AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented April 4, 1966

Title LACTONE PRECURSOR IN MILK FAT

Abstract approved
(Major Professor)

The occurrence of lactones in milk fat upon heating or storage has been recognized for many years. Lactones are important contributors to both normal flavor and certain off flavors of several dairy products. This investigation was undertaken to study the lactone precursors in milk fat.

Fresh raw milk fat was separated into polar and non-polar glyceride fractions by silicic acid chromatography. A polar glyceride fraction was isolated which accounted for 6.71% of the total petroleum ether soluble fraction. This fraction was found to contain traces of triglycerides, cholesterol, and monoglycerides. However, a component with an R_f slightly greater than normal diglycerides, but not clearly resolved, constituted the major part of the fraction. The infrared spectrum suggested a hydroxy triglyceride. Hydrolysis of this fraction at 140°C for 30 minutes in a sealed vial produced a series of even numbered δ-lactones from C_8 to C_{18} carbon atoms. γ-Undecalactone was tentatively identified from mass spectral data.

δ-Octadecalactone has not been reported previously in the literature.

The moisture content of the lactone precursor, dried over anhydrous Na_2SO_4, was determined by near infrared absorbance at 1.87 microns to be 0.175%. This water was sufficient to cause hydrolysis of the hydroxy esters to form lactones. No lactones were produced upon complete dehydration by calcium hydride. Thus, under the conditions studied, intramolecular alcoholysis appears to be an unimportant mechanism.

The polar fraction was subjected to BF_3 catalyzed methanolysis and the methyl esters were separated according to polarity by silicic acid chromatography. A polar fraction was isolated that appeared to be hydroxy methyl esters from infrared analysis. This fraction was converted to trimethylsilyl (TMS) ether derivatives for ease of isolation and identification.

The following hydroxy fatty acids were isolated and identified from milk fat as TMS ether methyl esters: 5-hydroxy octanoic, 4-hydroxy decanoic, 5-hydroxy decanoic, 5-hydroxy dodecanoic, 5-hydroxy tetradecanoic, 5-hydroxy pentadecanoic, 5-hydroxy hexadecanoic, and 5-hydroxy octadecanoic. Hydroxy heptadecanoic and hydroxy nonadecanoic were identified but the position of the hydroxy group could not be established. This is the first report of the direct identification of hydroxy acids in milk fat. All previous investigators have isolated and identified the lactones in milk fat and supposed they arose via lactonization of hydroxy acids in the classical manner.

The criterion for positive identification was comparison of retention times with authentic compounds, if available, and mass spectral analysis. None of the hydroxy acids identified from milk fat were available as standards, so it was necessary to synthesize standard compounds from the γ- and δ-lactones available. This was accomplished by converting the lactones to a TMS ether methyl ester. New information was obtained on the mass spectrometric fragmentation pattern for these compounds. This information was not available in the literature heretofore.

Possible contributions of this investigation may be as follows:

1. The new finding of δ-octadecalactone and its corresponding hydroxy fatty acid in milk fat.

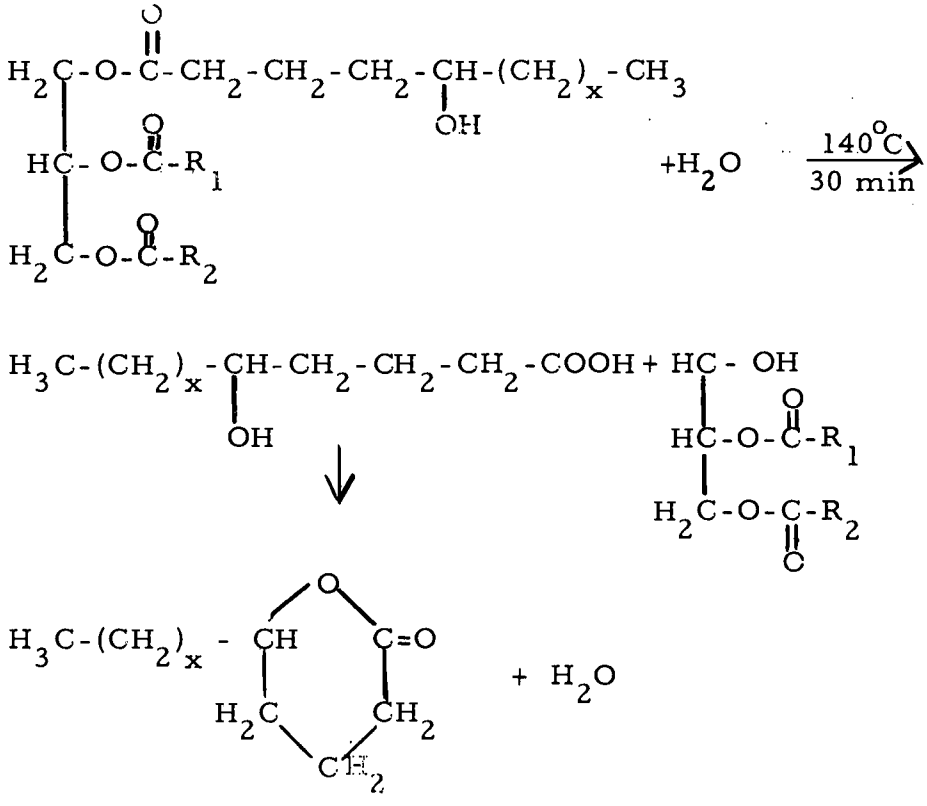
2. The identification of hydroxy fatty acids from milk fat.

3. The establishment of mass spectral fragmentation patterns of TMS ether methyl esters and correlation of important ions for structural identifications.

4. The applications of the TMS derivatives to study high molecular weight hydroxy compounds present in milk fat.

5. Of important significance is the evidence for the existence of hydroxy acids esterifed to glycerol in milk fat as the lactone precursor. The lactone precursor exists as a hydroxy triglyceride in milk fat. Heat and trace amounts of water are necessary to cleave the linkage of the hydroxy esters and subsequent lactone formation. The

following reaction is postulated for lactone formation in milk fat:



LACTONE PRECURSOR IN MILK FAT

by

CAROLYN JANE WYATT

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1966

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Date thesis is presented April 4, 1966

Typed by Gail Dailey

ACKNOWLEDGMENTS

I would like to thank Dr. E. A. Day for his support during this investigation. I would also like to express my thanks to the faculty and graduate students of the Department of Food Science and Technology for their assistance.

Special thanks are due Dr. R. L. Pereira for his guidance and assistance in the latter course of this study.

Thanks are due Dr. L. M. Libbey for his assistance in obtaining and interpreting the mass spectra.

Recognition is also due the Public Health Service, Division of Environmental Engineering and Food Protection, for providing the financial support for this research.

Finally, I would like to thank my husband, Bruce, for the patience and understanding he has shown throughout the course of this work.

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LACTONE PRECURSOR IN MILK FAT

INTRODUCTION

The occurrence of lactones in milk fat upon heating or storage has been recognized for several years. Lactones are important contributors to both normal flavor and certain off flavors of several dairy products. A butter-like flavor is imparted to margarine by the addition of δ-lactones. On the other hand, they have been shown to be responsible, in part, for the stale defect in stored fat-containing dairy products.

Since the identification of lactones in milk fat in 1956, study of these compounds has been hampered by a lack of adequate methodology. Identification and quantification were until quite recently extremely difficult; however some investigators have been able to measure the quantity of lactones in milk fat by use of thin-layer chromatography and isotope dilution.

Several workers have suggested that the precursors of lactones are hydroxy fatty acids esterified in milk fat. Direct evidence for the existence of these hydroxy acids in milk fat is still lacking. Conditions for hydrolysis and ring closures have not been established.

This investigation was undertaken to study the lactone precursors in milk fat by positively identifying the precursor fatty acids and to collect data regarding the glyceride structure of the precursor. In

addition, conditions for induction of hydrolysis and lactone formation were studied.

REVIEW OF LITERATURE

Lactones in Dairy Products

Keeney and Patton in 1956 (21, 22) first isolated δ-decalactone from heated milk fat and demonstrated its presence in evaporated milk, dried cream and dried whole milk. These workers identified δ-decalactone as the hydroxamate by a paper chromatographic technique. Tharp and Patton (45) subsequently found δ-dodecalactone in the steam distillate of milk fat. This compound was identified by liquid-partition column chromatography and gas-liquid chromatography (GLC) by comparison with authentic lactones. Boldingh and Taylor (3) reported the presence of a series of δ-lactones, including both saturated and unsaturated members, in heated butter fat. The series extends from C_8 through C_{16} and contains minor amounts of lactones with an uneven number of carbon atoms. Smaller quantities of the same lactones were found in unheated milk fat. Infrared analysis and partition chromatography ot the lactone anilides were used to characterize the molecular structure of the compounds reported. Muck et al. (33) isolated δ-C_{10}, C_{12}, and C_{14} lactones from evaporated milk. Their identification was based on data from gas-liquid and paper chromatography. No authentic compound was available for δ-tetradecalactone but identification was based on extrapolation of retention time data from other authentic lactones. Parliment et al.

(37) reported the mass spectrometric identification of δ-caprolactone from heated milk fat. Forss et al. (10) identified γ-dodecalactone in commercial Australian butter oil. Evidence was also obtained for the presence of γ-C_9, C_{10}, C_{11}, C_{13}, C_{14}, C_{15}, and C_{16} lactones and δ-C_{13} and C_{15} lactones. These compounds were isolated by thin-layer chromatography and identities were established by authentic R_f values and infrared analysis.

Several of the lactones are quite flavorful, having odors described as coconut (δ-C_{12}), peachy (δ-C_{10}), spicy (γ-C_{11}) and musky (pentadecanolide). The flavor imparted to a given food may be either desirable or undesirable depending upon the identity of the lactone, its concentration, and the nature of the food under consideration. Lactones in low concentration have been shown to be important in normal butter flavor (3, 36) and impart desirable butter-like flavor properties when added to margarine. Patents have been filed on the addition of δ-decalactone and δ-dodecalactone to margarine (4, 49).

Lactones have also been implicated in the stale flavor defect which develops during storage of certain fat containing dairy products (21, 22, 45).

Lactone Precursor in Milk Fat

Mattick and Patton, in 1959 (30) postulated that the δ-lactones resulted from ring closure of 5-hydroxy acids esterified in milk fat.

Boldingh and Taylor (3) reported that raw cream contains 4 ppm. of δ-dodecalactone and that this increases to 27 ppm. after heating at 140°C. These authors reported indirect evidence for 5-hydroxy acids in milk fat. Synthetic 5-hydroxy mono- and triglycerides were hydrolyzed by heat to give lactones as a product of the reaction. The anilides of the lactones isolated from milk fat were optically active which suggests that the precursors are natural intermediates in milk fat synthesis. Jurriens and Oele (19) reported the presence of bound lactones in a polar glyceride fraction from butterfat. Their evidence suggested the presence of hydroxy triglycerides but definite proof for the combination of the hydroxy fatty acids with glycerol was lacking. By the use of the isotope dilution technique, Jurriens and Oele (19) were able to quantify the γ- and δ-C_{10} to C_{16} lactones in the free and bound form. They also suggested the existence of hydroxy acids other than those yielding γ- and δ-lactones, as well as cis and trans unsaturated hydroxy fatty acids.

Boldingh and Taylor (3) have presented evidence for the presence of glyceride-bound β- and δ-keto acids in milk fat. They postulated that the former are precursors of methyl ketones and that the latter are intermediates in the formation of the δ-lactones. It was also postulated that δ-hydroxy acids bound to glycerides were the immediate precursor of the δ-lactones which are formed by heating milk fat. It was suggested that both the keto and hydroxy acids were products

of incomplete biological reductions in the chain-elongation sequence.

Possible Origin of Hydroxy Fatty Esters in Milk Fat

The hydroxy fatty esters that are found in milk fat could arise from several sources. They might occur as products of the reduction of keto esters, they could originate in the lipids of the animal's food or may be formed in the rumen by microbial action.

van der Ven (48) postulates that γ- and δ-hydroxy acids present in butterfat are formed from the corresponding keto acids. Tuynenburg Muys et al. (46, 47) reported that various micro-organisms are capable of converting δ-keto acids into optically active δ-hydroxy fatty acids and lactones. In addition, Francke (11) found that pigeon and bovine liver preparations can reduce δ-keto acids. He also found that the reduction proceeds via the coenzyme A ester of the keto acids, mediated by a mitochondrial enzyme.

