Fatty Acids of Cows' Milk. A. Techniques Employed in Supplementing Gas-Liquid Chromatography for Identification of Fatty Acids[1]

PAUL MAGIDMAN, S. F. HERB, R. A. BARFORD, and R. W. RIEMENSCHNEIDER,
Eastern Regional Research Laboratory,[2] Philadelphia 18, Pennsylvania

Milk fat methyl esters were subjected to distillation and silicic acid column chromatography to provide fractions of less complexity for gas-liquid chromatographic analysis. It was still necessary however to employ supplemental techniques for identification. Chromatograms were obtained with polyester columns of different polarity on all the fractions and necessary reference samples. While many of the components were identified in the usual way by plots of relative retention time versus number of carbon atoms, iodine values for total unsaturation and ultraviolet spectrophotometry for conjugated and nonconjugated polyunsaturated acids were essential for positive identification of some components. Similarly, examination by infrared spectrophotometry confirmed the presence or absence of conjugated diene as *cis-trans, trans-trans* or both. Isolated *trans* or terminal double bonds were also determined in this way. Gas-liquid chromatograms of some fractions showed incompletely resolved peaks attributable to the presence of methyl esters of odd-carbon atom, branched-chain, and unsaturated acids. Hydrogenation and rechromatographing provided more positive determination of the structure of these components. Further confirmation of identity of some peaks on the chromatogram was achieved by collection of the appropriate fractions and examination of the collected material. At least 60 fatty acids were identified, including several not previously reported, such as odd-numbered carbon chain length monoethenoid acids from C_{15} to C_{23}.

THE FATTY ACID composition of cows' milk has been of great interest over the years, principally owing to the importance of milk as a food, but also because its extreme complexity has presented a challenge to the chemist to unravel, and to the biochemist to explain the origin and biosynthesis of many of the unusual fatty acids. If one were to count the different acids that have been reported in milk fat as the result of all investigations to date the number would exceed fifty. Reviews by Jack and Smith (16) and Shorland and Hansen (29) adequately cover the literature prior to 1957. Some additional acids were added to the list since 1957 (1,8,27,28).

It was fully expected that gas-liquid chromatography (GLC) would be called upon to contribute its tremendous separating power to further elucidate the composition of milk fat. Since 1956, a number of papers (4,5,6,7,9,18,19,20,22,25,30,31) have been published in which GLC was employed either for studies on the total fatty acid methyl esters, or on fractions obtained by other means of fractionation. Although GLC certainly was useful in these studies, it was incapable of complete resolution of all the components. When the methyl esters of the total acids were subjected to GLC on a given column, evidence of only 25 to 30 components could be seen (17,24). The use of two columns and different stationary phases (15) indicated the presence of as many as 39 acids in human milk fat, some of which were unidentified. In all instances where a chromatogram was shown of a mixture of total milk fat esters, there was ample evidence of overlapping peaks. Furthermore, since not over half of the total number of components known to be present were in evidence, it is quite likely that many of the acids were present in concentrations too low to be detected.

The objective of this investigation was to study further the application of GLC, with supplementary means of prior fractionation, for identification of milk fatty acids. Fractional distillation and silicic acid column chromatography were employed to provide less complex fractions and concentrations of minor components which could be more readily resolved by GLC. In many instances however it was necessary to trap or collect fractions from the gas chromatograph and employ other means of identification. Evidence for the presence of at least sixty different acids was obtained, some of which had not been reported previously. To insure that this was not a unique sample, milk fat from a different herd was similarly investigated. The same fatty acids were identified. Details of the fractionation and a quantitative estimation of each individual acid in the latter sample is presented in another publication (11).

Experimental

The milk fat employed in this investigation was prepared from milk obtained from the dairy herd of the Agricultural Research Center, Beltsville, Md. The herd had been on winter rations for several months. The methyl esters of the milk fat were prepared by methanolysis with sodium methylate as catalyst and were distilled through a 30-in Vigreaux column under reduced pressure. Column efficiency was sacrificed for rapid throughput in order to avoid excessively long heating of the esters. Agitation in the still pot to lessen superheating was maintained by constant bleed-in of oxygen-free nitrogen through a fine capillary. Some of the methyl butyrate and any lower molecular weight esters were removed with the low boiling petroleum ether which was used in the recovery of the methyl esters from the methanolysis. Nine distilled fractions and an undistilled fraction were thus obtained. The undistilled portion was further separated into 7 fractions on a silicic acid column. All fractions were subjected to GLC and other supplemental examinations for identification of components.

