movable and can be replaced by a fatty acid at the last stage, this method offers better prospects for a synthetic approach to ceramide polysaccharides.

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Gas-Liquid Chromatography of Glycerides

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Abstract

Available techniques are reviewed for gas-liquid chromatography of mono-, di- and triglycerides and their chemical modification products. Major emphasis is placed upon optimum separation and quantitative estimation of the recorded peaks. Nonlinear rates of temp programming are shown to be superior to linear rates for maximum resolution of complex glyceride mixtures. The use of short columns improves the recoveries of the components but may not provide a sufficient number of theoretical plates for all types of separations. The quantitative interpretation of the results is facilitated by the utilization of a flame ionization detector which gives correct weight response for the combustible carbon content of these materials.

Introduction

TRIGLYCERIDES ARE THE MAJOR components of all fats and oils of animal and vegetable origin. Diglycerides are produced by the action of phosphatidase D on phosphatides, and mono- and diglycerides are released through partial hydrolysis of triglycerides by lipases. A chromatographic fractionation of all of these lipid classes is of interest because of the information it will yield on the structure of the fats and their metabolism. In recent years it has been customary to analyze them by gas-liquid chromatography (GLC) of the constituent fatty acids as the methyl esters. It is now possible, however, to elute triglycerides including tristearin as well as the mono- and diglycerides from GLC columns without prior hydrolysis of the glyceryl ester bonds.

Separation of Triglycerides

The feasibility of GLC for triglyceride analyses has been amply demonstrated in the past and much of the initial work was reviewed by Huebner (1). The advantages of using short narrow bore columns in combination with thermally stripped SE-30 (a methyl silicone polymer obtained from the General Electric Corporation) films have since been described (2). More recently preparative systems have been developed (3) which have permitted the recovery of many of the peaks. The recovered materials have been shown to be undecomposed triglycerides by reinjection into the gas chromatograph and by enzymatic and thin-layer chromatographic methods. During the last few years gas chromatography has been applied to the fractionation of the triglyceride mixtures of vegetable oils (2), butter oil (4), their molecular distillates (5,6), adulteration mixtures (7), and their chemical (8) and enzymatic (9) reconstitution products. Essentially identical systems have permitted a direct GLC analysis of naturally occurring neutral lipid mixtures (6).

No systematic investigation of the critical parameters involved in the GLC of triglycerides has yet appeared. The following presentation illustrates the effect of some common gas chromatographic variables upon the resolution and recovery of some synthetic and natural triglyceride mixtures.

Liquid Phases

A prerequisite for high temp separations is a stable high temp liquid phase. This limits the choice to the various silicone polymers of which the most popular has been the SE-30. Thermally stripped, i.e. preconditioned, SE-30 columns were used in the first practical demonstration of the use of GLC for the separation of natural triglycerides under moderate conditions (2).

Figure 1 shows the elution patterns recorded for butterfat and a mixture of butterfat and four simple triglycerides: trilaurin (C_{36}), trimyristin (C_{42}), tripalmitin (C_{48}) and tristearin (C_{54}). The separations are based on the carbon number (mol wt) of the glycerides. A nearly complete overlap is obtained between unsaturated and saturated triglycerides of comparable chain length. This is illustrated in Figure 2 by the elution patterns given by peanut oil and a mixture of peanut oil and tristearin in a ratio of 3 to 1.

Despite acknowledged differences in selectivity with respect to many other compounds, the thermostable fluoroalkyl silicone polymer QF-1-0065 (obtained from the Dow-Corning Corporation) yielded triglyceride elution patterns similar to those recorded on the SE-30 column. A selective retention effect with this phase was not observed for triglycerides having double bonds. Figure 3 compares the gas chromatograms obtained for coconut oil on the SE-30 and the QF-1 liquid phases. The dimethylpolysiloxane gum JXR (a silicone gum produced by Applied Science Laboratories), claimed to be somewhat more stable than either SE-30 or QF-1, possessed similar triglyceride separating characteristics. Because of high velocity and easy thermal degradation the polyester phases are less satisfactory (10). It would seem that with the development of dual column systems, many other liquid phases will become available for high temp applications.

To obtain the highest possible efficiency in the separation of the high mol wt triglycerides, small samples and thin film columns should be used. Although the actual film thickness is uncertain and probably steadily changing, it has been estimated (6) that the most efficient separations are obtained with coatings at the 1% (w/w, Chromosorb W) level. Columns containing 3 to 5% of liquid phase are not recommended for work with long-chain triglycerides because of the very high

