firmed by paper chromatography. The solids test readily picked up the small amount (2 mg. or less/l.) of silicate in subsequent chloroform fractions. The cholesterol percentage was determined by weight and found to be 20.3%, a value in agreement with the value in Table I. Evidently the very small amount of inorganic material did not influence the determination.

The ceramide peak (eluted with chloroform/methanol, 19/1) was collected as one main fraction and a small tailoff portion. The tailing portion was evidently mainly silicate (Fig. 9). The spectrum of the ceramide (Fig. 8) was altered slightly by silicate because of the very small amount of lipid. A small absorption band at 5.72 μ is apparent. This is not due to the solvent residue noted above. The weight of ceramide corresponded to 0.35% of the total material applied, a value slightly higher than usually obtained (0.30) due to the presence of silicate.

The cerebroside spectrum shown in Figure 10 is typical. This lipid was recovered after passage through DEAE that removed silicate. The small absorption band in the ester carbonyl region is not from solvent residue.

Figure 11 is the spectrum prepared from the first "sulfatide" fraction referred to above that was eluted from the DEAE column with the first chloroform/methanol/aqueous ammonia mixture. It was essentially all DEAE "fines" as shown by comparison with the spectrum of DEAE (Fig. 12).

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Column Chromatography of Fatty Acids[1]

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IN PART I, column chromatography of fatty acids is discussed, with emphasis being placed on practical aspects. Examples from the literature are quoted.

In Part II, experimental details are given of a new liquid-liquid chromatographic system and its use in isolating minor component fatty acids of menhaden oil.

The value of column chromatography of fatty acids depends on its combination with other analytical procedures.

Chromatography of fatty acids in liquid phase over a solid or another liquid phase is now used mainly for preparative purposes rather than for immediate quantitative analyses. The ultimate goal, of course, may still be analytical. Some circumstances that may favor the use of LC[2] rather than other methods of separation are pointed out in the following:

Amounts

Several grams of material can be chromatographed, depending upon the complexity of the mixture, for isolation and enrichment of components.

Examples. LSC and LLC are essential steps in a current preparation of methyl arachidonate (1). Individual fatty acids of rat lipids have been isolated by means of LLC in amounts large enough for determining their radioactivity in conjunction with chemical degradation (2). Odd-numbered (3) and other minor fatty acids of fish oils have been enriched by LLC so that other separation procedures could then be utilized for their isolation and ultimate identification (see Part II).

Instability

The temperature at which LC is carried out does not alter the structure of labile compounds. Although

[1] Work presented in Part II was supported by grant RG-4226 from the Division of General Medical Sciences of the U.S. Public Health Service, and by the Hormel Foundation.

[2] Abbreviations used in this article are: LC = liquid chromatography, i.e., LSC = liquid-solid (adsorption) chromatography, and LLC = liquid-liquid (partition) chromatography; PC = paper chromatography; GLC = gas-liquid chromatography.

certain adsorbents may induce structural changes, the commonly used adsorbents are safe in this regard. Precautions against autoxidation can be taken as efficiently as in other methods.

Examples. Highly unsaturated long-chain esters (4) and autoxidized esters (5) have been separated and isolated by LLC or LSC. These and other esters having particular structural features (6) require restraint in applying high temperatures.

Selective derivatization

Analyses often can be simplified by reacting the double bonds of the unsaturated components of a mixture so that they are converted into compounds which are more distinct from the saturated ones. Unaltered and derivatized portions may be separated by LC and individual compounds can be isolated.

Examples. Brominated (7), oxygenated (8), or mercurated (9) fatty acids or esters have been separated. Often it is possible to analyze both the reacted and the unreacted portion without mutual interference. When mercuration is used, the unsaturated esters are easily restored after separation (10).

Apparatus investment

Initial expenditures may be $700, of which a fraction collector is the major expense. Countercurrent distribution affords essentially the same type of fractionations as LLC, and possibly on a larger scale, but the apparatus is more costly.

Numerous treatises are available with chapters on chromatography of fatty acids (11,12,13). Therefore, practical aspects of LC will be emphasized here which may not be so obvious in more comprehensive treatments of the topic. Part I deals with some general procedures and outlines some typical examples. Part II gives the details of a new system for LLC (3) and describes its use in the course of isolating minor component acids of menhaden oil.

I. General Procedures

The Sample

In early applications of LC, free fatty acids were used because they were easily detected by acid-base indicators (14) and could be determined quantitatively by titrating the eluant fractions (15). The presence of carboxyl groups, however, is no longer a deciding factor. Methyl esters, hydroxamates (16), and other derivatives have been chromatographed. Among the derivatives, methyl esters are preferred because, with them, application of further analytical methods like GLC or recovery of the free acids is more convenient.

