TRIGLYCERIDE AND FATTY ACID ANALYSIS
BY GAS CHROMATOGRAPHY

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ABSTRACT

Extension of gas chromatographic methods to the analysis of high molecular weight compounds has permitted the study of natural lipid mixtures without a prior transformation into simple fatty acid esters. It is now possible to separate and measure in a single chromatogram the amounts of cholesterol, diglycerides, steryl esters and triglycerides present in a total lipid extract. The resolution of steryl esters and triglycerides has required a perfection in instrument design and in working procedures not ordinarily demanded of equipment used in isothermal steroid and fatty acid analyses. When applied to determinations of fatty acids, however, the advanced apparatus design and optimized operating parameters have yielded improved resolution and better quantification. When combined with thin-layer chromatographic methods for the separation of lipid classes and for a resolution of saturated and unsaturated homologues, the modified gas chromatographic techniques have permitted a nearly complete fractionation and quantification of all components of natural lipid mixtures.

The paper describes some of the advances made in the design of the gas chromatographs and in the preparation of high quality columns responsible for these achievements. The discussion of operating parameters is limited to the consideration of programmed temperature separation of mixtures of total neutral lipids, triglycerides and simple fatty acid esters.

INTRODUCTION

The invention of gas chromatographic techniques for the separation and quantitative estimation of fatty acids (1) completely revolutionized the field of lipid chemistry and for the first time provided an indication of the true complexity of natural fats and oils. This development led to the eventual introduction of the polyester liquid phases (2) which permitted the analysis of many complex mixtures of fatty acids with relative ease and reproducibility. These methods, which utilize heavy coatings of thermally unstable polymers as stationary phases, are best suited for the study of the C_{16} and C_{18} fatty acids which make up the bulk of the common animal fats and vegetable oils. They are unsuitable for the separation of natural fatty acid esters such as glycerides and steryl esters.

Successful gas chromatography of intact natural lipid esters (3) had to await the discovery of the thin-film techniques (4) used in steroid separations, and temperature programming. The separations presently achieved (5), however, have also required modifications in the basic design
of the gas chromatographs, the perfection of the dual hydrogen flame ionization detector, and the development of methods of dual column operation. The success of the studies with the high molecular weight materials and the subsequent refinements of the general conditions of gas chromatography have yielded better separations and higher recoveries also with the lower molecular weight compounds including the simple alkyl esters of fatty acids.

This paper discusses some of these developments and indicates how the improved methods of gas chromatographic analysis can be effectively combined with other chromatographic systems and chemical procedures of investigation to yield an essentially complete account of the composition of most mixtures of natural triglycerides.

GAS CHROMATOGRAPHY OF TRIGLYCERIDES

The procedures described here are chiefly those developed over the last few years in the author's laboratories (3,5). Short, thin-film columns prepared by coating diatomaceous earth supports with silicone polymers have been used for the resolution of natural triglyceride mixtures since 1961. The method has been gradually improved as new developments have taken place in instrumentation and column technology. The following is a brief account of the present state of the work together with a commentary on its more critical features.

1. Instrumentation

Effective gas chromatography of natural glycerides requires the optimum in apparatus design and operation. Although the basic principles of good design have been well known for some time, there has been considerable reluctance on the part of the manufacturers to incorporate all the features into any one instrument. Testing of gas chromatographic apparatuses for triglyceride separation in our laboratory has shown that only one of the four leading manufacturers examined had produced an instrument line which could be used for glyceride separation without modification. Although the unsatisfactory instruments were in several respects superior to the satisfactory instrument, they contained one or more design faults which rendered them unsuitable for these separations. The more adequate instrument makes are identified in the legends to the figures. Only one other line of gas chromatographs has been reported (6) to be capable of the separation of natural glycerides without modification.

a) Temperatures

Triglyceride analyses require temperature programmed operation with temperatures to 350°C. Linear temperature programmers with proportional heat input are of the most general application, but certain non-linear programs may be better suited for specific resolutions. The most useful temperature programs for metal columns have been those providing regular increases of 2-5°C per minute in the 200-325°C range of oven temperature. Glass and siliconized metal columns have permitted programs of up to 20°C per minute without any noticeable lack of equilibration of the solutes.
The column inlet heater should provide uniform heating of the inlet end of the column and the injector compartment and should be capable of maintaining its temperature setting without superheating despite the rise in the oven temperature. For special applications there may be a need for a programmed temperature operation of the injector heater also. Isothermal settings of 280-325°C, however, have proved adequate for most applications.