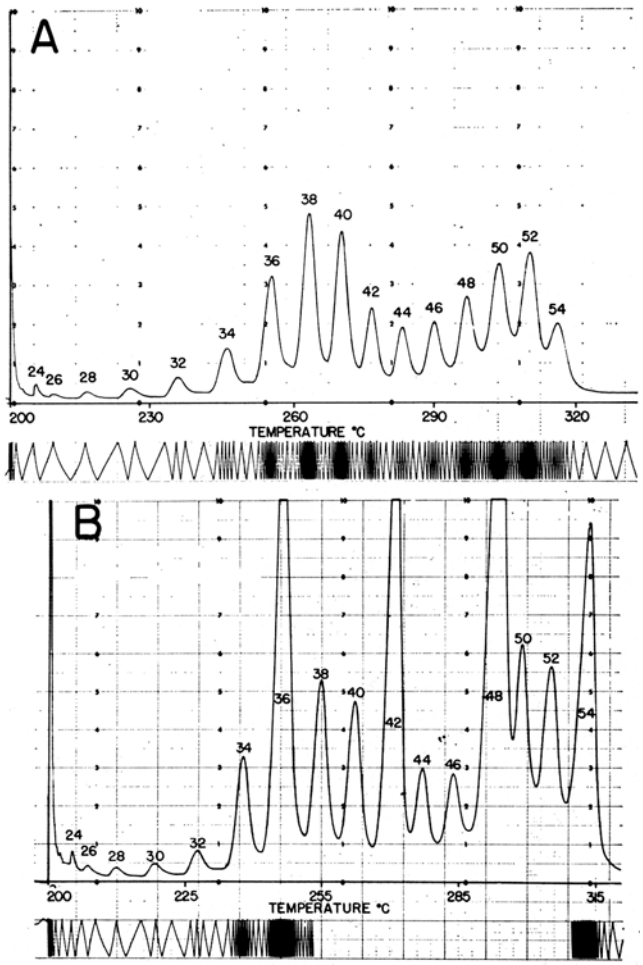

FIG. 1. GLC elution patterns recorded for butterfat (A) and a mixture of butterfat and four standard triglycerides (B): trilaurin (*36*), trimyristin (*42*), tripalmitin (*48*), and tristearin (*54*). Other carbon number assignments relative to standards. Conditions: stripped 2.25% SE-30 on Chromosorb W (60–80 mesh); 18 in. × ⅛ in. O.D. column; nitrogen flow rate 150 ml/min. Other conditions as indicated in the figure. Instrument: Aerograph Hy-Fi Model 600-B, F & M Linear Temperature Programmer Model 40 and Brown 1 mv recorder.

temps needed for their elutions. Such columns have been employed effectively, however, in preparative work with low and medium chain length triglycerides (3).

For best results the supports should be inactivated. Dimethyldichlorosilane seems to be a satisfactory siliconizing agent. Acid washed Chromosorb W, however, has been employed with singular success following multiple applications of the coating and intermittent thermal stripping (2). Thermal stripping of varying duration was necessary for most columns and produced the most stable and longest lasting coatings (6). With the availability of liquid phases of uniform mol wt and higher temp stability, direct application of the desired amt of stationary phase, however, should become practical.

Column Length

In steroid separations SE-30 columns of 5–6 ft in length are commonly employed (11). Such columns packed with supports containing about 1% stationary phase allow also the separation and recovery of the lower mol wt triglycerides. The medium and long-chain triglycerides are recovered partially or not at all. Under these conditions the peak separations are very great and suggest higher column efficiencies than those necessary for the resolution of triglycerides differing by two methylene units. Considerably shorter

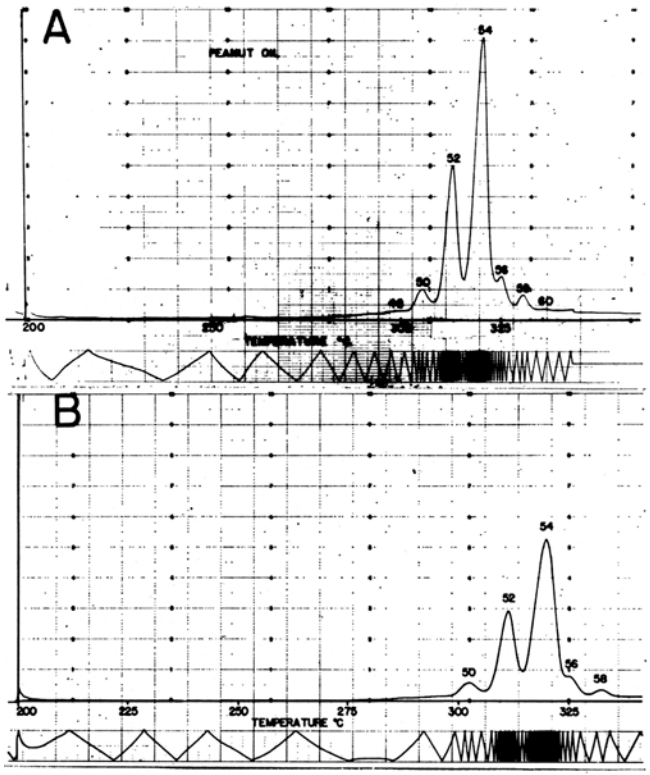

FIG. 2. GLC elution patterns recorded for peanut oil (A) and a mixture of peanut oil and tristearin (B). Chromatography conditions and carbon number assignments as in Figure 1.

columns can therefore be used. With the ⅛ in. O.D. column, a length of 12 to 18 in. provided a sufficient number of plates (3500 to 5000) for a complete partition of naturally occurring triglyceride mixtures. (The theoretical plates were calculated from temp programmed runs using the plate equation for isothermal conditions). As a result, the retention times and elution temps decreased greatly for all triglycerides. Columns as short as 6 in. have been tested since and further decreases in retention times and elution temps observed. Although these short columns permitted the most rapid and, what was later shown to be the most complete recovery of all the simple test triglycerides, they did not have enough theoretical plates for the partition of natural triglyceride mixtures. Figure 4 shows the separation of simple triglycerides on a 6 in. column containing 3% SE-30 as liquid phase. Even though the amount of the stationary phase has been tripled, the efficiency of such a column is low. The number of available theoretical plates (under conditions of temp programming) is approximately 400 (tristearin). For special separations, such as those involving triglycerides of odd and even carbon number fatty acids, more than the max 4000 to 5000 theoretical plates available in the 18 in. column may be necessary, and somewhat longer columns will have to be used.