Besides the common methods of esterification on a large scale, the procedures outlined below may be considered for esterification of small amounts of acids. Some of them have been described for micro amounts but all are certainly adaptable to samples of one or several grams.

2,2-dimethoxypropane + CH_3OH, aq HCl + Acid ⟶ Ester (17)

Benzene, CH_3OH, HCl + Acid or Glyceride ⟶ Ester (18)
(superdry)

$CH_3OH + BF_3$ + Acid ⟶ Ester (19)

N-Nitrosomethylurea + Ether, aq KOH ⟶ CH_2N_2 + Acid, Ether ⟶ Ester (20)

N-Methyl-N-nitroso-p-toluene-sulfonamide (Diazald) + Carbitol, ether, aq KOH ⟶ CH_2N_2 + Acid, Ether, CH_3OH ⟶ Ester (21)

In choosing one of the techniques, completeness of esterification, the volatility of the sample, the presence of unsaponifiables, and the number of transfers or extractions should be considered. The long-known esterification with diazomethane has been investigated in regard to complete reaction with fatty acids (21), and much of the aversion to its use because of explosion hazards and side reactions is largely overcome when it is used on a small scale with proper caution and discretion. The procedure lends itself also to preparation of methyl-C^{14} esters (22) and thus opens a convenient road to radioactive derivative methods (23).

The quantitative conversion of acids or esters into hydroxamates for subsequent chromatographic separations has been described (16). Other derivatives of fatty acids, generally containing chromophors, have been prepared to simplify detection of components during or after chromatography. They are hardly used any more.

Equipment

While it is unnecessary to discuss the common glass column, it appears warranted to mention some features of the coupled column. It was designed when particularly sharp separation of zones was required in certain types of adsorption chromatography. The advantage should not be overlooked whenever bands are in close sequence. For example, in chromatography of epoxy acids, the use of a coupled column led to detection of a minor component which otherwise had not been revealed (24).

The coupled column consists of different sized segments which are packed individually with stationary phase and then screwed together in a sequence which narrows the diameter of the column towards the end. Channels or mixing chambers between the segments smooth out the edges of a band so that irregularities are not transmitted to the lower part of the column (25,26). The instrument is commercially available (27), but requires modification for use with lipids and their solvents.

Some excellent experiments serve to introduce students to chromatographic procedures (28) but dispense with an automatic fraction collector. Such an instrument is almost a necessity with lipids and there are numerous sources for reliable models. The siphoning type of fraction collector is preferred in this laboratory.

Elution by solvent gradient is often applied in LSC and is probably more often used in LLC than is obvious from the literature. Several solvent mixers have been described (29,30,31), but the choice of manufactured instruments is rather limited (32). Optical devices for measuring the outflow of columns will be mentioned below.

Detection of Components

Locating components rapidly is important when a multitude of fractions has been collected blindly by volume. Weighing the lipid residue of the fractions is cumbersome, although with special evaporation equipment, automatic balances, and good planning, remarkable efficiency can be achieved. When spots of fractions are applied to chromatography paper or plates, the eluate immediately becomes amenable to the detecting reagents of these techniques. The loss of material is negligible and rather precise information about the width and maximum of a band is obtained. Secondary chromatography, especially in

a different system, helps further to identify and combine the fractions in the optimal way (33,34).

Acids can be titrated to achieve quantification, and special devices have been designed to improve the accuracy (15,29). However when the sample is to be recovered in pure form, extractions, evaporation of solvent, or separation from admixed stationary phase may involve loss of material.

Long-chain fatty acids or esters can be detected when the solvent is miscible with water, provided any turbidity arising from aqueous dilution is due to lipids only (3,35, see also Part II).

Immediate and continuous monitoring of columns has been accomplished interferometrically by comparing the refractivity of the outflowing solution with a standard solvent (36,37). A differential refractometer, combined with a recorder, is commercially available (38). Its use with LLC (39) and with countercurrent distribution (40) has been reported.

Adsorption Chromatography

Separation of fatty acids by chromatography over active carbon has been studied extensively by Tiselius and his school (41). Darco G-60 was selected as the preferential adsorbent from a wide variety of brands. On it, the adsorption isotherms of fatty acids are curved, as is the case with most other adsorbents. However, straight isotherms, i.e., distribution ratios independent of concentration, are desirable for the usual types of chromatography. When the distribution between two phases varies with concentration, the solute will migrate with different speeds according to its local concentration. This results in trailing and overlapping of compounds. The nonlinear adsorption isotherms of fatty acids on charcoal, however, were put to advantage in displacement and carrier displacement chromatography (41,42). These techniques operate, in principle, by complete saturation of the adsorbent with sample and known substances. In displacement chromatography the sample components emerge from the column as a sequence of bands, according to their adsorbability, until the whole column is saturated with a master displacer. The displacer chosen is a substance which is more strongly adsorbed than any component of the sample and it is added continuously with the solvent. In carrier displacement chromatography, individual displacers are added to the sample, one for each component, so that the bands of added and original compounds emerge alternately from the column. Thereby wider spacing of the compounds to be analyzed is accomplished and the sensitivity of the method greatly increased. Figure 1 represents an example of this latter method.[3]

Displacement chromatography was an important phase in the history of chromatography. The technique did not find broad application due to the requirement of rather elaborate apparatus and detailed knowledge about the sample. The potential of displacement chromatography should not be overlooked. Certainly the possibilities of the coupled column and the differential refractometer which were developed with these investigations should be appreciated more than they are at present.