A logical explanation for the presence of δ-hydroxy fatty acids appears to be via incomplete biosynthesis of fatty acids in the mammary gland. It is accepted that acetate is the precursor of fatty acids in milk fat synthesis (7, 8, 26, 38). While mammary gland enzymes are not completely characterized, there is some evidence that the malonyl coenzyme A pathway is an important route for the synthesis of fatty acids in the gland (14). Figure 1 illustrates the fatty acid synthetase complex and the normal sequence of reactions for the

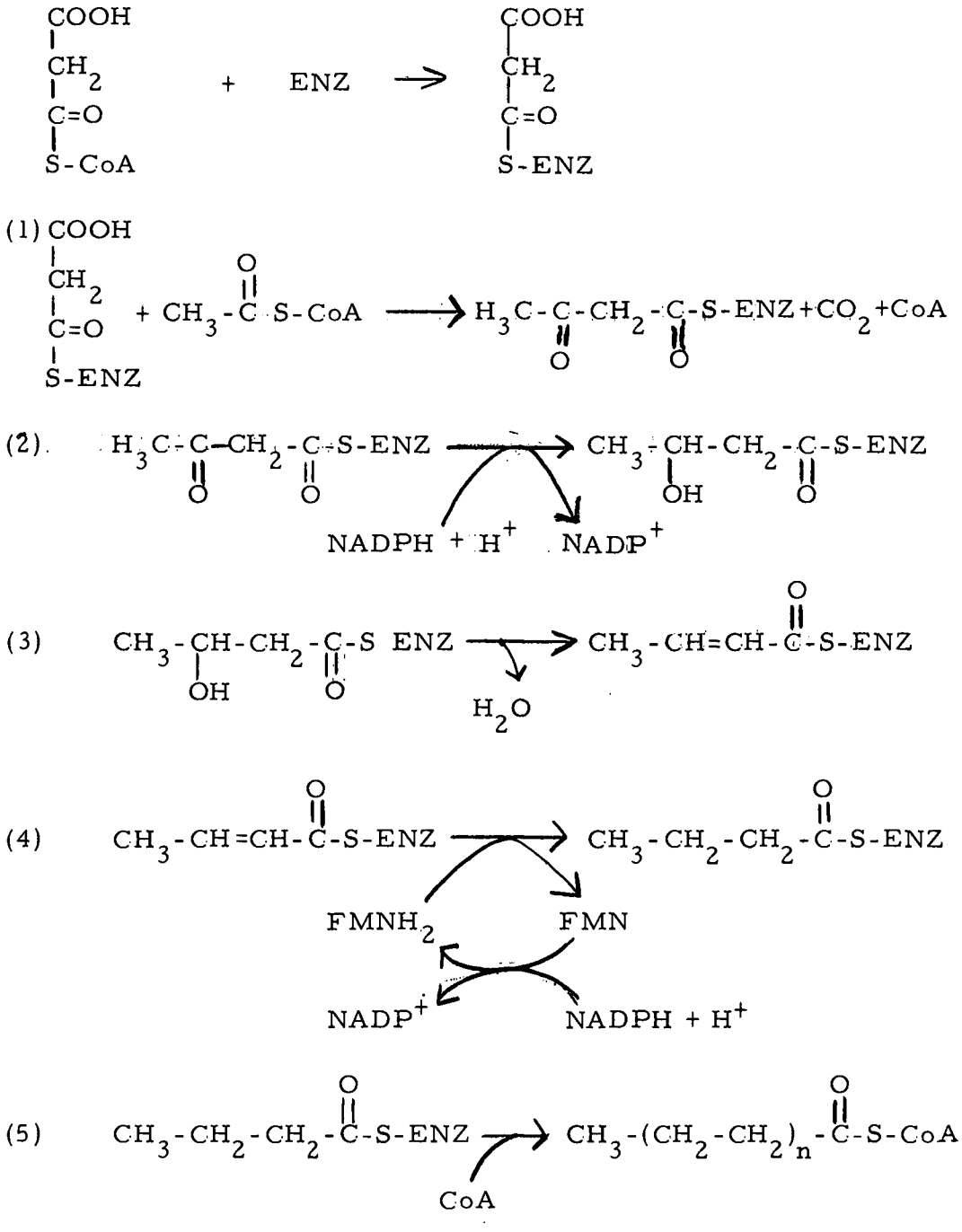
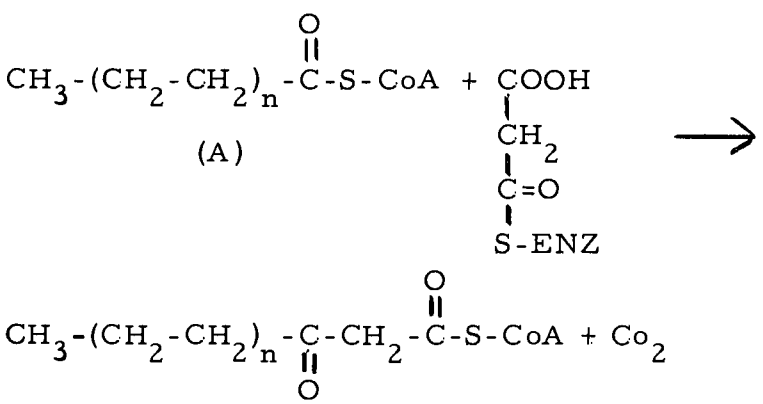


Figure 1. Fatty acid synthesis complex (6, p. 239)

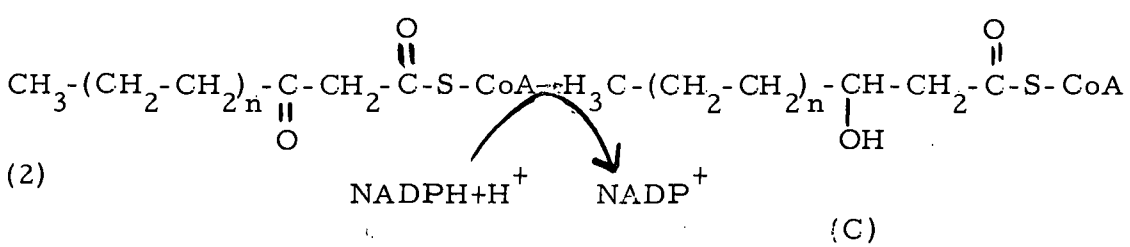
synthesis of a saturated fatty acid via the malonyl coenzyme A pathway. Both malonyl CoA and acetyl CoA become bound to the enzymes of fatty acid synthesis. The enzyme bound acyl groups undergo decarboxylation and condensation reactions, followed by reduction, dehydration and reduction again to form an enzyme-bound saturated compound. Reactions 1 through 5 may be repeated for chain elongation (6, p. 239). After several turns of the cycle reactions (1) and (2) are:

(1)



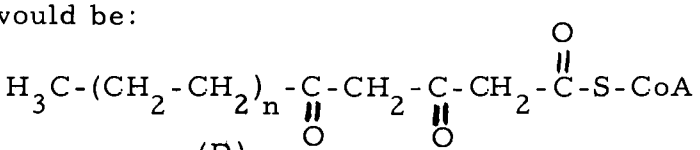
(A) (B)

(2)



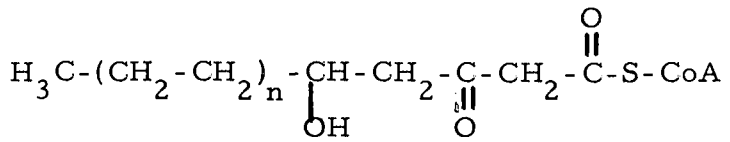
(C)

If compound B and C can "short circuit" the normal cycle and undergo reaction (1) with another mole of enzyme bound malonate, the products would be:



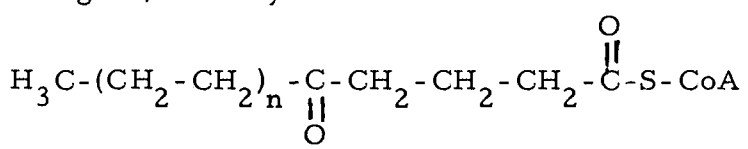
(D)

and



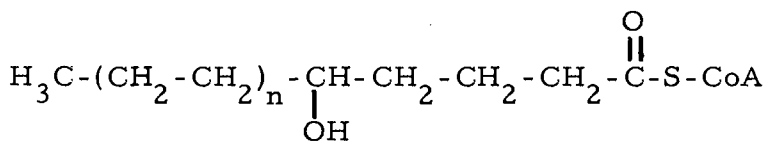
(E)

Compounds D and E proceeding through the normal pathway (reaction 1 through 5) would yield:



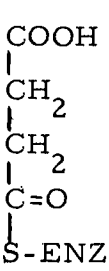
(F)

and

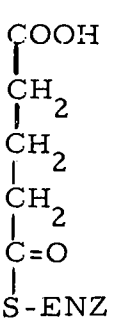


(G)

which would be available for triglyceride synthesis reactions. Unfortunately, this scheme cannot account for γ-hydroxy acids. It would be interesting to study the possibility that the succinyl (H) and glutaryl (I) thioesters of the enzyme could replace the malonyl derivative in reaction (1). These precursors would yield the γ- and δ-keto fatty acids in one step.



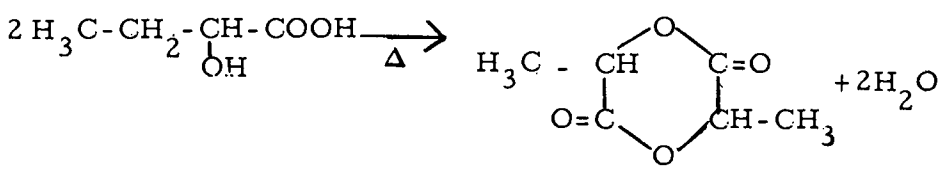
(H)



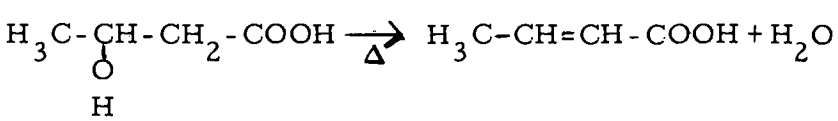
(I)

Chemistry of Hydroxy Acids and Lactones

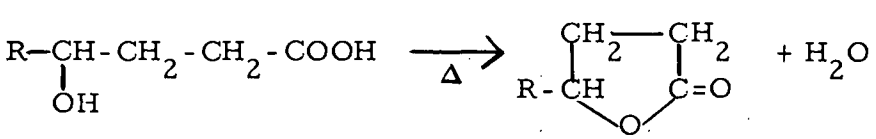
The dual function of an hydroxy acid is very clear. Both the hydroxyl and carboxyl groups show the usual properties of alcohols and acids. Thus such acids form salts, esters, and amides at the carboxyl group and undergo acylation or oxidation of the alcoholic group in the usual manner. In some reactions, the behavior of a hydroxy acid depends upon the location of the hydroxyl group (5, p. 385). For example, α-hydroxy acids form lactides upon heating.



When a β-hydroxy acid is heated, it easily loses water and forms an unsaturated acid.



Lactonization of the γ- and δ-hydroxy acids occurs readily upon heating.



The reaction is accelerated by mineral acids in the same way as ordinary esterification. Ring closure occurs more readily with a γ- than a δ-hydroxy group (5, p. 385).

The resultant ring system contains five or six members and is in accord with Baeyer's theory in that rings of five and six atoms are practically free from strain and are formed easily. The γ-lactones are characterized by great stability, being only partially converted to hydroxy acids by prolonged boiling with water, whereas δ-lactones react with water more readily. When a γ-lactone is hydrolyzed by alkali, the alkali salt is obtained, but the free acid liberated by acidification will change back to the lactone form much more readily than the δ-hydroxy acid (40, p. 745).

Synthesis and Mass Spectral Analysis of Trimethylsilyl Ethers

Within the last few years the use of the trimethylsilyl (TMS) ether derivatives of hydroxy compounds has opened new areas in which the GLC can be used for their analysis. Some of the areas in which the use of the TMS derivatives have proven useful have been reviewed recently (51). Wood et al. (50) described a quantitative method for the rapid gas-liquid chromatographic analysis of mono- and polyhydroxy stearates as their TMS derivatives. The TMS derivatives of the hydroxy esters are formed rapidly and quantitatively at room temperature. Polar materials are converted into volatile TMS ethers for ease of separation and determination.

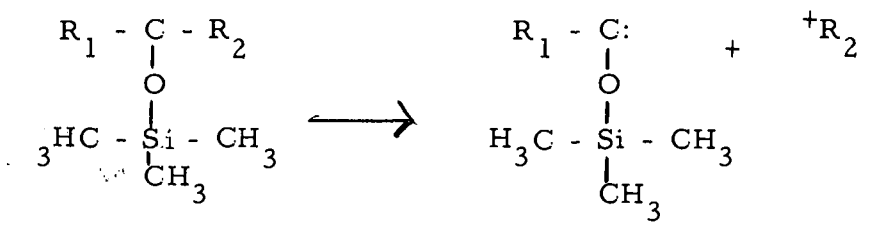
One usually encounters difficulty in interpretating mass spectra

of free hydroxy compounds. A compound with a free hydroxy group frequently loses water rapidly during the electron bombardment (13, 43, p. 12). This increases the liklihood of making an incorrect evaluation of the mass spectrum, since the parent ion may be absent. According to Sharkey et al. (42) the use of TMS derivatives produces distinct mass spectra that correlates with the molecular structure of primary and secondary alcohols. The major framentation pattern of normal aliphatic TMS ethers is as follows:

m/e	Structure	Relative Intensities
43	CH_3-Si-	17
73	$(CH_3)_3$-Si-	45
89	$(CH_3)_3$-Si-O-	16
103	$(CH_3)_3$-Si-O-CH_2-	25
P-15		100
P		0.9

where m/e is the mass to charge ratio and P is the molecular ion.

In addition common rearrangement ions of m/e 75, 61, 45 were found. Secondary TMS ethers prepared from secondary alcohols show intense peaks resulting from a break at the functional carbon in the following manner:



when R_2 is the larger hydrocarbon group (42).

EXPERIMENTAL

Preparation of Milk Fat

Raw sweet cream was cooled to 10°C and churned in a one gallon electric churn. The butter granules were drained and washed repeatedly with cold distilled water until the wash water was clear. The butter was melted at 40°C and the butter serum separated from the fat. The fat was washed several times with equal volumes of warm (40°C) distilled water. The fat was extracted with petroleum ether, dried over anhydrous Na_2SO_4, and the solvent removed on a rotary evaporator. The fat was fractionated immediately after preparation.

