acid-treated Florisil, which indicates that the interfering material had been removed by the procedure used for preparation.

One drawback of silicic acid chromatography is its failure to give clean separations of some classes of lipids such as lecithin and sphingomyelin. Acidic lipids also overlap with non-acidic lipid fractions (13, 16,17,18). Acid-treated Florisil undoubtedly shares these deficiencies, but it seems almost inevitable that any type of column used for preliminary fractionation of complex naturally-occurring mixtures will give some overlap of different lipid classes. The simplicity of operation and rapid flow rates of Florisil and acid-treated Florisil columns, coupled with the fact that they do provide separation of a number of different lipid classes, suggests their use for certain types of fractionation.

ACKNOWLEDGMENTS

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Special Methods of Purifying Fatty Acids

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Abstract

Preparative gas chromatography and thin-layer chromatography have been used successfully in several purification applications. Both methods are very good when small (10 mg or less) samples are involved. In fact, the low capacity of the methods is actually an advantage in such cases and is a major reason for using the methods. When larger samples are to be prepared by gas chromatography, the low capacity becomes a problem. However, by the use of conventional purification procedures to concentrate the desired component as much as possible, and the use of large columns (up to 1 in. diameter) gram quantities of rare fatty acids have been successfully prepared. Gas Liquid Chromatography has been an invaluable tool where other purification methods have failed.

Introduction

PREPARATIVE gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) have become important methods in purifying fatty acids and their derivatives. Separations either impossible or unduly difficult in the past have yielded to the new methods. However, even though the techniques appear very attractive, and indeed do have great potential, the practical application of them in the purification of lipids is often very difficult.

Typical applications will be described here. It is intended that these examples should illustrate the the scope of the methods and, especially in the case of relatively large scale GLC preparations, the examples will indicate detailed techniques useful in actually applying the methods. Auxiliary methods such as distillation and crystallization will also be set in proper perspective.

The first section describes concentration steps that should be taken prior to preparative chromatography. In the second section the preparation of uniformly carbon-14 tagged fatty acids is used to illustrate GLC purification of relatively small amounts of methyl esters. This is followed by an example of the use of thin-layer chromatography to prepare small amounts of radioactive cholesteryl palmitate. The last section covers in some detail the use of GLC to prepare larger amounts (1–10 g) of methyl esters.

Procedures

Concentration of Fatty Acids from Natural Sources

A desired fatty acid is often present in very low concentration in a very complex mixture. If any appreciable amount of the component is needed (such as a gram) chromatography techniques are at a disadvantage because of their inherently low capacity. It is usually worthwhile, therefore, to use conventional separation procedures to concentrate the component into a smaller sample. Every effort is made to purify to the greatest possible extent in this way—preferable to about 90%.

Although it is impossible to make general rules for the concentration procedure, certain guides have been listed below:

1) Select a source of the desired fatty acid which contains a reasonable concentration of the acid. The source selected will not necessarily be that which contains the highest concentration. Two factors which often lead to some other source are a) availability, and b) interference from components which are hard to separate.

2) Analyze by GLC, preferably using both polar and non-polar stationary phases.

3) Try to determine which impurities will be hard to separate or at least what their characteristics are. This may be done entirely by careful GLC with standards, or it may require attempted separations

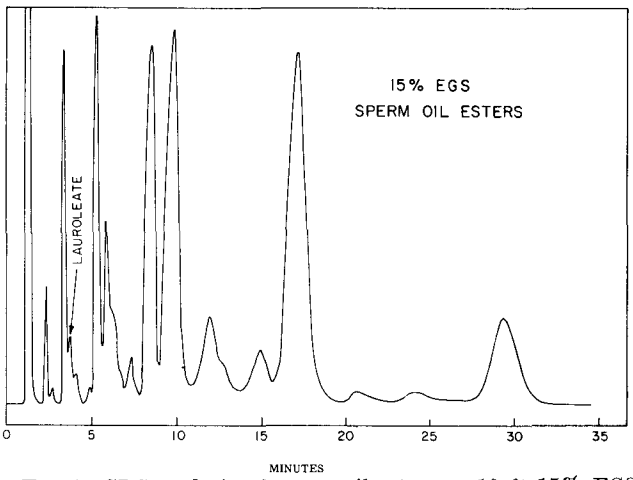

FIG. 1. GLC analysis of sperm oil esters on 10 ft 15% EGS column. Column temperature 185C. Detector 200C. Sample size 20 μg. Flow rate 90 ml per min.

on a test tube scale followed by GLC to determine how the impurities behave during crystallization, etc.

4) Sometimes the source may contain only minute amounts of the desired fatty acid in a wide range of other components. In such cases, it is often advantageous to perform a preliminary simple distillation. Such a distillation may considerably reduce the bulk of material used in the following steps.

5) The concentrate so obtained may contain components which boil close to the desired component, but which can be removed by crystallization (or sometimes extraction). This should be done if the bulk of the material is still considerable—i.e. if the resulting concentrate is large enough to handle conveniently.