The detector compartment should be maintained at a constant independent temperature, usually the maximum working temperature. A setting of 340-350°C has proved satisfactory. Although temperature variations of a few degrees may take place without any noticeable effect upon the resolution or recovery of the compounds, the retention times do change. For this reason, high quality work involving the measurements of physical constants on the separated components requires rigid control of all temperatures.

b) Injectors

The injector compartment should be joined directly to the oven without any cold junctions that require special inlet line heaters. The design should permit an on-column injection with a conventional 10 μl Hamilton syringe equipped with a 2 in. needle. The injector compartment should be of a small volume capable of accepting the injector end of the column in such a manner (Figure 1) as to allow the carrier gas to sweep around the end of the column and insure a rapid and complete entry of all sample into the column. The carrier gas should be preheated by passing through a stainless steel capillary wrapped around the injector block, and should enter the injector barrel just above the Swagelok fitting accepting the column. It has been shown (5) that on-column injection results in much sharper peaks, better resolution and higher recovery of the injected material. Furthermore, on-column injection allows a satisfactory admission of the solute to the column at temperatures of up to 70°C below those required for flash evaporation (325-350°C). The apparatus should be capable of dual, compensating column operation to minimize the effects of liquid phase bleeding at elevated temperatures. This requires that both columns be equipped with on-column injectors which are maintained at constant and identical temperatures throughout the operation.

c) Detectors

The detector should be located directly at the end of the column without any extra spacing which requires special detector line heaters and complicates the operation of the chromatograph. A dual, compensating flame detector is recommended. Those of the non-metallic construction give less noise and are free of competitive ion collection. The flame should be designed for operation at high flow rates and elevated temperatures. At the high column temperatures the argon ionization detector becomes easily contaminated with the bleed of the liquid phase and is less satisfactory. The hydrogen flame detector, however, may also show unusual effects which may be traced to silicone deposits on the flame jets or the collector assembly. The presence of these deposits is demonstrated in the loss of signal below a certain level of sensitivity. The base line appears extremely quiet and stable as the background noise, column bleed and the minor components are not recorded. This loss in sensitivity cannot be recovered by lowering the attenuation, and the instrument linearity is poor. Silica and any carbon deposits can be readily removed in the ultrasonic bath.
The extremely wide range of the hydrogen flame detector, which can be obtained with careful adjustment of hydrogen and air flows under normal operation, permits the use of a wide range of sample concentrations and a quantitative estimation of both major and minor components in the same run. It has been shown (7) that a linear response for trilaurin may be obtained in the range 0-45 µg and for tristearin in the range 0-20 µg. The latter compound has shown a different slope in the 20-45 µg range. Since under the usual operating conditions a full scale tristearin peak represents only 10-20 µg of triglyceride, this non-linearity occurs only in overloaded columns and does not present a problem in analysis.

d) Flow rates

High flow rates (150-300 ml/min of nitrogen or helium) of carrier gas obtained by means of low head pressures are necessary if the triglycerides are to be volatilized and brought off the column as symmetrical peaks at the lowest possible operating temperatures. The hydrogen flame detector is relatively insensitive to small changes in the flow rate of the carrier gas. Great fluctuations in the carrier flow, however, should be absolutely avoided as the concentration of the sample in the detector will change with the flow rate, which in turn will change the apparent detector response. Constant flow rates of carrier gas may be maintained by means of differential flow controls which automatically adjust the flows to compensate for the changes in column resistance during the temperature programming. Constant flows facilitate the operation of dual column systems and help to maintain equal base widths of the peaks during linear temperature increase.

2. Column Technology

Gas chromatography of triglycerides requires columns of especially high quality. Usually, however, those silanizing and support coating techniques which produce adequate packings for the resolution of sterols will also serve to prepare satisfactory columns for triglyceride work. For optimum performance, certain features of the physical makeup of the column and the mode of operation are important and deserve consideration. Specific columns and operating conditions are described in the legends to the figures used to illustrate various applications.

a) Columns

Glass, stainless steel, and aluminum tubes have been used with equal success as the column material. The glass columns, however, provide better peak resolution and allow faster programming rates than steel columns, which require more time for equilibration due to greater surface adsorption. Silanizing the interior of the steel tubes greatly reduces the adsorption and gives columns the performance of which is indistinguishable from that of glass columns. The dimensions of the columns employed have ranged from 6 in. to 8 ft. in length and 1/16-1/4 in. in diameter. Columns of 18-24 in. in length and 1/8 in. O.D. provide sufficient number of theoretical plates to completely resolve most mixtures of natural triglycerides, without impaired recoveries. The steel columns with 1/8 in. O.D. and 1/16 in. I.D. give the best resolution, but because of heavier wall require more time for heat equilibration and permit only low rates of programming. The
highest resolutions are obtained on thin-wall 1/16 in. O.D. steel columns which yield about 1000 theoretical plates per foot of column length. The decreased flow rates, however, may reduce the recoveries of the higher molecular weight materials. The great disadvantage of glass columns is the lack of adequate means of maintaining a satisfactory seal between the glass and the metal inlet and outlet ports during alternate heating and cooling cycles.

b) Packings

Of the large variety of chromatographic supports tried, the silanized diatomaceous earth preparations have yielded high quality columns most frequently. Mesh sizes of 60-80 and 100-120 have given maximum column efficiency even with short column lengths. Because of the requirement of high temperature stability and low polarity, satisfactory triglyceride separations have been realized only with the silicone liquid phases. SE-30 (General Electric silicone gum), QF-1 (Dow Corning fluoroalkyl silicone gum) and JXR (Applied Science polysiloxane polymer) have all given similar separations. The extremely low bleed of the JXR polymer makes possible the direct application of the desired thickness of coating of high temperature stability without extensive conditioning and stripping of the volatile material. Other silicone polymers of high thermal stability and of potential application in triglyceride separations have been introduced under the trade names of OV-1 and OV-17 (Applied Science Laboratories). These are phenyl-silicone polymers and have been claimed to give some 25-30% less rise in the base line than SE-30 when programmed to 340°C. Polysulfone (Applied Science Laboratories) is another polymer of high temperature potential with some separating characteristics similar to those of QF-1. It is a solid up to approximately 240°C and its thermal stability has been rated above that of JXR. Experimental techniques suitable for the preparation of the support, silanizing, and the application of the coatings have been described by Homing et al (4). However, excellent column packings can be obtained at a relatively low cost from reliable manufacturers of gas chromatographic supplies.

