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The Analysis of Glycerides by High Temperature Gas-Liquid Partition Chromatography

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GAS CHROMATOGRAPHY appears to be a powerful tool for the analysis of triglyceride mixtures. Even though higher molecular weight compounds are inherently more difficult to analyze by means of gas chromatography, useful quantitative analyses of triglyceride mixtures can be made if the operating conditions are carefully chosen.

Gas-liquid partition chromatography has been practically universally adopted as a means of determining the fatty acid composition of fats and oils. Analysis of fatty acid mixtures by gas chromatography has been found to be extremely rapid, sensitive, and reliable. Relatively little effort has been expended in using gas chromatography for the analysis of the triglycerides themselves. Presumably this is due to the extremely low volatility of triglycerides. It would be advantageous to analyze the actual glycerides of natural fats rather than their hydrolysis products. Perhaps an even greater need exists for a method capable of analyzing chemically altered fats and synthetic glycerides. It is hoped that this discussion will stimulate more interest in the analysis of glyceride mixtures by means of gas chromatography. Also, since gas chromatography is a highly flexible analytical tool, many other types of high molecular weight compounds could be analyzed by using the same general procedures which are used for glycerides.

Some preliminary investigations have shown that it is entirely feasible to analyze glycerides by means of high temperature gas chromatography. McInnes *et al.* (1) converted monoglycerides to allyl esters of their corresponding fatty acids and found that these volatile derivatives could be separated with an Apiezon M column operated at 240°C. Acetylated mono- and diglyceride mixtures were analyzed on a silicone grease column operated under isothermal conditions (2). Acetylated mono-, di-, triglyceride mixtures were analyzed on a silicone gum rubber column which was temperature programmed (3). Trimyristin was the largest glyceride glyceride eluted. Preliminary experiments by Fryer *et al.* (4) and Martin *et al.* (5) indicated that it may be possible to analyze natural fats containing glycerides as large as tristearin. Martin *et al.* (5) obtained approximately 12 peaks when they chromatographed margarine stock. Fryer *et al.* (4) obtained "fingerprint" chromatograms of various natural fats and oils.

Although the low volatility of triglycerides necessitates higher operating temperatures, these higher temperatures do not, in themselves, create any difficult instrumental problems. Several commercial gas chromatography detectors are now capable of operation at temperatures adequate for the analysis of glycerides. The thermal conductivity detector used in this study was of conventional design. The reference and detector cells used 50-ohm tungsten filaments operated at 12 volts. Both the detector and injection port were maintained at 370°C. for all triglyceride analyses. Helium carrier gas was preheated by passing it through a 12-in. length of stainless steel tubing contained in the column chamber. The rubber septum was cooled by passing air through a tube coiled near the top of the injection port. Operation of this detector at elevated temperatures decreased sensitivity, increased noise level, and increased the tendency for base line drift. Although these high-temperature effects are certainly undesirable, they did not seriously impair the value of high temperature gas chromatography.

Certain inherent characteristics of higher molecular weight compounds such as triglycerides may create serious analytical difficulties. Glyceride mixtures are likely to be highly complicated and contain components having a wide range of volatility. Thus, while the major fatty acids of lard differ by only four carbons (myristate to stearate), commercial monoglycerides prepared from lard are likely to contain components differing by 54 carbons (glycerol to tristearin). Isothermal analysis of this mixture at a temperature high enough to elute tristearin would result in such a rapid elution of the monoglycerides that resolution would be seriously impaired. This difficulty is minimized by temperature programming of the

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column, as this technique effectively spreads the first peaks apart and brings the later peaks closer together. Faster programming has been found to sharpen the peaks but to decrease separation, while programming from lower starting temperatures spreads the first peaks further apart but increases the total analysis time. In general however programming conditions are not especially critical, and only a few preliminary trials are needed to obtain a well-programmed chromatogram of a glyceride mixture.