Temperatures

The temperature requirements, up to 400C for the elution of tristearin, indicated in some of the earlier publications on triglyceride gas chromatography (1), would appear to be hazardous and much higher than necessary. Although the boiling points of these materials are high even at reduced pressures (e.g., 313C at 50μ for trimyristin) and would approach cracking temps (over 600C) when extrapolated to atmospheric pressure (12), they cannot be directly related to the chromatography conditions. The temp of emergence of a given glyceride peak also depends upon the type

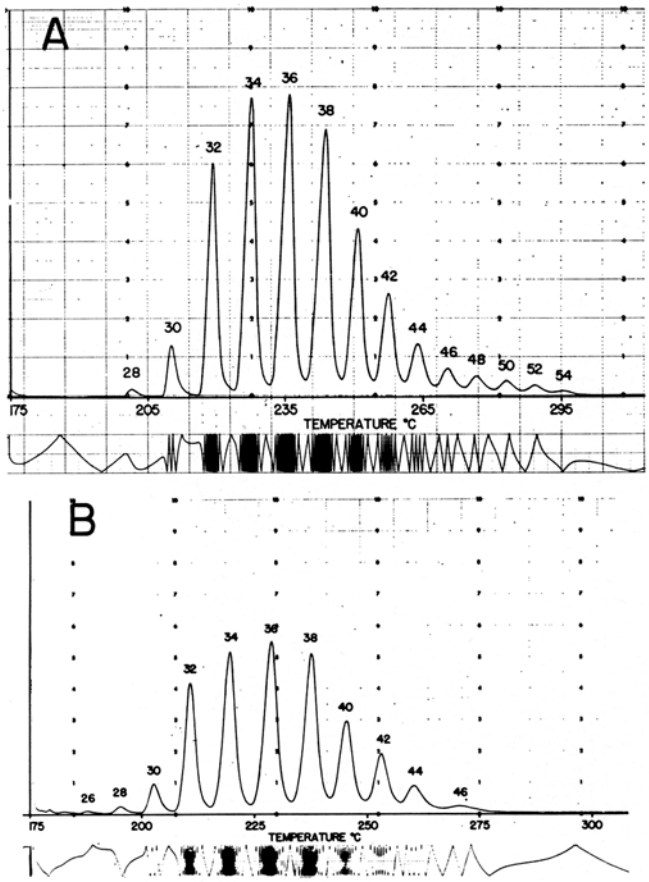

FIG. 3. Gas chromatographic separation of coconut oil on SE-30 (A) and QF-1 (B) liquid phases. Carbon number assignments and SE-30 column conditions as in Figure 1. QF-1 column: 1% QF-1 on Gas-Chrom P (100–120 mesh); 18 in. × ⅛ in. O.D.; nitrogen flow rate 100 ml/min. Other conditions as indicated in the figure. Instrumentation as in Figure 1.

and quantity of the liquid phase as well as the time allowed for elution. It is well known that gas chromatographic partitions do not have to be performed at the boiling point of the substances to be separated. Thus, the elution of methyl stearate at 185C under standard conditions of fatty acid analysis seems to be generally recognized as a readily accomplished task, yet the boiling point of this material has been extrapolated (12) to be 376.1C at atmospheric pressure. Obviously these high mol wt esters exhibit vapor pressures sufficiently high for effective GLC well below their boiling points. With aged 18 in. columns it has been possible to recover all the triglycerides of butter oil including the C_{54} component below 275C (4). This accomplishment compares favorably with the recovery of triolein and trilinolein at 290C in 15 to 20 min (13).

Another hazard has resulted from the popular belief that the injection port should be maintained at a temp considerably higher than the column to insure complete and rapid vaporization of all components. This requirement would seem to be carried over from the studies under isothermal conditions where one ought to strive for a plug type of introduction of the sample to avoid spreading the components over a long length of the column. With temp programming there is no need for complete vaporization of the sample prior to admitting to the column. Either on column injection or blowing of the sample onto the column in a partially evaporated state when performed at low enough column temp ensures that the sample enters the column in a narrow band.

Although satisfactory resolution of the simple triglycerides has been obtained under isothermal conditions (14), for the analyses of complex naturally occurring glyceride mixtures, temp programming is essential. Most of the programs thus far employed have been linear, a temp increase of 2–3C/min giving the best spacing. Due to continuously decreasing difference in volatility between the higher mol wt pairs in a homologous series, however, the longer chain glycerides are eluted at progressively closer intervals. To maintain an even spacing of the peaks, nonlinear temp programs are necessary. Figure 5 illustrates the effect of a short period of accidental cooling of the column under conditions of normal operation. The width of the peak about to emerge increases and there is a temporary return to the base line. This suggests a need for a leveling off in the heating rate.