c) Filling and conditioning of columns

The requirement of maximum efficiency demands that the short columns be uniformly packed and that the packing retains its structure during the initial conditioning and through the heating and cooling cycles of normal operation. Usually the narrow diameter (1/16-1/8 in.) steel coils are packed with the help of the suction of a water pump, but column loading under positive pressure (15-25 psi) of inert gas may also be used. Before filling the outlet end of a suitably shaped tube is closed with a light plug of siliconized glass wool, extending a few mm from the end of the tube into the column interior. If mechanical vibrators are used, care should be taken not to fracture the support particles and to expose adsorptive sites. The column is firmly filled to about 35 mm from the inlet end, and a small siliconized glass wool plug is pushed down the tube to rest against the packing. For a dual column instrument, two columns are packed simultaneously under closely similar conditions. Columns packed by gravity settling give very high flow rates but because of loose packing require longer column lengths to obtain column efficiencies comparable to those available in tightly packed short tubes.
Before use the columns are conditioned by heating to maximum operating temperatures and keeping there until the low molecular weight residues have been polymerized or bled off. To avoid contamination, the outlet end of the detector is left free and the carrier gas (slightly below its optimum flow rate) is vented without passing through the detector. Depending on the liquid phase, the time required for conditioning may vary from a few hours to several days. The first few runs seldom give entirely satisfactory recoveries and brief periods of additional conditioning are required.

The conditioned column is tested for its ability to resolve a standard mixture of trilaurin, trimyristin, tripalmitin and tristearin. Under properly adjusted working conditions, the four triglycerides should be eluted as symmetrical peaks with the approximate retention temperatures given in Figure 2. The base widths of the peaks should be narrow and the peak spacing sufficiently wide to accommodate two even carbon number triglycerides differing by two carbons between any two adjacent simple glycerides. Besides assessing resolution, the initial runs with the standard glycerides of known concentration also serve to determine the extent of the actual as well as the proportional recovery of the different glycerides. With the common flame ionization detectors, sample sizes range from 1-50 µg of total glyceride. The usual peak seen in separations of this kind is 0.5-2 µg for a single component. The limit of sensitivity is about 0.01 µg. The proportional recovery of the standard glycerides may be appraised from a comparison of the peak areas, which should reflect nearly correct weight proportions.

Exact recoveries of the various glycerides necessary for quantitative work may be determined by careful calibration of the system with accurately measured amounts of suitable standards. The correction factors derived from such calibrations (Table 1) may be used for work with natural glyceride mixtures to which they are applied on the basis of molecular weight. Because of gradual column deterioration and detector fouling it is necessary to recheck the calibrations periodically. For work with erucic acid rich oils and marine oils, the columns and the apparatus are tested with glyceride standards containing triarachidin and trierucin. Columns that do not show responses in the indicated range should be reconditioned or discarded. Since the performance of the column is determined using the complete system, it should be kept in mind that factors other than the column may be responsible for poor recovery and resolution.

3. Separation of Natural Glycerides

Work with standards has shown that the method separates glycerides according to the molecular weight or carbon number, although some resolution due to differences in molecular shape may also be demonstrated. Ordinarily, saturated glycerides are not resolved from their unsaturated homologues. For this reason the most effective separations are obtained with those natural glyceride mixtures which contain a large variety of fatty acid chain lengths. Coconut oil is made up of triglycerides containing C6 to C18 fatty acids. It is very easy to resolve (3) and may be used as a trial mixture for new columns. Similar elution patterns can be obtained for palm kernel oil, and the oils of the **Lauraceae**, **Lythraceae** and **Ulmaceae** plant families, all of which are rich in lauric acid (8).
The bovine milk fat, although somewhat more complex, contains a comparable range of triglycerides. The elution patterns recorded for triglyceride mixtures of this type have been shown by the author on several occasions (9,10). Figure 3 shows the elution patterns recorded for sheep and goat milk. Because of the presence of small amounts of odd carbon number and branched chain fatty acids in the milk fats, the return to base line between adjacent peaks is not as complete as for coconut oil. The two-hump elution patterns are very characteristic and resemble those observed for cow's milk. Figure 4 shows the elution sequences noted for dog and human milks. Due to a virtual absence of butyric acid from these fats, the first hump in the glyceride elution pattern does not appear. In all cases the milk fats contain a much wider range of glycerides than the body fats of the corresponding species.

Figure 5 shows the elution patterns recorded for the triglycerides of sheep suet and beef tallow. The large proportions of the C₅₀ and C₅₂ peaks indicate that most of the fat is made up of triglycerides containing two C₁₆ and one C₁₈ acid, and one C₁₆ and two C₁₈ acids. Comparable elution patterns are obtained for the triglyceride mixtures of other tissues of these animals.