Fractionation of Milk Fat

Milk fat was fractionated into polar and non-polar fractions by a modification of the procedure of Jurriens and Oele (19). Columns were prepared from 20 grams of silicic acid (dried 18 hours at 150°C), 10 grams Celite 545 (dried 18 hours at 150°C), and 6% water by weight. The packing was mixed well and a slurry made with redistilled petroleum ether (30-60°C). Ten grams of milk fat, dissolved in 2 ml. petroleum ether were applied to the top of the column. Milk fat glycerides were eluted from the column with 300 ml. 1:1 (v/v) petroleum ether: diethyl ether and the polar glyceride fraction was removed with 300 ml. diethyl ether.

Thin-layer Chromatographic Fractionation of the Polar Glyceride Fraction from Milk Fat

Alkaline thin-layer chromatographic plates were prepared and developed according to the procedure of Komarek et al. (25). The plates were developed successively with diethyl ether, until the solvent reached the center of the plate and then with 9:1 (v/v) hexane: diethyl ether. The lipid fractions were detected by spraying with bromothymol blue, recovered from the silica gel plates and rechromatographed to determine the completeness of separation.

Separation of Polar and Non-polar Methyl Esters

Methyl esters were prepared by subjecting the polar glycerides to BF_3 catalyzed methanolysis as described by Metcalfe (32). The methyl esters were separated into polar and non-polar fractions by a liquid-partition column chromatographic procedure described by Frankel et al. (12). The column, originally designed for separation of hydroperoxides, consisted of a stationary phase of 20% methanol in benzene supported on silicic acid. An automatic fraction collector was used to collect 10 ml. fractions of the mobile solvent. The composition of the mobile phase was increased in a step-wise gradient from 2% methanol in benzene to 100% methanol to elute completely the hydroxy methyl esters.

The fractionation was followed by testing each tube for the

presence of esters by the hydroxamic acid procedure (9, p. 253). The intensity of the color was assigned an arbitrary value ranging from 1+ to 3+. A plot of the intensity of the reaction vs. volume of eluant (ml) is shown in Figure 2. The tubes containing an individual fraction were combined and evaporated to dryness.

Preparation of Trimethylsilyl Ether Derivatives of Hydroxy Methyl Esters

The hydroxy methyl esters were converted to TMS derivatives and extracted as described by Sweeley et al. (44). A commercial solvent, reagent, catalyst system (Tri-Sil[1]) based on the following reaction was used:

$$2\ ROH + (CH_3)_3SiNHSi(CH_3)_3 \longrightarrow 2ROSi(CH_3)_3 + NH_3$$

The samples were analyzed by GLC and mass spectrometry as described below.

Gas-Liquid Chromatographic Analysis of the TMS Derivatives

The TMS derivatives were separated with an F and M Scientific Corporation Model 810 gas chromatograph equipped with a hydrogen flame ionization detector. A 5 foot x 1/8 inch stainless steel column was packed with 20% Apiezon L on 100-120 mesh Celite 545.

[1] Pierce Chemical Company, Rockford, Illinois.

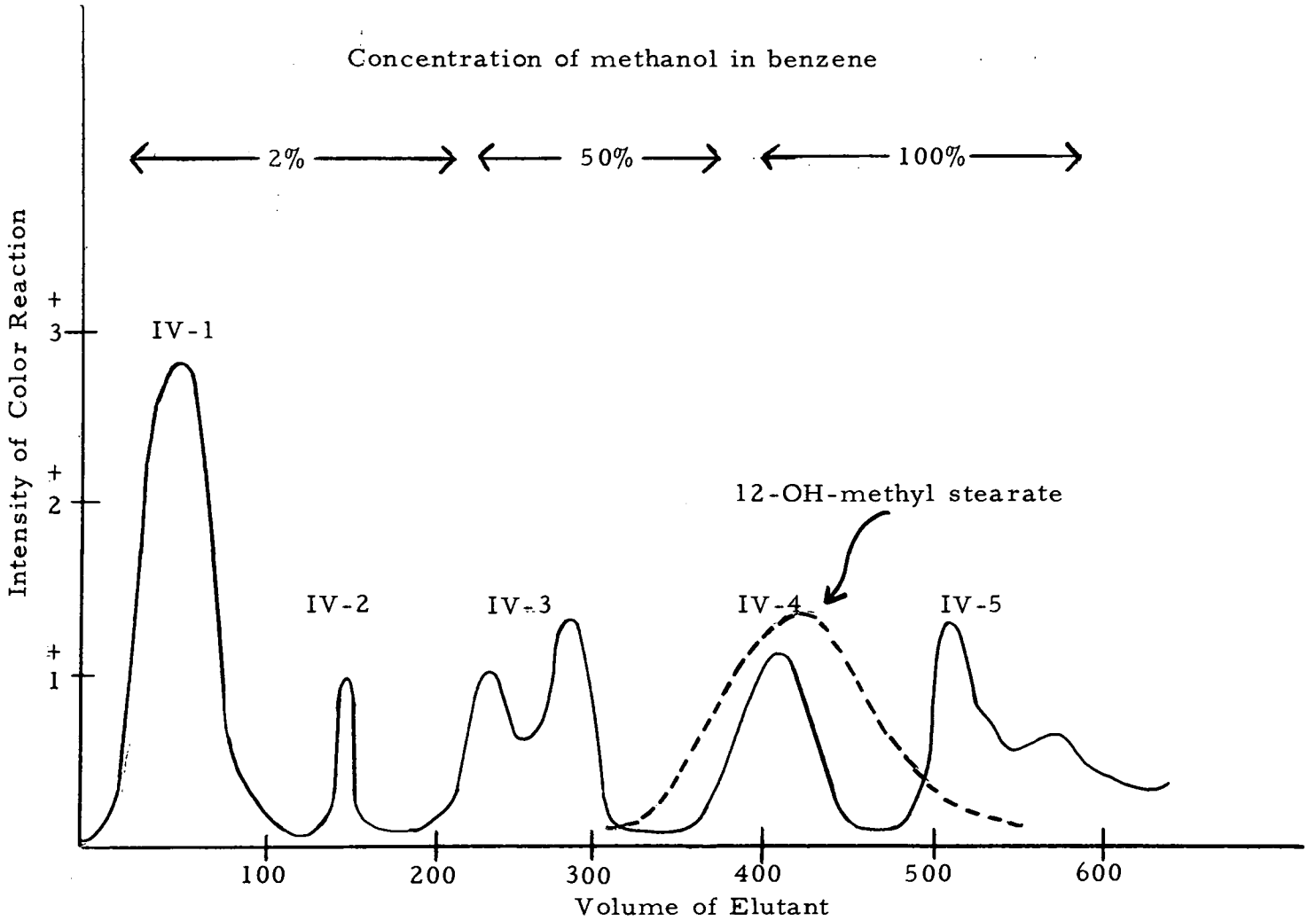


Figure 2. Separation of hydroxy and non-hydroxy methyl esters by silicic acid chromatography (stationary phase 20% methanol in benzene)

The column temperatures were 100°C and 230°C. An inlet pressure of 30 psi was required for a nitrogen flow through the column of 40 ml/min. The detector and injector port were maintained at a temperature of 10°C higher than the column temperature.

Mass Spectrometry of TMS Derivatives

The gas-liquid chromatograph described above, in conjunction with an Atlas MAT CH-4 Nier type mass spectrometer (a nine inch, 60 degree sector single focusing instrument) was used for final and positive identification of the compounds. Helium was used as a carrier gas for mass spectral analysis. The effluent from the GLC column was split with a "Swagelok" T-fitting at the end of the column and one portion was directed to the flame detector and the other to the mass spectrometer inlet. The ratio of the volume of the effluent gas entering the GLC detector to that entering the mass spectrometer inlet was 1:5. The mass spectrometer inlet system was equipped with the EC-1 gas inlet valve, which could be adjusted to regulate the amount of column effluent admitted to the mass spectrometer ion source. Mass spectra were obtained by magnetically scanning the GLC effluent at various points on the chromatogram and were recorded with a Honeywell model 1508 Visicorder. Usually a scan was taken at three points on the peak of interest, half-way up scale, at the top of the peak and mid-way down scale. The operating conditions for the mass spectrometer were:

Ionization current	60 μA
Accelerating potential	3000 V
Electron voltage	70 eV
Multiplier voltage	2.0-2.1 KV
Analyzer pressure when admitting sample	5×10^{-7} torr
EC-1 inlet temperature	240°C
Scanning speed	6.5 sec (m/e 24-450)
Viscorder chart speed	8 inches/sec

Synthesis of Standard TMS Derivatives

Hydroxy acids with the hydroxy group in the 4 and 5 position are not readily available since cyclic esterification of the 4 and 5 hydroxy acids occurs rapidly. Therefore, it was necessary to synthesize standard reference compounds to assist in the positive identification of the unknown compounds isolated from milk fat.

γ- and δ-lactones are commercially available in high purity and can be hydrolyzed by alkaline solutions. Three ml of lactone were refluxed for 30 minutes with 10 ml. of 1 N NaOH, and after cooling the sample was lyophilized.

The dried sodium salt of the hydroxy fatty acid was converted to the TMS derivative as described above. Warming was necessary to completely dissolve the sodium salt.

Esterification of the Standard TMS Derivative

Since the TMS ether linkage is labile to weakly acidic conditions, it was necessary to form the methyl esters by esterification with diazomethane under alkaline conditions (41). The standard methyl ester TMS derivatives were analyzed by gas chromatography and mass spectrometry as described above.

An Attempt to Prepare Unsaturated TMS Methyl Esters

Polar methyl esters were separated into hydroxy and non-hydroxy methyl esters on the plates coated with silica gel G. The solvent system was 50:50 (v/v) petroleum ether:diethyl ether. The hydroxy methyl ester fraction was eluted from the silica gel and converted to TMS ethers. The sample was rechromatographed on silver nitrate plates prepared from 30 grams of silica gel G and 60 ml of 12.5% aqueous silver nitrate (39, p. 136). The developing solution was hexane:diethyl ether 80:20 (v/v). This resulted in a separation of the methyl esters on the basis of unsaturation, the higher the degree of unsaturation, the lower the R_f value. The unsaturated fraction was eluted from the silica gel and subjected to GLC and mass spectrometry.

Hydrolysis of the Lactone Precursor Isolated from Milk Fat

The amount of water in the polar fraction of milk fat was determined by near infrared spectrophotometry using the method first described by Meeker et al. (31) and recently applied to milk fat by Kliman and Pallansch (23). The absorbance of a solution of fat in carbon tetrachloride at 1.87 microns, which is the characteristic absorption band for water, was measured. The reference standard was prepared by drying an aliquot of the fat solution over calcium hydride for 18 hours. The moisture content was determined from a standard curve prepared by adding known amounts of water to the reference solution. After evaporation of the solvent, both the dry reference and the moisture containing sample were sealed in Pyrex vials and subjected to hydrolytic conditions (140°C for 30 minutes). The lipid was extracted with diethyl ether and analyzed for the presence of lactones by GLC and mass spectrometry.

Determination of the Glyceride Structure of the Lactone Precursor

The lactone precursor was dissolved in 1 ml. of anhydrous diethyl ether. Anhydrous pyridine (1 ml.) and methyl-p-toluenesulfonate (1 ml.) were added to form the methyl ether derivative of all hydroxyls present in the fraction. The reaction was carried out for 30 minutes below 20°C. The pyridine salt of p-toluene sulfonic acid was

removed by filtration and the ether evaporated from the filtrate. Completeness of conversion was checked by thin-layer chromatography. The esters were reduced by lithium aluminum hydride and the acetate derivative of the alcohol moieties were formed (17).

Analyses were obtained with an F and M Model 810 gas-liquid chromatograph. A stainless steel column, 3 feet in length, 1/8 inch I.D. containing 25% SF-96 silicone grease on 35-80 mesh Chromosorb W was used for chromatographic separations at 205°C. Nitrogen was the carrier gas and the inlet pressure was 30 psi.

RESULTS AND DISCUSSION

Separation of the Lactone Precursor from Milk Fat

Exploratory investigations were conducted to determine which fraction in milk fat contained the lactone precursor. The isolated fractions were subjected to hydrolysis and evaluated for the presence of the typical lactone odor, which is characteristic of heated milk fat.

It was found that the lactone precursor was concentrated in the fraction which fails to crystallize from acetone at $-50^{\circ}C$, which agrees with the findings of Mattick et al. (30). Low temperature crystallization, however, is very time-consuming and requires large amounts of butterfat to obtain a useful quantity of the lactone precursor. Therefore, the separation scheme outlined in Figure 3 was devised.

