

G. Rao, E. Mayhood, M. Sullivan and T. Fielder rendered technical assistance. Partial support was obtained from the U.S. Public Health Services Grant FD 00049, the Illinois Agriculture Experiment Station and the Biomedical Support Grant at the University of Illinois at Urbana-Champaign.

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[Received June 16, 1972]