Figure 6 gives the elution patterns recorded for the triglycerides from chicken and goose fat. Again the triglycerides of C₅₀ and C₅₂ carbons predominate and there is a superficial similarity to the sheep and beef fat triglycerides as well as to the glycerides of the dog pancreas and rat liver shown in Figure 7. More extensive comparisons have shown that the differences in the glyceride elution patterns are frequently greater between different tissues in a single animal than between the same tissues of different species. Thus the glyceride mixtures of plasma, liver, kidney and heart show considerable similarities among some species, yet the glycerides of the kidney and the liver of the same animal may differ widely. The latter observation is illustrated with the elution patterns obtained for the kidney and liver of the same goose shown in Figure 8. Although these similarities or differences may frequently be very pronounced, the elution patterns could conceivably change with diet and even vary from one individual to the other, and are unlikely to provide any well defined characteristics.

The separations obtained with a number of the common vegetable oils were shown in one of the original publications from our laboratory (9). The predominance of the C₁₈ fatty acids in these oils results in a major peak for the C₅₄ glycerides with much smaller amounts contributed by the C₅₂ and C₅₀ glycerides which account for the lesser amounts of the C₁₆ acids also found in these oils. As shown by calculations of recovery (3) and by chromatography after hydrogenation, the areas of the eluted peaks are correct even for the most highly unsaturated oils. Thus Figure 9 shows identical elution patterns for raw and hydrogenated samples of linseed oil.

Figure 10 illustrates the resolution achieved for menhaden and herring oils. These oils are rich in unsaturated C₁₈, C₂₀, and C₂₂ fatty acids and contain triglycerides with carbon numbers of up to C₆₆. Because of the progressively decreasing retention times observed for the higher members of the homologous series under linear temperature programming, the longer chain glycerides show considerable overlap on the short columns.
Somewhat longer columns (24 in.) and narrower column diameters (1/16 in.) give better peak spacing. The elution pattern is not materially improved by a prior hydrogenation of the oil. Comparable elution patterns have been obtained for other marine oils and erucic acid rich plant oils (11).

The comparisons of the triglyceride elution patterns can be greatly upgraded by a preliminary separation of the triglyceride mixture on the basis of unsaturation on silica gel plates impregnated with silver nitrate (12). As a result of it, it is possible to compare the distributions among the saturated, and the mono-, di-, and tri-unsaturated glycerides of different tissues and animal or plant species. Figure 11 illustrates the separations obtained on silver nitrate plates for a sample of lard. In addition to the segregation based on the number of double bonds, there has also been a resolution of certain positional isomers, which may be separately recovered and analyzed in the gas chromatograph. Figure 12 shows the elution patterns recorded for the different bands and the original fat. The quantitative proportions of the bands may be determined from the peak area of tridecanoin which can be conveniently added to the triglyceride mixtures recovered from the plate.

The composition of the di- and tri-unsaturated glycerides can be further analyzed by subjecting a portion of the material recovered from the plate to a permanganate-periodate oxidation prior to gas chromatography. This oxidation quantitatively converts the unsaturated triglycerides into saturated glycerides containing azelaic acid residues (13). Following diazomethylation the oxidized glycerides behave in the gas chromatograph as triglycerides containing one, two, or three lauric acid residues. Figure 13 shows the elution pattern recorded for the oxidation products of whole lard. As it can be seen from the part of the print superimposed upon the standard glycerides, the overlap between triazelain and trilaurin is not complete, but it is close enough to permit effective analysis. Furthermore, the overlap with the corresponding even carbon number glycerides increases as the number of azelaic acid residues per molecule decreases.

This method of analysis is equally well suited for the determination of the triglyceride composition of vegetable oils, and fish oils. Because of high unsaturation, the fish oil triglycerides cannot be adequately resolved by the common techniques of silver nitrate thin-layer chromatography, as glycerides of more than 5 double bonds remain at the origin. This difficulty, however, is likely to be overcome by modifications in the gel and the developing solvents.

The application of these methods to the analysis of fats of industrial importance is illustrated with a sample of margarine prepared by partial hydrogenation of fish oils. Figure 14 shows the resolution obtained on the silver nitrate plate. The combination print given in Figure 15 shows the glyceride composition of the various bands recovered from the plate. As noted earlier, the proportions of the material in the various triglyceride bands can be effectively estimated by means of tridecanoin used as internal standard.

These gas chromatographic systems are equally effective for the resolution of total neutral and in certain cases total lipid mixtures. The chromatograms of total neutral lipid extracts of plasma and lymph show an
essentially complete resolution of all the lipid components present (14). There is no significant overlap between the steryl esters and the triglycerides. It has since been demonstrated that the total lipid extracts of these body fluids can also be analyzed in the gas chromatograph and good estimates for cholesterol, total phospholipid, cholesteryl esters and triglycerides obtained in a single run. Fig. 16 shows the neutral plasma lipids of man and dog. Under the conditions of gas chromatography the phospholipids are pyrolyzed to the corresponding propene diol diesters in about 60% yield. These diesters are eluted from the triglyceride column with retention times comparable to those of diglycerides, and emerge well ahead of the steryl esters. Eventually it may be possible to use gas chromatography for the determination of the lipid composition of plasma and other body fluids without prior extraction. The lipid esters apparently completely sublime from their lipoprotein complexes at the injector temperatures and appear as symmetrical peaks in the effluent. Figure 17 shows the elution pattern recorded for 3 µl of fasting dog plasma.