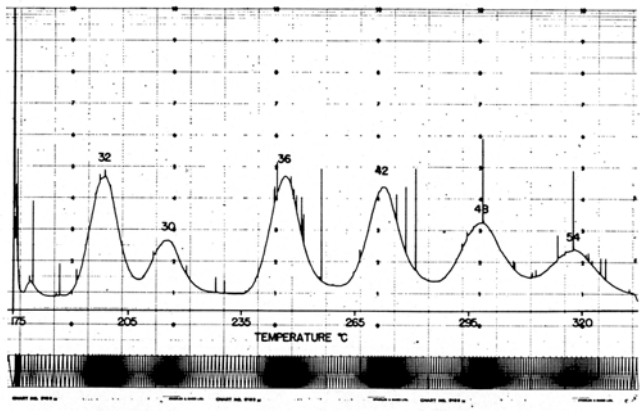

FIG. 4. Gas chromatographic separation of simple triglycerides on a low efficiency column: dotriacontane (*32*), tridecanoin (*30*), trilaurin (*36*), trimyristin (*42*), tripalmitin (*48*) and tristearin (*54*). Conditions: 3% SE-30 on Chromosorb W (60–80 mesh); 6 in. × ⅛ in. O.D. column; nitrogen flow rate 200 ml/min.; other conditions as given in the figure. Instrumentation as in Figure 1.

Simple powerstat controlled ovens permit some of the best approximations of the ideal temp programs. These allow rapid initial programming (15–20C/min), but the rate slows as the temp approaches the desired maximum. Furthermore, such nonlinear temp programs have special advantages for both the direct and the indirect oncolumn injection. Figure 6 shows the separation obtained for a sample of coconut oil in an oven controlled manually by powerstat. The temp program is indicated on the chromatogram. The small amts of the longer chain triglycerides seen in Figure 3 have been lost under these conditions. As the sample is deposited on the relatively cool column, the material remains in a narrow band. As the temp increases, the components have time to rearrange and line up in the order in which they ultimately emerge. The peaks are kept sharp and uniform by the rapidly increasing temp during the early part of the run. Each component is further separated as it moves down the column.

Other Conditions

Either direct or indirect on-column injection can be effectively accomplished only in those instruments in which the injection port is close to the end of the column (2 to 6 in.). The detector should be sensitive enough to permit analysis of small amts of material and located preferably directly at the end of the column. Both helium and nitrogen have been used as carrier gases with equal success. The optimum flow rates of the carrier gases have varied from about 100–300 ml/min depending on the pressure, the mesh size of the supporting material and the column length. Because of the high temp stability and great sensitivity

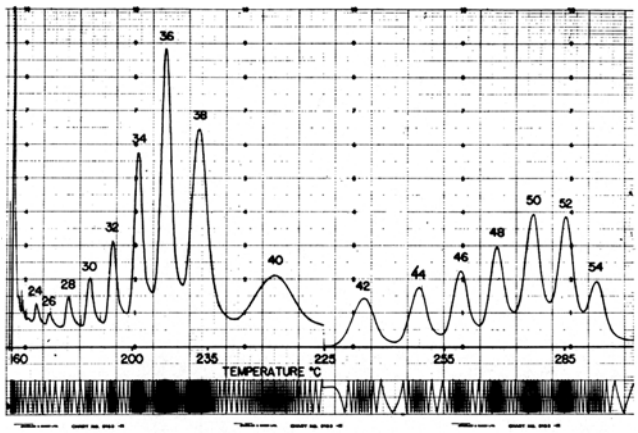

FIG. 5. Effect of changes in temperature programming upon the separation of butterfat triglycerides. Chromatography conditions as in Figure 1. After 18 minutes of programming the lid of the oven was momentarily opened.

for carbon rich materials the hydrogen flame ionization detector is preferred. This detector also allows a direct correlation of the recorded areas with the weights of the individual components of the test mixture.

Quantitation

The problems of quantitation in triglyceride gas chromatography encountered with a thermal conductivity detector have been discussed by Huebner (1) and some of these apply also when using the hydrogen flame ionization detector. In addition to the possible differences in the detector response to different glycerides, there is always the problem of the completeness of recovery of the individual components. Whether or not all the fat has been accounted for in terms of triglyceride peak area may be decided in a variety of ways. Thus, lack of early peaks might be taken as a good indication of the absence of decomposition or fragmentation of the material at the time of injection. The recovery of all and only the anticipated peaks for a mixture of authentic compounds serves a similar purpose. The use of CS_2 as the solvent is particularly advantageous as this solvent gives only negligible response in the hydrogen flame. An indication of loss is obtained by chromatography of weighed amts of standard triglycerides and determination of either absolute response or relative area proportions.

Figure 7 compares the elution patterns obtained for a mixture of saturated triglycerides under two different sets of conditions. After minor corrections were made for impurities, the areas recorded in the first part of the figure matched the weight percentages exactly. The data are given in Table I. The incomplete recovery of the longer chain triglycerides in the second part of Figure 7 is obvious. Only after further stripping of the liquid phase were correct area proportions noted. As a result of these observations, it has been found convenient (6) to proceed with the stripping of new columns (preconditioning) until correct area proportions are recorded for a mixture of trilaurin, trimyristin, tripalmitin and tristearin, although this might not be absolutely necessary when working with an internal standard.

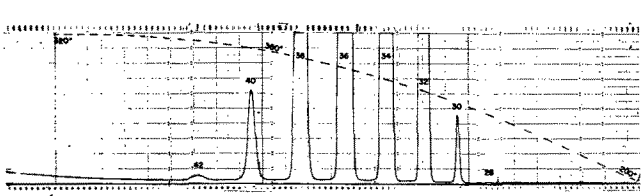

FIG. 6. GLC separation of coconut oil triglycerides in a powerstat controlled oven. Carbon number assignments as in Figure 3. Conditions: stripped 5% SE-30 on Chromosorb W (60–80 mesh); 2 ft. × ⅛ in. O.D. column; nitrogen flow rate 200 ml/min.; other conditions as given in the figure. Instrument: Aerograph Hy-Fi Model 600-C with L & N Speedomax H 1 mv recorder.