It was observed that if the polar glyceride fraction (II), which constituted 6.71% of the total petroleum ether soluble fraction, was separated by alkaline thin-layer chromatography (TLC), the lactone precursor (III) could be isolated in sufficiently high concentration for qualitative analysis. The TLC separation is shown in Figure 4. The polar glyceride fraction (II) contained traces of triglycerides, cholesterol and monoglycerides. A component with an R_f value slightly greater than the normal diglycerides, but not clearly resolved, constituted the major part of the fraction. Identification of each class

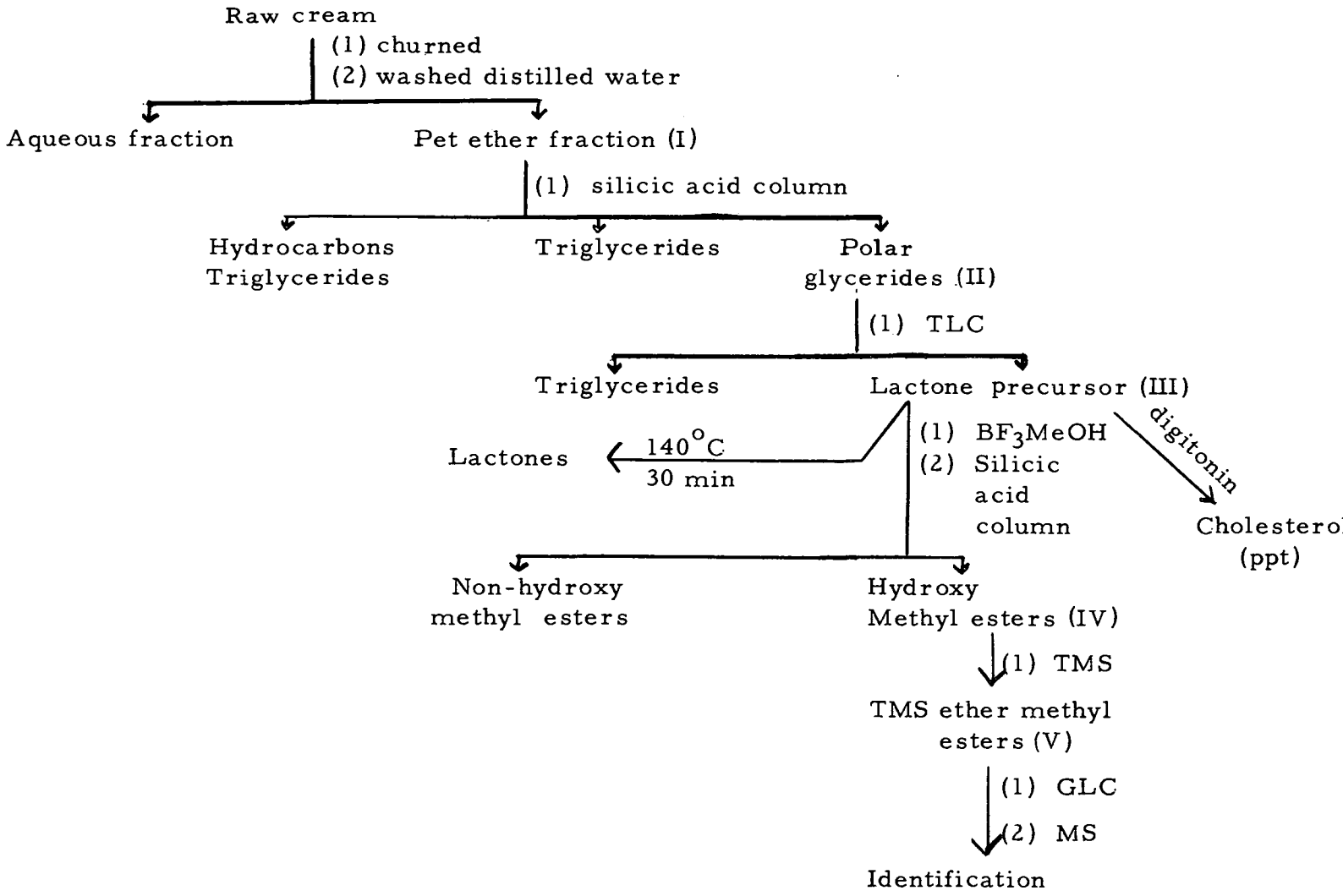


Figure 3. Schematic diagram of the separation of lactone precursor from milk fat

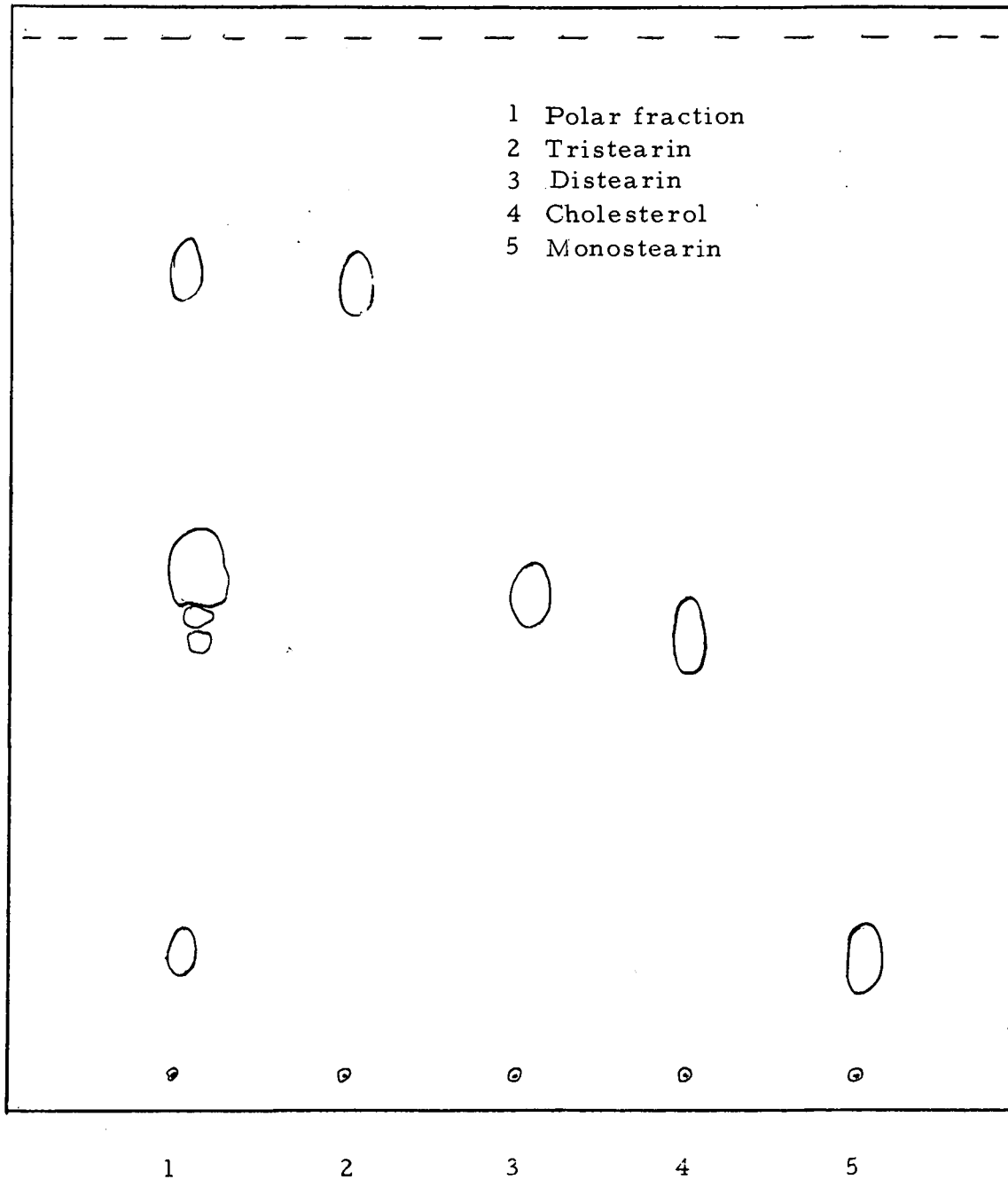


Figure 4. Thin-layer chromatogram of polar milk fat lipids

(Solvent system according to Komarek et al. (25))

was made by similar migration of reference compounds on TLC. The presence of cholesterol was confirmed by a positive Lieberman-Burchard reaction (24, p. 42). The majority of the cholesterol was subsequently removed from the lactone precursor fraction (III) by precipitation with digitonin (18, p. 210).

The solvent system described by Komarek et al. (25) was used for preparative TLC separation of the polar glycerides. This involves development of the plates with diethyl ether followed by 90:10 (v/v) hexane:diethyl ether. The solvent ratio was decreased to 80:20 and 50:50 in an attempt to resolve the lactone precursor and normal diglycerides. However, the polarity of the solvent had no effect on the resolution of the components; only the R_f values were changed.

Each fraction was removed from the TLC plates and heated to 140°C for 30 minutes in the presence of water. The fractions were evaluated for the presence of lactones by odor analysis. The diglyceride region was the only fraction that produced the typical lactone odor. Therefore, it was concluded that the lactone precursor had an R_f similar to normal diglycerides.

The hydrolyzed precursor was extracted with diethyl ether, dried over anhydrous Na_2SO_4 and evaporated to dryness. The extract was mixed with 10 grams Celite 545, packed into an empty chromatographic column and washed with 1:1 (v/v) diethyl ether: petroleum ether. The lactones were eluted from the column in the first 100 ml

while the glyceride esters remained absorbed on the Celite. The presence of lactones was confirmed by infrared analysis and GLC. Figure 5 illustrates the infrared spectrum of the hydrolyzed precursor from milk fat (A) and the spectrum of authentic δ-decalactone (B). The spectra are very similar and show the strong absorption at 1750 cm^{-1} due to the carbonyl absorption for lactones (2, p. 185; 34, p. 44). The retention data for the hydrolyzed precursor and authentic compounds obtained by GLC are shown in Table 1. The major components from the hydrolyzed lactone precursor were tentatively identified as δ-octalactone, δ-decalactone and δ-dodecalactone. The identity of these compounds was confirmed by mass spectrometry. The fragmentation patterns compared favorably with authentic spectra of the standard compounds and with published information (16, 28). It was therefore concluded that the diglyceride components as obtained by TLC contained the lactone precursor. Since the complete series of lactones expected were not identified in this experiment, a larger sample of the precursor was prepared as described earlier for further study.

An effort was made to obtain information on the glyceride structure of the lactone precursor (III). Figure 6 shows the infrared spectrum of the lactone precursor as obtained from alkaline preparative TLC plates. In addition, the spectrum of dipalmitin is shown in Figure 7. A comparison of the ratio of the hydroxyl (3460 cm^{-1}) to the

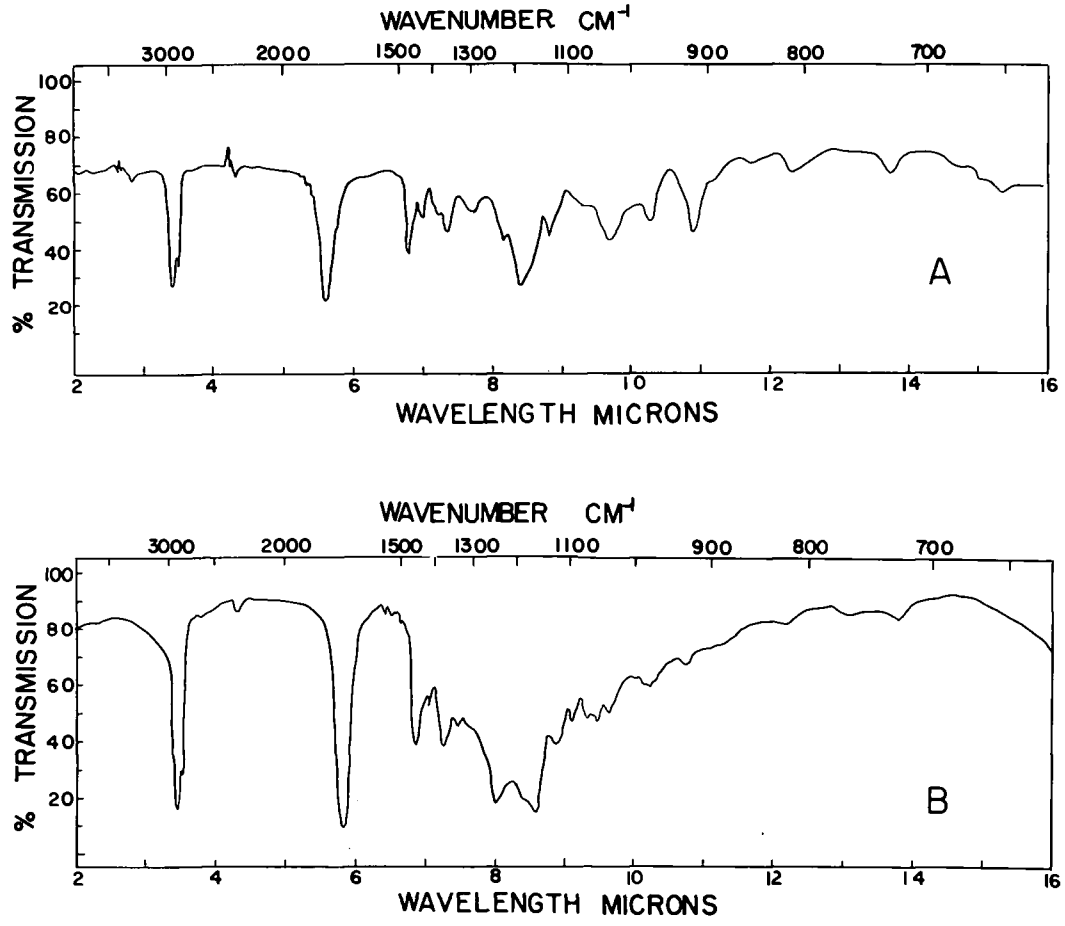


Figure 5. Infrared spectra of the hydrolyzed lactone precursor from milk fat (A) and authentic δ-decalactone (B)