Although it is generally agreed that isomers are more readily separated by adsorption than by partition, it appears uncertain to what extent and for which types of isomers this is valid with fatty acids. Some examples pertinent to this problem are cited in

[3] R.T. Holman, unpublished experiments.

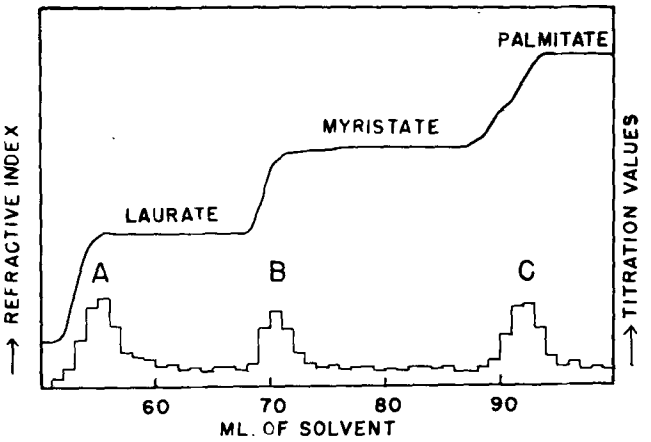

FIG. 1. Separation of isomeric C_{18} acids by carrier displacement chromatography. Sample: 15.3 mg. 2-amyl-3-methyldodecylic (A), 13.4 mg. 2-heptylundecylic (B), and 15.2 mg. 2-methylheptadecylic acids, dissolved in 5 ml. of 95% ethanol with altogether about 150 mg. of methyl laurate, myristate, and palmitate as carrier displacers. Column: 6 segments total volume 12.7 cm.^{3}, packed with 1 part Darco G-60 + 2 parts Hyflo Supercel. Eluant: 95% ethanol with 1% methyl stearate as master displacer (not in figure). Flow rate: about 15 ml./hr. at about 40 lb./in.^{2} pressure of N_2.

The increase of the refractive index was determined with an interferometer by micrometer readings without referring to absolute values. The titrations were carried out with 1 ml. fractions.

the following. Isomeric C_{18} acids[3] (Figure 1) and certain *cis-trans* isomers resulting from autoxidation of linoleic acid (43) or partial hydrogenation of linseed esters (44) could be resolved on charcoal or silicic acid. Similarly dicarboxylic acids, differentiated by double bonds in 2,3 or 3,4 position, could be separated on charcoal (45). Attempts to separate esters of *iso* acids from the corresponding *normal* esters on silicic acid succeeded with isobutyrate but failed with isovalerate (46). Long-chain normal and branched esters could not be separated by LLC either. A strict comparison by subjecting identical samples to both chromatographic methods has not been carried out, to the authors' knowledge.

Silicic acid became more important than charcoal in further development of LSC for fatty acids. Besides being the outstanding adsorbent for separation of lipids into groups, it has served many times in isolation of individual compounds. Silicic acid has been used to prepare linoleic and linolenic acids free of contaminants which would arise from bromination and debromination (47). The purity of these preparations was crucial to their use in determiiing standard alkali isomerization constants.

Adsorption chromatography on silicic acid also plays a role in the preparation of arachidonate (1). There the eicosatetraenoate is eluted while the eicosapentaenoate contaminant remains adsorbed.

Silicic acid was applied in LSC analysis of vernonia oil methyl esters and in the isolation of the major component, *cis*-12,13-epoxyoleate (24). The same esters were also separated by LLC so that a comparison is possible. Experimental conditions are listed in Table I.

It is seen that the relative amount of sample can be much larger in LSC than in LLC and this is a characteristic difference between the two methods. Adsorption is a surface phenomenon and therefore the capacity of a given volume depends on particle size. The liquids in a partitioning system do not have this variable.