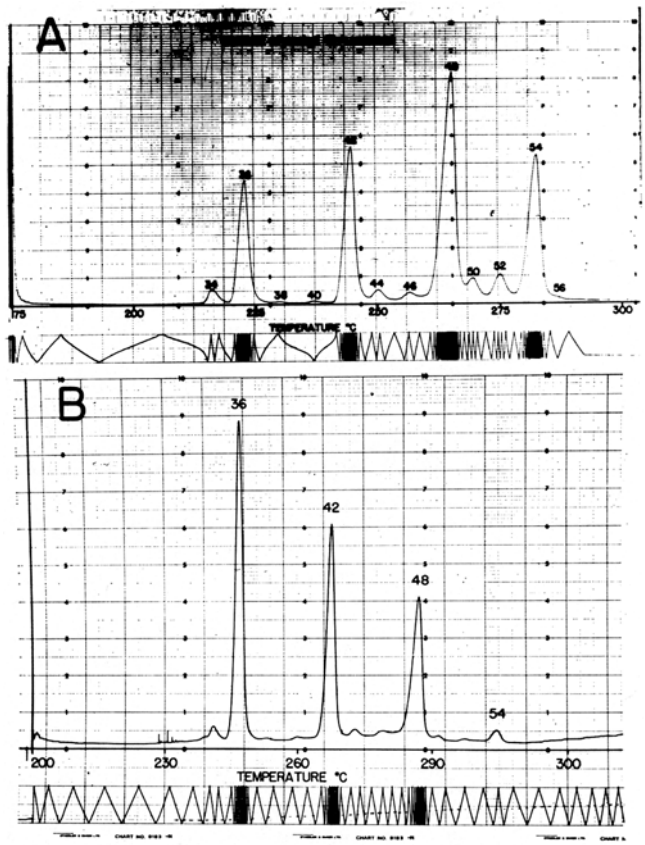

FIG. 7. Gas chromatographic separation of simple saturated triglycerides at the end (A) and during (B) the stripping of a 2.25% SE-30 column. Carbon number assignments and instrumentation as in Figure 1.

The recovery of an unknown triglyceride mixture may be checked by adding weighed amts of the standards to known weights of unknown and comparing the recorded area proportions. On properly bled and equilibrated columns proportionally correct quantitative results have been obtained using both saturated and unsaturated standards and a variety of natural oils (4,5,7,8,). Figure 8 shows the gas chromatograms used for the estimation of the recoveries of coconut oil (8) and a molecular distillate of butter oil (5). Tridecanoin (25% by weight) was added to the coconut oil and tripalmitin (5% by weight) to the distillate.

When depending on comparisons of total weight and area proportions, it is possible that the overall

TABLE I

Comparison of the Proportions of Weight and Peak Area of a Synthetic Triglyceride Mixture at Various Times of Stripping (Preconditioning) of the Liquid Phase

Glyceride	Weight %	Peak area proportions %		
		Beginning [a] of stripping	End of stripping	Aged [b] column
Trilaurin	15.2	43.0	18.1	15.2
Trimyristin	19.6	29.9	20.6	19.4
Tripalmitin	39.6	24.8	38.6	40.4
Tristearin	25.5	2.3	22.7	25.0

[a] See Figure 7B.
[b] See Figure 7A.

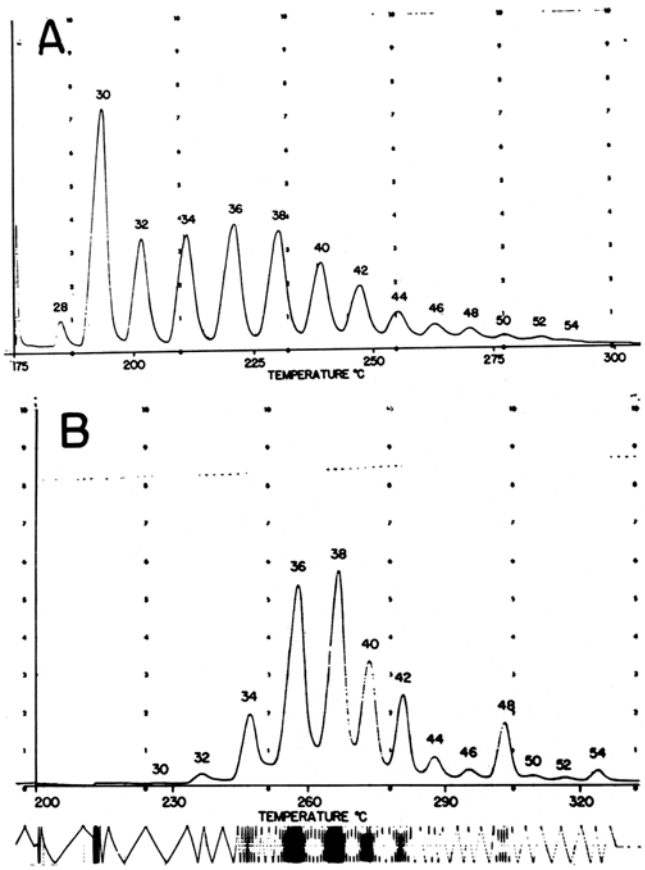

FIG. 8. Gas chromatographic estimation of triglyceride recovery of unknowns by means of internal standards. Chromatography conditions as in Figure 1: (A) coconut oil plus 25% tridecanoin on stripped 5% SE-30 column; (B) butter oil distillate plus 5% tripalmitin on stripped 2.25% SE-30 column.

