# Detection of Trace Fatty Acids in Fats and Oils by Urea Fractionation and Gas-Liquid Chromatography[1]

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## Abstract

The detection of trace fatty acids (<0.1%) in a fat or oil by gas-liquid chromatography is possible when the methyl esters are fractionated with urea to provide a number of less complex fractions. Identification and estimation of trace fatty acids is simplified by quantitative removal of other fatty acids having similar gas chromatographic retention times. A detailed knowledge of the order in which inclusion compounds are formed was obtained by fractionating a complex mixture of marine and vegetable fatty acids. In addition, lanolin was fractionated to determine the preferential order in which saturated, branched chain (*iso-, anteiso-*) and hydroxy acids form inclusion compounds. Using urea fractionation and gas chromatography, 52 trace fatty acids were tentatively identified in butter, 30 in lard, and 26 in walnut oil.

## Introduction

ALTHOUGH GAS-LIQUID CHROMATOGRAPHY (GLC) is a powerful analytical tool for determining the fatty acid composition of fats and oils, it does not achieve complete resolution of all fatty acids, both major and minor, so that they can be qualitatively determined from a single chromatogram. The detection of $C_{20}$ and higher molecular weight trace fatty acids present in a complex oil is made difficult by the presence of other high molecular weight fatty acids which elute with increasing peak widths as the retention time increases. Also, the presence of $C_{16}$ to $C_{20}$ trace fatty acids is masked by major fatty acid peaks. The need for fractionation of complex mixtures prior to GLC is clearly indicated. Such fractionations have been carried out successfully by urea complexing, a technique that is not destructive and requires only simple equipment and inexpensive reagents. Urea fractionation has been used in this laboratory to detect the minor fatty acids of peanut oil (1) and olive oil (2).

Formation of inclusion compounds by complexing with urea is widely used in the analysis of fats and oils, often without a precise knowledge of the preferential order in which fatty acids are complexed with urea. Inclusion compounds are formed by urea "host" molecules joining together to form a spiral "trap" around a fatty acid or ester "guest" molecule. Inclusion compounds are formed more readily with increasing fatty acid chain length, and with greater difficulty as the chain adds unsaturation, branching, and hydroxyl or other functional groups. Excellent reviews of inclusion compounds and their use in the separation of fatty acids have been presented by Swern (3,4). Schlenk's (5) data point out some of the separations which can be achieved with urea. Dissociation temperatures of urea complexes depend upon the stability of the inclusion compounds at higher temperatures and indicate the order of stability of urea complexes (3,6,7). It is impossible to predict precisely the preferential order in which urea forms inclusion compounds with fatty acids in a complex mixture.

Patton et al. (8) used polar and nonpolar GLC columns to detect 17 fatty acids present in milk. GLC would not resolve the trace fatty acids known to be present in milk (8–12). Herb et al. (13) collected fractions from a nonpolar GLC column and gas chromatographed them on a polar column, detecting 29 fatty acids in lard. Magidman et al. (14) used distillation and silicic acid chromatography to obtain less complex fractions, detecting 64 fatty acids in cow's milk. The milk fat fractions were gas chromatographed on two polar columns of different polarity. If the relative retention time of a peak agreed with known references, the assigned identity was considered correct. Sen and Schlenk (15) determined the fatty acid structure of mullet oil by using distillation followed by gas chromatography.

The present paper describes the use of urea fractionation followed by GLC for analysis of a complex mixture of marine and vegetable fatty acids, lanolin (degras) fatty acids, butter, lard, and walnut oils. The complex mixture of marine and vegetable fatty acids and the lanolin fatty acids were fractionated with urea to establish the order in which 25 common fatty acids, branched acids, and hydroxy acids formed urea inclusion compounds. A standard urea fractionation procedure was then used to determine the fatty acid composition of butter, lard, and walnut oil with particular emphasis on the trace and unusual fatty acids present in these oils.

## Experimental

### Sample Preparation

The complex mixture of marine and vegetable fatty acids was prepared by mixing appropriate amounts of commercially available menhaden, herring, sardine, coconut, jojoba, olive, and peanut oils. The marine oils contributed a wide variety of long chain unsaturated fatty acids to the mixture, jojoba contributed $C_{20}$ and $C_{22}$ monoene acids, coconut oil contributed short chain saturated fatty acids, peanut oil contributed long chain saturated fatty acids, and olive oil contributed additional linoleic acid.

Lanolin (USP), sweet cream butter, lard (antioxidant free and without added stearin or hydrogenated lard), and French walnut oil were obtained from commercial sources.

The complex mixture and the other samples were saponified and the unsaponifiables extracted with ethyl ether according to AOAC method 26.049 (16). The soaps were acidified with HCl and the fatty acids extracted with diethyl ether-petroleum ether (1 + 1). Petroleum ether was redistilled through glass (BP 30–60C). The extract was dried with anhydrous sodium sulfate, and the solvent was evaporated on a steam bath. The last traces of solvent and moisture were removed on the steam bath under a vacuum of

[1] Presented at the AOCS Meeting in New Orleans, 1964.