Apparatus for GLC. The GLC apparatus and thermal conductivity detector were the same as described in earlier work (12). It was found advantageous to use a loosely packed plug of fine stainless steel wool in the portion of the sample injection chamber which is heated independently (usually 100–125C higher than column temperature). This served effectively to

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[2] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

wipe any sample left on the end of the syringe needle and allowed instantaneous volatilization of the sample. The exit section from the detector was independently heated 15–25C above the column temperature. This section consisted of a short length of small bore stainless steel (ss) tubing which had a ss ball joint welded to the end for easy clamping to a receiver. The receiver was simply a glass tube 12 x 3/8 in ID fitted with a corresponding socket and bent to a U-shape. Each leg of the U-tube was filled to a depth of about 3 in with 1/8 in diameter ss balls. During the collection of fractions, the receiver was cooled in a Dewar flask containing dry-ice in alcohol. When the same peak material was collected from a number of GLC separations on the same sample, the receiver, in the interim, was kept in the cold Dewar flask and the contents protected from autoxidation by leading a slight flow of oxygen-free nitrogen through the receiver. This receiver permitted condensation of the emerging component vapors and easy extraction of collected material with solvent, such as low boiling petroleum ether.

Columns for GLC. During the period of this investigation a number of coiled ss columns of different size and with different packings were employed. Some of these are described in connection with various chromatograms. In general, a column 15 ft x 3/8 in OD containing 16% (w/w) diethylene glycol adipate polyester crosslinked with pentaerythritol (LAC-2-R446, Cambridge Industries Co., Cambridge, Mass.) coated on 42–60 mesh Chromosorb was employed for esters of acids up to C_{18}, particularly when collection of components was required. For esters of acids C_{18} or greater, a column 8 ft x 1/4 in OD containing 20% ethylene glycol succinate polyester (11) coated on the same mesh Chromosorb was employed.

Supplemental Methods of Analysis. Iodine values were determined by the Wijs method as modified for small samples (21). The ultraviolet spectrophotometric method was used to determine or confirm the presence of conjugated and nonconjugated polyunsaturated acids (21).

Infrared techniques and interpretations were quite similar to those described earlier (28) except that micro cells were employed for the small fractions collected from GLC. Hydrogenations were conducted by the micromethod of Ogg and Cooper (23).

Results and Discussion

A typical gas-liquid chromatogram of the methyl esters of the total milk fatty acids (Fig. 1) shows overlapping peaks and only evidence for 26–28 components, hardly half of the total number known to be present. The positions of only a few of the components are indicated on the chromatogram as reference guides.

Numerous instances of coincident, or nearly the same, retention for certain fatty acid methyl esters have been reported in GLC analysis with a given column and stationary phase. Fortunately the same components do not often coincide in retention when two columns are employed, each with a stationary phase of different polarity. Most investigators have employed polar versus nonpolar columns. However it is often advantageous to use two columns with highly polar stationary phases which differ only in degree of polarity, as ethylene glycol succinate (EGS) vs. diethylene glycol adipate (DEGA). Similarly, a highly polar column operated at two widely different temperatures will often produce a difference in relative retention of the saturated and unsaturated components.

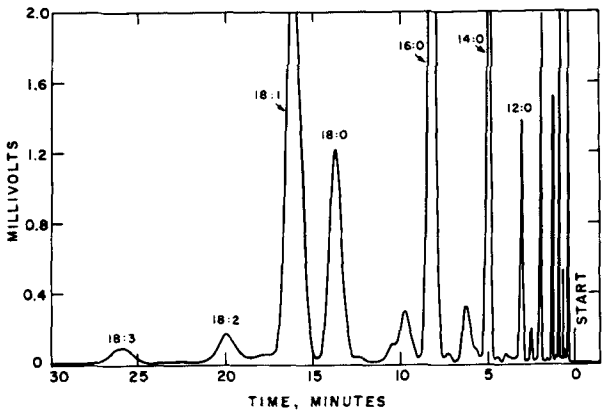

FIG. 1. Chromatogram of total methyl esters of milk fat. Column 8 ft x 1/4 in, temp 200C, stationary phase—25% EGS polyester.