Although each peak obtained by chromatographing a natural fat will contain only triglycerides of the same molecular weight, these triglycerides usually contain an assortment of many different fatty acids esterified to glycerol. Thus, a singe peak may represent a mixture of trimyristin, dilaurostearin, and lauromyristopalmitin. A gas chromatography column will attempt to separate this type of mixture, but separation between glycerides of the same molecular weight can usually be achieved only when the differences in fatty acid composition are very great. The usual result is that heterogeneous tri-glycerides merely contribute to undesirable peak broadening.

Another difficulty which becomes significant when high molecular weight materials are analyzed is that the relative difference in volatility between any pair of components in a homologous series decreases as the molecular weight increases (5). A column may be capable of completely separating trioctanoin from trihexanoin but will not resolve tristearin from tripalmitin.

The major problem in the programmed temperature analysis of glycerides is the necessity of obtaining complete elution of all components before the column packing itself is eluted or the glycerides are decomposed. In this connection various methods can be used to extend the applicability of gas chromatography for high molecular weight compounds. Silicone gum rubber is one of the most stable column coatings available and is widely used for high-temperature analyses. Its primary disadvantage is that it is very nonpolar and is therefore only capable of separations on the basis of volatility. The stability of silicone rubber columns may be improved by a preliminary bleed-off procedure to eliminate the more volatile components present in silicone rubber. Very thin liquid phase coatings have been successfully used by Vandenheuvel *et al.* (6) to lower the column temperature required for the elution of steroids. Columns containing a very small proportion of liquid phase coating have a tendency to overload easily and thereby produce skewed peaks. Sensitive ionization detectors will eliminate this problem by permitting the use of smaller samples, but impose other problems such as susceptibility to column bleeding and nonlinearity of response. In addition, the small samples which must be used with very lean columns makes it impractical to perform further analyses on the separated fractions. Most solid supports must be "deactivated" to decrease absorption by uncoated surfaces when an extensive bleed-off procedure or a lean liquid phase coating procedure is used. Very short columns can be used to elute higher molecular weight compounds but these columns will have lower separation efficiencies. Higher gas flows also can be used but they reduce separation efficiency and decrease the response of thermal conductivity detectors. A combination of all these range-extending methods should be made if a glyceride as large as tristearin is to be separated from closely related glycerides.

In this study 8.0 g 100–120 mesh Gas-Chrom P [a] was covered with carbon tetrachloride and approximately 2 ml. silicon tetrachloride was added. After 30 min. the support was dried on a rotary evaporator. The "deactivated" support was stirred into 100 ml. of a 3.0% solution of silicone rubber SE-30[b] in methylene chloride. After 20 min. it was filtered and then dried at 130°C. for two hours. This coating procedure is similar to that used by Horning et al. (7). A 30-in. length of ¼-in. OD stainless steel tubing was filled with this packing and fitted to the detector. This silicone rubber column was preconditioned at 400°C. in the presence of air for one hr. It was then programmed from 250°C. to 400°C. over a 30-min. period with a helium flow of 30 ml./min. This temperature programming procedure was repeated approximately ten times with increasing helium flow rates until a flow of 200 ml./min. was finally used. It is estimated that a 3% silicone rubber coating remained after this conditioning procedure. Incompletely conditioned silicone rubber columns appear to have the unique property of drastically reducing the apparent thermal conductivity response of higher molecular weight compounds. When an incompletely conditioned column is programmed this loss in response occurs even before bleeding causes a noticeable base line drift. Bleed-off or conditioning progress may be followed by chromatographing a mixture of compounds having a wide range of molecular weights and noting the temperature where peaks become smaller than normal or are absent. For example, if a triglyceride test mixture of tributyrin through tristearin is chromatographed, trilaurin and lower molecular weight glycerides may be completely eluated but trimyristin and higher molecular weight glyceride peaks may be absent. This column would only be suitable for analyzing mixtures containing components more volatile than trimyristin. More extensive conditioning would be necessary if higher molecular weight compounds were to be analyzed.