losses of the unknowns may approximate those of the added standards, thus obscuring the extent of the true recovery. Occasionally the uncertainty of the area-weight relationship may be further compounded by unequal responses of the short and long chain glycerides in the detector. Whether or not this has been the case may not always be readily ascertained, and low recoveries may be confused with low response. Recoveries may be calculated by comparing the sum of the individual peak areas to the total area response recorded when an open tube is substituted for the chromatographic column. While at first this technique may appear to be reliable, except for difficulties in reproducible sampling, closer examination reveals a number of drawbacks. Complications may arise when dealing with mixtures of heterogeneous materials which may not give additive responses. Therefore, in practice this method is limited to mixtures of homologs or near homologs. Furthermore, any deficiencies in the recoveries are distributed over the whole series of compounds, regardless of individual recoveries. While heat stable saturated triglycerides may be assumed to reach the flame unchanged, highly unsaturated materials are known to decompose readily, and what is detected as a peak in the flame may have only a remote relationship to what was admitted to the flash evaporator. Other materials may remain trapped on the column. Recoveries for individual peaks, are therefore necessary, and these are obtained when dealing with known materials eluted at the rate of one component per peak.

When more than one component per peak is encountered and the substances are present in an unknown ratio and differ in stability, this technique fails.

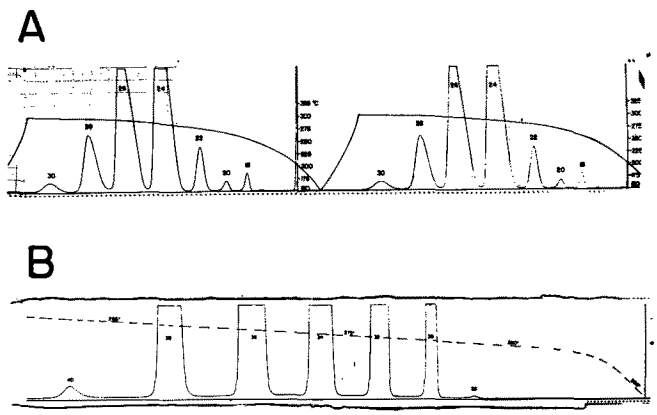

FIG. 9. Preparative gas chromatographic separation of short chain triglycerides. (A) a repeat sequence with synthetic medium chain length triglycerides; (B) a section of a repeat sequence with coconut oil. Carbon number assignments as in Figure 1. Conditions: partially stripped 5% SE-30 on siliconized Chromosorb W (60–80 mesh); 2 ft. × ¼ in. O.D. column; nitrogen flow rate 200 ml/min.; other conditions as given in the figure. Instrumentation: Aerograph Autoprep 700 with splitter and hydrogen flame ionization kit. Split ratio 4.5 to 1.

Such may be the case with mixtures of natural triglycerides separated by means of temp programmed gas chromatography on the basis of their carbon numbers where both saturated and unsaturated triglycerides may occur in one peak. This is illustrated by a study of butterfat adulteration with a vegetable fat (7). In addition to the predominantly saturated butterfat triglycerides, peaks 50, 52 and 54 now contain the largely unsaturated C_{50}, C_{52} and C_{54} glycerides of the plant oil (2) which may have different thermal stability or adsorbability and may not yield the peak proportions anticipated on the basis of the overall weight proportions. It has been found (4) that in such cases the correctness of the estimated peak areas may be ascertained by making use of the readily accessible data on the fatty acid composition. Table II illustrates the calculation of the theoretical carbon number for the pure butterfat and shows how it can be experimentally estimated from the fatty acid proportions determined separately and independently. By this means it is possible to arrive at a reliable estimate of the actual recoveries if the triglyceride mixture is complex enough. It has been demonstrated that the recoveries obtained for the common vegetable oils and butterfat are of the order of 95 to 100% of the theoretical carbon yield (4,7,8). Ultimately preparative gas chromatography systems could be used and the recoveries determined by direct weighing of the col-

TABLE II

Calculation of Fatty Acid Carbon Recovery (4)[a]

Theoretical fatty acid carbon number (TCN)	Fatty acid carbon number for experimentally determined triglyceride distribution (ECN)
Formula:	
$TCN = 3\Sigma_k$ (Mole % F. A.) × (X)	$ECN = \Sigma_n$ (Mole % T. G.) × (X′)
X is the number of carbon atoms per fatty acid residue	X′ is the number of fatty acid carbon atoms per triglyceride residue
k is the number of fatty acids	n is the number of triglyceride types
Example:	
$TCN = 3\Sigma_{12}$ (7.8 × 4) + ... + (0.5 × 20) = 439050	$ECN = \Sigma_{31}$ (4.00 × 54) + ... + (0.51 × 24) = 421811

$$\text{Recovery of theoretical carbon yield} = \frac{ECN \times 100}{TCN} = \frac{421811 \times 100}{439050} = 96\%$$

[a] Dividing the TCN and ECN by 10,000 (the total number of triglycerides) gives the carbon number of the average triglyceride. The latter value can be correlated to the molecular weight of the average triglyceride estimated from the saponification value.