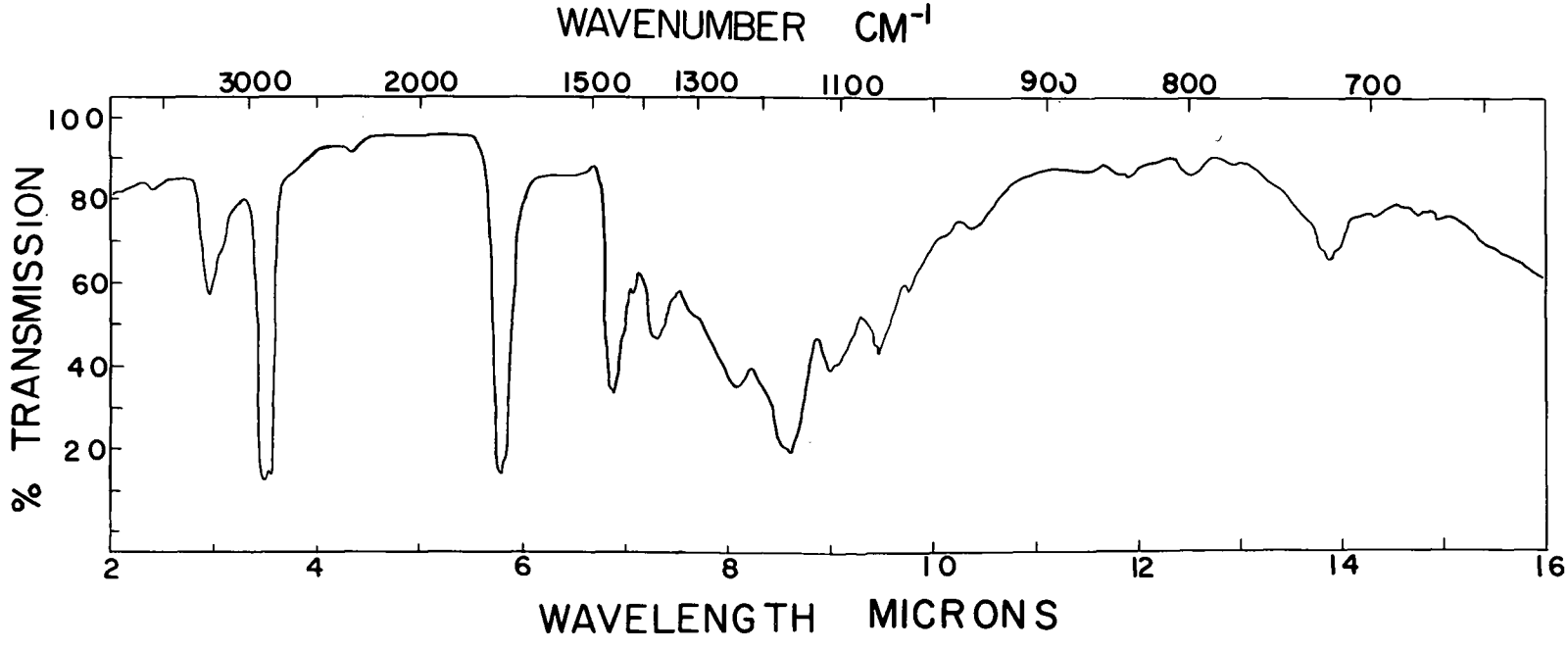


Figure 6. Infrared spectrum of the unhydrolyzed lactone precursor (III)

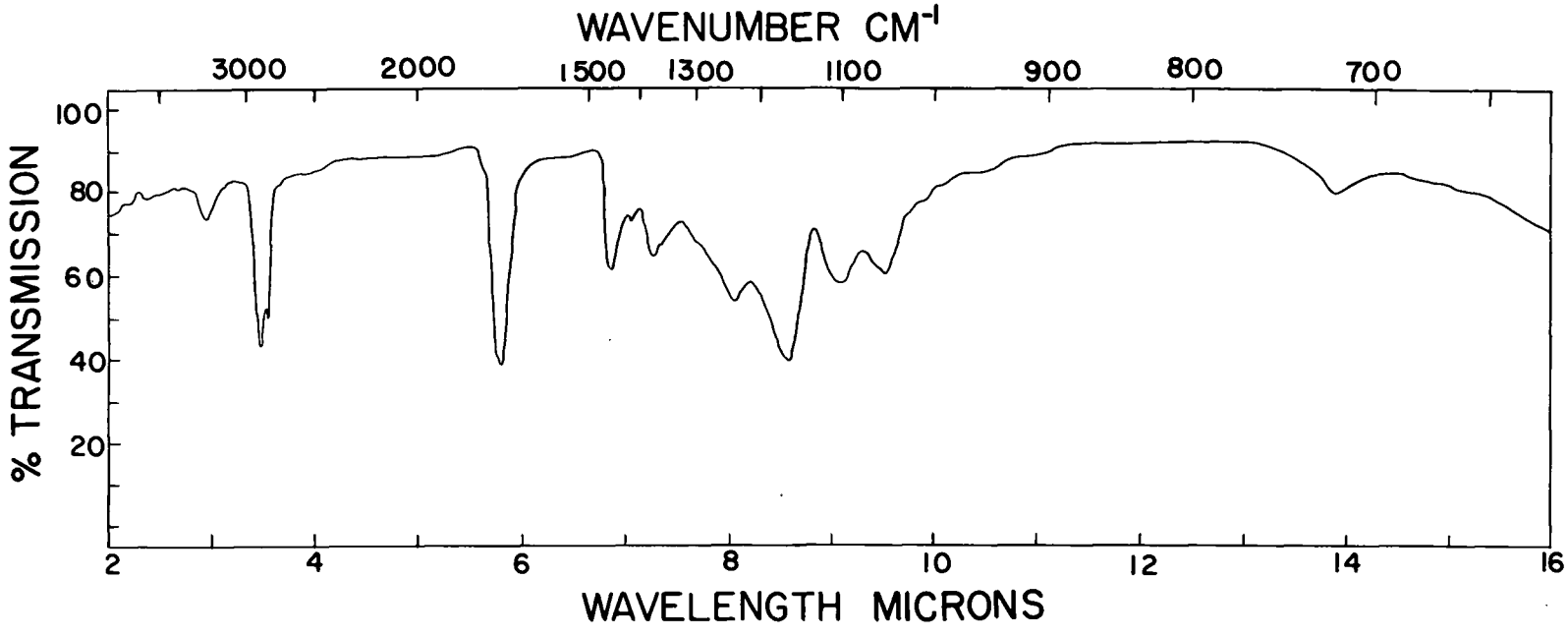


Figure 7. Infrared spectrum of authentic dipalmitin

carbonyl (1725 cm^{-1}) absorptions was made. Authentic dipalmitin, which has two moles of carbonyl per mole of hydroxy exhibits a ratio of 3.9 which compares quite favorably with a value of 4.2 obtained from a published spectrum (35). The ratio of the precursor is 2.1, which indicates more moles of carbonyl per mole of hydroxy than diglycerides. This strongly suggests a hydroxy triglyceride. Jurriens and Oele (20) reported the R_f value of a synthetic 1-mono-5 (hydroxy acyl)-triglyceride to be like that of diolein on silica gel G.

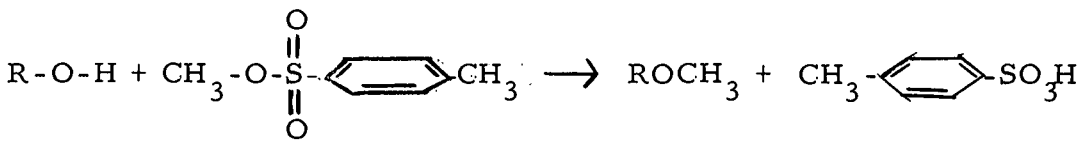
Table 1. Gas chromatographic analysis of a preliminary study of hydrolyzed lactone precursor (III) from milk fat (Column temperature 150°C)

Tentative identification	Retention times (minutes)	Retention time of authentic compounds (minutes)
δ-octalactone	16.01	15.81
δ-decalactone	19.18	19.39
δ-dodecalactone	23.49	23.65

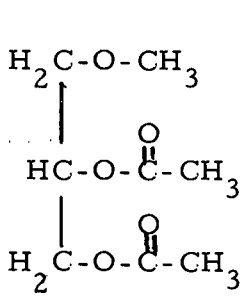
Identification of the Glyceride Structure of the Lactone Precursor

The evidence from TLC and infrared spectrometry presented above indicated that the lactone precursor (III), isolated from preparative TLC plates, was a mixture of diglycerides and a component tentatively identified as a hydroxy triglyceride. The polar glyceride mixture was reacted with methyl-p-toluenesulfonate to form the

methyl ether of all free hydroxy groups:

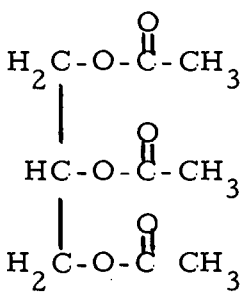


After reduction of the esters and formation of the acetate derivatives of the alcohol moieties, the following compounds were in question:



(1)

and



(2)

Compound 1 would come from diglycerides and compound 2 from any triglyceride present in the mixture. Triacetin (2) was positively identified by gas chromatography and mass spectrometry. Authentic triacetin was used for comparison of retention times and mass spectra. In addition, the spectra were compared to published results (1). It was concluded that the presence of triacetin (2) definitely established the existence of triglycerides in the lactone precursor. The presence of triglycerides, coupled with the chromatographic behavior of the isolated fraction and of a synthetic monohydroxy acyl triglyceride (20), can be taken to be presumptive evidence that the structure of the lactone precursor is a monohydroxy acyl triglyceride.

Identification of Lactones from the Lactone Precursor

The lactone precursor (III) was hydrolyzed at 140°C for 30 minutes and the lactones, as a product of the reaction, were isolated by extraction with diethyl ether. The lactones were separated and identified by GLC and mass spectrometry. The quantitative data are presented in Table 2. The percentages were computed from the peak areas, obtained by triangulation, assuming linear detector response by weight. A series of δ-lactones with even carbon numbers from C_8 to C_{18} were identified. The lactones were identified by coincidence of retention times with authentic lactones, if available, as well as mass spectral analysis. The retention data are given in Table 3. Prominent mass spectral parent and fragmentation ions of the fractions separated from the hydrolyzed lactone precursor are presented in Table 4. The fragmentation pattern of the δ-C_8, C_{10}, C_{12}, and γ-C_{11} lactones are in agreement with the spectra of standard compounds run in this laboratory, as well as those published by McFadden *et al.* (28) and Honkanen *et al.* (16). However, spectra are not available for the δ-C_{14}, C_{16}, and C_{18} lactones.

These compounds were tentatively identified by taking into consideration the known fragmentation pattern for the available δ-lactones. The most favored cleavage is the rupture of the bond between the side chain and the ring. The intense peaks at m/e = 85 and 99

Table 2. Quantitative relationship of lactones identified from milk fat

Compound	Mole percent
δ-octalactone	10.57
δ-decalactone	18.59
γ-undecalactone	2.45
δ-dodecalactone	14.53
δ-tetradecalactone	14.02
δ-hexadecalactone	20.40
δ-octadecalactone	18.53

Table 3. Lactones identified by gas-liquid chromatographic analysis of hydrolyzed lactone precursor (III) in milk fat (column temperature 230°C).

Compound	Retention time (minutes)	Authentic retention time (minutes)
δ-octalactone	3.17	3.08
δ-decalactone	6.66	6.60
γ-undecalactone	10.48	10.40
δ-dodecalactone	15.44	15.44
δ-tetradecalactone	18.21	21.95 *
δ-hexadecalactone	25.60	24.39 *
δ-octadecalactone	38.21	36.58 *

*By extrapolation

Table 4. Prominent fragmentation and molecular ions of the lactones separated from the hydrolyzed lactone precursor (III)

Compound	m/e	Source	Reference
δ-octalactone	99	P-R	16, 28
	114	P-28	
	124	P-18	
	142	P	
δ-decalactone	99	P-R	16, 28
	114	P-56	
	128	P-42	
	134	P-36	
	152	P-18	
	170	P	
γ-undecalactone	85	P-R	16, 28
	100	P-84	
	114	P-70	
	128	P-56	
	148	P-36	
	166	P-18	
	184	P	
δ-dodecalactone	99	P-R	16, 28
	114	P-84	
	162	P-36	
	180	P-18	
	198	P	
δ-tetradecalactone	99	P-R	
	208	P-18	
	226	P	
δ-hexadecalactone	99	P-R	
	170	P-84	
	218	P-36	
	236	P-18	
	254	P	
δ-octadecalactone	99	P-R	
	198	P-84	
	264	P-18	
	282	P	

(P-R) are formed by this type of cleavage and are characteristic of γ- and δ-lactones respectively. The intensity of the parent (P) is generally very low or completely non-existent; however, P-18 due to the loss of water is generally seen. The expected ions were observed as shown in Table 4.

δ-Octalactone has been reported in milk fat by Boldingh and Taylor (3). However, in a recent quantitative study of lactones from butterfat by Jurriens and Oele (19) it was not found. These workers reported the presence of a series of γ- and δ-lactones, saturated and unsaturated with both odd and even carbon numbers from C_{10} to C_{16}. On the other hand, in their bound lactone fraction, which is much more like the fraction isolated in this study, they found only the even numbered C_{10} to C_{16} δ-lactones. No mass spectra consistent with unsaturated lactones were found in this study. Tentative evidence was obtained for the presence of γ-undecalactone on the basis of the mass spectra. No suggestion of the presence of any other γ-lactones was found.