Another technique which seemed generally more effective and advantageous was the use of a "new" EGS column versus the same or a similar column after it had been in operation at 200C or higher for several weeks. It was observed that a new EGS column, even after conditioning, undergoes a slow but fairly constant rate of "bleed" or decomposition. Succinic anhydride was isolated as one of the products. As the column "aged" in operation, the stationary phase seemed to become less polar in character and the retention times for the unsaturated esters changed at a different rate than the saturated. For this reason, when analyzing mixtures of components with close retention times, it was found necessary to chromatograph pertinent reference esters the same day in order to avoid misinterpretation of the chromatogram. The change in retentions on the aged column effected resolution of certain esters which had the same retention on the new column. For example, Figure 2 is a

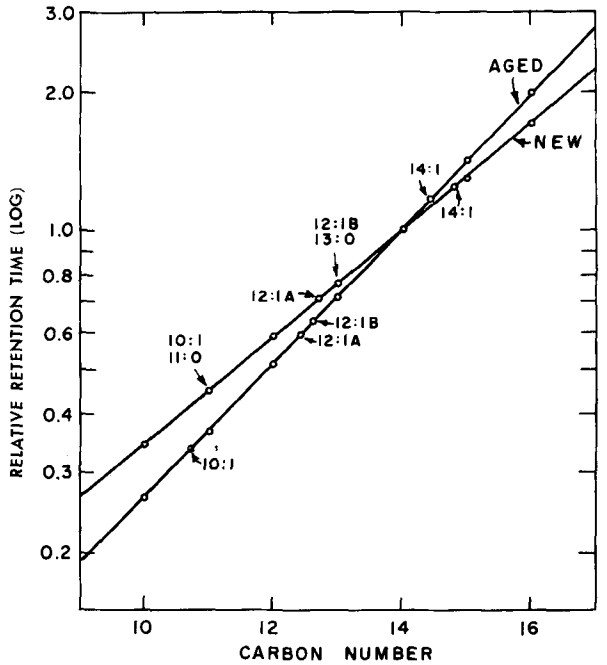

FIG. 2. Relationship between log retention time relative to methyl myristate and carbon numbers for some straight chain saturated and monounsaturated methyl esters on a "new" and "aged" column. Column 17 ft x 1/4 in, temp 203C, stationary phase—20% EGS polyester.

plot of the log of retention times relative to 14:0 [3] versus carbon numbers (2,33) for a new and aged column. The esters 10:1 and 11:0 and also 12:1B and 13:0 are found as coincident peaks on a new column at carbon number 11.0 and 13.0, respectively. However on an aged column both pairs are separated with carbon numbers of 10.7 and 11.0 for the former, and 12.6 and 13.0 for the latter pair. This technique was employed in a similar manner for all fractions. Hydrogenation of the sample followed by GLC is known to be a valuable supplementary technique in identification and was always employed for confirmation of the presence or absence of unsaturation as well as indicating the chain length of the compound.

The Distilled Fractions. Gas-liquid chromatograms were obtained on each of the distilled methyl ester fractions with columns of different polarity. Since it would be impracticable to present the great number of chromatograms obtained, only typical examples will be shown to illustrate the application of the techniques for identification.

The identification of the methyl esters of the normal straight chain saturated acids of even- and odd-numbered carbon atoms in the distilled fractions of milk fat esters offered no particular difficulty. These esters were identified by comparing the relative retentions of the peaks of the sample with those of known reference saturated esters on a new and on an aged column. When the relative retention of a peak on the new column coincided with a reference saturated ester it was tentatively identified as that acid. If on the aged column its relative retention again agreed with the reference, the assignment of identity was considered correct. For further confirmation the peaks representing 11:0 and 13:0 esters were trapped and independently identified by mixed melting points and comparison of their infrared curves with those of authentic 11:0 and 13:0 esters.

In the designation of branched chain esters, 13:0br, 14:0br, etc., the number refers to the total carbon atoms in the acid and the assumption was made that they were saturated and had only one methyl branch in the *iso* or *anteiso* position. This is consistent with the branched chain acids previously reported present in butter fat with the exception of a 20 carbon atom multibranched acid (10,29). Another C_{20} multibranched acid has been reported which has a retention time similar to 17:0 (32); therefore, the assignment of chain length should be considered tentative. A branched chain ester has been shown to immediately precede its normal saturated ester on a gas-liquid chromatogram (3,10,17). The identifications of the branched chain esters were based on the appearance in this position of certain peaks on the chromatogram before and after hydrogenation of the fraction. When the log of the relative retention time of each of these esters and of the normal saturated esters were plotted against chain length, curves were obtained which were similar to those reported by others (3,10,17) for the normal and branched chain esters. Additional evidence that branched chain acids were present was obtained by trapping the material designated as 14:0br. This ester was examined in the carbon-hydrogen stretching region of the infrared spectrum using LiF prisms. The ratio of the methyl band at 2872 waves/cm to the methylene band at 2853 waves/cm of the collected sample was considerably greater than that found for reference 14:0 indicating that it was indeed a branched chain ester.