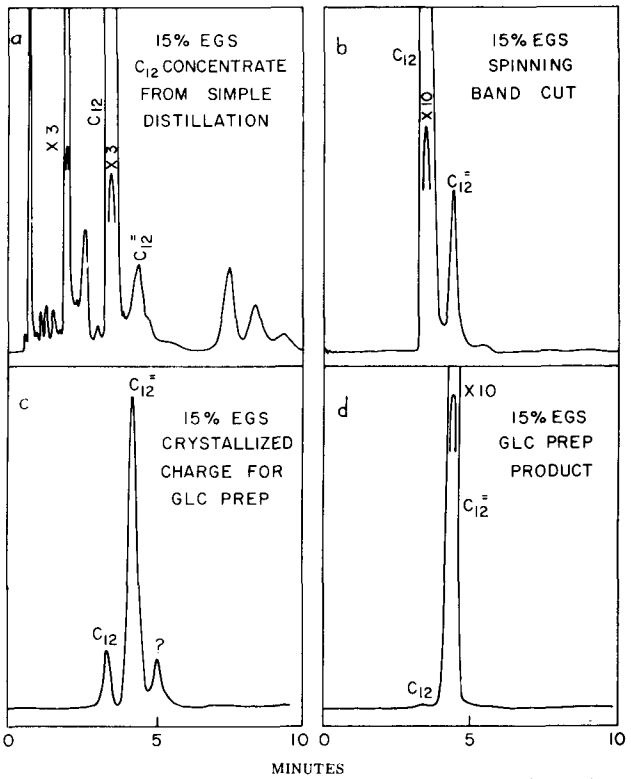

FIG. 2. GLC analysis of methyl lauroleate in various stages of purification on 10 ft 15% EGS column. Temperature 185C. Detector 200C. Flow rate 90 ml per min. *a* is the C_{12} concentrate from simple distillation of the sperm oil esters. *b* shows the heart cuts from a subsequent spinning band distillation. *c* is the fraction after crystallization and *d* shows the pure lauroleate after preparative chromatography.

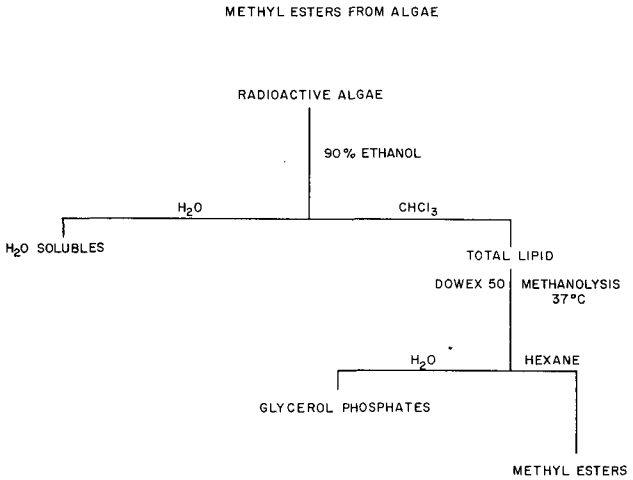

FIG. 3. Process for preparing methyl esters from algae.

6) Carefully fractionate the mixture in a spinning band still. Analyze the cuts by GLC and select the very good ones for preparative GLC.

A good example of such a procedure was the preparation of 1 g of 99.5% pure methyl lauroleate from sperm oil. The starting material was 6 kg of sperm oil. This oil was transesterified to obtain the methyl esters. Figure 1 is a chromatogram of the esters. Note that the mixture contained only a small fraction of a percent of the desired component. Other GLC analyses indicated that the only close boiling impurities would be methyl laurate and methyl tridecanoate. As can be seen from Figure 1, the bulk of the esters are much higher in molecular weight than the lauroleate. Thus, a simple distillation was performed. Four choice cuts from this distillation were combined and the analysis is given in Figure 2a. (About 50 g was obtained.)

Because crystallization at this point to remove laurate would have resulted in working with only about 5 g of the material in the spinning band column, this material was distilled prior to crystallization. Analyses of the better cuts from the spinning band are shown in Figure 2b. All the impurities except the laurate and a small amount of what is probably tridecanoate were removed. Approximately 15 g of this concentrate was obtained. This was crystallized from acetone at dry ice temperatures. The mother liquor from the crystallization was analyzed and the chromatogram is shown in Figure 2c. The preparative GLC in this case was fairly straight forward. Two grams were available, and the sample was injected in 75 mg portions into a 1 in. diam, 5 ft long preparative column containing 26% EGS on GAS-CHROM P (80/100 mesh). The retention time for the lauroleate was seven minutes and a sample was injected every 12 min. One g of 99.5% pure methyl lauroleate was obtained. The product analysis is shown in Figure 2d.

Uniformly C^{14} Labelled Methyl Esters

Uniformly labelled fatty acids were prepared by growing algae in the presence of $C^{14}O_2$ (1, 2), extracting the lipids and purifying the fatty esters by GLC.