TABLE I

Isothermal Gas Chromatographic Analyses of Reference Fatty Acid Methyl Ester Mixture and Applications of Correction Factors[a]

| Acid | True wt., % | Found % | | |
|---|---|---|---|---|
| | | Uncorrected peak area | Molecular wt., correction[b] | Square root correction[c] |
| Mixture A[d] | | | | |
| 16:0 | 20.00 | 19.79 | 18.42 | 19.10 |
| 18:0 | 20.02 | 18.62 | 19.13 | 18.87 |
| 18:1 | 19.99 | 20.78 | 21.21 | 20.99 |
| 18:2 | 20.00 | 21.27 | 21.56 | 21.42 |
| 18:3 | 19.99 | 19.54 | 19.68 | 19.61 |
| Mixture B[e] | | | | |
| 12:0 | 11.49 | 10.49 | 7.42 | 8.48 |
| 14:0 | 10.86 | 10.51 | 8.44 | 9.50 |
| 16:0 | 11.90 | 11.86 | 10.58 | 11.28 |
| 18:0 | 14.20 | 15.09 | 14.87 | 15.09 |
| 20:0 | 22.16 | 23.32 | 25.13 | 24.56 |
| 22:0 | 29.38 | 28.69 | 33.56 | 31.25 |

[a] Average of triplicate analyses with ±2% precision.
[b] M. W. Corr. = (M. W. Methyl Ester/M. W. Methyl Stearate) × Area of Methyl Ester Peak.
[c] Sq. root M. W. Corr = Area × (M. W. Methyl Stearate)$^{1/2}$/(M. W. Methyl Ester)$^{1/2}$.
[d] Purchased from the Hormel Institute, Austin, Minnesota.
[e] Weighed from individual Hormel standards.

6–10 mm Hg. The fatty acids were converted to methyl esters by the $BF_3$ method (17) and extracted with ethyl ether-petroleum ether (1 + 1), and the solvent was evaporated on the steam bath under vacuum.

### Urea Fractionation of Complex Mixture of Marine and Vegetable Fatty Acids

A total of 250 g of methyl esters was separated into 25 fractions by a modification of the standard urea fractionation (see below). The complex fatty acid composition of this mixture required additional fractions to establish the preferential order in which inclusion complexes were formed. The ratio of esters to solvent was kept constant at approximately 1 to

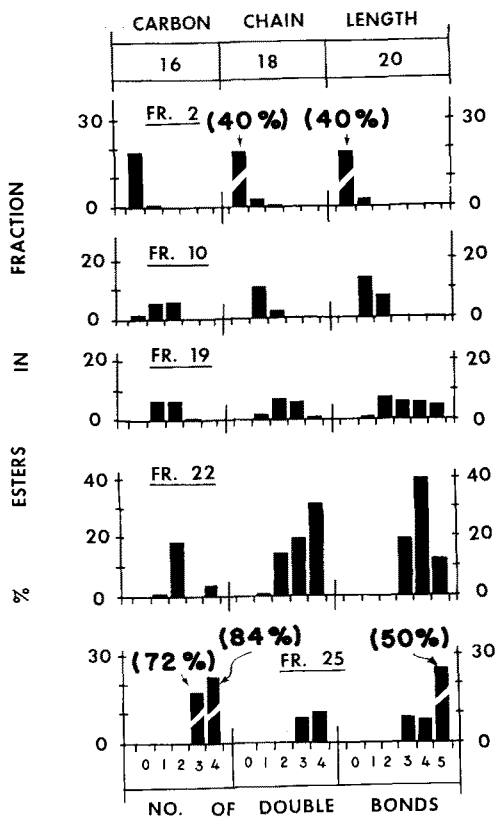


FIG. 1. $C_{16}$, $C_{18}$, and $C_{20}$ fatty acid composition of fractions 2, 10, 19, 22, and 25 from a complex mixture of marine and vegetable fatty acids. FR = urea complexed fraction.

20. This necessitated reducing the total volume after each fractionation by evaporating the filtrate on a steam bath under nitrogen.

### Urea Fractionation of Lanolin

Thirty grams of methyl esters were separated into 15 fractions by a modification of the standard urea fractionation procedure starting with a solution containing 50 g of urea in 6,000 ml of methanol. For fractions 2–5, no additional urea was added but the volume of filtrate was reduced by about 1,500 ml after each fractionation so that the filtrate from the 5th fraction contained about 1,200 ml. Fractions 6–10 were obtained by addition of 15 g of urea to the previous filtrate, followed by evaporation of about 125 ml of solvent on the steam bath prior to cooling and crystallization of adduct. Fractions 11 and 12 were obtained from 600 ml of filtrate by adding 100 g of urea prior to each fractionation. Fractions 13 and 14 (final adduct and filtrate, respectively) were obtained from the filtrate of fraction 12 by complexing with excess urea as described below.