A mixture of simple triglycerides [c] from triacetin to tristearin was made and 1.7 μl. aliquots were chromatographed. A helium flow of 145 ml./min. was used for all glyceride analyses. A typical chromatogram of this mixture is shown in Fig. 1. The peaks are numbered according to the total number of fatty acid carbons present in the molecules. The peaks seen between the major simple triglyceride peaks are due to mixed triglyceride impurities. This analysis required 25 min. Injection of a larger sample would have increased the peak heights but also would have overloaded the column to an objectionable extent. This chromatogram illustrates the feasibility of analyzing very wide boiling point range mixtures by means of the programmed column temperature procedure. It also illustrates the continuously decreasing difference in volatility between higher molecular weight pairs in a homologous series. Even though the rate of programming was decreased as the temperature increased, the glycerides were eluted at closer and closer intervals. This phenomenon severely limits the separating ability of a gas chromatographic column for high molecular weight compounds.

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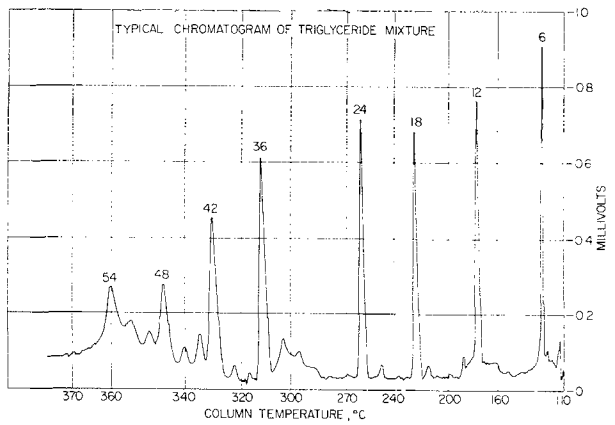

FIG. 1. Typical chromatogram of synthetic triglyceride mixture.

The peak areas from four analyses of this mixture were determined by triangulation. Previous gas chromatographic analyses of the individual triglycerides had shown that the major impurities were triglycerides differing by only two carbon units. In other words, the major impurities in trilaurin were found to be dilaurocaprin and dilauromyristin. Therefore the true area response of each major triglyceride was assumed to be the total area of the simple triglyceride peaks and their adjacent homologues. Table 1 compares the average relative peak areas from four chromatograms with the weight and molecular compositions of the synthetic mixture. The average deviation from the mean peak areas was 1.2%.

TABLE I

Comparison of Relative Peak Area with Composition of a Synthetic Triglyceride Mixture

Glyceride	Actual analysis			Adjusted analysis		
	Molar %	Peak Area %	Weight %	Molar %	Peak Area %	Weight %
Triacetin	12.9	8.1	5.1	.....	.....	.....
Tributyrin	13.7	11.7	7.5	.....	.....	.....
Trihexanoin	10.5	12.0	7.4	17.9	17.6	11.6
Trioctanoin	14.6	13.7	12.6	24.9	20.1	19.8
Trilaurin	13.4	16.5	15.6	22.8	24.2	24.5
Trimyristin	12.0	16.0	15.9	20.4	23.4	25.0
Tripalmitin	8.2	10.1	12.1	14.0	14.8	19.0
Tristearin	14.6	11.9	23.7	.....	.....	.....