The algal strain, chlorella scenedesmus, was employed in all preparations. A continuous process was used to grow the culture in an inorganic medium and in the presence of 5% carbon dioxide in nitrogen. The culture was surrounded by a water cooled jacket

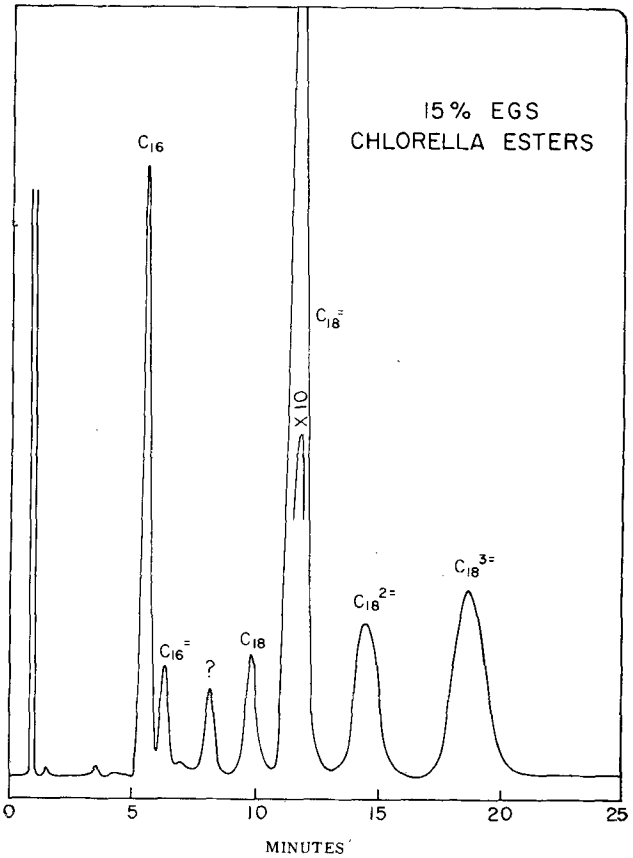

FIG. 4. GLC analysis of chlorella esters on 10 ft 15% EGS column. Column temperature 190C. Detector 250C. Sample size 5 μg. Flow rate 100 ml per min.

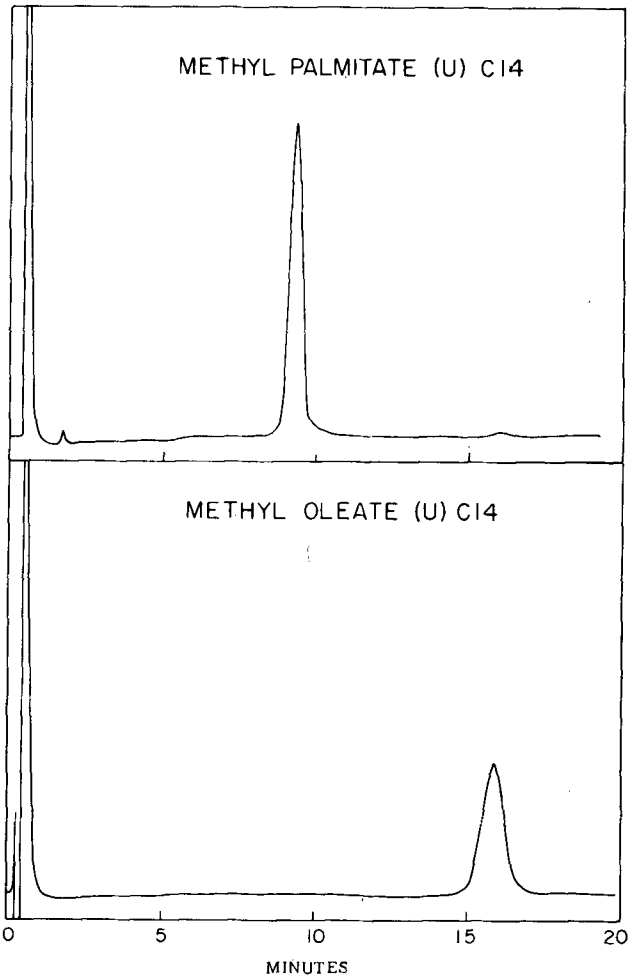

FIG. 5. GLC analysis of pure uniformly tagged methyl palmitate (top) and methyl oleate (bottom). A 10 ft 15% EGS column at 190C was used. Detector 250C. Sample size 0.5 μg. Flow rate 70 ml per min.

and illuminated by a 20 w warm-white fluorescent lamp.

The C^{14} labelled chlorella were grown in 150 ml of medium starting with 0.1 mg suspension of algae in a closed gas-circulating system operated by a valveless roller pump. Nitrogen containing 5% CO_2 (with 15 mc of $C^{14}O_2$ in it) was circulated through the system for a fixation period.

Figure 3 is a schematic diagram for the extraction (3) of the C^{14} labelled chlorella. The isolated lipids were deacylated by methanolysis using the procedure of Dawson (4). Because of the easily oxidizable nature of the extracted material, extracts were exposed to air as little as possible.

Samples of the total lipids and of the methyl esters from deacylation of the lipids were examined by radioautography on Whatmann AE-30, using the methods of Mumma and Benson (5). This was done to determine the completion of the extraction and methanolysis procedure, and also to determine what product distribution was being obtained from the chlorella.