### Standard Fractionation Procedure for Butter, Lard and Walnut Oil

A 25 g mixture of methyl esters was dissolved in 400 ml of methanol, 25 g of urea was added, and the mixture was heated with intermittent stirring until the urea dissolved. The solution was held at room temperature for 3 to 4 hr, with intermittent stirring during the first ½ hour to permit the complex to crystallize and equilibrate at room temperature. The urea-complexed esters were removed by filtration through a stainless steel powder funnel holding a pledget of pyrex wool and were rinsed two times with 10 ml portions of urea-saturated methanol. The adduct esters were recovered by breaking the complex with 150 ml of 1% HCl solution, transferring the methyl esters into a separatory funnel with 50 ml of methyl alcohol, and extracting with 150 ml of petroleum ether. The methyl esters were extracted a second time from the aqueous phase with a 150 ml portion of ethyl ether and finally with a 150 ml portion of petroleum ether. The esters were washed free of acid with small (10 ml) portions of distilled water and dried with anhydrous sodium sulfate; the solvent was evaporated on a steam bath, and the last traces of solvent and moisture were removed under a vacuum of 6–10 mm Hg. The esters were cooled and weighed to obtain fraction 1.

Fraction 2 was obtained by adding 20 g of urea to the filtrate from fraction 1; methanol was added if necessary to maintain a constant volume of about 425 ml. The sample was heated, stirred, cooled, and filtered as above to obtain fraction 2. Fractions 3–7 were obtained in a similar manner.

An 8th and a 9th fraction (final adduct and filtrate, respectively) were obtained after recovery of the esters in the filtrate from fraction 7. The filtrate was transferred to a 1,000 ml separatory funnel, 400 ml of 1% HCl was added, and the esters were extracted as above. These esters were complexed with excess urea by adding 10 parts of urea (passed through a No. 30 sieve), 3 parts of methanol, and 1 part of esters, warming to 50C on a steam bath, and stirring vigorously for 30 sec. The mixture was stirred again after 5 min, removed from the steam bath, and stirred four additional times while cooling to room temperature. After final stirring, the complex was allowed to stand at room temperature for 4 hr. The mixture

was then filtered through a medium porosity fritted glass Buchner funnel. The adduct was washed 3 times with 25 ml of urea-saturated methanol, and the washings were saved. The final adduct (fraction 8) was recovered in the same manner as fraction 1. The final filtrate (fraction 9) was recovered in the same manner as the filtrate esters from fraction 7.

#### Hydrogenation

A portion of each fraction was hydrogenated at room temperature for 20 min in a Parr reaction apparatus (Parr instrument Company, Moline, Ill.) at a pressure of 50 psi of hydrogen. One part of 10% palladium on charcoal catalyst was used to 10 parts of sample dissolved in 10 volumes of absolute methanol.

#### Gas Chromatograhy

An Aerograph dual column gas chromatograph, Model A-350B, with thermal conductivity cell was used for the analyses. Two well-conditioned ¼ in. by 6 ft aluminum columns packed with 20% (w/w) polydiethylene glycol succinate (DEGS) on acid-washed Chromosorb W (80–100 mesh), and two 3/16 in. by 9 ft aluminum columns packed with 8% (w/w) Apiezon L on Chromosorb P (60–80 mesh) were used. The instrument was either operated isothermally or programmed, or a combination of both techniques was used depending on the particular need.

#### Identification

Chromatographic peaks were identified by (a) comparing logarithmic plots of retention time against chain length with plots obtained from known standards, (b) comparing chromatograms of nonhydrogenated and hydrogenated samples, and (c) injecting known standards or previously identified secondary standards with the fraction and establishing identities by observing increases in peak area. In addition, branched chain and hydroxy acids in lanolin were tentatively identified by using (a) $C_{15}$ *iso*, $C_{16}$ *iso* and $C_{17}$ *anteiso* reference standards furnished by Dr. R. P. Hansen of New Zealand, (b) $C_{16}$ and $C_{18}$ hydroxy standards obtained from commercial sources, and (c) logarithmic plots of programmed retention times to tentatively identify homologous series of components as suggested by Miwa (18). Multiple branched fatty acids in milk fat and lard were tentatively identified by logarithmic plots of programmed retention times. The presence of 3,7,11,15-tetramethylhexadecanoic acid (19) and 2,6,10,14-tetramethylpentadecanoic acid (20) has been reported in milk fat.