GAS CHROMATOGRAPHY OF FATTY ACIDS

Conventional gas chromatographic analyses of fatty acids are performed on the methyl esters using heavy coatings of liquid phases. Under the isothermal conditions generally employed, these methods are best suited for the determination of the common C_{12} to C_{18} saturated and unsaturated fatty acids. They are not fully satisfactory for the scanning of acid mixtures containing both short and long chain homologues. Positional and steric isomers of the common unsaturated fatty acids are not resolved and there is difficulty in the quantitative estimation of peaks of long retention times. These problems may not be serious when dealing with extracts of total lipids in which the unusual acids make up negligible proportions. Accurate estimates of all fatty acids become extremely important when attempting to reconstruct the overall composition of a lipid mixture from the fatty acid determination of completely resolved lipid classes and individual ester groups. Attempts to solve this problem have been made by seeking new liquid phases that would be capable of resolving all fatty acids, and by incorporating into the present gas chromatographic systems those design and operation features which have proved successful in the separations of other compounds. Other improvements in the methods of determining fatty acids have been sought through short cuts in the preparation of fatty acid esters. The determination of free fatty acids presently appears to be of limited practical applicability and will not be discussed.

1. Column Technology and Instrumentation

Since stable liquid phases of polarity high enough for the resolution of fatty acids have been slow in forthcoming, the major improvements in the gas chromatography of fatty acids have been made by designing instruments that permit work with volatile phases. In these efforts the dual column systems have proved most satisfactory and have allowed temperature programmed operations of up to 50°C above the normal working limit of the common liquid phases. With increasing interest in programmed temperature separations there has been a gradual switch towards the use of columns with thinner coatings of stationary phase.
a) Liquid phases

Polar liquid phases in the form of various polyesters have been most useful for fatty acid separations. Columns containing 10-25% (w/w) liquid phase have been applied extensively to the separation and identification of fatty acids in the form of methyl esters (1). These phases are limited to temperatures not exceeding 220°C. As the upper limit is approached a significant amount of the liquid phase volatilizers and bleeds into the detector. Several techniques have been developed to stabilize the polyesters and thereby raise their maximum useful temperature limit. By introducing various terminating agents and by a judicious choice of the esterification catalyst it has been possible to increase the upper limit of the working range to about 250-270°C. The most useful method of decreasing the bleed rate of the polyester columns in the working range has been the copolymerization of ethylene glycol, succinic acid and dimethylsiloxane monomers (EGSS-X, Applied Science Laboratories). The mixed silicone-polyester polymers are less polar than the simple polyesters but are still capable of maintaining sufficient selectivity for the unsaturated fatty acids to give complete separations. The utilization of 1% coatings of this phase permits temperature programming with single columns in the range of 150-225°C, without significant baseline elevation (15). The capacity of such columns is sufficient for work with flame ionization detectors. As a 10% coating this stationary phase is ideally suited for the isothermal separation of methyl esters in the C₁₄ to C₂₄ range, all of which can be eluted within 30 minutes. The shorter chain fatty acids can be effectively determined at selected lower isothermal temperatures. Another polar ester packing to which specific attention has been called is the tetracyanoethylated pentaerythritol (TCEPE). Its upper thermal limit is only 150°C, but when used as a 3% coating on Aeropak 30 in a 10 ft. x 1/16 in. O.D. column (Wilkens Instrument and Research) it has been claimed to permit complete resolution to all the common fatty acid esters within 35 minutes at 105°C.

Non-polar liquid phases have generally higher thermal stabilities than the polyesters, but have the inability to separate the C₁₈ and higher unsaturated fatty acids which contain 2 or more double bonds. Because of their greater temperature stability, non-polar liquid phases are excellent choice for separating saturated fatty acids with chain length larger than 22 carbons. Columns packed with 10-17% Apiezon L grease have been widely used (1). Separations of the C₁₈ homologues are possible, however, also with the non-polar liquid phases when packings of about 3% liquid phase are used in combination with 1/16 in. O.D. x 20 ft. columns (Wilkens Instrument and Research) which generate over 20,000 theoretical plates (calculated for methyl stearate). Of the non-polar phases the silicone polymers (SE-30, JXR) give the lowest bleed rates and are particularly well suited for temperature programming and quantitative work which does not require a resolution beyond the molecular weights. A stable material recommended for preparative scale gas chromatography of fatty acid esters because of low bleed rate (up to 250°C) is the beta-cyclodextrin acetate. This phase, however, will not resolve methyl stearate and methyl oleate.

b) Columns and supports

Although the fatty acid esters are relatively non-polar and highly volatile, the gas chromatographic analysis of their mixtures can be
considerably improved by adopting the techniques developed for dealing with more polar and much less volatile compounds. Thus silanizing the interior of metal columns or the use of glass columns improves peak shape and resolution. Significant improvements in column efficiency are realized by using siliconized supports of narrow mesh sizes. Gas Chrom Q (Applied Science Laboratories), Aeropak (Wilkens Instrument and Research), and the high performance Chromosorbs (Johns Manville) are all well suited for preparing high quality columns. Narrow bore 1/8 in. O.D. columns and the Micropak (Wilkens Instrument and Research) columns (1/16 in. O.D.) of 10 to 20 ft. length give vastly improved resolutions with most liquid phases because of the greater number of theoretical plates provided per foot of column length.

c) Instrumentation

Advanced equipment design and better methods of operation have facilitated the realization of good fatty acid separations even with poor packings and badly packed columns. Of major significance has been the replacement of the flash vaporization chamber with an on-column inlet. This change has allowed the application of the ester mixtures to the column in a narrow zone giving much sharper peaks and better resolution, and has permitted work at considerably lower temperatures.