TABLE I

Conditions for Chromatography of *Vernonia anthelmintica* Methyl Esters

Size of sample (g.)	Dimensions of column (cm.)	System	Phases
100	100 x 5.5	Adsorption	Ether-hexane / SiO_2
25	80 x 3	Adsorption	Ether-hexane / SiO_2
5	90 x 1.25	Adsorption	Ether-hexane / SiO_2
0.44	27 x 1.5	Partition	Hexane / Acetonitrile on hyflo
0.32	30 x 1.5	Rev. polarity partition	Acetonitrile / Trimethylpentane on hyflo

The separations to be accomplished with vernonia esters were relatively simple inasmuch as epoxy esters were to be separated from hydroxy and nonoxygenated esters. Epoxyoleate accounts for 79–80% of all esters and it is virtually the only compound of the epoxy type; little attention had to be given to the separation of homologs and vinylogs, which is usually done by partition chromatography.

Partition Chromatography

Distribution equilibria of solutes in two liquids are less affected by concentration than are adsorption equilibria. Still, concentrations must be kept low with long-chain fatty acids and solvents must be used which prevent self-association in order to obtain satisfactory separations. The results obtained by column partition chromatography resemble those from countercurrent distribution, and it has been debated how close a parallel can be drawn between the two processes (48).

Long-chain fatty acids or esters are separated in "reversed phase systems." The term "reversed" reflects the historical development: adsorption chromatography used polar adsorbents; from it, partition chromatography was developed using polar stationary liquids; this was finally followed by partition chromatography with solvents of reversed relative polarity, i.e., stationary liquid phases that are less polar than the mobile phases.

The last step was mandatory for making LLC applicable to the common fatty acids of more than 12 C atoms. Their solubilities and partition coefficients provide for better separation when reversed polarity phases are used. The principle was first utilized with paper that had been impregnated with rubber latex (49) and soon afterwards applied to rubber columns (50). In both cases fatty acids were the examples.

Polymers like rubber (39,50), polyethylene (51), or polytetrafluoroethylene (52) need no other support when used as stationary phases in the column. Their definition as "liquid" phases is justified when the deciding criterion is dissolving *versus* adsorbing a solute. Particularly after swelling with solvent, they dissolve rather than adsorb a solute. For other stationary liquid phases like hydrocarbons (53) or silicone oil (3), a solid support is needed. Diatomaceous earth was found very suitable for this purpose since it is virtually inert as an adsorbent and holds liquids well by capillarity. However, mechanical washing-out of the nonpolar liquids (bleeding) must often be minimized by silanizing the diatomaceous earth (15, 53). This process renders the material water repellent by modifying surface groups of high polarity.

Normally the stationary phase must be saturated with developing solvent before filling the column (54) and often the temperature must thereafter be kept constant. Also, it is usually necessary to presaturate the mobile solvent with stationary phase so that the column will not be depleted by solubility elution. These precautions are less important when silicone oil is used as stationary phase (see Part II).

Isolation of C-14 fatty acids from rat lipids may serve as an example of reversed polarity LLC (2). Amounts and purity of the preparations had to be so that measurement of specific radioactivity combined with chemical degradation would permit convincing conclusions in regard to the metabolism of the acids. The component of interest in this example was arachidic acid which, in the original lipids, had been arachidonic acid. The arachidic acid was obtained, together with stearic and behenic acids as the major contaminants, after a complicated preparative and analytical history.

Silanized Celite (55) was loaded with 78.8% (by weight) of mineral oil of specified viscosity. The powdery material was suspended in acetone-H_2O (2:1 by volume) that had been saturated with mineral oil. The slurry was homogenized mechanically, heated to about 50°, and packed in a jacketed column which was kept at 35°. The packing procedure required precautions to prevent air pockets. The column, after packing and settling, measured 31 x 3.4 cm. The fatty acid mixture (169 mg.) was incorporated in an aliquot of prepared stationary phase, slurried with acetone-H_2O (1:1) and transferred to the top of the column. The mobile solvent was saturated with mineral oil and elution proceeded as seen from Figure 2. All effluent was titrated under N_2.

The titrated fractions were pooled, acidified, extracted, and chromatographed over adsorbent silicic acid to eliminate oil and indicator. After evaporation of the solvent from the middle fractions and crystallization of the residue, 52 mg. of pure arachidic acid were obtained with which the further analytical procedures, i.e., degradation and radioactive measurements, could begin.

This LLC procedure is impressive. Although it is nearly a minor event in the whole analytical procedure, it is an indispensable one. In spite of recent

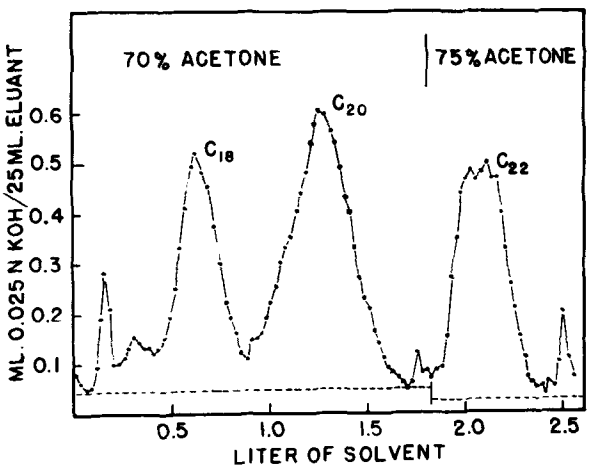

FIG. 2. Separation of saturated fatty acids by reversed polarity partition chromatography. Sample: 169 mg. acids. Column: 31 x 3.4 cm., mineral oil on silanized Celite. Solvent: aqueous acetone. The base line indicates blank value of titrations.