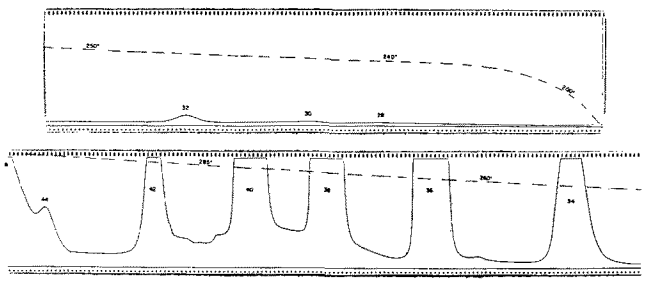

FIG. 10. Preparative gas chromatographic separation of butter oil triglycerides. Carbon number assignments as in Figure 1. Chromatography conditions as in Figure 9.

lected material, but so far only short chain triglycerides have been recovered from gas chromatographs in weighable quantities.

For many problems the separations provided by these columns are unsatisfactory. Butterfat cannot be completely resolved on account of the presence of odd carbon number triglycerides which emerge between peaks of adjacent even carbon number triglycerides. For preparative gas chromatography with an automatic fraction collection it is necessary that a reliable and reproducible return to the base line be obtained between any two adjacent peaks. This may be corrected to a considerable extent by selecting a more suitable temp program. A greatly increased number of theoretical plates, on the other hand, is not likely to improve the elution pattern, as there is reason to believe that triglycerides with multiple double bonds will be eluted ahead of their saturated counterparts resulting in undesirable peak broadening and possibly new overlaps (14,15).

Preparative Gas Chromatography

Although each peak obtained by chromatographing a natural fat contains only triglycerides of the same carbon number or mol wt, these triglycerides usually contain an assortment of many different fatty acids esterified to glycerol. In order to identify the glycerides it is necessary to isolate the individual peaks and to determine their fatty acid compositions. This has been successfully accomplished (3) by repetitively injecting and collecting samples from an essentially analytical column (¼ in. O.D. × 2 ft). To avoid the incineration of the whole sample the effluent stream was split. A split ratio of 4.5:1 was found to allow the collection of 20 to 50 mg of each peak with some 10 to 20 injections of a triglyceride mixture of a few peaks,

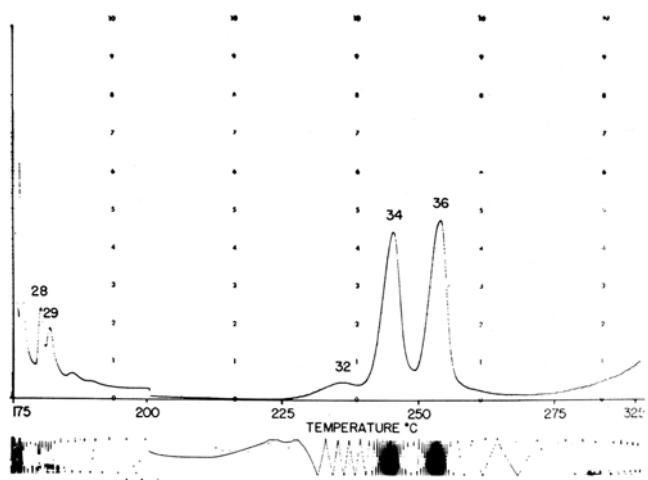

FIG. 11. Gas chromatographic separation of corn oil diglycerides. Carbon number assignments based on fatty acid composition. Chromatography conditions as in Figure 1.

proportionally higher number of injections being necessary for the collection of similar quantities of triglycerides from mixtures containing 10 or more components. Attempts to further increase the load resulted in serious cross-contaminatoin of the eluted peaks. The first part of Figure 9 shows a repeat sequence obtained (3) with synthetic, medium chain length triglycerides (MCT) (obtained from Drew Chemical Corporation, Boonton, N.J.) in the Aerograph Autoprep. A representative section of a similar sequence for coconut oil is given in the second part of this figure.

The lack of linear temp programming facilities in the standard Autoprep A-700 proved to be beneficial. The selection of fixed powerstat settings allowed programming rates not easily reproduced with a linear programmer. The return to base line between adjacent peaks is absolutely complete, thus allowing a reliable operation of the recorder pen activated fraction collector. With heavier coating however, recoveries are excellent only for the lower mol wt triglycerides. The longer chain glycerides are incompletely eluted under the present working conditions. This incomplete recovery of the longer chain triglycerides is further demonstrated in Figure 10, which shows the separations obtained with butterfat triglycerides under similar experimental conditions. Some difficulty is seen to arise also with the odd carbon number triglycerides. The separations obtained, however, are encouraging. They show that the temp program is correct and suggest that adjustments in the amount of the stationary phase will result in acceptable separations. However, our work with the preparative gas chromatograph has been limited only to the investigation of the triglyceride structures of the lower mol wt synthetic triglycerides (MCT) and the molecular distillates of butter oil and coconut oil, for which these columns are satisfactory.