Figure 3 is a chromatogram of distilled fraction which was principally esters of C_{10} acids. An attenuation of 8-fold was used for the 10:0 peak. The 10:1

[3] A "shorthand" designation of structure of each fatty acid is used throughout this paper, 18:0 = saturated C_{18} acid; 18:2 = C_{18} with 2 double bonds; 15:0br = branched chain C_{15} acid, etc.

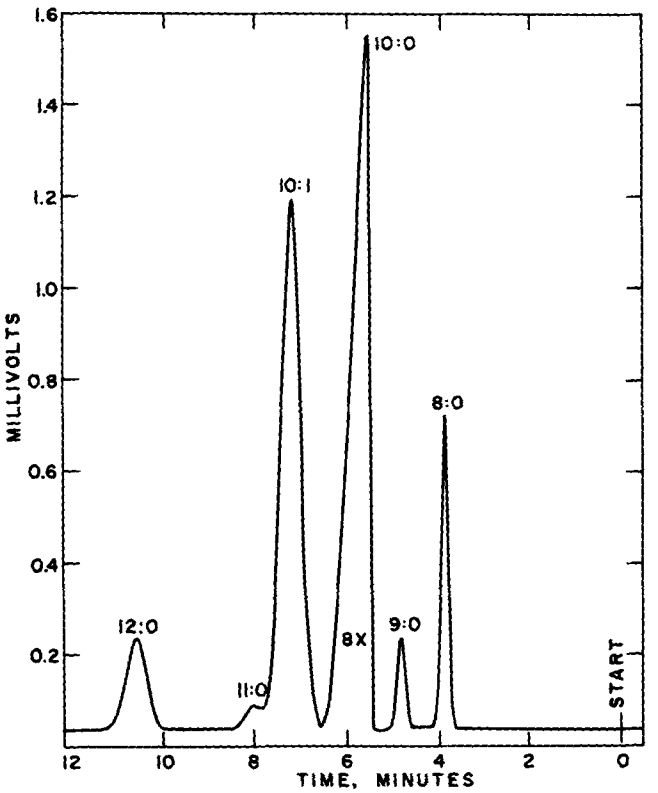

FIG. 3. Portion of chromatogram of a distilled fraction containing predominantly C_{10} methyl esters of milk fat. Column 15 ft x ⅜ in, temp 200C, stationary phase—16% LAC-2-R446. 8X—attenuation.

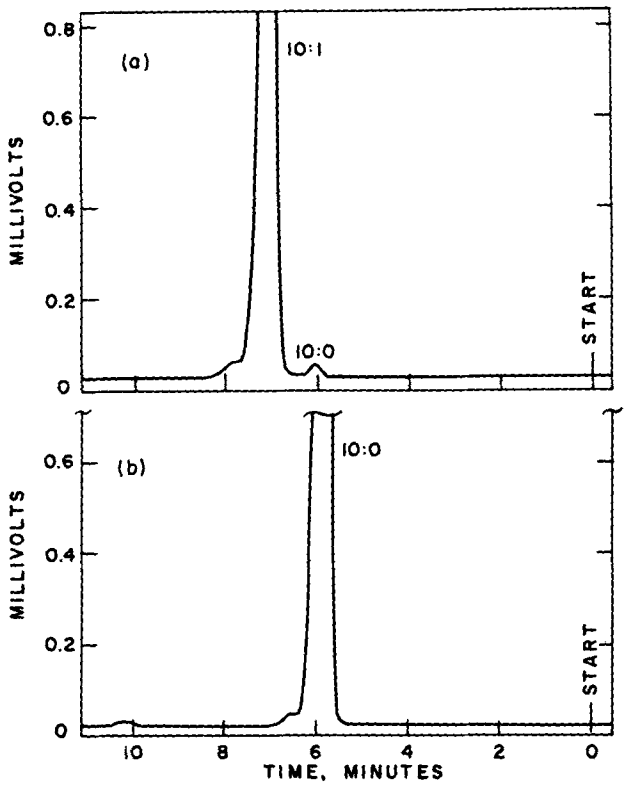

FIG. 4. Chromatograms of collected fraction of methyl decenoate. (a) Before hydrogenation. (b) After hydrogenation. Column same as in Fig. 3.