Figure 4 is a GLC analysis of the methyl esters obtained. Preparative GLC was actually a convenience in working with the milligram amounts of material available. The esters were fractionated by preparative GLC through a 9-foot column of 15% SE-30 silicone rubber gum. The trapping system consisted of shock cooling to form an aerosol and electric precipitation in a Cottrell-type trap. The compounds were isolated according to carbon number. Two major fractions, the C_{16} and C_{18}, were obtained. The vapor outlet exhausted into a charcoal trap and the complete preparative unit was installed in a walk-in hood. The unsaturated C^{14} random-labelled methyl esters were separated according to their degree of unsaturation by preparative thin-layer chromatography, using silver nitrate impregnated silica gel. The materials were analyzed on a Beckman GC-2 with a flame detector.

Figure 5 shows chromatograms of methyl palmitate-U-C^{14} and methyl oleate-U-C^{14}. The purity was above 95%.

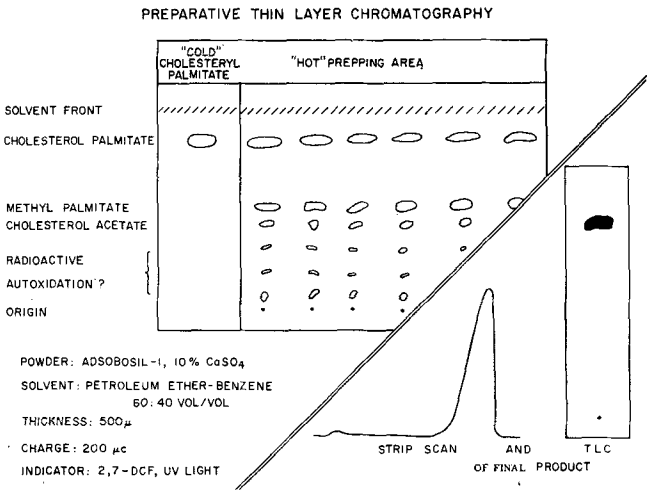

FIG. 6. TLC purification of radioactive cholesterol palmitate.

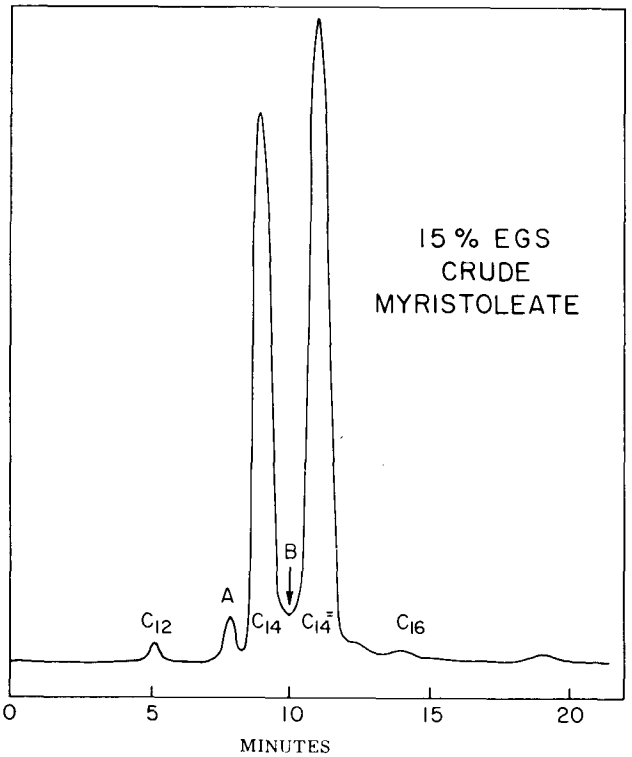

FIG. 7. GLC analysis of crude methyl myristoleate on 9 ft 15% EGS column. Column temperature was 180C. Detector 225C. Flow rate 60 ml per min.

Preparation of Cholesteryl Palmitate-1-C^{14}

A good example of the use of preparative thin-layer chromatography is a procedure used for the purification of radioactive cholesteryl palmitate-1-C^{14}.

Radioactive cholesteryl palmitate was synthesized by the ester interchange procedure of Mahadevan and Lundberg (6). Cholesteryl acetate was reacted with methyl palmitate-1-C^{14} with sodium methoxide as catalyst.

Millicurie amounts were prepared using a 15 ml pyrex centrifuge tube as the reaction vessel. The tube was connected to an ice trap, manometer and vacuum pump. The evolution of methyl acetate was observed by the effervescence and the reaction was completed in 45 min. Redistilled benzene was added to the tube and the contents were applied directly to preparative TLC plates. The plates were developed with a mixture of petroleum ether and benzene, 60:40 (V/V).

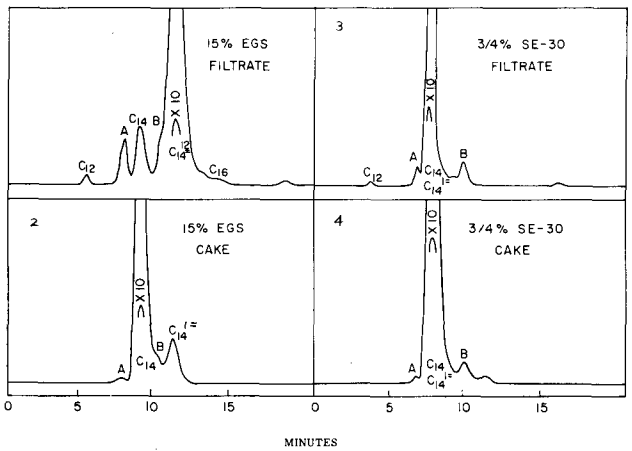

FIG. 8. GLC analysis of cake and filtrate from crystallization of crude methyl myristoleate. Sample size was 5 μg. EGS column was 9 ft and operated at 180C and 60 ml per min. SE-30 column was 6 ft and operated at 160C and 75 ml per min.

