

Trace Constituents in Milk Fat: Isolation and Identification of Oxofatty Acids

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ABSTRACT

Ca. 1% of the glycerides of milk fat contain oxofatty acids. The isolation, fractionation, and characterization of oxofatty acids were accomplished using the following sequence of steps: (A) transmethylation, (B) conversion into 2,4-dinitrophenylhydrazones, (C) adsorption of the 2,4-dinitrophenylhydrazones on magnesium oxide to eliminate the colorless lipid, (D) fractionation of the 2,4-dinitrophenylhydrazones into non-oxofatty acid and oxofatty acid fractions on alumina, (E) separation of the oxofatty acid 2,4-dinitrophenylhydrazones into saturated and unsaturated classes by argentation column chromatography, (F) separation of these classes by chain length using liquid-liquid column and thin layer partition chromatography, (G) resolution of positional isomers by thin layer chromatography, (H) regeneration of the positional isomer 2,4-dinitrophenylhydrazones, and (I) analysis of the parent oxofatty acids by gas liquid chromatography-mass spectrometry. In this manner, 36 saturated and 11 unsaturated oxofatty acids were identified tentatively or positively. The saturated oxofatty acids ranged in chain length from C₁₀-C₂₄, predominantly C₁₈ and C₁₆, and generally contained an even number of carbon atoms. The unsaturated oxofatty acids ranged from C₁₄-C₁₈, with C₁₈ predominating.

INTRODUCTION

Application of a method for the direct isolation of carbonyl compounds from fats and oils (1) revealed that milk fat contained a class of nonvolatile carbonyl compounds subsequently identified as glycerides containing one or more esterified oxofatty acids (OFA). This class constituted ca. 1% of the milk lipids and was by far

the major carbonyl-containing moiety. Studies in this laboratory and elsewhere (M. Keeney, private communication) have shown that OFA occur in both animal and vegetable lipids in varying concentrations, usually, however, lower than that in milk fat. They also occur in normal amounts in the milk fat of cows fed a synthetic diet containing little lipid, indicating that OFA cannot be derived from the feed (2).

The problem of identifying the OFA in milk fat was undertaken first by Keeney, et al., (3) who reported the occurrence of six saturated isomeric oxostearic acids using the Beckman rearrangement of the oximes to locate the oxo-group. They also reported that OFA down to C₁₀ occur in lesser amounts but did not identify them. Details of the methods used were not communicated. In the only other study of OFA in milk fat, van der Ven (4) used Girard T reagent to extract the OFA, reduced them to hydroxy fatty acids, and determined those hydroxy acids which lactonized. In this manner, three 4-OFA and three 5-OFA were identified indirectly.

In a problem related to the biosynthesis of OFA in milk fat, Katz and Keeney (5) isolated an oxostearic acid fraction from the lipids of rumen digesta which consisted mainly of 16-oxostearate. They also reported that the 8-oxo-through the 15-oxopositions were present.

Aside from the work on milk fat and rumen lipids, relatively few OFA have been identified in biological material: lactarinic (6-oxostearic) acid from the mushroom fungus *Lactarius refus* (6,7); 13-oxodotriacontanoic acid from the cochineal insect, coccerin (8); licanic (4-oxo-9,11,13-octadectrienoic) acid in the seed fat of *Licania rigida* (9) and other species of this genus (10); 9-oxo-*trans*-2-decenoic acid, so-called Queen Substance from bees (11), which also has been considered to be the sex attractant for drones (12); 8-oxohexadecanoic acid which occurs in small amounts in the oil of the spores of *Lycopodium* species of the clubmoss *Lycopodium* (13); 6-oxotetradecanoic acid from hydrolyzed lac resin (14); 10-oxooctadecanoic acid from the lipids of the Tubercle bacillus (15); and 17-oxo-*cis*-20-hexacosenoic, 15-oxo-*cis*-18-tetracosenoic, and 19-oxo-*cis*-octacosenoic acid from the seed oil of *Cuspidaria pterocarpa* (16).