peak material was collected, a portion was chromatographed as shown by Figure 4a, and a portion was hydrogenated and chromatographed (Figure 4b). An iodine value and an infrared absorption curve were also run on the collected material. The iodine value found was 137; theory for 10:1 is 137.8. The absorption maxima at 990 and 908 waves/cm, shown in Figure 5, are characteristic of terminal double bonds (vinyl group) and are not evident in the curve for methyl oleate which has an internal double bond. It is clear from the gas-liquid chromatograms and the other data that the identity assignment of 10:1 containing a terminal double bond is reasonable. Comparable collection and treatment of 12:1 and 14:1 indicated that the isomer with a terminal bond was present but that the ester with an internal double bond was predominant and had both the *cis* and *trans* configuration.

Another example where there was serious overlapping in retention is shown in Figure 6a. In this chromatogram of distilled fraction the identity assignments shown were made from consideration of a number of independent analyses in conjunction with the relative retentions. Iodine values along with ultraviolet and infrared spectrophotometric absorption were determined on the distilled fraction and on a collection of material from the GLC represented by 16:1, 17:0br (isomers A and B), 17:0, and 17:1. The retention of a 16:2, if present, would probably overlap in this region but UV analysis indicated only slight absorption, probably insignificant, in the diene region. Infrared analysis showed the presence of isolated *trans* double bonds, presumably some of the 16:1 since the *trans* isomer has been reported (1). A portion of the distilled fraction was hydrogenated and chromatographed (Figure 6b). Consideration of this chromatogram in relation to Figure 6a indicates the presence of the assigned acids.

The Undistilled Fraction. The undistilled portion of methyl esters remaining from the distillation would of course be expected to contain higher molecular weight components including polyunsaturated esters. Figure 7, a gas-liquid chromatogram of this fraction, confirms this and also shows evidence of unresolved peaks. This complexity was further verified by ultraviolet and infrared analysis. The peaks are labeled to show the esters that were later identified in the fraction. Since evidence of odd carbon atom monoethenoid acids had already been obtained in some of the distilled fractions, it seemed probable that higher molecular weight members of the series would be present also in this portion. Previous work (13,14,26) had shown that silicic acid adsorption chromatography allowed substantial fractionation of such mixtures on the basis of degree of unsaturation of the components, the saturated being eluted first, then those with 1, 2, 3, 4 or more double bonds successively with some overlapping. This type of fractionation was employed to further simplify the undistilled methyl

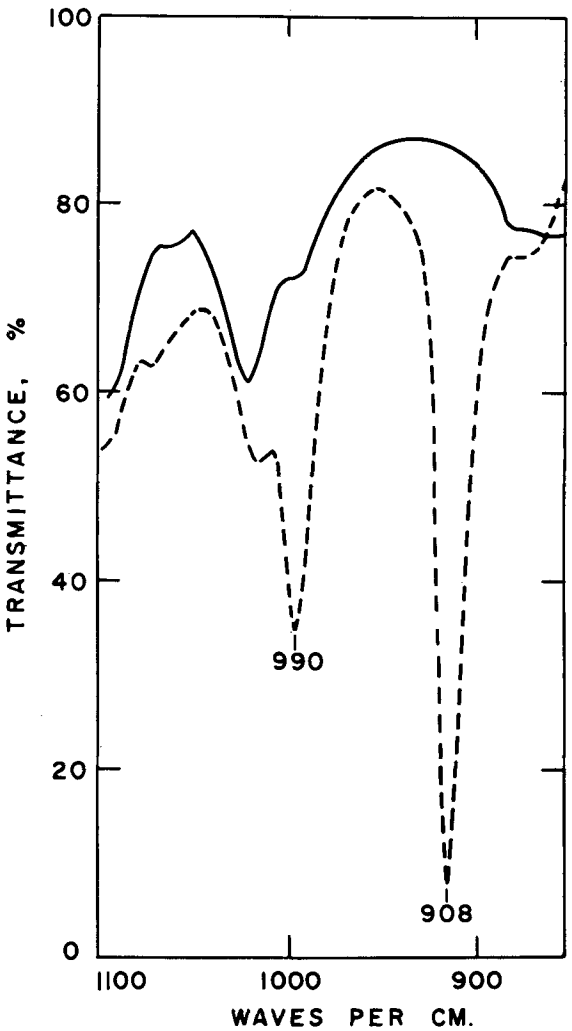

FIG. 5. Portion of infrared spectrum showing absorption maxima for vinyl group in methyl decenoate. - - - - - - - - Methyl decenoate. ———— Methyl *cis* 9, octadecenoate.