#### Component Calculations

Fatty acids were estimated quantitatively by triangulation of peak areas for programmed chromatograms and by the retention time-peak height method (21) for isothermal chromatograms. Two standard mixtures of fatty acid methyl esters were examined by isothermal analysis to determine whether uncorrected peak areas were satisfactory or whether correction factors based on molecular weight or square root of molecular weight (22–24) were desirable. The results (Table I) indicated that calculations based on uncorrected peak areas were preferable. Quantitation of temperature programming was not checked with standard mixtures; it was assumed that response would be similar to that obtained isothermally. Reproducibility was ±2% with a relative error (amount present-amount found/amount present) of ±4% at the 20% level. High purity standards for unusual fatty acids (branched chain, hydroxy, etc.) were not available. We were primarily interested, however, in determining the presence or absence of these fatty acids and their approximate concentration.

TABLE II

Fatty Acid Composition of USP Lanolin (% of Total Saturated, Branch and Hydroxy Acids)

| Acid[a] | % | Acid | % | Acid | % |
|---|---|---|---|---|---|
| 8:0 hy | 1.2 | 16:0 hy | 22.7 | 22:0 i | 4.3 |
| 10:0 hy | 2.2 | 17:0 | [b] | 22:0 hy | [b] |
| 12:0 hy | 0.8 | 17:0 a | 2.0 | 23:0 | 0.2 |
| 12:0 | 0.7 | 18:0 | 1.7 | 23:0 a | 4.0 |
| 12:0 i | 0.1 | 18:0 i | 3.5 | 24:0 | 3.3 |
| 13:0 | < 0.1 | 18:0 hy | 3.8 | 24:0 i | 4.3 |
| 13:0 a | < 0.1 | 19:0 | 0.6 | 25:0 | 1.8 |
| 14:0 | 2.2 | 19:0 a | 4.0 | 25:0 a | 4.4 |
| 14:0 i | 1.5 | 20:0 | 0.6 | 26:0 | 2.3 |
| 14:0 hy | [b] | 20:0 i | 5.4 | 26:0 i | 1.5 |
| 15:0 | 0.5 | 20:0 hy | 1.8 | 27:0 | 0.9 |
| 15:0 a | 1.0 | 21:0 | 0.1 | 27:0 a | 0.9 |
| 16:0 | 3.3 | 21:0 a | 4.9 | 28:0 | 0.5 |
| 16:0 i | 2.4 | 22:0 | 3.5 | 28:0 i | 1.5 |

[a] First number indicates number of carbon atoms in fatty acid moiety; second number, the number of double bonds; i = *iso*; a = *anteiso*; hy = hydroxy (assumed to be in the α position).
[b] Interfering peak prevents accurate estimation.

#### Cumulative Distribution Curves

The fatty acid composition of each urea fraction was determined by gas chromatographic analysis, and cumulative distribution curves were drawn for each fatty acid present in the mixture. The percentage of a particular component present in each urea fraction was found by dividing the amount present in the fraction by the amount present in all fractions. A cumulative distribution curve was obtained by plotting the amount present in fraction 1 on the fraction 1 axis, the total amount present in fractions 1 and 2 on the fraction 2 axis, and so forth until 100% was reached. The plots were prepared so that the abscissa (urea fractions) also indicated the cumulative percentage of esters removed as urea fractionation progressed. A horizontal dashed line was drawn through the cumulative distribution curves at the 50% level to indicate the order in which fatty acid methyl esters were removed from the mixture by formation of urea inclusion compounds.

## Results and Discussion

#### Complex Mixture of Marine and Vegetable Oils

Long chain saturated fatty acids were concentrated in the first four fractions and were quantitatively removed from the mixture by urea fractionation, whereas the highly unsaturated fatty acids were concentrated in the last fractions (21–25). There were five pairs of fatty acids with nearly equal equilibrium constants: (a) 16:0 and 24:1, (b) 14:0 and 22:1,

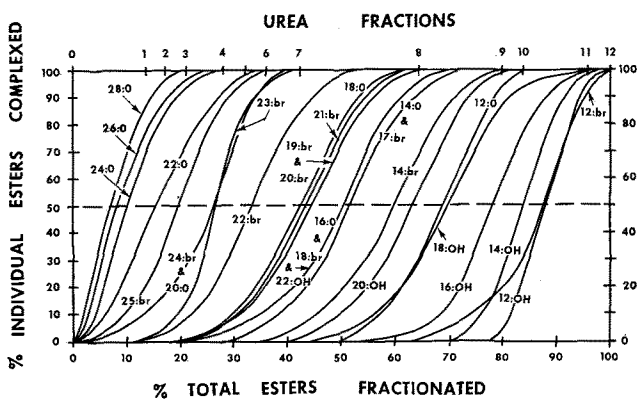


FIG. 2. Cumulative distribution curves for fatty acids present in lanolin; OH = hydroxy, br = branched (even chain length acids are *iso*, odd chain length acids are *anteiso*).