This report concerns a reinvestigation of the

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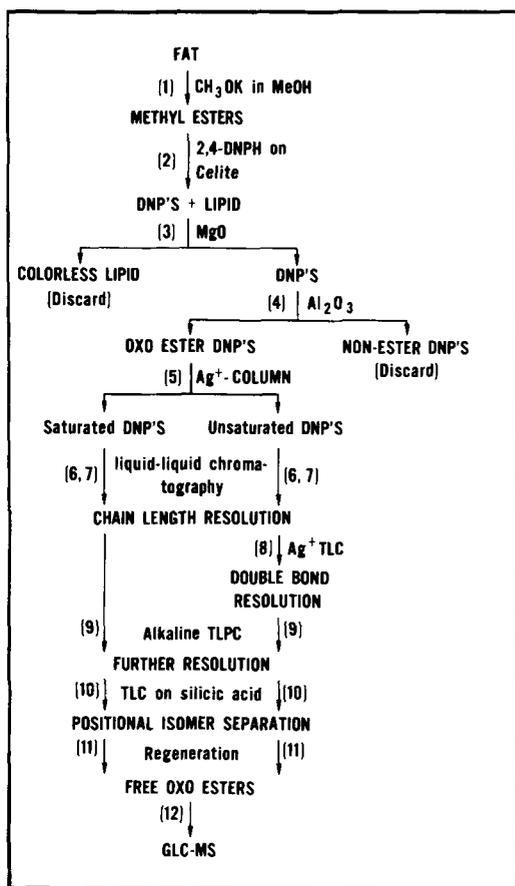


FIG. 1. Schematic of steps used in the isolation, fractionation, and characterization of oxofatty acids from milk fat. DNPH = 2,4-dinitrophenylhydrazine, GLC-MS = gas chromatography-mass spectrometry, DNP = 2,4-dinitrophenylhydrazones, TLC = thin layer chromatography, and TLPC = thin layer partition chromatography.

OFA of milk fat using new, improved wet microchemical techniques and mass spectrometry. Our analysis has revealed that the OFA fraction is highly complex, containing over 60 components, 47 of which have been either positively or tentatively identified.

EXPERIMENTAL PROCEDURES

The methods for isolating, fractionating, and identifying the OFA in milk fat are outlined in Figure 1 and are described in the following steps:

Step 1—Transmethylation

Milk fat (46 g), prepared from mixed herd winter milk obtained from the USDA, Beltsville, Md., was transmethylated by the proce-

dures of Luddy, et al., (17) using 460 ml 0.5 N methanolic potassium methoxide. Following transmethylation, 460 ml 0.5 N HCl was added and the esters extracted with 5 x 500 ml portions of carbonyl-free n-hexane (18, 19).

Step 2—Preparation of 2,4-Dinitrophenylhydrazones (DNPs)

The hexane extract was divided into 3 equal portions and passed over three 30 g columns of Celite impregnated with 2,4-dinitrophenylhydrazine, prepared according to Schwartz and Parks (19), to derivatize the carbonyl compounds. Analysis of the effluents indicated that reaction had been incomplete. The effluents were, therefore, recycled over the columns which resulted in quantitative derivatization relative to results obtained on a small aliquot.

Step 3—Adsorption of DNPs on MgO

The combined effluents from step 2 were evaporated to ca. 300 ml and passed over a 50 g column containing Seisorb 43: Celite 545 (1:1) (1). Nitrogen pressure was applied to force the solution through the column so that an unbroken stream of effluent issued from the column continuously. The sides of the column were washed down with hexane and the column washed under pressure with 1 liter hexane to remove the last traces of colorless lipid. The total time involved to this point was ca. 30 min. The DNPs then were desorbed with a 25% solution of nitromethane in chloroform and the solvent evaporated under nitrogen until the odor of nitromethane was absent.

Step 4—Fractionation of DNPs on Al₂O₃

The residue from step 3 was dissolved in 50 ml hexane and passed over a 50 g column of 8% hydrated Al₂O₃ (19). Aldehyde and ketone DNPs containing no ester function (band I) were eluted with hexane:benzene (1:1) until all color below the next (major OFA) band was removed. The major OFA band (yellow-orange, band II) was eluted with benzene. The next band (light red, band III) which contained geometric isomers of the major OFA band (see "Results and Discussion") was eluted with dichloromethane. Spectrophotometric analysis of band II in chloroform at its absorption maximum (365 nm) indicated 185 μ moles and that of band III (adsorption maximum, 355 nm) of 32 μ moles. Band I was discarded.