progress in other methods for analysis and preparation of lipids, LLC still was necessary when the metabolic origin of eicosatrienoic, octadecadienoic, and arachidonic acids (56,57) was studied. The references cited above are examples for integration of LLC with many other techniques for one analysis. The number of instances demonstrating this necessity could be greatly enlarged.

Patterns of Separation

It is common to all partitioning processes (countercurrent distribution, LLC, and PC) that palmitic and oleic acids; myristic, palmitoleic, and linoleic acids; and other vinylog-homolog acids migrate at the same rate. In reversed polarity LLC, long-chain compounds travel more slowly than their short-chain homologs and saturated compounds travel more slowly than their vinylogs. The changes in migration rates caused by taking away 2 CH_2 (stearic ⟶ palmitic) or by taking away 2 H (stearic ⟶ oleic) are nearly equal so that palmitic and oleic acids are virtually inseparable. The equivalency must, and actually does, prevail within many types of lipids including esters, glycerides, alcohols, and aldehydes as well as acids. Changing the functional groups of all compounds in a mixture does not bring about new patterns for their separation. The rule of superpositions is particularly closely fulfilled by those acids which are prominent in amount and occurrence and it may be relied upon in analyses (58,59). In the latter reference, chromatographed fractions of superimposing components were hydrogenated so that differentiation by chain length became possible in a second chromatogram.

Secondary chromatography has been avoided in the analysis of certain seed oils. Original and hydrogenated acids were chromatographed over mineral oil as the stationary phase. The saturated acids were also chromatographed after eliminating unsaturated ones by ozonization. All eluates were titrated and the results correlated to render a complete analysis of the common acids. The mixtures also contained oxygenated acids which were quantified by the same principle, using acetylated castor oil as the stationary phase (60).

Derivatization for Improved Separation

The lack of selectivity in LLC may be partly overcome by bromination (7), oxidation (8), and similar reactions specific for double bonds (61). The more recent work on mercuration of fatty esters will be discussed in some detail.

The reaction of mercuric acetate with double bonds (62) yields, in methanol, acetoxymercuri-methoxy compounds from unsaturated esters (63). Interest of lipid chemists in this reaction was revived when mercurated fatty esters were used in PC (64). Mercuration in connection with chromatography has not been fully exploited yet, but the following may indicate its potential.

Mono-, di-, and polymercurated esters have been separated on silicic acid by fractional elution with ether, n-butanol + acetoacetate 1:1, and methanol + acetic acid (10).

Small amounts of saturated esters can be separated from unsaturated esters after mercuration by normal polarity LLC in petroleum ether over nitromethane on silicic acid. The stationary phase is impregnated at the end of the column with diphenylcarbazone as an indicator for the arrival of mercury compounds there. By then the saturated components have been eluted from the column and can be recovered for further separation and analysis (65).

The reaction rates of *cis* and *trans* double bonds with mercuric acetate are quite different. When a mixture of oleate and elaidate was reacted with an amount of mercuric acetate insufficient for complete derivatization, the derivative of the former was the predominant product and enrichment of the components was already 90% after one extraction (9). Although this model experiment did not involve column chromatography, the combination of this differential reaction with chromatography promises separations which are otherwise difficult.

II. Liquid-Liquid Chromatography in the Separation of Odd-Numbered Menhaden Fatty Acids

Menhaden oil is a complicated mixture of lipids, containing numerous odd-numbered acids which could not be isolated by a single method such as distillation. In order to obtain samples of individual components sufficient in amount and purity to prove their structure, an efficient system of LLC was developed in our laboratory. Although the same vinylog-homolog superpositions prevail here as in other partitioning methods, the resolution obtained permits isolation of odd-chain components that are associated with even-numbered ones.

The System

Attempts to extrapolate the PC system, aqueous acetic acid/silicone oil (66), to column chromatography failed until Hyflo Supercel was substituted for cellulose, and acetonitrile for acetic acid. Cellulose powder cannot be loaded with silicone as efficiently as can diatomaceous earth. The advantages of acetonitrile are that it dissolves a smaller amount of silicone, provides better resolution, and is less of a nuisance to handle than acetic acid. However acetonitrile was found more suitable for the separation of esters than of acids.