Separation of Mono- and Diglycerides

Preliminary investigations have shown that it is entirely practicable to analyze mono- and diglycerides by means of gas chromatography. McInnes et al. (16) have 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 isothermally at 240C. Huebner (17) found that mono- and diglycerides, as such, could not be eluted from a column containing 23% silicone grease, but that acetylation assured their complete elution. Monoglycerides up to monoarachidin and diglycerides up to myristopalmitin were separated in the temp range of 284 to 317C. Small extreneous peaks, however, appeared before most of the major peaks suggesting that thermal changes of some sort took place on the column. Of great interest is Huebner's observation that the alpha-glyceride diacetates were eluted slightly faster than the beta-isomers. Similarly, the alpha, alpha-diglyceride monoacetates migrated ahead of the alpha, beta-derivatives. To what degree these differences in the migration rates of the positional isomers apply to higher mol wt triglycerides and whether or not they can be exploited in GLC separations remains to be established.

The suitability of stripped SE-30 columns for direct gas chromatography of unacetylated diglycerides has been demonstrated recently with diglyceride mixtures obtained from molecular distillates of corn oil and human plasma (6) and the phosphatidase D hydrolysis products of phospholipids (supplied by N. H. Tattrie of the National Research Council of Canada).

Except for a proportionally diminished response when compared to equal weights of triglycerides, no special problems anticipated from dehydration or heat isomerization were noted. Figure 11 shows a chromatogram of the diglycerides of corn oil (6). The mixed diglycerides of palmitic, oleic and linoleic acids were isolated from molecular distillates of corn oil by adsorption chromatography (18). Under the same GLC conditions serious losses occurred with the monoglycerides and there were signs of tailing and dehydration. The separations of the unmodified mono- and diglycerides appear to be based on mol wts but not enough work has been done to determine if positional isomers can be resolved.

Judging from work with other hydroxy compounds, both trifluoroacetylation and silylation should be satisfactory for the preparation of mono- and diglyceride derivatives suitable for gas chromatography. Wood et al. (19) have just prepared the silyl ethers of the monoglycerides of the C_8 to C_{18} fatty acids and have shown that these derivatives can be readily separated at low temps and that they are suitable for the resolution of the isomeric 1- and 2-monoesters. In this preliminary report no data are given on the separation conditions or the application of this method to fat hydrolyzates. Any equilibria shifts taking place during the preparation of the acetyl or silyl derivatives of the mono- and diglycerides seem to have remained ignored.

Separation of Oxidized Glycerides

Although these results suggest that gas chromatographic methods may be used in a direct way for tri- and diglyceride studies, the conditions are extreme and other approaches to the problem may be needed. Natural glyceride mixtures are readily distilled under conditions of molecular vacuum (5), and systems can be envisaged that would allow the operation of gas chromatographs at reduced pressure. Thus far, however, relatively little effort has been expended in developing gas chromatographs to operate under conditions comparable to molecular distillation (20).

A more limited approach, but of particular interest for the study of the structure of unsaturated vegetable oils, has been based on the permanganate-periodate degradation procedure. Oxidation at the site of unsaturation, followed by diazomethylation, GLC separation, identification and estimation of the remaining triglyceride structure has given satisfactory results (21,22). The separations of these materials were particularly effective (22) on a 4-ft SE-30 column which provided a larger number of theoretical plates than the 18 in. column used earlier (21). A total of 10 triglyceride types can be recognized in fats containing glycerides of palmitic, stearic and unsaturated fatty acids (oleic, linoleic and linolenic) which give rise to azelaic acid on oxidation. In terms of retention time, the methylated azelayl residue in a glyceride molecule is equivalent to a residue of lauric acid. Tripalmitin and distearoazelain, for example, emerge at about the same time. On this basis relative carbon numbers, comparable to the carbon numbers of undegraded triglycerides, have been assigned to the oxidized glycerides and the peak identities established (22). After correcting for differences in the combustible carbon content, good quantitations with the flame ionization detector were obtained for all derivatives including triazelain which contains six ester groups. The method has already been extensively applied in the analysis of animal and vegetable fats (22–24). Since all the unsaturated acids are estimated together in one group as azelaoglycerides, complete glyceride structures are not obtained, but this technique allows considerably more insight into the structure of the fat than a simple GLC based on mol wt which for most of these fats yields only a few triglyceride peaks.

An alternative procedure for the partial oxidation of glycerides is the reductive ozonolysis method of Privett and Nickell (25). Thus far the products of this reaction sequence do not appear to have been tried in gas chromatographic separations.

Further refinements in the GLC of both intact and partially oxidized glycerides may be introduced by a preliminary segregation of the fat on the basis of polarity and unsaturation. The small quantities of material necessary for the subsequent GLC can be conveniently obtained from preparative thin-layer chromatography (TLC) on reversed phase or silicic acid-silver nitrate plates. Litchfield et al (26) used a combination of TLC and GLC to characterize the triglyceride composition of *Cuphea llavia* seed fat. Silver nitrate plates were used for the separation of the triglycerides according to the number of double bonds/molecule. The recovered fractions were analyzed by GLC to determine the molecular weight of the triglycerides present. The triglyceride composition of the total fat was then calculated from these results. Using the common seed oils as an example, it has been shown (27) that consecutive applications of chromatographic fractionations based on polarity, unsaturation and mol wt can yield triglyceride groups of progressively decreasing complexity. Although pure triglycerides are not obtained, the mixtures are simple enough for a meaningful enzymatic analysis which can provide practical information for the assignment of complete glyceride structures.

Gas chromatography promises to yield much valuable information on the composition of natural fats but much more work is needed before complete analysis is possible.

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