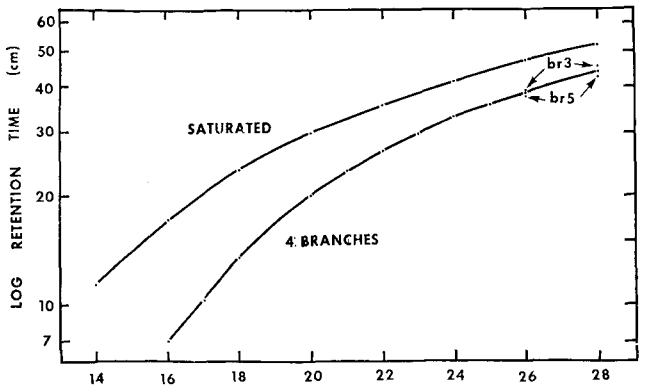


FIG. 3. Semilogarithmic plot of retention time vs. chain length for butter fraction 9 hydrogenated and temperature programmed at 3C/min (165–245C) on a 20% DEGS polyester column. The retention time of the $C_{19}$ tetrabranched acid could not be precisely determined due to interfering peaks.

(c) 12:0 and 18:1, (d) 10:0 and 16:1, and (e) 14:1 and 18:3. It would be impossible to separate these fatty acids by urea fractionation. Addition of the naturally occurring *cis* double bond to a methyl ester was observed to be equivalent to decreasing the chain length by 2 to 8 carbon atoms, depending on chain length and number of double bonds present.

The bar graph (Fig. 1) illustrates the separations that were achieved. Percentages on the vertical axis apply to individual fatty acid esters in each fraction. Fraction 2 contained approximately 20% of the palmitate, 40% of the stearate, and 40% of the arachidate present in all fractions. All of the arachidate was removed prior to fraction 10 and all of the palmitate prior to fraction 19. The $C_{16}$ fatty acid with 4 double bonds was concentrated in the last fractions (21–25) with 4% in the 22nd fraction and over 80% in fraction 25.

Routine GLC methods do not adequately resolve all of the fatty acid esters of a complex oil such as menhaden oil for quantitation of the fatty acids. The following $C_{18}$ acid peaks may elute together: 18:0 and 16:2, 18:1 and 16:3, 18:3 and 20:1, and 18:4 and 20:2. In all cases, the individual acids of these pairs were separated almost quantitatively into different fractions by urea fractionation.

TABLE III

Fatty Acid Composition of Butter Oil (% of Total Fatty Acid Methyl Esters)

| Acid[a] | | Acid | | Acid | |
|---|---|---|---|---|---|
| Saturated | % | Branched contd. | % | Dienes contd. | % |
| 8:0 | 0.69 | 19:0 br | 0.01 | 22:2 | 0.14 |
| 10:0 | 1.88 | 20:0 br | 0.01 | 24:2 | 0.02 |
| 11:0 | 0.12 | 21:0 br | 0.01 | 26:2 | 0.0004 |
| 12:0 | 2.96 | 22:0 br | 0.02 | | |
| 13:0 | 0.10 | 23:0 br | 0.01 | Polyenes | |
| 14:0 | 11.2 | 24:0 br | 0.02 | 18:3 | 1.03 |
| 15:0 | 1.52 | 25:0 br | 0.004 | 18:4 | 0.10 |
| 16:0 | 27.8 | 26:0 br | 0.004 | 20:3 | 0.05 |
| 17:0 | 0.71 | | | 20:4 | 0.07 |
| 18:0 | 12.1 | Monoenes | | 20:5 | 0.02 |
| 19:0 | 0.05 | 10:1 | 0.48 | 22:3 | 0.03 |
| 20:0 | 0.02 | 12:1 | 0.05 | 22:4 | 0.04 |
| 21:0 | 0.06 | 13:1 | 0.003 | 22:5 | 0.02 |
| 22:0 | 0.04 | 14:1 | 0.75 | | |
| 23:0 | 0.01 | 15:1 | 0.02 | Multibranched[b] | |
| 24:0 | 0.02 | 16:1 | 1.84 | 16:0 br3 | 0.01 |
| 25:0 | 0.02 | 17:1 | 0.2 | 17:0 br3 | 0.01 |
| 26:0 | 0.02 | 18:1 | 30.3 | 18:0 br3 | 0.16 |
| 27:0 | 0.00004 | 19:1 | 0.14 | 19:0 br4 | 0.02 |
| 28.0 | 0.00004 | 20:1 | 0.52 | 20:0 br4 | 0.14 |
| | | 21:1 | 0.01 | 21:0 br4 | 0.02 |
| Branched[b] | | 22:1 | 0.02 | 22:0 br4 | 0.02 |
| 12:0 i | 0.01 | 23:1 | 0.05 | 23:0 br4 | 0.01 |
| 13:0 i | trace | 24:1 | 0.0008 | 24:0 br4 | 0.10 |
| 14:0 i | 0.03 | 25:1 | 0.0008 | 25:0 br4 | 0.10 |
| 15:0 i | 0.14 | 26:1 | 0.0008 | 26:0 br3 | 0.01 |
| 15:0 a | 0.23 | | | 26:0 br4 | 0.04 |
| 16:0 i | 0.2 | Dienes | | 27:0 br4 | 0.04 |
| 17:0 i | 0.36 | 14:2 | 0-04 | 28:0 br3 | 0.02 |
| 17:0 a | 0.23 | 16:2 | 0.02 | 28:0 br4 | 0.12 |
| 18:0 i | 0.02 | 18:2 | 2.22 | 28:0 br5 | 0.01 |
| | | 20:2 | 0.12 | | |