Preparation of Columns

As in most other LLC procedures, purification of the phases is desirable. Acetonitrile is refluxed over P_2O_5 and then distilled from fresh P_2O_5. About 10% of yellow residual liquid is discarded. The stationary phase, silicone oil (Dow Corning No. 200, 10 cs.) is distilled preferably in a molecular still (Consolidated Electrodynamics Corporation) or conventionally. In the former case, the fraction used distilled from 150° to 200°/20 to 1.5 μ and had n_D^{25} 1.4002; in the latter case, distillation temperature was 195° to 230°/250 μ n_D^{25} 1.4007. They represent about 66% of the total. The forerun, amounting to 20% (n_D^{25} 1.3975), is discarded. Undistilled silicone is about 10 mg. % soluble in 85% acetonitrile and, when contaminating the fatty esters, gives trailing peaks in GLC. These difficulties are negligible with the higher molecular weight fraction of silicone.

Hyflo Supercel is soaked with an equal weight of silicone which has been mixed with the same volume of absolute ether. The resulting paste is brought to dryness in a rotary evaporator at 50°/14 mm. It is then sieved to break up lumps and dried under high vacuum. The material can be stored in this form without precautions. Before filling the column, the

prepared stationary phase is again sieved into aqueous acetonitrile of the desired concentration. The slurry is poured through a separatory funnel into columns of 1 cm. or less in diameter, or directly into wider columns. In both cases the columns are first partly filled with solvent. Drainage and supply of liquid are regulated so that the powdery phase settles by flow rather than gravity. Narrow columns are further settled by gentle suction. Homogeneous packing is verified by running some p-aminoazobenzene through the column and observing the colored zone. An estimate of the volume of the mobile phase in the column (interstitial volume) can be made in this way.

Silanizing the Hyflo Supercel, equilibrating the mobile solvent with silicone, and thermostating the column are of little, if any advantage.

Packed columns have been used eight to ten times with solvent concentrations varying between 65 and 85%. There is some loss of silicone by mechanical leakage, but not enough to affect the performance of the column. In one case, a packing of 90 cm. length shrank 5 cm. with passage of about 40 l. of solvent, mainly due to tighter settling of the stationary phase. This column was used further with 25 l. of solvent.

Flow rate can be widely varied and pressure is never necessary. Rates differing by a factor of 10 did not affect the retention volumes in successive experiments using the same packing. Full capacity flow was seldom used.

Application of Samples

Difficulty is often encountered in applying samples to the column in small volumes of solvent. The following procedures are practical. Solid esters are dissolved in warm solvent and soaked into a wad of cotton, the solvent evaporated, and the cotton then placed as an even layer on the column. For liquid esters it is preferable to dissolve and apply them in pure acetonitrile and then to lower gradually, within one interstitial volume, the concentration of the eluant from 100% to the desired level. The formation of droplets and their coagulation to large globules on top of the column can so be avoided. This method also gives good protection against autoxidation of the sample.

Development and Monitoring of the Column

The volume required to elute a given substance can be predicted from the logarithmic rule of separations. As in GLC, the log volumes of eluant in LLC are in linear relationship when plotted for homologs. Such diagrams are also useful in determining the most suitable concentration of solvent for the desired separation.

To locate the substance in the eluant, 5 ml. of water are mixed with 2-ml. aliquots and, after at least 10 min., the turbidity is measured densitometrically in a spectrophotometer (Spectronic, Bausch & Lomb, or similar). It was found practical to test ten or more fractions at the same time for better compatibility of the relative values instead of standardizing the procedure more rigorously (35). GLC or other methods can be applied for analysis of the peaks and the fractions are pooled accordingly.

Recovery of the Samples

While fractions are being combined, the acetonitrile is poured through a funnel with a plug of cotton in the stem which holds back droplets of silicone. The greater part of the solvent is taken off under vacuum in a rotary evaporator. The opaque aqueous residues are further diluted with water and acidified with HCl. They are then extracted with petroleum ether, which is washed with water and evaporated. The crude esters are distilled alembically under high vacuum at 100°–120° in test tubes which have a small notch to prevent mixing of residue and distillate, and a bulb to collect liquid distillate. The tubes measure 16 x 150 mm. for samples up to 0.5 g. and 25 x 200 mm. for up to 4 g. They are inserted horizontally as far as the notch into tightly fitting holes of an electrically heated aluminum block. After distillation the tubes are cut for easy removal of the distilled portion, leaving behind any silicone or other residue introduced during the procedure.

The LLC procedure described above has been used in the course of isolating minor component acids in pure form for identification.