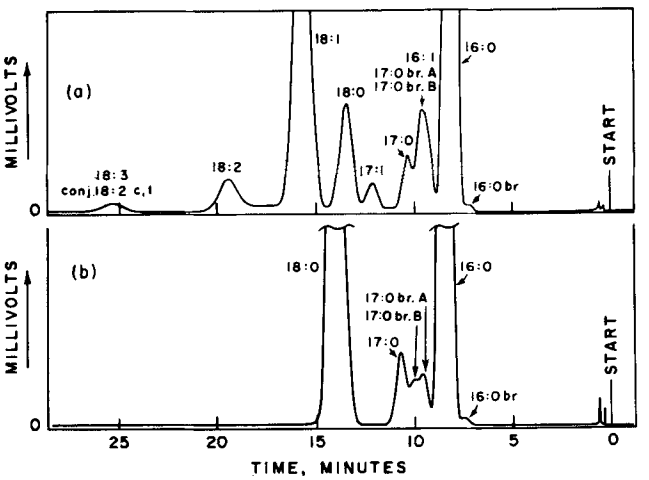

FIG. 6. Chromatograms of a distilled fraction of milk fat methyl esters. (a) Before hydrogenation. (b) After hydrogenation. Column 8 ft x ¼ in, temp 200C, stationary phase—25% EGS polyester, *c,t* = *cis-trans*.

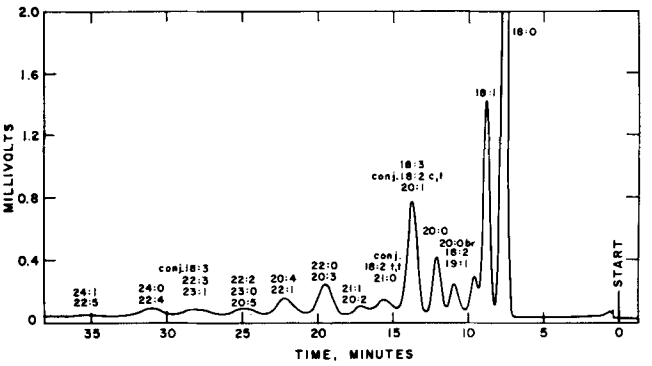

FIG. 7. Chromatogram of undistilled fraction of milk fat methyl esters. The peaks are labeled to show the esters that were later identified in the fraction. Column 8 ft x 3/16 in, temp 225C, stationary phase—25% EGS polyester, *c,t* = *cis-trans* and *t,t* = *trans-trans*.

ester fraction. Seven fractions were thus obtained, the unsaponifiable material coming off in the last (7th) fraction. These were then subjected to GLC analysis. Chromatograms of Fractions 1 to 3 are shown in Figure 8 to illustrate separation achieved by the silicic acid column and to show evidence of even- and odd-carbon saturated acids up to 26:0 inclusive, and even- and odd-carbon monoethenoid acids 18:1 to 24:1 inclusive.

Fraction 3 (Figure 9) was rechromatographed on a different column using a larger sample and for comparison a hydrogenated portion of the sample was run on the same column. It is evident from the shift in retentions on hydrogenation that the series of monoethenoid acid esters was correctly designated. The presence of 20:0br ester is also indicated in the hydrogenated portion. The peak for this component was masked by the 19:1 peak in the chromatogram of the unhydrogenated fraction. The odd-carbon chain length monounsaturated fatty acids shown in Figures 8 and 9 along with the 15:1 and 17:1 found in other fractions constitute a homologous series of monoenes 15:1 to 23:1 which have not previously been reported in cows' milk fat. However since completion of this work it has come to our attention that Hansen, Shorland, and Cooke (8A) have identified 17:1 in butterfat.

