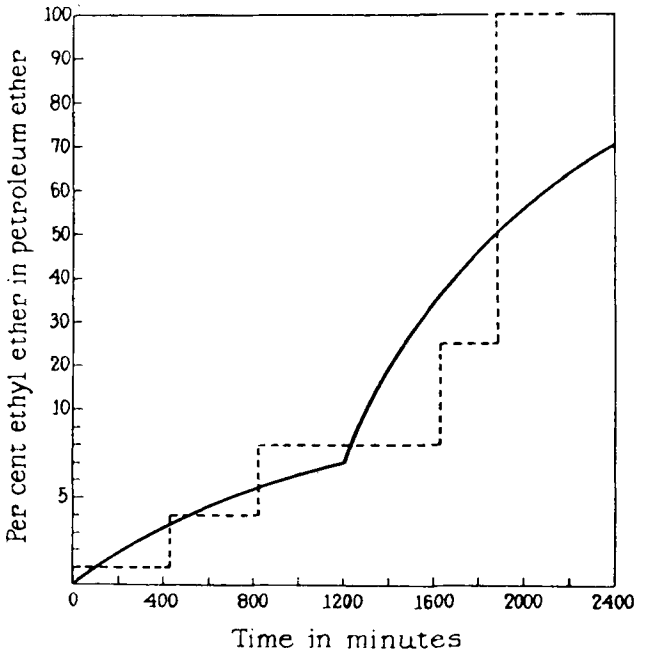

FIG. 2. Solvent composition change with time for a typical gradient (solid line) and stepwise (broken line) elution. In the gradient scheme, 10 per cent ethyl ether in petroleum ether is added at a constant rate to a constant volume of petroleum ether for a period of 20 hours, followed by the addition of ethyl ether (2).

for specific separations. An excellent apparatus for this purpose has been described by Hirsch and Ahrens (2) and is pictured in Fig. 1.

When compounds of somewhat different polarity are to be separated, step-wise elution offers a simple and adequate approach. In this technique the column is simply eluted with pre-determined quantities of different specific mixtures of the solvent pair. An illustration of the solvent changes with time for these two approaches is seen in Fig. 2.

Obviously a third choice is available which utilizes a combination of the continuous gradient and step-wise elution techniques. An operation of this type could be applicable to special situations, but generally one chooses one or the other as a starting point.

In both step-wise and gradient elution, the usual practice is to start with the least polar mixture or member of the pair and increase the polarity by the addition of the more polar material.

In addition to the above considerations, other factors play an important role in the selection of the eluting solvents. The absorbent may have a high affinity for the eluant but the value of the eluant may also be enhanced if it is a good general solvent for the materials to be eluted. Other general requirements are that the eluant be easily removed from the sparated material and that it should not react so as to irreversibly change the nature of the solute.

In this discussion we have considered only the major elements of column chromatography. There are many factors involved in the selection or development of a specific technique and it should be understood that this cannot be considered to be a complete treatise from the standpoint of theory or application. However it will serve as a means of introduction of the subject and to outline some of the problems associated with its use as a laboratory tool. For those who wish a more thorough and authoritative treatment of fundamentals, the work of Cassidy (3) is highly recommended as a starting point.

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Isolation of Neutral Lipids by Column Chromatography

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THE USE of column chromatography for the separation of lipids has been a valuable addition to the techniques for study of these important biological compounds. Historically alumina was the first adsorbent to be applied to lipid problems (1, 2, 3, 4, 5, 6, 7). With the introduction of silicic acid chromatography by Trappe in 1940 (8, 9), a new concept of the use of columns for lipid separations was initiated. More recently (10), Florisil has been introduced as an adsorbent potentially valuable for neutral lipid class separations. Although alumina may have some value in certain specific investigations, its use has been largely supplanted by silicic acid for lipid studies. Therefore this discussion will be oriented mainly toward the use of the latter for neutral lipid separations, but Florisil will also be discussed since it appears to have some advantages over silicic acid.

The value of silicic acid as an adsorbent for lipid separations is clearly demonstrated by a number of methods presently available. After the introduction of this adsorbent, Börgstrom (11) presented a method for separation of several lipid classes by elution with various mixtures of petroleum ether and benzene. This development was followed by other adaptations (12, 13).