[a]First number indicates number of carbon atoms in fatty acid moiety; second number the number of double bonds; i = *iso*; a = *anteiso*; br = *iso* and/or *anteiso*; last number indicates number of methyl branches for multibranched acids.

[b]Tentatively identified in appropriate urea fractions by semilogarithmic plots of GLC retention times.

### Lanolin (degras)

Lanolin (64% unsaponifiables) was chosen for fractionation because it contains branched chain (*iso, anteiso*) and hydroxy acids, and it was desirable to know the preferential order in which these fatty acids form urea inclusion compounds. Previous attempts to fractionate lanolin with urea were only partially successful (25). Quantitative analysis is difficult because of the large number of fatty acids and unsaponifiable constituents present, the relative insolubility of long chain constituents and the formation of intractable emulsions (26).

A cumulative distribution curve for lanolin fatty acids, shown in Figure 2, indicates the relative ease of urea adduct formation for the saturated, branched, and hydroxy acids in lanolin. Addition of a methyl group (*iso* or *anteiso*) was equivalent to decreasing the chain length of the unbranched, saturated acid by 2 to 4 carbon atoms, whereas introduction of a hydroxyl group was equivalent to decreasing the chain length by 6 carbons.

Trace amounts of monounsaturated fatty acids ($C_{14}$ to $C_{20}$) were detected in the urea fractions. In addition, the last three urea fractions, comprising 4% of the total fatty acids, contained 7 unidentified components in relatively large amounts which formed a homologous series (carbon number-log retention time plot) and may be multibranched ($C_{20}$ to $C_{28}$) fatty acids. Multibranched acids have been found in sheep lipids (27), and multibranched paraffins are also believed to be present in wool wax (28).

Weitkamp (29) established that wool wax contains a series of even chain length *iso* acids and a series of odd chain length *anteiso* acids. The $C_{12}$ to $C_{28}$ branched acids of these series were identified by GLC analysis of the urea fractions. Separation of *iso* and *anteiso* acids on the polar column was similar to that reported by Farquhar et al. (30). The relative amounts of saturated, branched and hydroxy acids in lanolin are shown in Table II.

### Butter Oil

The urea fractionation procedure was applied to butter oil to further test its effectiveness in concentrating minor constituents for detection by GLC. About 64 fatty acids were reported to be present in milk (12). A normal GLC analysis will resolve only 20–25 peaks; the other fatty acids are present in small amounts, and either do not produce identifiable peaks, or are obscured by major peaks. By urea fractionation, GLC column overloading, and temperature programming, it was possible to detect and estimate the amounts of 84 fatty acids present in butter.

The total fatty acid composition of butter oil determined by GLC of individual urea fractions (Table III) agrees well with values reported by Herb et al. (12). Of the 84 acids we detected, 52 were present in amounts less than 0.1% and 33 are reported for the first time. We did not determine $C_{10}$ and lower fatty acids (totally or partially lost during removal of solvent), trans isomers, conjugated dienes and trienes, and keto acids.

Sharp and well-defined gas chromatographic peaks for components greater than $C_{20}$ were obtained from the urea fractions when relatively large amounts (40 mg) were chromatographed, even though the $C_{16}$–$C_{18}$ peaks were greatly overloaded. Gas chromatographic analysis of individual fractions suggested that a series of branched chain acids were present in butter oil. The $C_{13}$ to $C_{18}$ *iso*-acids and the $C_{15}$ and $C_{17}$ *anteiso*-acids were tentatively identified from plots of carbon numbers versus retention time. It was not possible to determine whether $C_{19}$ and higher branched acids were of the *iso* or *anteiso* type.

Analysis of the final urea adduct and filtrate indicated a series of saturated multibranched fatty acids from $C_{16}$ to $C_{28}$ may be present. Two members of this series have been previously identified as 3,7,11,15-tetramethylhexadecanoic acid (19) and 2,6,10,14-tetramethylpentadecanoic acid (20). Tri- and pentabranched $C_{24}$, $C_{26}$ and $C_{28}$ fatty acids were tentatively identified in the last 3 fractions. A semilogarithmic plot of retention time vs. chain length (DEGS column) for the final filtrate hydrogenated, produces smooth curves for the tentatively identified tetrabranched series as well as for the reference saturated series (Fig. 3). If the tetrabranched curve is displaced three carbon units to the left, it almost matches the saturated curve. It would not be expected that hydroxy acids, keto acids, or free acids would be found in the final filtrate, and it is not expected that complex oxidation products would be components of such a regular series.