The replacement of the thermal conductivity cell and argon ionization detectors with flame ionization units has resulted in a more satisfactory interpretation of the detector response and a more rapid quantitation of the eluted components. Flame ionization detection in combination with dual column operation has been most effective in temperature programming of columns containing thermally unstable liquid phases. Other improvements in instrumentation of the type described for triglyceride separations are valuable also in work with fatty acid esters but have not been repeated here.

2. Method of Chromatography

Wide ranges of fatty acid methyl esters may be scanned on suitable columns by the use of temperature programming. Excellent separations of the methyl or butyl esters of fatty acids on the basis of molecular weight are obtained on 5% SE-30 columns (Figure 18). This column provides the most effective means presently available for the quantification of the total fatty acids recovered from a particular lipid preparation using an internal standard (methyl heptadecanoate). The elution can be completed within 30 minutes using a temperature range of 160 to 300°C and selecting temperature increments and chart speeds to suit the ester mixture at hand. Complete separations of all fatty acids can be made on 1% EGSS-X liquid phase in the temperature range 125°C to 215°C using a 12 ft. column (15). Good batches of this packing yield excellent separations of not only the monoenes and dienes but also of trienes and tetraenes of various chain lengths. These packings, however, are quite variable and frequently batches are found that give no resolution of anything. Highly efficient packings prepared with this phase also allow the resolution of C₁₆ and C₁₈ dimethyl acetics from the C₁₆ and C₁₈ fatty acid methyl esters. Both the SE-30 and the EGSS-X packings are ideal for instrumental area integration even with single column instruments.
Temperature programmed runs with the diethylene glycolsuccinic acid polyester liquid phases (DEGS) may be obtained in dual column, dual flame detector instruments. Good resolution of the methyl esters of milk fat triglycerides, for example, has been achieved in some 20 minutes in the temperature range of 100-235°C, when programmed at rates of 4 to 6°C/min. A run with the methyl esters of menhaden oil may be completed in about 30 minutes giving complete separations of all the saturated and unsaturated fatty acids including the C22:5 and C22:6 esters. Although the efficiency of the polyester columns may be somewhat increased by decreasing the thickness of the coating to about 6% and thus also minimizing the column bleed, the advantages gained are offset by the shortened life time of the column. A loading of about 10% liquid phase appears to provide a satisfactory compromise. Baseline stability in the compensated analysis is very good until the end of the run when a tendency to drift below scale develops. This can be avoided by levelling the program off at about 210°C and completing the run under isothermal conditions. Certain preparations of ethylene glycol adipate have been shown (2) to possess a considerably higher thermal stability than the diethylene glycol succinate polymer, as have the stabilized polyesters. The advantages that such esters may offer in programmed temperature analyses of fatty acids, however, have remained uncertain.

The separations obtained by temperature programming the Apiezon liquid phases are comparable to, and offer no advantage over, those noted for silicone gum columns. The work with capillarity columns has been largely qualitative in nature and has been restricted to isothermal separations of the short and medium chain length fatty acid esters. It is applicable to the separation of positional and geometric isomers of unsaturated fatty acids and some branched chain acids. Apiezon L and the polyester liquid phases have been most satisfactory for these studies.

### 3. Identification of Fatty Acid Esters

Relative retention times are conveniently used for tentative identification of fatty acid esters because considerable data of this type has been published. For a systematic characterization of the esters the gas chromatographic data may be expressed in terms of the equivalent chain lengths (ECL) or the modified equivalent chain length (MECL). The ECL is obtained by plotting the logarithm of retention times of normal saturated monocarboxylic acid esters against the number of carbon atoms in the corresponding acids. The MECL is based upon a plot with the monounsaturated rather than the saturated esters, and utilizes retention times relative to methyl stearate rather than absolute values. The chain lengths of other compounds may be measured by comparing the retention times of the standards to that of the unknown. The use of two or more columns is recommended.

Although these gas chromatographic techniques are quite helpful for determination of the tentative identity of unknown fatty acid esters, other methods should be used to verify this information. Oxidation with permanganate-periodate, reductive ozonolysis and mass spectrometry have all been demonstrated to provide much of the required information about the structure of unsaturated and saturated fatty acid esters. The consideration of these techniques is beyond the scope of the present review.
4. Preparation of Fatty Acid Esters

The fatty acid esters necessary for the gas chromatographic analysis may be conveniently obtained by transmethylation of the lipids or by a methylation of the fatty acids recovered after saponification. Both techniques have been extensively employed and a variety of reagents utilized (16). Special advantages have been claimed for 5% perchloric acid solution in methanol as a rapid (10 min.) methylaing agent for free fatty acids at low temperatures (55°C). Because of the small quantities of lipid usually involved in work with biological materials, the preparations of the methyl esters are best conducted by means of transmethylation. Saponification and extraction of the acids inevitably results in losses due to foaming and numerous transfers.