Step 5—Argentation Column Chromatography

Separation of band II into saturated and unsaturated OFA DNPs was effected by Ag⁺ column chromatography. Absolute separation was not achieved, but the majority of the satu-

rated and unsaturated DNPs moved with their respective classes. Supplemental testing with iodine monochloride, hydrogenation, and mass spectrometry was sufficient to establish whether the OFA was unsaturated.

Silicic acid (10 g) (Mallinckrodt's 100-200 mesh) was slurried with 10 ml 10% solution of AgNO_3 in acetonitrile, poured into an evaporating dish, heated 1 hr at 100 C, cooled, and ground briefly to eliminate clumps. The silicic acid was slurried in hexane, poured in a column (20 x 2 cm), and packed under air pressure. The DNPs dissolved in a minimum of hexane were applied to the column. The majority of the saturated DNPs was removed as a single band with dichloromethane:hexane (1:1), and the remaining bands on the column were removed with 5% methanol in chloroform. The "saturated" DNP fraction was rechromatographed as above and separated into a major and a minor band (ca. 9%). The major band was collected and designated as the saturated fraction. The minor band was pooled with the "unsaturated" DNP fraction from the initial chromatogram. This was designated as the unsaturated fraction. Spectrophotometric analysis (at 365 nm, $E = 22,500$) gave an estimate of ca. 69% saturated and 31% unsaturated DNPs.

Band III from step 4 was subjected to the above procedure giving a fraction comprised of 55% saturated and 44% of unsaturated DNPs.

Step 6—Column Partition Chromatography

The saturated fraction from band II was chromatographed on a 25 g acetonitrile-Celite column as described by Corbin, et al. (20). Fractions (15 ml) were collected. Separation was followed visually and spectrophotometrically at 340 nm. The fractions comprising a given peak were pooled.

The unsaturated fraction from band II and the saturated and unsaturated fractions from band III were chromatographed similarly.

Step 7—Thin Layer Partition Chromatography (TLPC) of Saturated Fractions on Neutral Plates

Each peak from the saturated fractions obtained in step 6 was streaked across a 20 x 8 in. TLPC plate and developed in the hexane system described by Schwartz, et al. (21). The bands were scraped from the plate, the color eluted with benzene, and any contaminating bands were pooled with the appropriate subsequent fraction. All fractions then were freed of stationary phase (polyethylene glycol 400) by passing the benzene solution over a column of 8% hydrated alumina (19) (ca. 1 g in a disposable pasteur pipette), and the column was eluted with benzene until all color was removed.

Step 8—Thin Layer Argentation Chromatography of Unsaturated Fractions

Mylar sheets (8 x 8 in.) precoated with silica gel (Baker-Flex 1B, J.T. Baker Co., Phillipsburg, N.J.) were drawn through a 10% silver nitrate solution in acetonitrile and dried 10 min at 100 C. The peaks obtained for the unsaturated fractions in step 6 were streaked across the sheet and the chromatogram developed repeatedly (usually 3 times) in chloroform:benzene (3:2) to resolve the derivatives more or less according to the degree of unsaturation. The bands were scraped from the sheet and eluted with chloroform or ethyl acetate.

Step 9—TLPC on Alkaline Plates

Each band obtained in steps 7 and 8 was streaked across the origin of a 20 x 8 in. alkaline TLPC plate (21) and developed in hexane and hexane:benzene (65:35). This system afforded further separation according to chain length which was not attained on either the partition column or on the neutral TLPC plates. This was especially evident with the longer chain ($>C_{16}$) OFA DNPs. At the same time, some DNPs gave different colors (see "Results and Discussion"). The bands were scraped from the plate, eluted with benzene, and purified on small alumina columns as in step 7.