It is probably naïve to expect a thermal conductivity detector to respond equally to all glycerides within as great a molecular weight range as was used in this synthetic mixture. If these data are mathematically adjusted to include only the trihexanoin through tripalmitin glycerides, a fairly good agreement between peak area % and molar % composition is obtained (Table 1). It is possible that the lower apparent response of tristearin was due to slight column bleeding rather than to a lower thermal conductivity response of tristearin. Triacetin and tributyrin may actually have a low molar thermal conductivity response. The data of Fryer *et al.* (4) also suggest a lower response for tributyrin. Quantitative analysis of glyceride mixtures containing from 18 to 48 fatty acid carbons will be sufficiently accurate for most purposes if the peak area % is assumed to be directly proportional to the molar composition. Unfortunately this molar response relationship imposes an additional difficulty to the analysis of triglycerides. Higher molecular weight triglyceride peaks are likely to be smaller and consequently more difficult to measure

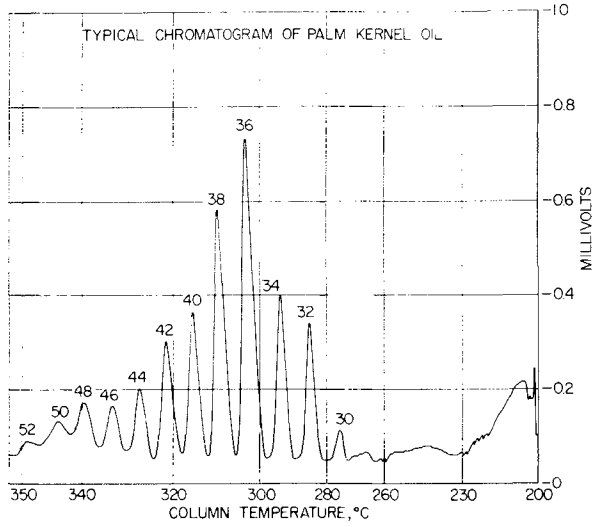

FIG. 2. Typical chromatogram of palm kernel oil.

accurately. Nevertheless gas chromatography is a highly useful tool for the analysis of glyceride mixtures, as will be seen in the subsequent chromatograms.

Analysis of natural fats is more difficult than that of simple triglyceride mixtures. Triglycerides found in natural fats normally differ by only two carbon increments rather than by six carbon increments. Furthermore the heterogeneity of naturally occurring triglycerides tends to produce broader peaks.

The chromatogram shown in Fig. 2 was obtained from 2.0 μl. of refined palm kernel oil. The total analysis time was 22 min. The total fatty acid carbon contents of the peaks were determined by comparison of the retention temperatures with those of known glycerides. Since palm kernel oil contains a preponderance of lauric acid, the large peak corresponding to glycerol esterified with 36 fatty acid carbons is not unexpected.

A chromatogram from 2.0 μl. butteroil is shown in Fig. 3. This chromatogram required a total analysis time of 24 min. Butteroil has a more diverse fatty acid composition than does palm kernel oil and therefore it can be expected that its triglycerides are more heterogeneous mixtures. Consequently the triglyceride peaks from butteroil tend to be broader than for palm kernel oil. It is interesting to note that the

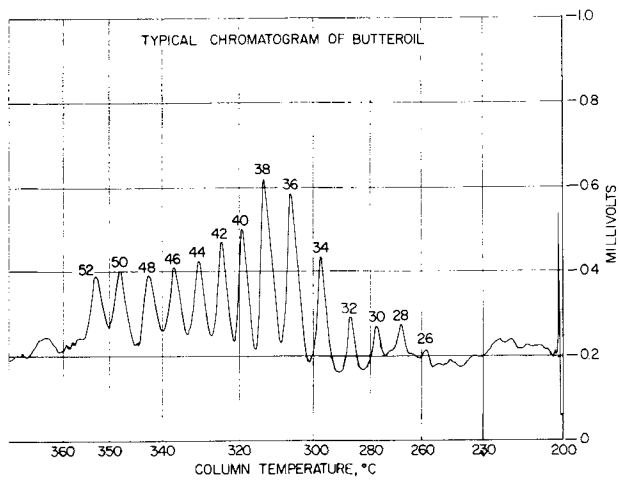

FIG. 3. Typical chromatogram of butteroil.