Useful in quantitative work combining thin-layer and gas chromatographic systems is the finding that complete transesterifications of all lipids may be obtained in the presence of the silica gel scrapings from thin-layer plates, including those containing silver nitrate. Refluxing or heating in sealed tubes with 10% (w/v) sulfuric acid in dry methanol for 1-2 hours at 80°C is sufficient to convert all lipids to the methyl esters except sphingomyelin which requires about 16 hours. The time for the transesterification of the sphingomyelin may be shortened by increasing the reaction temperature to 110°C. Nitrogen atmosphere and a crystal of hydroquinone protects the lipids from peroxidation. The use of methanolic sulfuric acid for the procedure is preferred in view of the reports claiming contamination from the use of methanolic HCL as a methylating agent in the ultramicroanalyses. Nevertheless, transmethylations with methanolic HCL as well as BF₃-methanol have been found highly satisfactory in a number of laboratories. The methanolic sulfuric acid procedure has been adopted as a tentative method by the American Oil Chemists' Society for the preparation of the methyl esters of C₈-C₂₄ saturated and unsaturated fatty acids (17).

The quantitative analysis of short chain fatty acids or mixtures of short and long chain fatty acids are improved by a preparation of the butyl esters. These esters are somewhat less volatile than the methyl esters and permit more satisfactory concentration during the evaporation of organic solvents. The butyl esters can be prepared by substituting butanol for methanol in the common esterification mixtures catalyzed by mineral acids. Figure 19 shows a programmed temperature separation of the butyl esters of fatty acids derived from milk fat triglycerides on diethylene glycol succinate. The fatty acid esters should be analyzed as soon as prepared. They may be kept in an atmosphere of nitrogen in a screw cap vial at low temperature for 24 hours. For longer storage they should be sealed in a glass ampule under vacuum and placed in a freezer.

CONCLUSION

The described techniques permit the accumulation of a fantastic detail of information regarding the composition of any fat. Such efforts, however, may not be wisely invested unless the source of the fat is adequately defined and reproducibly sampled. An analysis of a total lipid extract of a whole organ is of little biochemical interest. An effective tissue and cellular fractionation must precede any lipid extraction. There
may be a further need for the isolation of individual lipoproteins or lipoprotein groups, if the design and purpose of the diverse mixtures of natural lipid esters is to be determined.

REFERENCES


TABLE I
Calibration Factors for Simple Triglycerides under Optimum Operating Conditions

<table>
<thead>
<tr>
<th>Triglyceride</th>
<th>Cn</th>
<th>Double Bonds</th>
<th>Weight %/Area %</th>
<th>Litchfield et al (7)</th>
<th>Breckenridge (5)</th>
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<tbody>
<tr>
<td>Trioctanoin</td>
<td>24</td>
<td>0</td>
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<td>1.00</td>
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<tr>
<td>Tridecanoin</td>
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<td>1.00</td>
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<td></td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Trimyristin</td>
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<td></td>
<td>0.96</td>
<td>1.00</td>
</tr>
<tr>
<td>Tripalmitin</td>
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<td>0</td>
<td></td>
<td>0.98</td>
<td>1.00</td>
</tr>
<tr>
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</tr>
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<td>3</td>
<td></td>
<td>1.34</td>
<td></td>
</tr>
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</table>

LEGENDS TO FIGURES 1 TO 19

Fig. 1. Positioning of Column in the Injector Assembly. A, carrier gas inlet; B, packed column; C, Swagelok nut (front and back ferrules not shown); D, injector barrel; E, injector nut and silicone septum.

Fig. 2. GLC of Standard Triglycerides. Peaks identified by the total number of carbon atoms in the fatty acid moieties. Instrument 1(A): Aerograph 204-1B; Columns: stainless steel tubes.
20 in. x 1/8 in. O.D., packed with 3% (w/w) JXR on Gas Chrom Q (100-120 mesh) and assembled as shown in Fig. 1; Nitrogen flow rate: 120 ml/min. at room temperature; Injector, 280°C; Detector, 340°C; Sample: 1 µl of 1% (w/v) solution in chloroform. Attenuation 1 x 10. Temperature program as shown. Chart speed, 6 min/in.

Instrument 2(B): Beckman GC-4 with modified on-column inlet and special heater; Columns: stainless steel tubes, 20 in. x 1/8 in. O.D., packed with 3% (w/w) JXR on Gas Chrom Q (100-120 mesh); Nitrogen flow rate: 120 ml/min. at room temperature; Special injector, 280°C; Inlet line, 300°C; Detector line, 325°C; Detector, 340°C; Sample: 1 µl of 1% (w/v) solution in chloroform. Attenuation 5 x 10. Temperature program as shown. Chart speed, 0.2 in/min.

Fig. 3. GLC of Triglycerides of Sheep (A) and Goat (B) Milk. Experimental conditions as given for Instrument 1 in Fig. 2. Peak identities as in Fig. 2.

Fig. 4. GLC of Triglycerides of dog (A) and Human (B) Milk. Experimental conditions and peak identities as in Fig. 3.