Step 10—Resolution of Positional Isomers by Thin Layer Chromatography (TLC)

The bands from the saturated fraction, cut and purified from the plates in step 9 and presumably containing OFA DNPs of a given chain length, were streaked across the origin of a silica gel sheet and the chromatogram repeatedly developed with 5% ethyl acetate in hexane to resolve positional (oxogroup) isomers. A clean cut separation of the 2-oxo- through 8-oxo positions was achieved. The 9-oxo- through 12-oxo positions were not as cleanly resolved, but relatively pure fractions were obtained by scraping successive narrow segments from the partially resolved band. The 13-oxo- through 16-oxo positions (in the stearates) could not be resolved. Each band was scraped from the plate, eluted with chloroform, and checked for saturation or unsaturation using iodine monochloride (22). Saturated DNPs are not retarded on TLPC plates, whereas most unsaturated DNPs are retarded from 30-80%, depending usually upon the degree of unsaturation. All bands also were purified by dissolution in 10% dichloromethane in hexane and passage over a small Al_2O_3 column as described in step 7. The column was washed with 5 ml 10% dichloromethane in hexane and the effluent discarded. The colored band then was eluted with dichloromethane.

Step 11—Regeneration of OFA

A volume of a dichloromethane solution of each purified fraction (obtained in step 10) containing ca. 5 μ g OFA DNP was transferred to a mp capillary and the solvent removed under vacuum. A 1% solution (10 μ liter) of concentrated hydrochloric acid in acetone (prepared each day) was added and the DNP dissolved by repeatedly drawing up and expelling the solution with the hypodermic syringe. The solution was allowed to stand for 15 min to complete the regeneration (M. Keeney, private communication).

Step 12—Gas Liquid Chromatography-Mass Spectrometry (GLC-MS) of the Regenerated OFA

The entire solution from step 11 was injected into a gas chromatograph and the effluent vapor analyzed by MS. The LKB-9000 spectrometer was used. OFA with 16 carbons or less were chromatographed on a 5 ft x 1/8 in. stainless steel column packed with 7.5% ethylene glycol adipate and 2% phosphoric acid on 90-100 mesh Anakrom ABS (Analabs, North Haven, Conn.). OFA containing 17 or more carbons were chromatographed on a 10 ft x 1/8 in. stainless steel column packed with 3% OV-1 on 100-200 mesh Chromosorb Z. Both columns were operated isothermally, the former at a temperature of 160-180 C, depending upon chain length, and the latter at 190-210 C, also depending upon the chain length. Helium was the carrier gas and was maintained in all instances at 40 psi. Other standard operating conditions were: flash heater and separator, 230 C; ion source, 290 C. Mass spectra were obtained at a constant accelerating voltage of 3500 V with an electron energy of 70 ev and a scanning time of 4.5 sec over a m/e range of 12-450. Chromatographic peaks were scanned repeatedly, and the strongest area at the apex was compared to a similar area of an authentic compound when available.

Additional Methodology

Hydrogenation: In a few instances, hydrogenation of unsaturated OFA was conducted to compare their spectra with those established for saturated OFA. The OFA was regenerated, as in step 11, and the excess acetone removed under vacuum. The residue was taken up in the minimum volume of dimethoxypropane, transferred to a microcolumn of Celite impregnated with palladium chloride and hydrogenated as described by Schwartz, et al. (23).

Location of double bond position: The position of the double bond in monounsaturated OFA was determined directly on the DNP

using the periodic acid column procedure of Wehrauch and Schwartz (24) when the mass spectrum indicated that the double bond occupied a position between the oxogroup and end of the chain. When the mass spectrum indicated that the double bond was between the ester and oxogroups, a potassium permanganate-periodic acid column procedure was employed on the DNP (J.L. Wehrauch and D.P. Schwartz, unpublished data) because the former procedure does not give the semialdehyde. Briefly, this procedure consisted of application of the unsaturated OFA DNP in dichloromethane to a column of Celite (0.5 g) which had been ground (on the day of use) with 12 drops of a saturated aqueous solution of potassium permanganate and 3 drops of a saturated aqueous solution of potassium carbonate. The OFA DNP was permitted to remain on the column for 30 min. It then was eluted and passed over the periodic acid column system (24).