### Lard

A total of 44 fatty acids was detected in lard (Table IV), with 30 acids present in amounts less than 0.1%. The presence of all fatty acids reported by Magidman, et al. (31) was confirmed, with the exception of the conjugated *cis-trans* and *trans-trans* isomers of linoleate, and the $C_{18}$ branched isomer. In addition, 18 fatty acids not previously reported were detected. Components tentatively designated as $C_{20}$ to $C_{28}$ tetrabranched even chain length acids and a $C_{28}$ tribranched acid were detected in very small amounts.

### Walnut Oil

The fatty acid composition of a walnut oil of French origin was determined (Table V). Some 36 fatty acids were detected, 26 in amounts less than 0.1%. There was also an indication that tetrabranched $C_{16}$ and $C_{20}$ fatty acids and arachidonic acid may be present in very small amounts. Grieco and Piepoli (32) report 12.1% linolenic acid in Italian walnut oil compared to our value of 14.8% for the French walnut oil.

### Artifacts

Since the completion of this study, the possibility of erroneously identifying artifacts as methyl esters of fatty acids by GLC analysis was emphasized by Johnston and Roots (33). We reinvestigated the reagent, saponification, methanolysis, and urea fractionation blanks, and artifacts were detected in each. The total residue from fivefold blanks was injected into the chromatograph and programmed at 4° per minute from 150 to 250C. Artifacts were present in such minute quantities that it is believed they would be detected only in the final filtrate and final adduct. However, it does explain the presence of several unidentified peaks which did not behave in a predictable manner in the urea fractions. Most of the artifacts had retention times longer than $C_{20}$.

In future work, clean-up procedures (e.g., column chromatography, thin-layer chromatography, or sublimation) should be added to procedures for preparation of final filtrate and final adduct. Present knowledge of the possible existence of artifacts does not detract from the identifications and qualitative estimations in this study. It emphasizes the necessity of carefully examining reagents and blanks carried through the entire procedure in trace analysis and the need for more rigid specifications for reagents to keep pace with the use of more sensitive analytical methods.

TABLE IV

Fatty Acid Composition of Lard[a] (% of Total Fatty Acid Methyl Esters)

| Acid[b] | | Acid | |
|---|---|---|---|
| Saturated | % | Monoenes contd. | % |
| 12:0 | 0.08 | 18:1 | 43.5 |
| 13:0 | 0.01 | 19:1 | 0.06 |
| 14:0 | 1.27 | 20:1 | 0.34 |
| 15:0 | 0.02 | 21:1 | 0.004 |
| 16:0 | 24.0 | 22:1 | 0.02 |
| 17:0 | 0.29 | 24:1 | 0.0001 |
| 18:0 | 13.0 | Dienes | |
| 19:0 | 0.03 | 18:2 | 11.2 |
| 20:0 | 0.34 | 20:2 | 0.54 |
| 21:0 | 0.004 | 22:2 | 0.02 |
| 22:0 | 0.04 | Polyenes | |
| 23:0 | 0.02 | 18:3 | 1.30 |
| 24:0 | 0.02 | 20:3 | 0.08 |
| Branched[c] | | 20:4 | 0.13 |
| 23:0 br | 0.008 | 20:5 | 0.04 |
| 24:0 br | 0.004 | 22:3 | 0.02 |
| 25:0 br | 0.02 | 22:4 | 0.05 |
| 26:0 br | 0.02 | 22:5 | 0.02 |
| | | Multibranched[c] | |
| Monoenes | | 20:0 br4 | 0.02 |
| 12:1 | 0.02 | 22:0 br4 | 0.02 |
| 14:1 | 0.03 | 24:0 br4 | 0.03 |
| 15:1 | 0.01 | 26:0 br4 | 0.03 |
| 16:1 | 2.82 | 28:0 br3 | 0.09 |
| 17:1 | 0.22 | 28:0 br4 | 0.20 |

[a] Lower fatty acids not determined.
[b] First number indicates number of carbon atoms in fatty acid moiety; second number the number of double bonds; br = branched (*iso* or *anteiso* position not determined); last number indicates number of methyl branches for multibranched acids.
[c] Tentatively identified in appropriate urea fractions by semilogarithmic pots of GLC retention times.