Isolation of Normal Odd-numbered Acids (3)

The occurrence of C_{19}, C_{17}, and C_{15} acids in menhaden oil was first indicated by PC of fractions obtained by distillation of hydrogenated methyl esters (66). However positive identification of these acids was lacking. The samples for separation by LLC were selected on the basis of PC of hydrogenated aliquots after distillation of nonhydrogenated esters. The occurrence of odd-numbered normal acids in saturated form was established by PC in peroxidic system (67) and by GLC. Portions rich in C_{19}, C_{17}, and C_{13} esters were then hydrogenated. In the fraction containing C_{15} esters there were substantial amounts of unsaturated C_{16} esters. Therefore the isolation of C_{15} esters could be simplified by oxidizing the sample (68), since oxygenated esters are eluted from the reversed polarity system well ahead of C_{14} and C_{15} esters. The cromatograms in Figure 3 represent the isolation of the normal odd-numbered acids. Fractions were pooled as indicated in Figure 3 and the recovered esters alembically distilled, saponified, and crystallized once or twice at −20° to −40°. Between 40 and 80 mg. of each was obtained in a purity of better than 99%, according to GLC. Their data are given in Table II.

Isolation of Isopentadecanoic and Isoheptadecanoic Acids

The possibility of isomers being present was suggested when a C_{15} fraction, pure according to PC and saponification value, was redistilled in a spinning band column (Podbielniak). Some fractions solidified at about 10° while others stayed liquid. GLC of peaks of the LLC chromatograms shown in Figure 3 indicated that branched esters are somewhat enriched by LLC towards the front of the bands. Such fractions were pooled and the greater part of the straight-chain esters was removed by crystallization from acetonitrile at −20°. Isoheptadecanoate and isopentadecanoate in the mother liquors were finally separated from other isomers and traces of normal esters by preparative GLC. After saponification and recrystallization 40 to 50 mg. of these iso acids were obtained, free of detectable contaminants. Their physical constants are given in Table II.

Isolation of Unsaturated Components and Identification of Heptadecenoic Acids

In chromatographing a nonhydrogenated C_{17}-enriched fraction, a column prepared as described earlier was used but N_2 was bubbled through the solvent

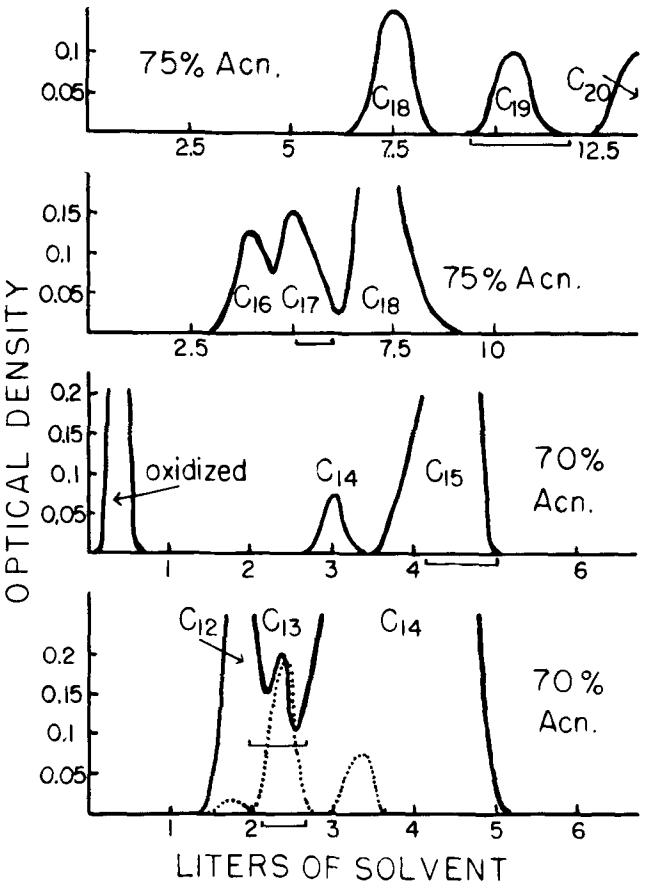

FIG. 3. Separation of saturated fatty esters by reversed polarity partition chromatography. Samples: 0.6 g. C_{18-20}, 0.8 g. C_{16-18}, 1.2 g. C_{14-15}, and oxygenated C_{16}, 3.9 g. (0.4 g.) C_{12-14} esters. Column: 90 x 2.2 cm., about 135 g. silicone on 135 g. Hyflo Supercel. Solvent: aqueous acetonitrile (Acn). Flow rate: approximately 180 ml./hr. Fractions: 15 ml. Two chromatograms were necessary to isolate C_{13} ester.

container at the head of the column and a current of CO_2 was directed into the collection tubes. The fractions were stoppered and placed in a refrigerator for further protection against autoxidation. The curve

TABLE II

Identification of Minor Component Acids of Menhaden Oil[a]