Jurriens and Oele (19) report δ-dodecalactone and δ-tetradecalactone to be in the highest concentration, 10.8 and 10.0 ppm respectively. It can be seen from Table 2 that, in this study, the C_{16} and C_{18} δ-lactones were present in relatively higher proportions than the remaining lactones. The δ-octadecalactone has not been reported previously, and the mass spectrum of this compound is

illustrated in Figure 8. The fragmentation pattern is in complete agreement with previously reported mass spectral structure correlations of δ-lactones (16, 28). The peaks m/e = 99 and 264 for P-R and P-18 respectively were prominent.

Effect of Moisture on Hydrolysis of the Lactone Precursor

It would seem possible that hydrolysis of the hydroxy ester could occur by intramolecular alcoholysis with the formation of lactones in the absence of water.

The moisture content of the lactone precursor (III) was measured to be 0.175%. This water was sufficient to cause hydrolysis of the hydroxy esters to form lactones. The lactones were identified by GLC and mass spectrometry. No lactones were produced after complete dehydration by calcium hydride. Therefore, under the conditions studied, intramolecular alcoholysis appears to be an unimportant mechanism for lactone formation.

Identification of Hydroxy Methyl Esters

The separation of hydroxy and non-hydroxy methyl esters on a silicic acid column is shown in Figure 2. The methyl esters (1.66 grams) were applied to the column and the components were eluted and weighed. The fractions were numbered according to increasing elution volumes (IV-1 to IV-5) as shown in Figure 2. Authentic normal

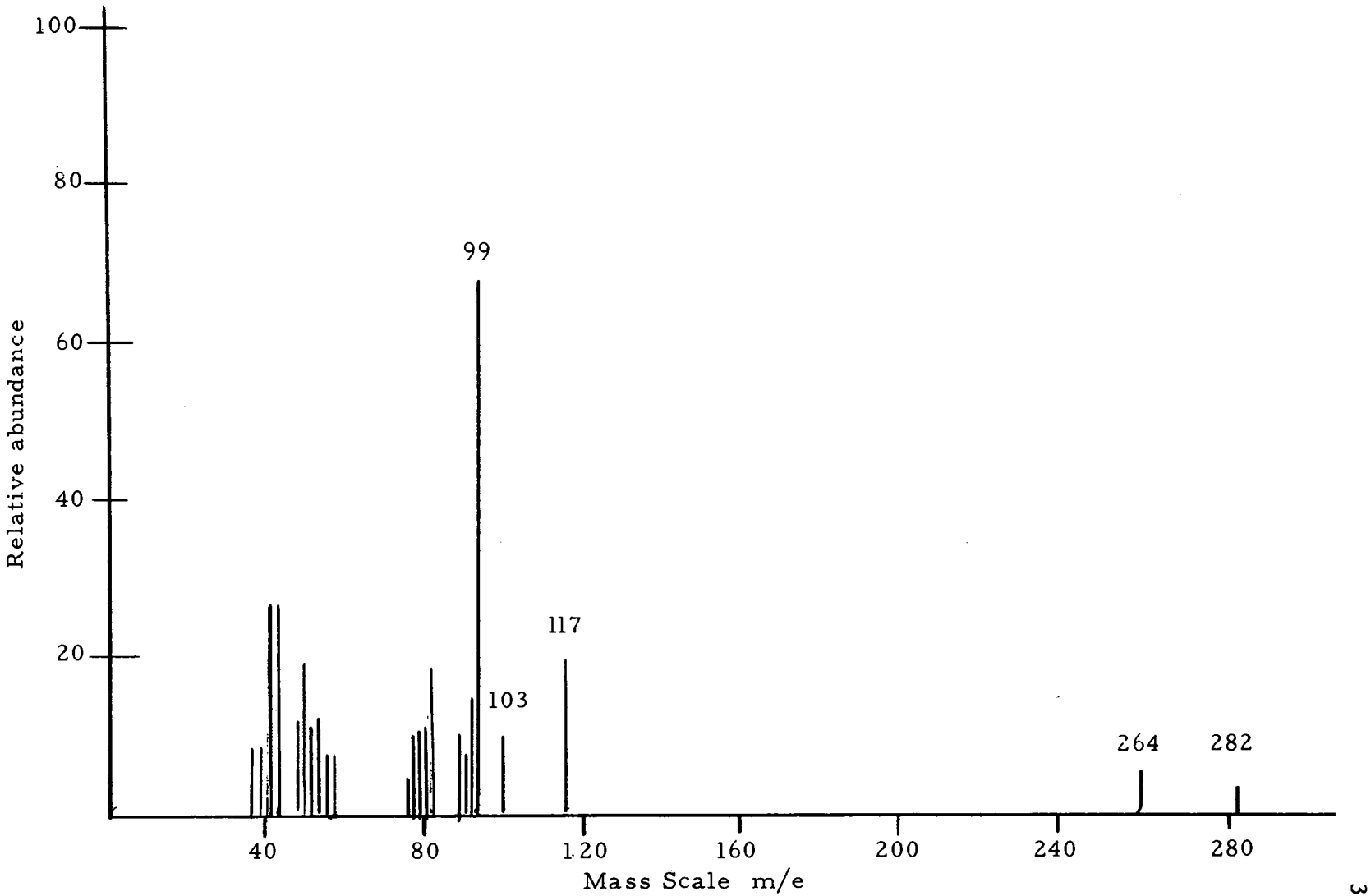


Figure 8. Mass spectrum of δ-Octadecalactone

methyl esters (C_4 to C_{18}) had an elution volume similar to fraction IV-1 and 12-hydroxy methyl stearate was eluted between fraction IV-3 and IV-4. The quantitative and qualitative data are presented in Table 5. An infrared spectrum was obtained for each fraction and the functional groups present in the individual fractions are presented in Table 5.

Table 5. Tentative composition of polar methyl esters separated by silicic acid chromatography

Fraction No.	Weight (grams)	Functional group from I.R.	Wave number (cm^{-1})	Composition
IV-1	1.30	-C=O	1725	Normal methyl esters
IV-2	0.02	-C=O	1725	Normal methyl esters
IV-3	0.06	-C=O -OH	1725 3460	Hydroxy methyl esters
IV-4	0.05	-C=O -OH	1725 3460	Hydroxy methyl esters
IV-5	0.12	-C=O -OH	1725 3460	Hydroxy methyl esters
Standard		-C=O -OH	1725 3460	12-hydroxy methyl stearate

The fractions were also analyzed by GLC and it was concluded from infrared spectra and GLC retention data for authentic methyl esters compared with data for the unknown fractions, that fractions IV-1 and IV-2 contained only normal milk fat methyl esters. The

preliminary GLC separation of fractions IV-3, IV-4, and IV-5 indicated no clearcut separation by chain length so the fractions were pooled. The infrared spectrum of the pooled fractions, shown in Figure 9, is quite suggestive of the presence of hydroxy methyl esters. The strong absorption band at 3460 cm^{-1} is due to the hydroxy stretching (2, p. 96; 34, p. 30) and the band at 1725 cm^{-1} is due to the ester carbonyl (2, p. 179, 185; 34, p. 44). The band at 1110 cm^{-1} is attributed to the secondary hydroxyl group (2, p. 96; 34, p. 31). The spectrum compares favorably with that of 12-hydroxy methyl stearate and 3-hydroxy methyl octanoate (15). There were several components in the combined fraction with extremely long retention times. Hydroxy methyl esters are very difficult to chromatograph under normal conditions because of the polarity conferred by the hydroxy group. These compounds have extremely long retention times and are eluted in broad peaks. Because of the difficulty in chromatographic resolution of the free hydroxy methyl esters, the possibility of converting the hydroxyl group to a derivative was investigated. The pooled fraction was converted to TMS ether derivatives as described earlier and analyzed by GLC and mass spectrometry.

The retention times of the TMS ether derivatives of the polar methyl esters and of standard compounds are shown in Table 6. Tentative identifications of the derivatives were made by a comparison with authentic retentions times and identifications were later

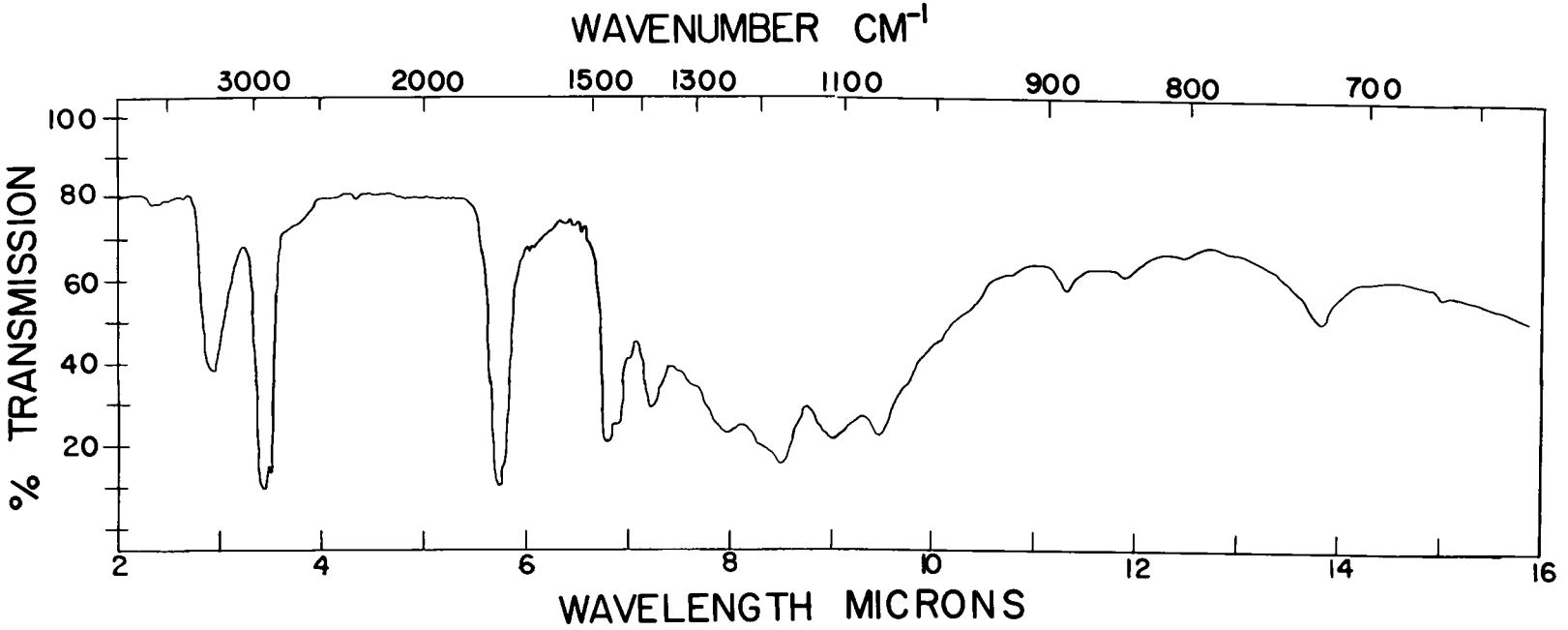


Figure 9. Infrared spectrum of hydroxy methyl esters separated from milk fat

confirmed by mass spectrometry.

Table 6. Retention data of TMS ether methyl esters isolated from the lactone precursor (III) from milk fat

Compound	Retention time (minutes)	Authentic retention time (minutes)
5-TMS-Methyl octanoate	16.50	15.44*
4-TMS-Methyl decanoate	18.04	18.13
5-TMS-Methyl decanoate	18.78	18.17
4-TMS-Methyl undecanoate	-----	20.16
5-TMS-Methyl undecanoate	-----	20.08
5-TMS-Methyl dodecanoate	21.78	21.82
5-TMS-Methyl tetradecanoate	26.50	26.42*
5-TMS-Methyl pentadecanoate	27.88	29.26*
5-TMS-Methyl hexadecanoate	30.65	32.11*
5(?)-TMS-Methyl heptadecanoate	35.52	34.95*
5-TMS-Methyl octadecanoate	38.37	38.21*
5(?)-TMS-Methyl nonadecanoate	42.43	42.27*

*By extrapolation

The major fragmentation peaks characteristic of the authentic TMS ether methyl esters are shown in Table 7. The mass spectra of 5-TMS-methyl decanoate and 5-TMS-methyl dodecanoate are shown in Figures 10 and 11 respectively. These spectra illustrate

the typical fragmentation pattern observed in the TMS derivatives. The parent ion is frequently weak or non-existent, however P-15 is usually prominent. Probably the most useful fragment ion other than the P or P-15 for identifying the TMS ether methyl esters was the R_2 ion which is m/e = 189 and 203 for the 4 and 5 position, respectively, of the TMS ether linkage. Unfortunately, these peaks were not always prominent in the unknown spectra, in which cases the position of the ether linkage could not be ascertained. The major fragmentation pattern of the compounds isolated from milk fat are shown in Table 8.