Figure 6 shows the preparative plate used.

The plates consisted of 500 μ layers of Adsorbosil-1 and were visualized with 2,7-dichlorofluorescein (DCF). A standard "cold" cholesteryl palmitate was used to compare the migratory rate of the "hot" cholesteryl palmitate-1-C^{14}. The spots representing hot material were scraped off the plates into a Soxhlet thimble and extracted with solvent.

Figure 6 insert shows the analytical results for the purified cholesteryl palmitate-1-C^{14}.

Purification of Gram Quantities by Preparative Gas Chromatography

Many fatty acids and their derivatives can be obtained in a highly purified form by a combination of careful fractional distillations and low temperature crystallizations. In fact, the isolation of methyl lauroleate previously described could have been done by these methods, but certain of the materials contain impurities which cannot be completely removed by the above purification steps. Such impurities have been successfully removed from several different fatty acid methyl esters using relatively large scale preparative gas chromatography. In some cases it is necessary to combine column chromatography with gas chromatography.

Our work has been limited to producing only one to five g quantities of a specific product, using 5/8-in. and 1-in. diam gas chromatographic columns and several different liquid phases. The charge materials usually were highly concentrated by previous steps and contained 90–95% of the product. A thermal conductivity cell, designed to accept the total flow of carrier gas from the column, was used for component detection. Samples were injected into a vaporizer maintained at 300–325C and positioned just ahead of the column inlet. The vaporizer was packed with stainless steel ball bearings to provide a large surface area for heat transfer and a high mass for a heat source. Several schemes for trapping were used, which will be discussed later. Helium was used as carrier gas in all cases.

The most extensive work has been done in the purification of methyl myristoleate. The myristoleate purification exemplifies the use of preparative gas chromatography with a combination of columns and in conjunction with column chromatography.

The myristoleate charge for preparative gas chromatography was obtained by fractional distillation followed by low temperature crystallization and contained approximately 90% methyl myristoleate. High grade "red oil" methyl esters were fractionated by distillation. A chromatogram of the C^{14} cut from this distillation is shown in Figure 7. The gas chromatographic results on two different columns for the filtrate and the cake from the crystallization are shown in Figure 8. The filtrate was the charge for the preparative gas chromatograph.

Sections 1 and 2 of Figure 8 show the filtrate and cake analysis respectively with a 15% EGS column. Myristoleate was highly concentrated in the filtrate which contained also two unidentified components, A and B, as the major impurities. Component B is not separated from myristoleate on an EGS column and is barely detected as a slight shoulder on the leading edge of the myristoleate peak. Section 2 shows the cake, which was highly concentrated with respect to methyl myristate. Here impurity B is seen as a shoulder on the back edge of the myristate peak.

Sections 3 and 4 of Figure 8 show the filtrate and

cake analyses respectively with a ¾% SE-30 column. By comparing the analyses for the filtrate and cake it can be seen that methyl myristate cannot be separated from methyl myristoleate with an SE-30 column. However, component B emerged after the myristoleate and was quite well separated from it. Another minor impurity can be seen between myristoleate and component B in the filtrate. Component A emerged before myristoleate and was only partially separated from it.

It was apparent from the chromatograms that all the impurities in the myristoleate filtrate could not be removed with EGS or SE-30 columns. It was therefore decided to run the filtrate first through an EGS preparative column and then through an SE-30 preparative column.

The EGS column was 1 in. diam and 5 ft long, packed with 26 wt % EGS on 80–100 mesh GAS-CHROM P and operated at 180C and 20 psig. Samples (100 μl) were injected to give chromatograms of the type shown in Figure 9.

Samples were injected approximately every 20 min. The resolution of the components was quite bad as compared to an analytical run. Column length was decreased, column diameter increased, and a large sample constituting an overload was injected. However, by cutting at the points shown by the dashed lines in the chromatogram, a product containing only methyl myristoleate and component B was obtained. The gas chromatographic analysis results for this product using both a 15% EGS and ¾% SE-30 column are shown in Figure 10. The chromatograms from both columns show that B was the only impurity left.

The product from the EGS preparative column was then rerun on an SE-30 preparative column ⅝ in. diam and 5 ft long. The column was packed with 14 wt % SE-30 on 100–120 mesh GAS-CHROM P and was operated at 212C and 16 psig. The sample size was 50 μl. A chromatogram of one of these runs is shown in Figure 11. Samples were run every 7–8 min and the cuts were made at the points shown by the dashed lines. The analytical results for this product (on a ¾% SE-30 column) are shown in Figure 12a. The impurity B was completely removed and the methyl myristoleate was pure by gas chromatographic standards. From approximately 4 g of initial charge, 1.7 g of product were obtained.