The gas-liquid chromatogram of Fraction 6 (from the silicic acid column separation) is shown in Figure 10. The presence of acids containing 2, 3, 4, and 5 double bonds in the sample was shown independently by ultraviolet spectrophotometric analysis, which of course does not differentiate between acids of different chain length and same number of double bonds. The material represented by 20:3 and 20:4 (Figure 10) was collected as one fraction. A portion was chromatographed and is shown in Figure 11a, and a portion was hydrogenated and chromatographed (Figure 11b). From consideration of these two chromatograms it was determined that about equal areas are represented by the two peaks and that the components are predominantly C_{20}. The peak representative of 18:0 in the hydrogenated portion is probably due to a small amount of conjugated 18:3 which is coincident with 20:3. Small amounts of impurities show up as minor peaks in this chromatogram, probably because the amount chromatographed was about twice that of the unhydrogenated portion. The two peaks were assigned 20:4 and 20:3 from their relative retentions, although the retention of a conjugated 20:2 probably would coincide with that of 20:3. However, examination of the collected material of the two peaks by ultraviolet spectrophotometry before alkali isomerization indicated only about 3% conjugated diene and 1% triene; after alkali isomerization, the analysis showed only 32% tetraene, 6% triene, and 17% diene (calculated as C_{20} acids). The iodine number was about 15% lower than theory for an equal mixture of 20:4 and 20:3. Admittedly, owing to the

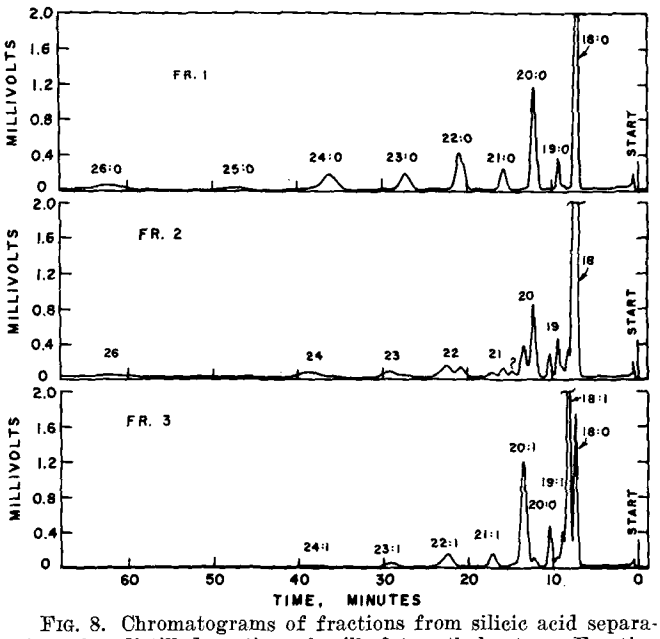

FIG. 8. Chromatograms of fractions from silicic acid separation of undistilled portion of milk fat methyl esters. Fraction 1, long chain saturated esters. Fraction 2, mixture of long chain saturated and monounsaturated esters. Fraction 3, principally long chain monounsaturated esters. Column 8 ft x ¼ in, temp 220C., stationary phase—25% EGS polyester.

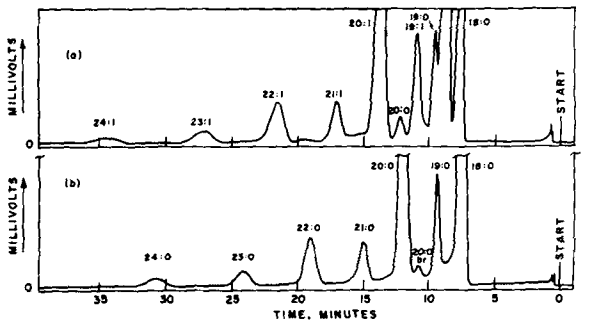

FIG. 9. Chromatograms of Fraction 3 from silicic acid separation of undistilled portion of milk fat methyl esters. (a) Before hydrogenation. (b) After hydrogenation. Column same as in Fig. 7.

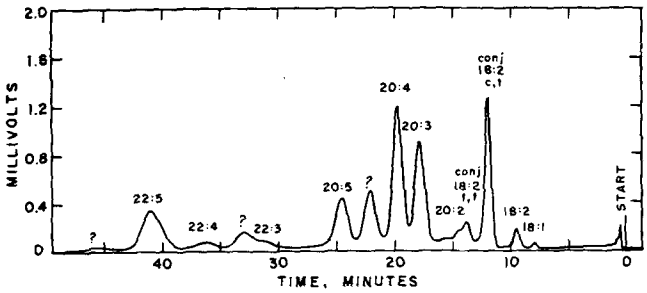

FIG. 10. Chromatogram of Fraction 6 from silicic acid separation of undistilled portion of milk fat methyl esters. Column same as in Fig. 8. *c,t* = *cis-trans* and *t,t* = *trans-trans*.