### Detection of Fatty Acids Present in Trace Amounts

In Tables III–V a total of 108 fatty acids was found to be present in amounts <0.1% in milk, lard and walnut oil. The detection and quantitative measurements of these low concentrations would be impossible without prior fractionation. The separations of fatty acids present in complex mixtures by urea fractiona-

TABLE V

Fatty Acid Composition of Walnut Oil from France (% of Total Fatty Acid Methyl Esters)

| Acid[a,b] | | Acid | |
|---|---|---|---|
| | | Branched | |
| Saturated | % | contd. | |
| 14:0 | 0.008 | 23:0 br | 0.02 |
| 15:0 | 0.008 | 24:0 br | 0.001 |
| 16:0 | 7.04 | 25:0 br | 0.01 |
| 17:0 | 0.06 | Monoenes | |
| 18:0 | 1.82 | 14:1 | 0.002 |
| 19:0 | 0.0004 | 16:1 | 0.2 |
| 20:0 | 0.34 | 17:1 | 0.008 |
| 21:0 | 0.008 | 18:1 | 15.50 |
| 22:0 | 0.08 | 19:1 | 0.04 |
| 23:0 | 0.02 | 20:1 | 1.70 |
| 24:0 | 0.03 | 22:1 | 3.82 |
| 25:0 | 0.0002 | 24:1 | 0.2 |
| 26:0 | 0.004 | Dienes | |
| Branched[c] | | 18:2 | 53.7 |
| 16:0 br | 0.004 | 20:2 | 0.2 |
| 17:0 br | 0.008 | 22:2 | 0.1 |
| 18:0 br | 0.008 | Polyenes | |
| | | 18:3 | 14.8 |
| 20:0 br | 0.008 | 20:3 | 0.2 |
| 21:0 br | 0.008 | 22:3 | 0.01 |
| 22:0 br | 0.002 | | |

[a] First number indicates number of carbon atoms in fatty acid moiety; second number the number of double bonds; br = branched (*iso* or *anteiso* position not determined).
[b] Trace amounts of $C_{16}$ and $C_{20}$ tetrabranched fatty acids and arachidonate were also present.
[c] Tentatively identified in appropriate urea fractions by semilogarithmic plots of GLC retention times.

tion was demonstrated to be extremely valuable for detecting fatty acids present in trace amounts. This was accomplished by separating all of the fatty acids present in the original sample so that in most cases each peak on a gas chromatogram represented only one fatty acid. The long chain saturated and polyunsaturated fatty acids present in small amounts were readily detected by overloading the GLC column and temperature programming the instrument. Except for lanolin acids, it was impossible to detect *iso* and *anteiso* fatty acids without prior urea fractionation of these acids eluted together with more prevalent monounsaturated fatty acids having one less carbon atom.

Urea fractionation does not provide a quantitative means of separating individual fatty acids. However, formation of urea complexes proceeds in a predictable manner. A more detailed knowledge of the order in which it proceeds aids in assigning the proper identity to GLC peaks, and should also aid in establishing the identity of abnormal fatty acids present in heated or used fats.

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# Phase Behavior of Aqueous Systems of Monoglycerides

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## Abstract

Monoglyceride-$H_2O$ systems in the range above about 5% $H_2O$ exhibit a varied phase behavior with a number of mesomorphic states reminiscent of those found for soap-$H_2O$ systems. There are fluid neat, stiff and "short" viscous isotropic, and plastic or stringy middle states.

In the case of saturated monoglycerides, neat states prevail at low molecular weight as with monolaurin, but viscous isotropic and finally middle states appear at higher chain length as with monoarchidin and monobehenin.

The phase diagrams for systems of unsaturated compounds resemble those for systems of saturated compounds, with corresponding phase regions occurring at lower temperatures. The monoelaidin-$H_2O$ system closely resembles a shifted monostearin-$H_2O$ system, neither system showing middle. Middle phase appears in a small island for monoolein and monolinolein near 90C and 20% water.

Water dissolves negligible amounts of monoglycerides so that phases which are nearly 100% $H_2O$ exist for substantially all systems and conditions when $H_2O$ content is 60% or more.

## Introduction

THE FORMATION OF MESOMORPHIC states in anhydrous polar long-chain compounds is well recognized (1). Much more familiar is the formation of such states, specifically neat and middle, in aqueous systems of such compounds (2). While the phenomena are more common with ionic compounds, they have been recognized in nonionic materials (3). A lengthy study of gels in aqueous monoglyceride systems has been reported (4). Except for certain crystal dispersions they were said to be "amorphous by x-ray diffraction." No mention of mesomorphic states was made.

Lawrence (5) reports the formation of liquid crystalline phase in aqueous systems of 1-monolaurin but without presenting a phase diagram.

Because of the interest in monoglycerides as familiar components of foods, their membership in the family of nonionic polar long-chain compounds, and the intriguing nature of the gel states reported, a systematic study of monoglyceride-$H_2O$ systems was launched. It was soon discovered that these systems exhibit a variety of mesomorphic states quite analogous to those of soap-$H_2O$ systems, and show profound variations of phase behavior with composition.

There is increasing evidence of the widespread occurrence and fundamental significance of such mesomorphic states (6) and their importance technically and biologically is not to be underestimated.

## Experimental

Several 1-monoglycerides were prepared by recognized methods and purified by solvent crystallization. The following compounds, all substantially free