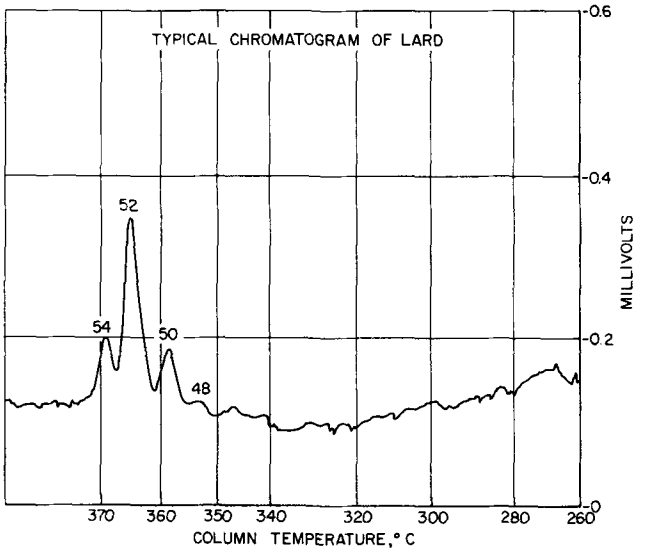

FIG. 4. Typical chromatogram of lard.

lowest molecular weight triglyceride peak found in butteroil contained 24 fatty acid carbons, even though butteroil contains a sizeable amount of butyric and caproic acids. Butteroil is apparently composed of triglycerides containing both long- and short-chain fatty acids in the same molecule.

Fig. 4 shows a chromatogram obtained from 0.6 μl. lard in a total analysis time of 12 min. The analysis of this type of very high molecular weight mixture is much more difficult than the preceding analyses. The triglycerides contained in lard have a lower thermal conductivity response and their elution temperature is close to the point where column bleeding becomes a serious problem. Even so, gas chromatography may be used to obtain useful information from this type of glyceride mixture.

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Monitoring Eluates from Chromatography and Countercurrent Distribution for Radioactivity[1]

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UNIQUE ANSWERS to problems as to the mechanism of organic reactions and of biological transformations are frequently provided by the use of radioactive isotopes. Their application usually involves three steps: 1) Addition of a known, radiochemically pure compound; 2) fractionation and reisolation of intermediates or reaction products; 3) isotopic assay of radiochemically pure isolated components. Chromatography affords a powerful tool for carrying out the second step. However, isotopic assay of the isolated products in step 3 has frequently posed a problem because of the difficulty of assaying radioactivity on the small amounts of individual components available.

The present review of methods for monitoring eluates for radioactivity is limited to a consideration of C^{14}- and H^3-labeled compounds and is restricted to 1) liquid-liquid chromatography (LLC), 2) countercurrent distribution (CCD), and 3) gas-liquid chromatography (GLC). Paper chromatograms have long been assayed for radioactivity, but the well-established techniques (1), which have been developed, are not discussed in this review.

Liquid-Liquid Chromatography. Liquid-liquid chromatography may be monitored by discontinuous or continuous methods of assay. One of the simplest procedures of discontinuous assay is illustrated by Fig. 1 for the chromatography of methyl oleate exposed to tritium gas (2). This reaction yields the 9,10-tritiostearate (3). Chromatography of the fatty acids was carried out by the liquid-liquid partition chromatographic procedure of Nijkamp (4), which employs a methyl alcohol-isooctane solvent system on a silicic acid column. Alternate 1-ml. eluate fractions were (a) titrated in a nitrogen atmosphere with 2/10 *N* potassium hydroxide to a thymol blue endpoint using a Gilmont microburet and (b) diluted with 15 ml. of scintillation solution for assay of radioactivity with an Automatic Tricarb Scintillation Spectrometer (Packard Instrument Company). The time of counting may be continued over a sufficient period of time to attain the desired statistical accuracy of results. If absolute measurements of radioactivity are required rather than relative values, the presence,

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