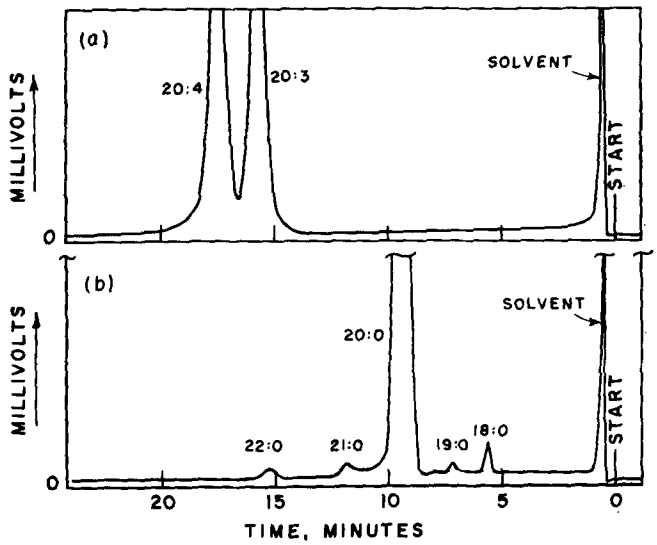

FIG. 11. Chromatograms of collected fraction containing 20:3 and 20:4. (a) Before hydrogenation. (b) After hydrogenation. Column same as in Fig. 7.

handling of very small samples collected from GLC for analyses, absolute percentages would hardly be expected. Yet, if the 20:4 and 20:3 components had the usual one-methylene-interrupted double bond systems which would conjugate normally, it seems reasonable that the ultraviolet spectrophotometric analysis would have approximated a 1:1 ratio of the two and would have accounted for a higher percentage of the material, more in agreement with the iodine value which indicated 85% as 20:4 and 20:3. Hence the only reasonable interpretation is that isomers were present which had one or more isolated double bonds. This seemed to apply more to the 20:3 than to the 20:4, as judged by low triene compared to tetraene after alkali isomerization. The apparent diene (17%) found after isomerization was probably produced from 20:3 isomers which had one double bond isolated from the other two by more than one methylene.

In all, evidence was obtained for the presence of at least 60 different acids, including the following: normal saturated even- and odd-carbon from 4:0 to 26:0; branched chain saturated 13:0br, 14:0br, 15:0-brA, 15:0brB, 16:0br, 17:0brA, 17:0brB, 18:0br, 20:0-br; even-carbon monoethenoid from 10:1 to 24:1; odd-carbon monoethenoid from 15:1 to 23:1; diethenoid 18:2, 20:2, and 22:2; conjugated *cis-trans* 18:2 and *trans-trans* 18:2; triethenoid 18:3, 20:3, and 22:3; conjugated 18:3; tetraethenoid 20:4 and 22:4; and pentaethenoid 20:5 and 22:5 acids.

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Fatty Acids of Cows' Milk. B. Composition by Gas-Liquid Chromatography Aided by Other Methods of Fractionation[1]

S. F. HERB, PAUL MAGIDMAN, FRANCIS E. LUDDY, and R. W. RIEMENSCHNEIDER,
Eastern Regional Research Laboratory,[2] Philadelphia 18, Pennsylvania

A fresh sample of cows' milk was converted directly to the methyl esters by methanolysis and the esters were fractionated into eleven distilled fractions and an undistilled portion. The latter, which contained the bulk of the polyunsaturated and long chain esters, was further fractionated by adsorption chromatography on a silicic acid column. Each fraction from the distillation and adsorption chromatography was analyzed by gas-liquid chromatography on columns containing polyester stationary phases of different polarity. Twenty-seven minor components, including some not previously reported, were present each in less than 0.1%. The fatty acid distribution of the major components fell within the range generally reported.

PUBLISHED quantitative data on the fatty acids of cows' milk fat have generally been limited to less than half of the number of acids that have been reported in the fat owing to the difficulties of determining many of the minor components. Typical fatty acid analyses of various milk fats by methods other than gas-liquid chromatography (GLC) are given in a review by Jack and Smith (10). Many of the minor fatty acids with an estimation of the amounts found are reported by Shorland and Hansen (18). In the past few years, a number of papers have been published in which GLC has been employed to determine the fatty acids in various fractions of milk fat. The analysis of the total fatty acids of cows' milk fat or butterfat by GLC has also been attempted (2,4,11,14,15,17,19,20). The results were, in general, comparable to those determined by other methods, accounting for less than half of the total number of fatty acids. The difficulties of employing GLC to determine all acids present in milk fat were clearly recognized by Patton, *et al.* (15) who stated in part: "The problem of revealing the C_{19} to C_{26} fraction

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[2] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.