This product was next analyzed by TLC, using Absorbosil-1 as the adsorbent. The left-hand side of Figure 13 shows the TLC analytical results for the myristoleate product from the SE-30 preparative column. The developing solvent was a mixture of petroleum ether, diethyl ether, and acetic acid (90/10/1). TLC showed a number of minor impurities not shown by gas chromatography. These were evidently decomposition products that did not emerge from the gas chromatographic column. The impurities were removed by liquid-solid column chromatography, using silica gel as the immobile phase and n-pentane as the mobile phase. The column was ⅜ in. diam with a 3-in. packed section. Approximately 7 ml cuts were taken. Every third or fourth cut was analyzed quickly by gas chromatography on an SE-30 column in order to estimate the amount of myristoleate in the cut. After the first six cuts, very little myristoleate was eluted. Eighteen cuts were taken in all and TLC analysis of these cuts showed that some were pure myristoleate. The right hand side of Figure 14 shows the TLC analysis results for the

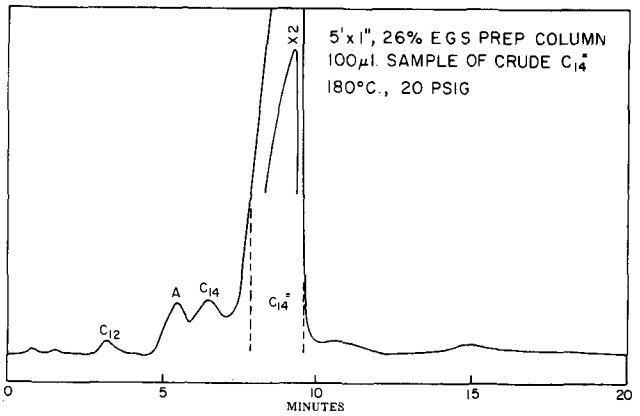

FIG. 9. GLC trace from preparative run of crude myristoleate. Column was 5 ft long, one inch diameter packed with 26% EGS. Temperature was 180C, pressure 20 psig, flow rate 1 liter per min. 100 μl sample was used.

combined myristoleate from the 18 cuts. All of the impurities present before purification by column chromatography were removed. Finally, this product was analyzed again by gas chromatography using a ¾% SE-30 column. The result is shown in Figure 12b. This chromatogram is similar to that for the myristoleate before it was purified by column chromatography. The use of two types of preparative gas chromatographic columns (an EGS and an SE-30 column) and then column chromatography gave methyl myristoleate which was pure both by GLC and TLC analysis.

The first six cuts from the silica gel column contained 71 wt % of the charge to the column while

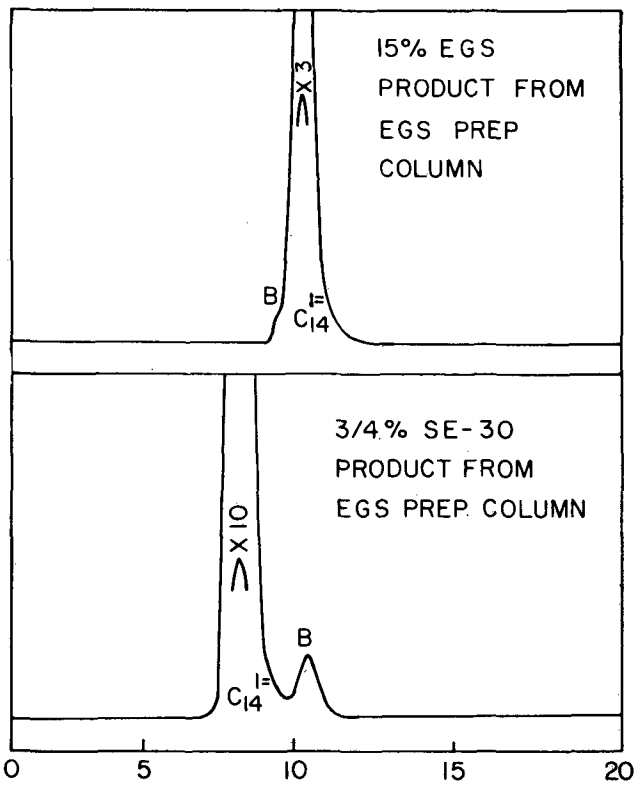

FIG. 10. GLC analysis of product from EGS column preparation. Top was analysis on 15% EGS (9 ft) at 180C and 60 ml per min. Bottom was analysis on ¾% SE-30 (6 ft) at 160C and 75 ml per min. Sample size was 5 μg in both.