Fig. 5. GLC of Triglycerides of Sheep Suet (A) and Beef Tallow (B). Experimental conditions and peak identities as in Fig. 3.

Fig. 6. GLC of Triglycerides of Chicken (A) and Goose (B) fat. Experimental conditions and peak identities as in Fig. 3.

Fig. 7. GLC of Triglycerides of Dog Pancreas (A) and Rat Liver (B). Experimental conditions and peak identities as in Fig. 2.

Fig. 8. GLC of Triglycerides of Goose Kidney (A) and Liver (B). Experimental conditions and peak identities as in Fig. 3.

Fig. 9. GLC of Triglycerides of Raw (A) and Hydrogenated (B) Linseed Oil. Experimental conditions and peak identities as in Fig. 3.

Fig. 10. GLC of Triglycerides of Menhaden (A) and Herring (B) Oils. Injector, 325°C; Nitrogen flow rate: 200 ml/min. at room temperature. Other experimental conditions and peak identities as in Fig. 3.

Fig. 11. Separation of Lard by TLC with Plates Coated with Silica Gel G Impregnated with Silver Nitrate and Developed with 0.7% Methanol in Chloroform. Usually two peaks are seen for each mono, di, tri, tetra, and polyunsaturated glyceride class.

Fig. 12. GLC of Triglycerides of Lard Before and After Silver Nitrate TLC. Experimental conditions and peak identities as in Fig. 3.

Fig. 13. GLC of Oxidation Products of Whole Lard. A, oxidation products only; B, oxidation products plus standard triglyceride mixture. Experimental conditions as in Fig. 3. Peak identities as explained in the text.
Fig. 14. Separation of the Triglycerides of a Fish Oil Margarine by TLC with Plates Coated with Silica Gel G Impregnated with Silver Nitrate and Developed with 0.7% Methanol in Chloroform. Band identities as in Fig. 11.

Fig. 15. GLC of Triglycerides of the Fish Oil Margarine Before and After Silver Nitrate TLC. Experimental conditions and peak identities as given in Fig. 10.

Fig. 16. GLC of neutral Plasma Lipids of Dog (A) and Man (B). Experimental conditions as in Fig. 3. Peak identities as explained in the text.

Fig. 17. GLC of Total Plasma Lipids of Dog. Peak identities as in Fig. 16. Instrument 3: Aerograph Ry-Fi 600 D; Column: stainless steel tube, 18 in. x 1/8 in. O.D., packed with 3% (w/w) JXR on Gas Chrom Q (100-120 mesh) and assembled as shown in Fig. 1. Nitrogen flow rate: 300 ml/min. at room temperature; Injector, 290°C; Detector, equal to column temperature; Sample: 3 µl of whole dog plasma. Attenuation 1 x 16. Temperature program as shown. Chart speed, 1 x 3 in/min.

Fig. 18. GLC of Fatty Acid Methyl Esters. C₁₄ to C₂₄ saturated fatty acids. Instrument 3: Aerograph Ry-Fi 600 D. Column: stainless steel tube, 5 ft. x 1/8 in. O.D., packed with 5% (w/w) SE-30 on silanized Chromosorb W (60-80 mesh) and assembled as shown in Fig. 1. Nitrogen flow rate: 60 ml/min. at room temperature; Injector, 250°C; Detector, temperature equal to column temperature; Sample: NIH standard mixture F, 1 µl of a 1% (w/v) solution in chloroform. Attenuation 1 x 16. Temperature program as shown.

Fig. 19. GLC of Fatty Acid Butyl Esters. C₄ to C₂₀ saturated and unsaturated acids. Instrument 4: F & M High Efficiency Gas Chromatograph, Model 402 with modified injector inlet. Columns: glass U tubes, 4 ft. x 1/4 in. O.D., 1/8 in. I.D., packed with 20% (w/w) diethylene glycol succinate (DEGS) on Gas Chrom P (80-100 mesh); Injector, 230°C; Hydrogen Flame detector, 230°C; Sample: fatty acids of bovine milk fat triglycerides, 1 µl of a 1% (w/v) solution in chloroform. Attenuation 10 x 32. Temperature program as shown.
FIGURE 3

FIGURE 4
FIGURE 11

SATURATES
MONOENES
DIENES
TRIENES
TETRAENES
POLYENES
FIGURE 12

ORIGINAL

SATURATES

MONOENES

TRIENES

TEMPERATURE °C

SENSITIVITY DOUBLED
FIGURE 14

SATURATES
MONOENES
DIENES
TRIENES
TETRAENES
POLYENES
FIGURE 15

ORIGINAL

SATURATES

MONOENES

TRIENES

TEMPERATURE °C

200 225 250 275 300 325 350 375

200 225 250 275 300 325 350 375

200 225 250 275 300 325 350 375

200 225 250 275 300 325 350 375
FIGURE 16

A

B

FIGURE 17

PYROLYSIS PRODUCTS OF PHOSPHOLIPIDS

TEMPERATURE °C
DR. CARPENTER: I would like now to call on Dr. Donald J. Lisk who is head of the Pesticide Residue Laboratory here at Cornell University. He has been doing a great deal of work in this area and of course his pesticide and residue studies are of vital interest to us in our large animal research at the present time. Dr. Lisk.