Table 7. Major fragmentation ions (m/e) of standard TMS ether methyl esters

Compound	Source			
	P	P-15	R_1	R_2
4-TMS-Methyl decanoate	274	259	187	189
5-TMS-Methyl decanoate	274	259	173	203
4-TMS-Methyl undecanoate	288	273	201	189
5-TMS-Methyl undecanoate	288	273	187	203
5-TMS-Methyl dodecanoate	302	287	201	203

A peak, m/e = 147, due to the formation of $(CH_3)_3SiOSi(CH_3)_2$ in the heated high vacuum inlet system from other trimethylsilanes,

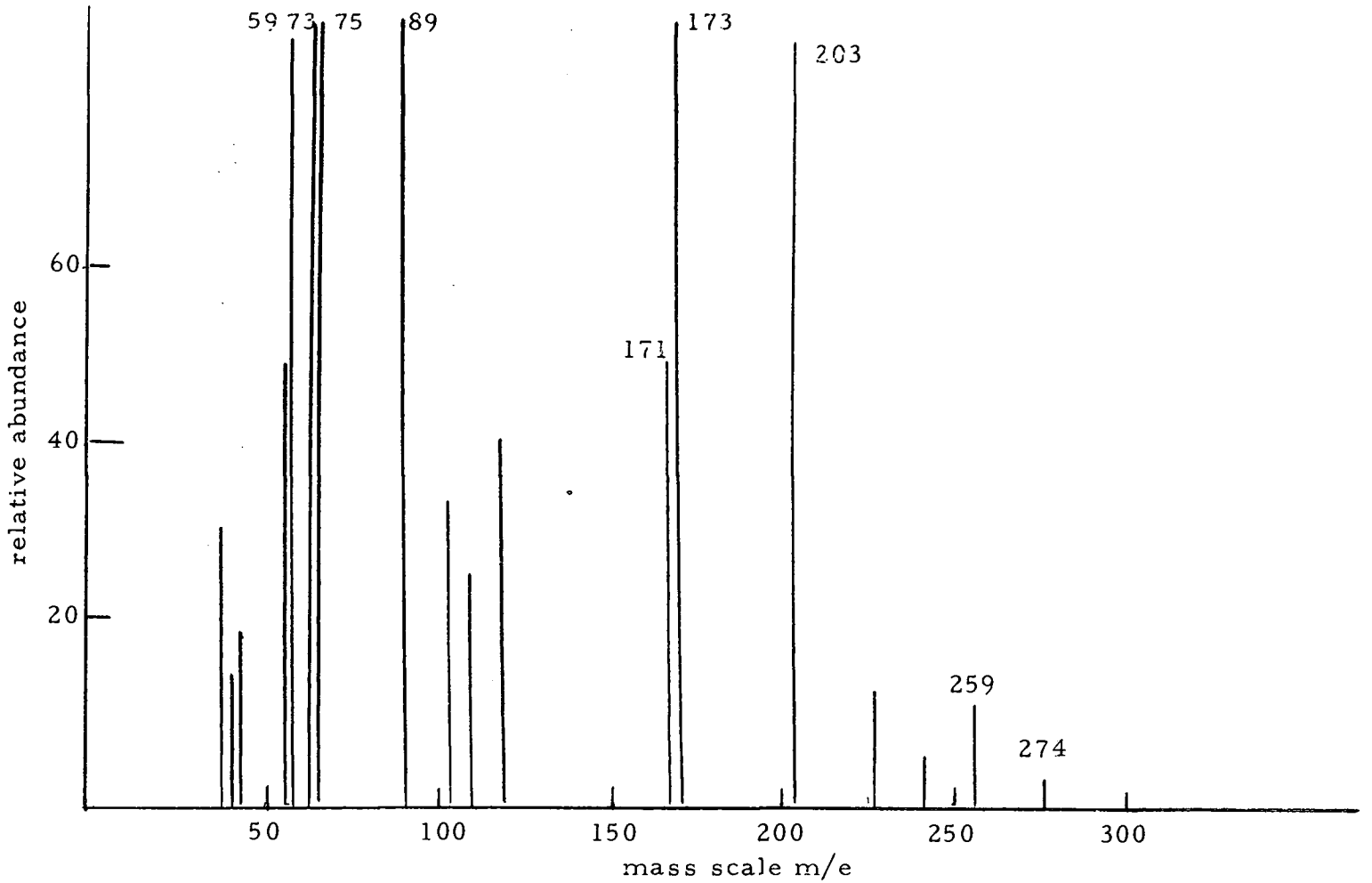


Figure 10. Mass spectrum of 5-TMS-methyl decanoate

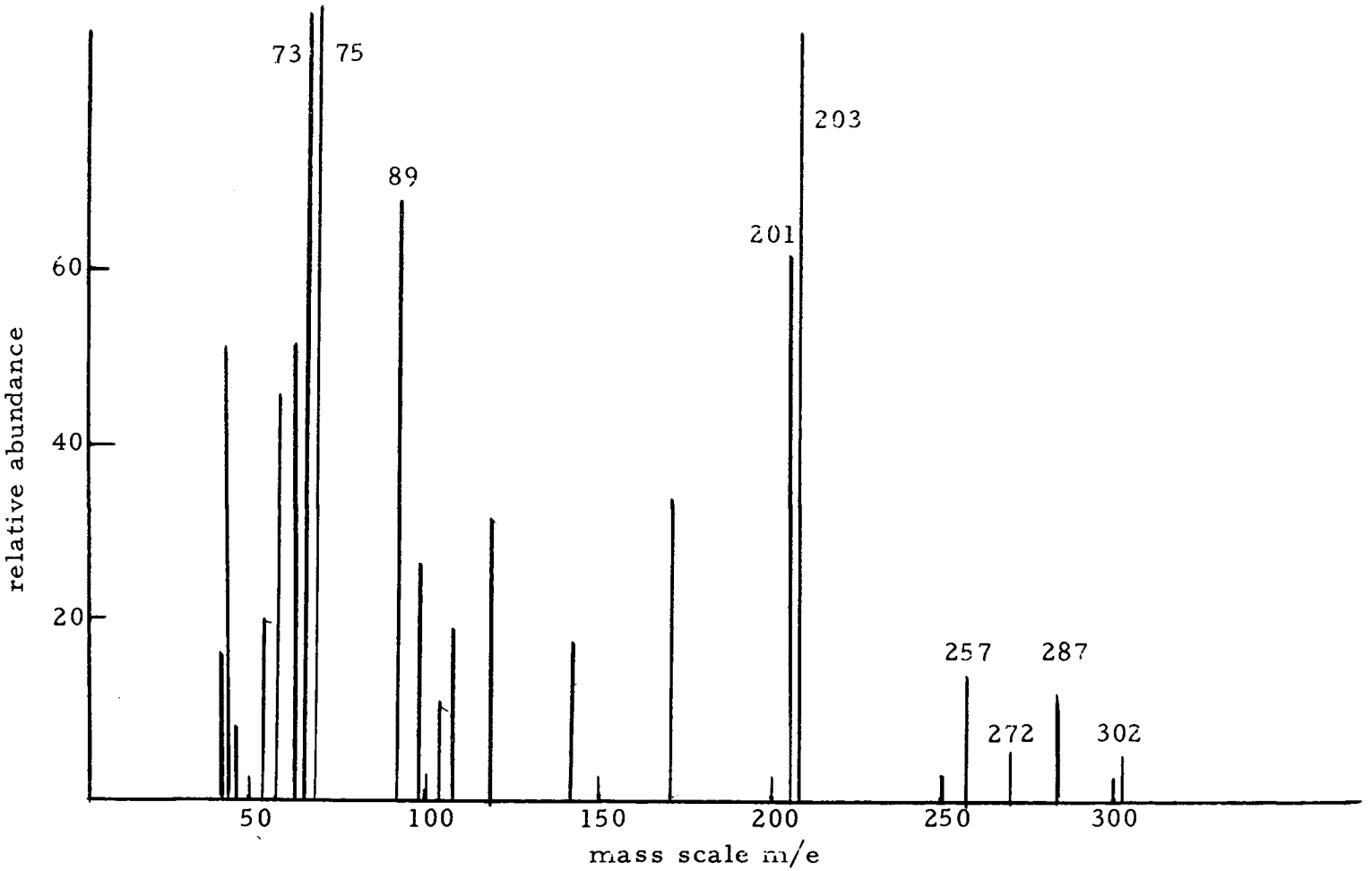


Figure 11. Mass spectrum of 5-TMS-methyl dodecanoate

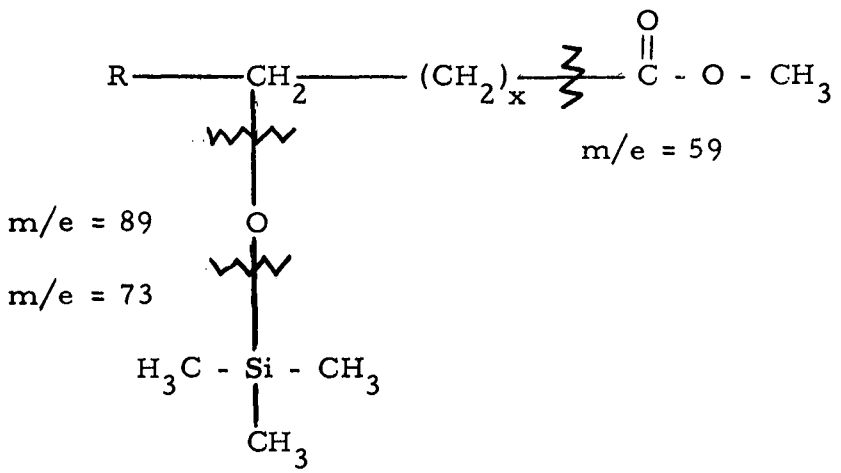
Table 8. Major fragmentation ions (m/e) of compounds identified from milk fat as TMS ether methyl esters

Compound	Source					
	P	P-15	P-30	R_1	R_2	Other
5-TMS-Methyl octanoate[1]		231		145		89, 79, 75, 73
4-TMS-Methyl decanoate[2]		259		187	189	89, 79, 75, 73
5-TMS-Methyl decanoate[1]		259			203	201, 89, 75, 73 59
5-TMS-Methyl dodecanoate[1]	302	287	272	201	203	89, 79, 75, 73
5-TMS-Methyl tetradecanoate[1]		315	300	229	203	89, 79, 75, 73
5-TMS-Methyl pentadecanoate[2]		329			203	201, 89, 79, 75 73
5-TMS-Methyl hexadecanoate[1]		343			203	201, 103, 89, 79, 75, 73
5(?)-TMS-Methyl heptadecanoate[2]		357	342			89, 79, 75, 73
5-TMS-Methyl octadecanoate[1]		371			203	201, 103, 73, 75, 59
5(?)-TMS-Methyl nonadecanoate[2]	400	385				75, 73, 59

1 - Positive identification

2 - Tentative identification

was observed in the spectra of TMS derivatives (29, p. 60). In addition to those ions listed in Tables 7 and 8, m/e = 89, 73, 59, and the rearrangement ion m/e = 75 were prominent in all the spectra of TMS ether methyl ester derivatives. The origin of these fragments is shown below:



Also, m/e = P-47 and P-31 were commonly observed. P-31 could be due to the loss of CH_3-O- and P-47 could be due to a rearrangement involving the trimethylsilyl group. One possibility would be the loss of $SiOH_3$. The compounds listed in Table 7 are standards that were synthesized as described previously. However, not all of the suspected compounds were available as standards, so the theoretical fragmentation pattern was calculated on the basis of the pattern established from the known compounds. This theoretical breakdown was used as a basis for identification of unknown compounds and is shown in Table 9.

Table 9. Major fragmentation ions (m/e) predicted for certain TMS ether methyl esters

Compound	Source			
	P	P-15	R_1	R_2
5-TMS Methyl octanoate	246	231	145	203
4-TMS-Methyl dodecanoate	302	287	215	189
5-TMS-Methyl tetradecanoate	330	315	229	203
5-TMS-Methyl pentadecanoate	344	329	243	203
5-TMS-Methyl hexadecanoate	358	343	257	203
5-TMS Methyl heptadecanoate	372	357	271	203
5-TMS-Methyl octadecanoate	386	371	285	203
5-TMS-Methyl nonadecanoate	400	385	299	203

The following hydroxy fatty acids were found to be present in milk fat: 5-hydroxy octanoic, 4-hydroxy decanoic, 5-hydroxy decanoic, 5-hydroxy dodecanoic, 5-hydroxy tetradecanoic, 5-hydroxy pentadecanoic, 5-hydroxy hexadecanoic, 5(?)-hydroxy heptadecanoic, 5-hydroxy octadecanoic, and 5(?)-hydroxy nonadecanoic. These compounds were identified as TMS ether methyl ester derivatives. Positive identification was based on mass spectral fragmentation patterns and correlation of retention times by GLC. Not all of the compounds identified above were available as standards for establishment of retention data. A plot of log retention times vs. carbon number was prepared from available standards and missing values obtained by

extrapolation. The identities of compounds tentatively identified by retention data were confirmed by mass spectrometry. Figure 12 demonstrates the excellent fit of the retention times of the compounds from the lactone precursor to the line drawn through the standard compounds. Table 10 illustrates the quantitative relationship of the compounds identified.

A search was made for the presence of unsaturated hydroxy fatty acids in milk fat. Figure 13 illustrates the schematic separation of the unsaturated polar methyl ester fraction. The identity of these esters was established by GLC and mass spectrometry. No TMS ether derivative of an unsaturated compound was found, as judged by the complete absence of fragmentation ions at m/e = 89, 75, 73, and 59 from all gas-liquid chromatographic peaks. The composition of gas-liquid chromatographic peaks identified is shown in Table 11. All of these acids have been previously reported in milk fat with the possible exception of methyl nonenoate, although the quantitative relationship is not representative of whole milk fat fatty acids. In addition, there were several unidentified peaks present.