Reference oxoacids: Synthesis of 6-oxomyristic, 7-oxomyristic, 6-oxopalmitic, 7-oxopalmitic, 8-oxopalmitic, 8-oxoheptadecanoic, and 8-oxostearic acids were carried out according to Hünig, et al. (25,26). The acids were converted to methyl esters overnight in methanolic hydrochloric acid. The 4- and 5-oxodecanoates and 4- and 5-oxododecanoates were prepared from the corresponding γ - and Δ -lactones by transmethylation in methanolic hydrochloric acid, extraction with hexane, passage of the hexane extract over a chromic acid column (27) to oxidize the hydroxy ester to the oxoester, conversion of the oxoester to the DNP, and purification by preparative TLC.

RESULTS AND DISCUSSION

The saturated OFA positively or tentatively identified are listed in the first section of Table I. A compound is listed as being identified positively only when an authentic sample was identical both chromatographically and mass spectrometrically.

The oxostearates comprised the major portion of the total oxoesters which confirms the findings of Keeney, et al. (3). Ca. 85% of the oxostearates are comprised of the 13-oxo-, 9-oxo-, and 10-oxoisomers in decreasing prevalence. The 11-oxoisomer was found in only trace amounts. Keeney, et al., (3) reported the order of prevalence to be 9-oxo-, 10-oxo-, and 13-oxostearates. They also found a significant amount (ca. 10%) of the 11-oxoisomer. The occurrence of all oxostearates (8-oxo through 13-oxo) reported by Keeney, et al., (3) with the exception of the 12-oxoisomer, was confirmed in the present study.

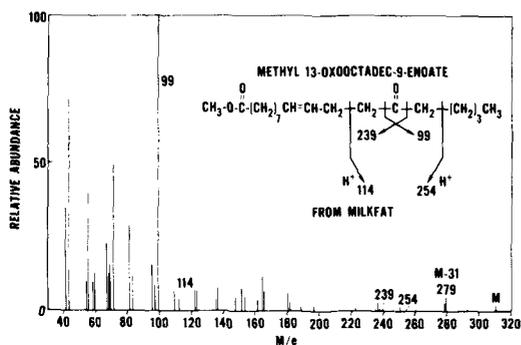


FIG. 2. Mass spectra of authentic methyl-9-oxooctadec-12-enoate and an oxofatty acid methyl ester isolated from milk fat.

The oxopalmitates occurred in the next highest concentration (ca. 20% of the total saturates) and was comprised predominantly of the 11-oxoisomer.

The oxomyristates, oxolaurates, and oxodecanoates were found in decreasing amounts. The most abundant of these oxoesters were 9-oxomyristate, 5-oxolaurate, and 5-oxodecanoate. The occurrence in milk fat of 4-oxolaurate, 5-oxodecanoate, and 5-oxolaurate indirectly identified as lactones by van der Ven (4) is substantiated by our data. However, the other reported oxoacids (4-oxodecanoate, 4-oxoundecanoate and 5-oxooctanoate) were not detected.

Very small amounts of odd carbon oxoacids were identified and were estimated to occur in the amount of ca. 1 $\mu\text{g/g}$ milk fat.

OFA with more than 18 carbons were found, their amounts decreasing with increasing chain length. Only traces of the oxotetracosanoates were detected.

Unsaturated OFA: Analysis of the unsaturated OFA fraction yielded only 11 identifiable compounds. These are given in the second section of Table I.

Methyl 9-oxooctadec-12-enoate and methyl 13-oxooctadec-9-enoate were the predominant unsaturated OFA. Besides those unsaturated OFA given in Table I, the presence of the following OFA was indicated: a C_{15} monounsaturate, a C_{16} diunsaturate, a C_{17} mono-, di-, and triunsaturate, a C_{18} di- and triunsaturate, a C_{19} mono- and diunsaturate, and a C_{20} mono-unsaturate.