Acid	Melting point[b] (°C.)	Long spacing[c] (Å)
n-Tridecanoic	41.2–41.5 (41.5–41.8)	35.0 (35.0)
n-Pentadecanoic	52.0–52.5 (52.3)	39.9 (39.9)
n-Heptadecanoic	60.8–61.0 (61.0–61.2	44.0 (44.0)
n-Nonadecanoic	68.0–68.2 (68.0–68.2)	48.8 (48.8)
Isopentadecanoic	51.5–52.0 (50.5–51.5)	33.2 (33.2)
Isoheptadecanoic	59.8–60.2 (60.8–61.2)	29.7 (29.7)
n-Heptadecenoic	+ H_2 ⟶ n-Heptadecanoic (m.p., GLC) + CH_2N_2, O_3, H_2, then GLC:[d] 39% n-octanol + 41% azelaic ester half aldehyde 11.5% n-undecanal + 8.2% adipic ester half aldehyde IR: 15% *trans*; alk. isom. of mother liquor ~ 3% diene	

[a] Constants of synthetic compounds are given in parentheses.
[b] Mixed m.p.'s were unaltered.
[c] The limit of error is ±0.05 Å.
[d] Area percentages are given. Identification was made by comparison with authentic samples.

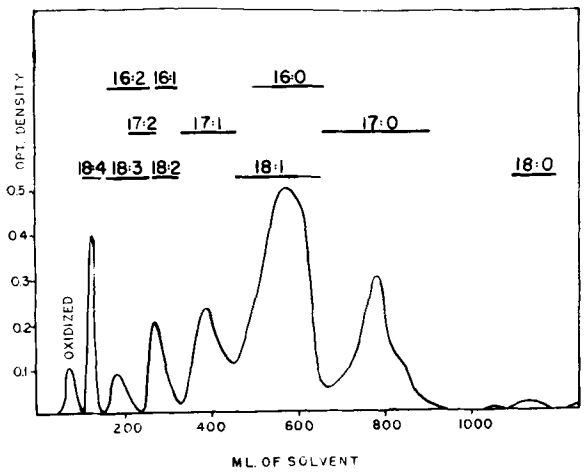

FIG. 4. Reversed polarity partition chromatogram of "C_{17}" fraction of menhaden esters. Sample: 0.2 g. Column: 90 x 1.1 cm., silicone oil on Hyflo Supercel. Solvent: 75% aqueous acetonitrile. For further explanation, see text.

in Figure 4 shows the densitometric results of the chromatogram.

Many fractions were analyzed by GLC before and after hydrogenation. By comparing the various data, it was possible to locate with certainty the number of carbon atoms and level of unsaturation of the components in each peak. GLC also indicated the occurrence of several isomers which have not yet been identified. They are present at all levels of unsaturation and their relative amount increases with the number of double bonds.

Chromatography of the C_{17}-enriched fraction was repeated in a large column and more than one gram of heptadecenoic esters was collected. They were saponified and recrystallized twice from acetonitrile at −20° to −35°. This procedure yielded 0.3 g. of straight-chain acid. Ozonization showed it was a mixture of 80% 9,10- and 20% 6,7-heptadecenoic acid. The positional isomerism is not an artifact. Oleic acid was ozonized concurrently with the heptadecenoic acids and analysis of the fragments proved that formation of over-oxidized products was entirely prevented. Therefore the fragments listed in Table II must be considered genuine and the positions of the double bond can be derived from them.

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Countercurrent Distribution

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IN COUNTERCURRENT distribution a mixture of substances is fractionated by repeated partition or distribution between two immiscible liquid phases. The distributions are performed in a discontinuous and quantitative manner so that the process is adaptable to mathematical analyses.

Apparatus for countercurrent distribution has evolved from the small metal instruments described by Craig in 1944 (1) to completely automatic glass equipment with many tubes. With such equipment separations are made that would be practically impossible if the distributions were performed individually in separatory funnels.

The subject of countercurrent distribution has been reviewed by Craig (2), Weisiger (3), and Hecker (4). Its applications to lipids have been described by Dutton (5, 6).

Modern instruments consist of a train of glass cells connected in series in such a way that they are suitable for automatic operation (7). The operation of the cells is illustrated in Fig. 1 (7). Each cell contains lower phase solvent to bring the interface to *a* in position C. The amount of upper layer may be varied to suit the partition coefficients of the solutes being resolved. Equilibration of the phases is accomplished by rocking between positions A and B. The phases separate in position B. On tilting to C the upper layer decants through *c* to *d*. On tilting to A the contents of *d* flow through *e* to the adjoining cell.

An instrument with 220 such tubes and with a robot for automatic operation has been described by Craig (7). The 200-tube automatic apparatus used at this Laboratory is shown in Fig. 2.

Craig (1) has also described the operation of countercurrent distribution instruments using fundamental, single withdrawal, and recycle procedures. In