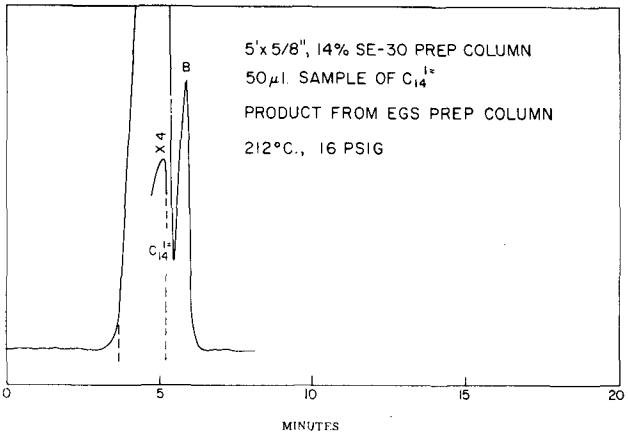

FIG. 11. GLC trace from preparative run of myristoleate which had previously been treated in the EGS column. This column was 5 ft by 5/8 in. packed with 14% SE-30. Temperature was 212C, pressure 16 psig, flow rate 600 ml per min. 50 μl sample was used.

the next twelve cuts contained only 4% of the charge. The yield by column chromatography was 75%. The column was cleared with a more polar solvent (a mixture of chloroform, methanol, and water). The material collected amounted to another 19% of the charge, resulting in a total of 94% of the initial charge accounted for. Gas chromatographic analysis of the last material washed off the column showed it to be essentially all myristoleate by measurement of the myristoleate peak size. TLC analysis of this material also showed it to be mostly myristoleate,

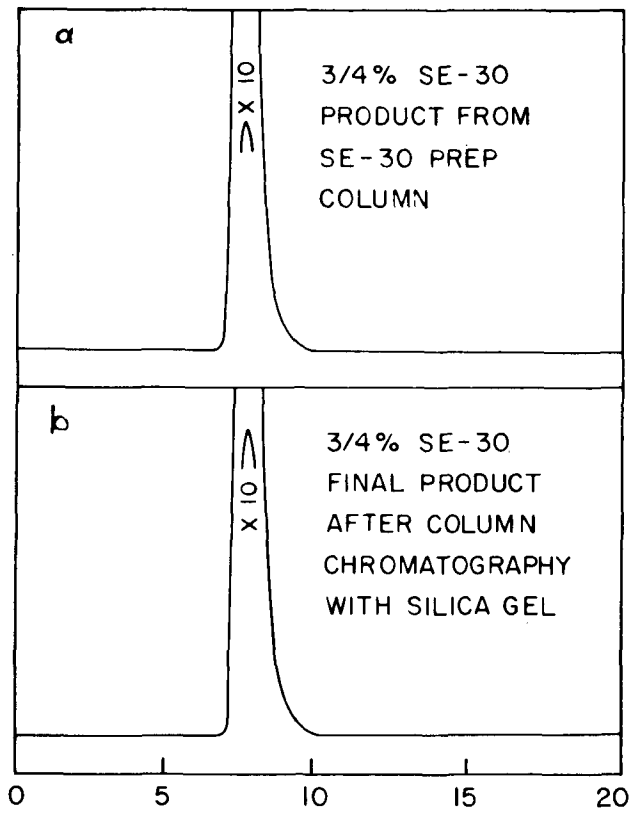

FIG. 12. GLC analysis of methyl myristoleate product before (top) and after (bottom) passing through silica gel. Column was 6 ft of 3/4% SE-30 run at 160C and 75 ml per min. Sample size was 5 μg.

but showed the original impurities in it. Evidently methyl myristoleate tails badly in a silica gel column when n-pentane is used as the eluting solvent.

The yield for this batch of myristoleate from each of two preparative gas chromatographic columns was about 70%, resulting in a combined yield from the two columns of about 50%. These yield values are based on the amount of myristoleate in the original charge. Such results are typical. The yield on a single pass through a column varied from 65–75%. The amount of product lost as waste material due to the position of the cut points does not account for these relatively low yields.

Fogging in the traps used for gas chromatography is a problem. Generally a train of ordinary cold traps with an electrostatic precipitation at the end is required for maximum recovery. The carrier gas flows in through the center tube and out through the annulus of the trap. Different combinations of these traps have been tried. One trap in a wet ice bath in series with an electrostatic precipitator or two traps, the first at room temperature and the second in a wet ice bath in series with a precipitator were equally efficient. A glass wool plug after the precipitator has been used with little or no improvement in yield. At times a trace of fog can be seen escaping from the electrical precipitator.

We have concluded that poor yields are not due completely, if at all, to poor trapping efficiency. Perhaps the methyl esters are partially retained or destroyed in the column system. The stainless steel ball bearing packing used in the vaporizer has been observed to be coated with carbon after extensive use. If high temperature vaporization does cause partial destruction of methyl esters, on-column injection might solve the problem. This has not been tried yet.

The actual running time on the two preparative columns was 24 man-hr. All operations including column chromatography, analysis, removal of solvent, etc., required 40–48 man-hr of actual work on the chromatographic steps to produce 1.2 g of high purity methyl myristoleate. If the yields from the preparative chromatographic columns could be increased, one might expect to obtain about 3 g of myristoleate with the same amount of effort by preparative gas chromatography.