Both saturated and unsaturated OFA DNP fractions contained bands which regenerated, but the fragmentation pattern was not interpretable outside of being recognized as methyl esters of OFA. Another set of bands was not methyl esters, and a third set of bands did not

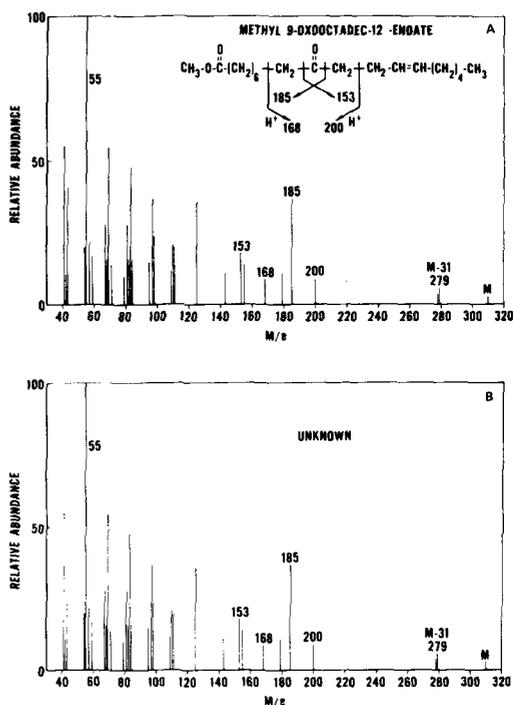


FIG. 3. Mass spectrum of an oxofatty acid methyl ester isolated from milk fat and tentatively identified as methyl 13-oxooctadec-9-enoate.

regenerate under the conditions used in step 11. In addition, a series of bands displaying a lavender color on the alkaline TLPC plates was seen, and also a series of blue bands was observed. The former were reminiscent of the color shown by alk-2,4-dienal or alk-2,4-dienone DNPs but were not investigated further. Both classes were present in small amounts, probably less than 1% of the total OFA.

Mass spectrometry: The mass spectra of the oxofatty acids isolated from milk fat are given in Table I. The location of the carbonyl group is made readily as both α - and β -cleavage occur on either side of the carbonyl group. The molecular ion which is usually less than 1% of the base peak is readily discernible. Mass spectra of the methyl esters of the oxostearates were published by Ryhage and Stenhagen (28). Our spectra were generally in good agreement with theirs.

For monounsaturated OFA, the mass spectrum reveals also the side of the carbonyl group in which the double bond occurs. Figure 2 shows mass spectra of authentic methyl 9-oxooctadec-12-enoate and an OFA from milk fat with an identical spectrum as examples of unsaturated OFA with the double bond between the carbonyl group and the end of the chain. A

TABLE I
Mass Spectra of Oxofatty Acids Positively or Tentatively Identified in Milk Fat