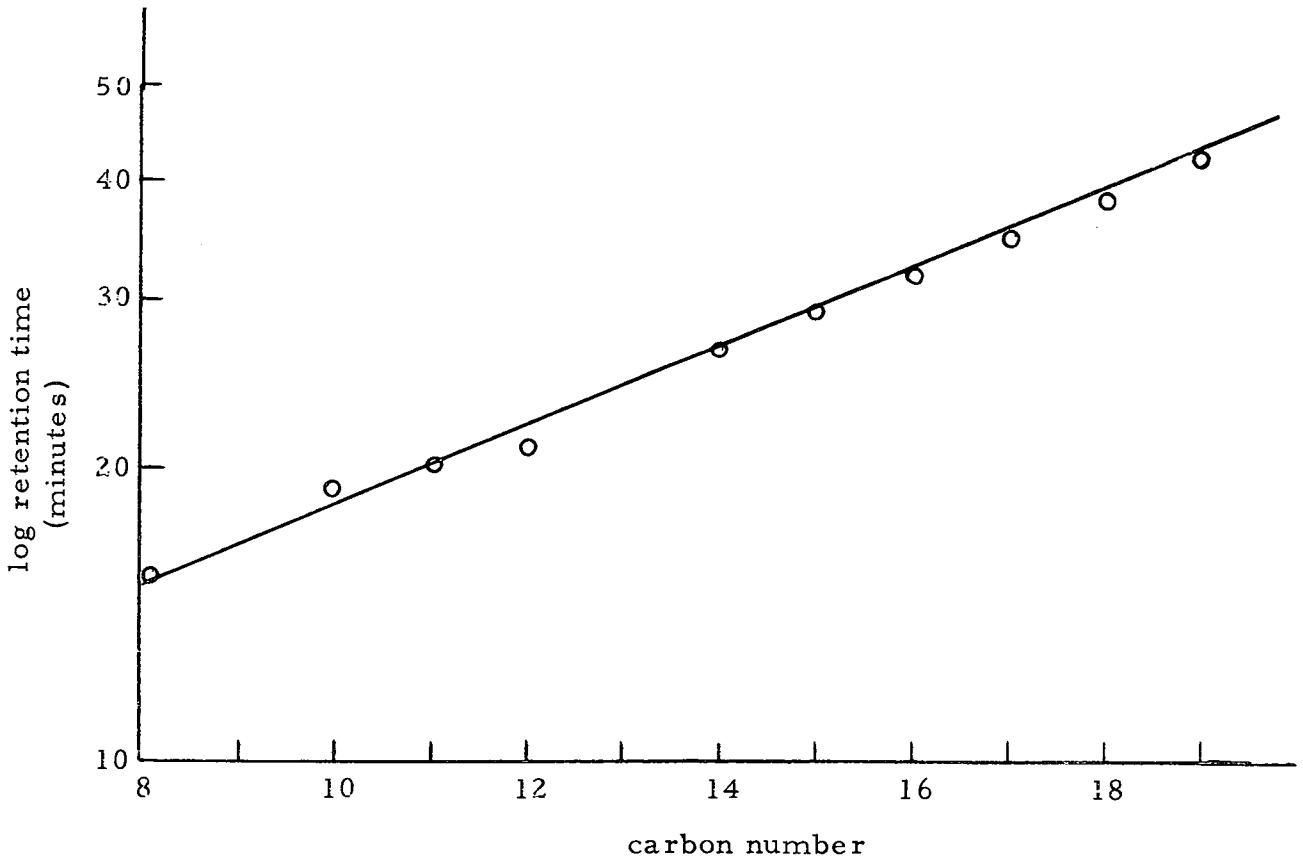


Figure 12. Log plot of retention data vs. carbon number for TMS-ether methyl esters identified from milk fat

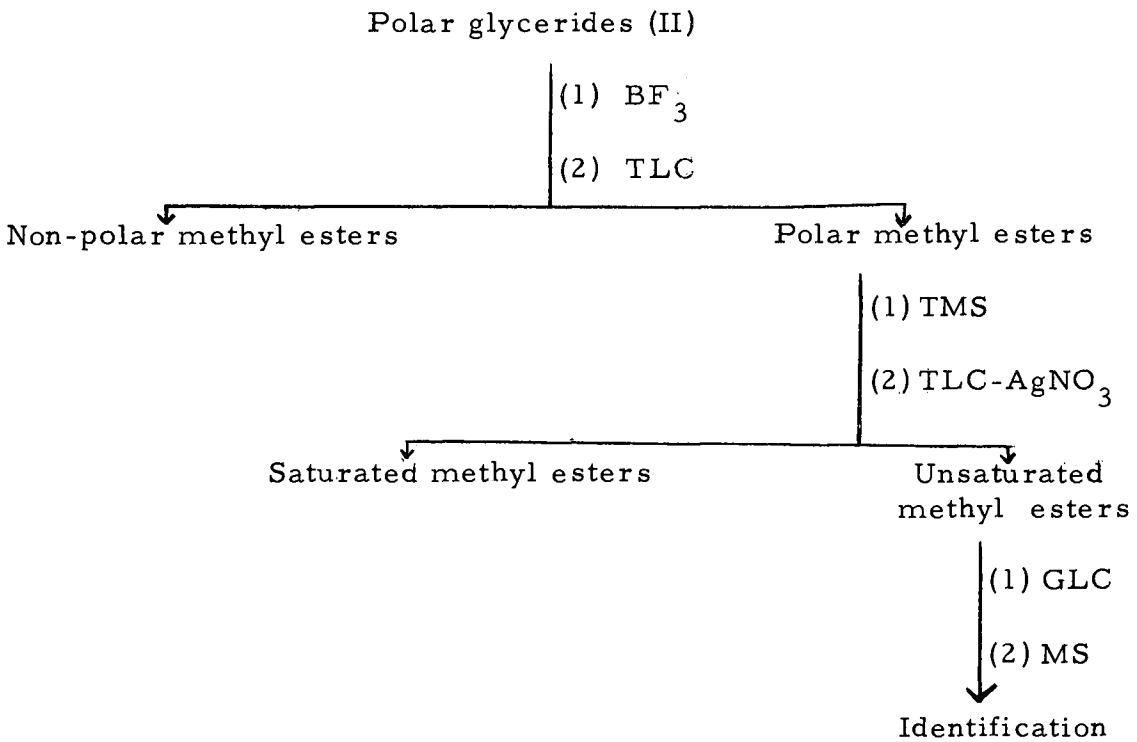


Figure 13. Schematic diagram of the separation of unsaturated polar methyl esters

Table 10. Quantitative relationship of the TMS ether methyl esters identified from milk fat

Compound	Mole - percent
5-TMS-Methyl octanoate	7.58
4-TMS-Methyl decanoate	8.98
5-TMS-Methyl decanoate	10.39
5-TMS-Methyl dodecanoate	13.04
5-TMS-Methyl tetradecanoate	14.43
5-TMS-Methyl pentadecanoate	4.53
5-TMS-Methyl hexadecanoate	15.98
5(?)-TMS-Methyl heptadecanoate	1.06
5-TMS-Methyl octadecanoate	16.52
5(?)-TMS-Methyl nonadecanoate	2.46

Table 11. Retention data of unsaturated methyl esters from milk fat (column temperature 220°C)

Compound	Retention time (minutes)	Mass Spectra Confirmation
Methyl nonenoate	1.54	Positive
Methyl decenoate	3.33	Positive
Methyl dodecenoate	5.04	Positive
Methyl tetradecenoate	6.91	Positive
Methyl hexadecenoate	14.06 *	Positive
Methyl octadecenoate	29.26 *	Positive
Methyl octadecadienoate	29.26 *	Positive

* By extrapolation

SUMMARY AND CONCLUSIONS

Fresh raw cream was churned until the emulsion separated. The butter granules were washed with distilled water and dissolved in petroleum ether (30 - 60°C). The aqueous fraction was removed and the milk fat was separated according to polarity by silicic acid chromatography. A polar glyceride fraction was isolated which accounted for 6.71% of the total lipid fraction. This polar fraction was further separated by thin-layer chromatography (TLC) and found to contain traces of normal triglycerides, cholesterol and monoglycerides. However, a component with an R_f slightly greater than normal diglycerides, but not clearly resolved, constituted the major part of the fraction. The infrared spectrum suggested a hydroxy triglyceride. These fractions were recovered from the silica gel and heated to 140°C for 30 minutes in the presence of water. Only the diglyceride region produced lactones as determined by GLC, infrared and mass spectrometry. A series of lactones from the lactone precursor were identified as δ-octalactone, δ-decalactone, γ-undecalactone, δ-dodecalactone, δ-tetradecalactone, δ-hexadecalactone, and δ-octadecalactone. δ-Octadecalactone has not been previously reported in the literature. Semi-quantitative calculations were made from the peak areas of the gas chromatograms.

A moisture content of 0.175% as determined by near infrared

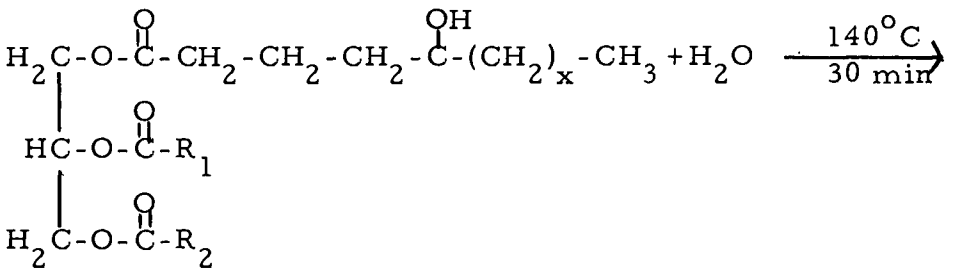
absorbance at 1.87 microns, was found to be sufficient to cause hydrolysis of the hydroxy esters to form lactones. No lactones were produced upon complete dehydration by calcium hydride. Thus, under the conditions studied, intramolecular alcoholysis appears to be an unimportant mechanism for lactone formation.

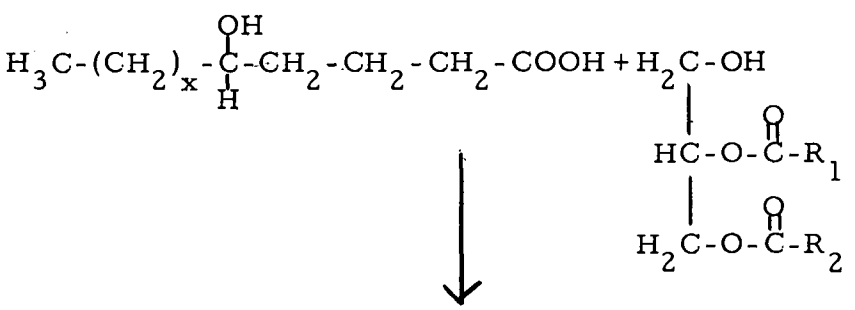
Methyl esters of the lactone precursor were prepared by transmethylation with BF_3-methanol reagent. Hydroxy and non-hydroxy methyl esters were separated on a silicic acid column impregnated with 20% methanol in benzene. The polarity of the solvent was gradually increased from 2% methanol in benzene to 100% methanol to separate the hydroxy methyl esters. The hydroxy methyl esters were converted to trimethylsilyl ether derivatives for ease of identification. A series of compounds were identified by GLC in combination with mass spectrometry. The criterion for positive identification was coincidence of retention times with authentic compounds, if available, and by mass spectral analysis. A semi-quantitative analysis of the compounds was made from peak areas of the gas-liquid chromatograms.

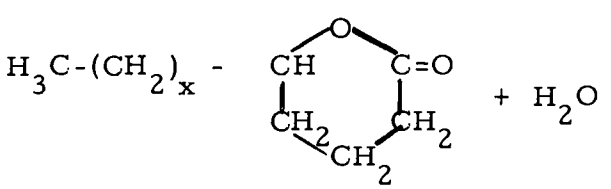
The following hydroxy fatty acids were identified in milk fat as the TMS ether methyl esters: 5-hydroxy octanoic, 4-hydroxy decanoic, 5-hydroxy decanoic, 5-hydroxy dodecanoic, 5-hydroxy tetradecanoic, 5-hydroxy pentadecanoic, 5-hydroxy hexadecanoic, and 5-hydroxy octadecanoic. Hydroxy heptadecanoic and hydroxy

nonadecanoic were identified but the position of the hydroxy group could not be established.

These hydroxy acids exist in milk fat esterified to glycerol and the following mechanism occurs for lactone formation:







A summary of the compounds identified from the lactone precursor is as follows:

Hydroxy acid	Mole percent	Lactone	Mole percent
5-hydroxy octanoic	7.58	δ-octalactone	10.57
4-hydroxy decanoic	8.98	-----	-----
5-hydroxy decanoic	10.39	δ-decalactone	18.59
-----	-----	γ-undecalactone	2.45
5-hydroxy dodecanoic	13.04	δ-dodecalactone	14.53
5-hydroxy tetradecanoic	14.43	δ-tetradecalactone	14.09
5-hydroxy pentadecanoic	4.52	-----	-----
5-hydroxy hexadecanoic	15.98	δ-hexadecalactone	20.40
5(?)-hydroxy heptadecanoic	1.06	-----	-----
5-hydroxy octadecanoic	16.52	δ-octadecalactone	18.53
5(?)-hydroxy nonadecanoic	2.46	-----	-----

In general, the agreement between the lactones and the corresponding hydroxy acids is quite good. There are differences in the quantitative and qualitative data which are probably due to the isolation techniques used since these compounds exist in milk fat in very minute quantities and, due to their high molecular weight, are difficult to separate.

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