It was noted above that a 1-in. diam EGS column was overloaded with 100 μl samples. This may not seem to be a large sample considering the size of the column, but our work has indicated that in this case it is. The first chromatogram in Figure 14 illustrates one preparative run with a 100 μl sample on a 1-in. EGS column. The myristoleate peak is assymetrical with a diffuse front and a sharp back. This indicates a nonlinear isotherm with the partition coefficient increasing with increasing concentrations of the myristoleate. This causes the back portion of the band to move more slowly than the front portion and spreads the band out. The effect is more pronounced when the sample size is increased to 500 μl (0.5 ml sample on a 7-ft by 5/8-in. diam column) as shown in Figure 14.

Roughly equivalent results were obtained with a 1000 μl (1 ml) sample on the 1-in. diam column, where the myristoleate peak was badly skewed and about five times as wide as with the 100 μl sample and spread over into the C_{16} methyl esters region. As the sample size is increased, the two minor peaks

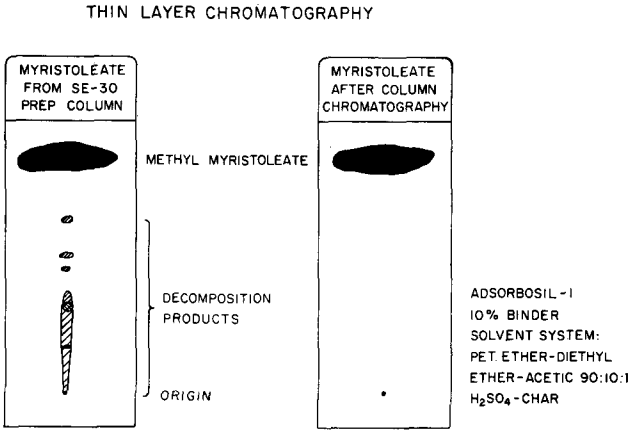

FIG. 13. TLC analysis of methyl myristoleate before and after silica gel treatment.

for methyl myristate and component A broadened appreciably until they finally merged into one shoulder on the front of the myristoleate peak.

It is not clear whether the relatively small samples are required because the stationary phases (polyesters) are easily overloaded or whether the separations performed were so critical that near optimum conditions were required.

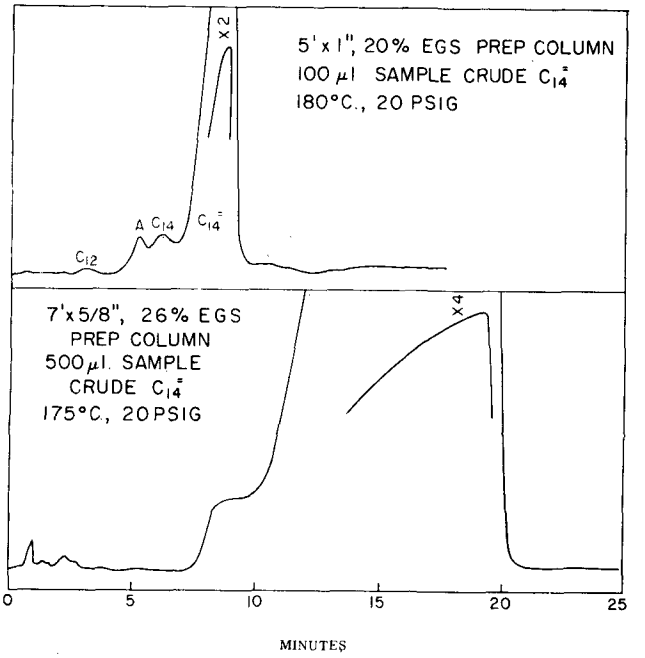

FIG. 14. Comparison of GLC traces from preparative runs without severe overloading (top) and with overloading (bottom).

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Lipid Composition of Beef Brain, Beef Liver, and the Sea Anemone: Two Approaches to Quantitative Fractionation of Complex Lipid Mixtures

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Abstract

Two new schemes for fractionation of complex lipid mixtures are presented. Their use for the study of lipids of beef brain, beef liver, and the sea anemone are described. Apparatus and techniques for working in an inert atmosphere, evaporation of solutions in the cold under nitrogen, use of infrared spectroscopy for examination of lipids and their hydrolysis products, preparation and elution of diethylaminoethyl (DEAE) cellulose and silicic acid-silicate columns, and general column combinations that can be used to fractionate complex lipid mixtures are considered in detail. The first scheme, employing DEAE cellulose columns followed by thin layer and paper chromatographic examination of the fractions, was applied to liver lipids. The many components, some of them new lipids not previously detected, are clearly seen with this technique but are not seen when paper or thin layer chromatography alone or silicic acid chromatography are used.

The second scheme employing DEAE for initial fractionation, followed by complete separation on silicic acid and silicic acid-silicate columns, was applied to lipids of the sea anemone and beef brain. Typical lecithin and phosphatidyl ethanolamine were isolated, but sphingomyelin was not found. A new sphingolipid, ceramide aminoethylphosphonate, with a free amino group and a direct carbon to phosphorus bond was isolated and characterized. The methods used for quantitative isolation, the infrared spectra, and the amounts of cholesterol, ceramide, cerebroside, galactosylglyceride, sulfatide, sphingomyelin, lecithin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, triphosphoinositide, phosphatidic acid, cardiolipin, and ganglioside of beef brain are presented. Finally, the types of lipid-nonlipid interactions disclosed by column chromatography and their potential application to biological problems are discussed.