	Mass spectrum, m/e (relative intensity)
Saturated n-oxofatty acids	
Methyl-5-oxodecanoate	43(100), 112(61), 71(60), 101(59), 99(57), 129(42), 144(36), 169(19), 200(M ⁺) (0.14)
Methyl-4-oxolaurate	98(100), 130(65), 115(56), 71(43), 111(23), 141(21), 197(9), 228(M ⁺) (0.4)
Methyl-5-oxolaurate	57(100), 112(74), 55(55), 43(48), 144(38), 129(37), 127(29), 197(12), 228(M ⁺) (0.3)
Methyl-7-oxolaurate	43(100), 55(59), 71(57), 69(43), 99(43), 172(13), 157(9), 197(4), 228(M ⁺) (0.4)
Methyl-5-oxomyristate	112(100), 43(67), 55(58), 144(56), 101(47), 129(44), 155(22), 225(12), 256(M ⁺) (0.5)
Methyl-6-oxomyristate	158(100), 141(77), 143(53), 156(24), 225(7), 197(5), 256(M ⁺) (1.3)
Methyl-7-oxomyristate	57(100), 115(41), 125(37), 127(29), 142(14), 172(14), 157(10), 225(1.3), 256(M ⁺) (0.4)
Methyl-9-oxomyristate	43(100), 55(71), 71(62), 99(52), 114(15), 185(15), 200(8), 225(6), 256(M ⁺) (0.2)
Methyl-4-oxopentadecanoate ^a	130(100), 115(59), 111(41), 239(9), 169(4), 270(M ⁺) (1)
Methyl-5-oxopentadecanoate ^a	112(100), 43(63), 55(60), 144(58), 41(47), 129(43), 169(17), 239(10), 270(M ⁺) (0.3)
Methyl-4-oxopalmitate ^a	98(100), 130(91), 55(62), 115(50), 43(54), 57(49), 197(10), 253(4), 284(M ⁺) (0.5)
Methyl-5-oxopalmitate ^a	112(100), 144(67), 43(51), 55(51), 57(47), 129(40), 183(15), 253(10), 284(M ⁺) (0.5)
Methyl-6-oxopalmitate	158(100), 143(42), 169(42), 184(15), 235(7), 253(4), 284(M ⁺) (1.0)
Methyl-7-oxopalmitate	43(100), 55(90), 71(73), 172(38), 155(35), 170(23), 157(21), 253(3), 284(M ⁺) (0.13)
Methyl-8-oxopalmitate	141(100), 171(86), 156(68), 154(64), 144(50), 186(46), 253(13), 211(9), 284(M ⁺) (0.13)
Methyl-9-oxopalmitate	127(100), 143(87), 142(71), 185(36), 200(26), 168(26), 253(21), 284(M ⁺) (1.9)
Methyl-11-oxopalmitate	43(100), 71(62), 55(58), 99(50), 114(16), 213(10), 228(7), 253(5), 284(M ⁺) (0.3)
Methyl-8-oxohepta decanoate	171(100), 170(64), 186(53), 267(7), 169(7), 298(M ⁺) (1.4)
Methyl-5-oxostearate	112(100), 144(66), 43(51), 55(49), 57(46), 129(37), 211(7), 281(6), 312(M ⁺) (0.24)
Methyl-8-oxostearate ^a	171(100), 154(75), 169(73), 184(59), 186(59), 281(15), 312(M ⁺) (1.6)
Methyl-9-oxostearate	43(100), 55(96), 71(71), 155(24), 170(20), 185(19), 200(16), 281(4), 312(M ⁺) (0.2)
Methyl-10-oxostearate	55(100), 43(99), 57(90), 156(51), 141(46), 214(24), 199(24), 281(14), 312(M ⁺) (0.75)

Methyl-11-oxostearate	43(100), 127(19), 142(14), 213(7), 281(9), 228(6), 312(M ⁺) (0.8)
Methyl-13-oxostearate	43(100), 55(66), 71(61), 99(49), 114(17), 241(10), 256(7), 281(5), 312(M ⁺) (0.6)
Methyl-16-oxostearate	47(100), 55(57), 43(54), 72(49), 41(42), 209(13), 283(10), 281(3), 312(M ⁺) (1.5)
Methyl-11-oxononadecanoate ^a	43(100), 141(37), 156(36), 228(19), 213(18), 295(8), 326(M ⁺) (0.5)
Methyl-9-oxoicosanoate ^a	43(100), 185(13), 183(9), 200(9), 198(8), 309(4), 340(M ⁺) (0.5)
Methyl-11-oxoicosanoate ^a	43(100), 170(30), 155(23), 228(15), 213(13), 309(7), 340(M ⁺) (0.8)
Methyl-15-oxoicosanoate ^a	43(100), 99(40), 114(16), 269(8), 284(7), 309(6), 340(M ⁺) (0.7)
Methyl-11-oxodocosanoate ^a	43(100), 198(11), 228(11), 183(10), 213(7), 337(4), 368(M ⁺) (0.41)
Methyl-12-oxodocosanoate ^a	184(100), 189(94), 242(71), 227(55), 337(80), 368(M ⁺) (9.4)
Methyl-13-oxodocosanoate ^a	170(100), 155(87), 256(59), 241(45), 337(37), 368(M ⁺) (4.4)
Methyl-14-oxodocosanoate ^a	141(100), 156(97), 255(41), 270(39), 337(59), 368(M ⁺) (7.0)
Methyl-15-oxodocosanoate ^a	127(100), 142(44), 269(19), 284(11), 337(56), 368(M ⁺) (6.6)
Methyl-14-oxotetracosanoate ^a	184(100), 169(93), 270(53), 255(44), 365(16), 396(M ⁺) (5)
Methyl-15-oxotetracosanoate ^a	155(100), 170(96), 269(21), 284(11), 365(16), 396(M ⁺) (5)
Unsaturated n-oxofatty acids	
Methyl-5-oxotetradec-9-enoate ^a	55(100), 112(75), 41(71), 144(39), 129(28), 153(6), 223(5), 231(2), 254(M ⁺) (1.5)
Methyl-5-oxohexadec-10-enoate ^a	55(100), 112(75), 41(71), 144(39), 129(28), 153(6), 223(5), 231(2), 254(M ⁺) (1.5)
Methyl-9-oxotetradec-5-enoate ^a	43(100), 99(87), 71(57), 223(4), 222(4), 254(M ⁺) (3), 198(1.5)
Methyl-7-oxohexadec-10-enoate ^a	157(100), 172(50), 153(42), 251(11), 250(10), 282(M ⁺) (6)
Methyl-11-oxohexadec-7-enoate ^a	99(100), 43(89), 71(64), 94(47), 55(44), 251(4), 282(M ⁺) (2), 226(1.8), 211(1.3)
Methyl-11-oxohexadec-9-enoate ^a	99(100), 43(92), 71(65), 94(47), 55(47), 251(4), 282(M ⁺) (2.1), 226(1.6), 211(1.4)
Methyl-9-oxooctadec-12-enoate ^a	55(100), 41(56), 69(53), 185(29), 153(16), 200(8), 168(7), 279(6), 310(M ⁺) (2)
Methyl-9-oxooctadec-13-enoate ^a	43(100), 55(92), 41(81), 185(36), 153(22), 279(15), 200(9), 310(M ⁺) (6)
Methyl-9-oxooctadec-15-enoate ^a	55(100), 43(75), 41(67), 185(34), 153(19), 168(8), 200(8), 279(8), 310(M ⁺) (4)
Methyl 13-oxooctadec-7-enoate ^a	99(100), 43(72), 71(49), 55(45), 41(37), 279(5), 310(M ⁺) (2), 254(0.7), 239(0.6)
Methyl-13-oxooctadec-9-enoate ^a	99(100), 43(72), 71(50), 55(40), 41(35), 279(5), 310(M ⁺) (2), 254(0.7), 239(0.6)

^aTentative identification.

similar spectrum has been reported by Kleiman and Spencer (29) for methyl 17-oxo-*cis*-20-hexacosenoate.

Figure 3 is the mass spectrum of an unsaturated OFA isolated from milk fat in which the double bond occurs between the ester and carbonyl functions. It tentatively has been identified as methyl-13-oxooctadec-9-enoate based upon the spectra and identification of methyl azelaaldehyde following oxidation on the potassium permanganate-periodic acid column.

Geometrical isomerism in the DNPs: The well known geometrical isomerism in DNPs was evident also with the DNPs of some of the OFA. This phenomenon created some confusion and also a great deal more work, as many additional bands had to be scraped from the plates, purified, regenerated, and examined in the mass spectrometer. Although two isomers for each OFA DNP undoubtedly were present, they were not always separable. The DNPs of the OFA with the oxogroup in the 2-8 positions were separable into 2 isomers. The 9 position and higher were not. The closer the oxofunction was to the ester group, the more marked the separation of isomers became. In this study, the 5-oxoposition occurred most frequently among the positions up to 9-oxo (Table I); consequently, the geometric isomers of the DNP derivative of the 5 OFA were encountered most frequently. The main isomer was always the slower moving spot or band on the plates. The minor, faster moving spot or band usually moved as if it had two more carbon atoms. Thus, it was not unusual to find, for example, some 5-oxodecanoate as a contaminant in the oxolaurate band. The problem was complicated further by reformation of two isomers from each isomer cut from the plate, thereby doubling the number of isomer bands each time a separation was made.

Despite the shortcoming of geometrical isomerism in the DNPs, it is felt that the information obtained using the methods described more than justified the additional work involved.

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