Despite the efforts of many investigators, silicic acid separations were often found unreliable. Published methods worked well in the hands of their authors but their use by others often required modification. This prompted Hirsch and Ahrens to initiate a systematic study of all factors involved and to develop a standardized method for application of the technique to lipid class separations (14). This method is one of the most effective yet described. Using mixtures of

TABLE I

Elution Schemes *

A. Stepwise elution of 8 major classes of lipides

Fraction	Eluant
I	50 ml. of 1% ethyl ether in petroleum ether (60–70°)
II	75 ml. of 1% ethyl ether in petroleum ether
III	225 ml. of 1% ethyl-ether in petroleum ether; then, 60 ml. of 4% ethyl ether in petroleum ether
IV	240 ml. of 4% ethyl ether in petroleum ether; then, 200 ml. of 8% ethyl ether in petroleum ether
V	450 ml. of 8% ethyl ether in petroleum ether; then, 50 ml. of 25% ethyl ether in petroleum ether
VI	200 ml. of 25% ethyl ether in petroleum ether
VII	300 ml. of ethyl ether
VIII	400 ml. of absolute methanol

B. Stepwise elution of 3 major classes of fatty acid esters

Fraction	Eluant
I	350 ml. of 1% ethyl ether in petroleum ether; then, 60 ml. of 4% ethyl ether in petroleum ether
II	300 ml. of ethyl ether
III	400 ml. of absolute methanol

C. Gradient elution of 8 major classes of lipides

Fraction	Eluant
I	Upper reservoir: 1200 ml. of 10% ethyl ether in petroleum ether; then, 1000 ml. of ethyl ether ↓ Lower (mixing) reservoir: 600–800 ml. of petroleum ether
II	Then, 400 ml. of absolute methanol

* Reprinted from (14).

ether and petroleum ether in a stepwise elution (Table I, Schemes A and B) these workers were able to quantitatively separate synthetic mixtures composed of seven major classes of neutral lipids. An elution diagram is seen in Fig. 1; representative quantitative data are in Table II. Excellent separations are obtained in all cases except for the tailing of cholesterol into diglyceride. However the authors demonstrated that this tail was due, in part, to impurities in the cholesterol as a more complete resolution was obtained with the recrystallized sterol (Fig. 2). The small amount of tailing that remained was thought to be caused by the particular adsorption properties of cholesterol, as the infrared spectrum of eluted material was not altered by passage through the column. Extension of the technique to the separation of naturally occurring plasma lipids is illustrated by the elution diagram in Fig. 3. In this case elution is completed by the addition of methanol to remove phospholipids.

TABLE II

Separation of Synthetic Mixtures into Major Lipide Classes *

Charge	Cholesteryl palmitate—31.7 mg. Tripalmitin—24.2 mg. Cholesterol—17.2 mg. Monopalmitin—49.5 mg.	Cholesteryl palmitate—31.7 mg. Tripalmitin—24.2 mg. Cholesterol—17.2 mg. Dipalmitin—38.2 mg.
Fractions I, II, & III (cholesterol-ester)	Weight = 31.1 mg. Esterified cholesterol** = 31.2 mg. % recovery = 98.1	Weight = 30.6 mg. Esterified cholesterol** = 30.4 mg. % recovery = 96.5
Fraction IV (triglyceride)	Weight = 24.8 mg. Total cholesterol = 0 mg. % recovery = 102.5	Weight = 24.6 mg. Total cholesterol = 0 mg. % recovery = 101.7
Fraction V (cholesterol)	Weight = 17.1 mg. Total cholesterol = 15.5 mg. % recovery of cholesterol (in Fractions V and VI) = 96.5	Weight = 15.9 mg. Total cholesterol = 14.5 mg. % recovery of cholesterol (in Fractions V and VI) = 94.7
Fraction VI (diglyceride)	Weight = 1.9 mg. Total cholesterol = 1.1 mg.	Weight = 39.5 mg. Total cholesterol = 1.8 mg. % recovery (weight-cholesterol) = 98.7
Fraction VII (monoglyceride)	Weight = 48.1 mg. Total cholesterol = 0 mg. % recovery = 97.2	Weight = 0.3 mg. Total cholesterol = 0 mg.

* Reprinted from (14).
** Expressed as milligrams of cholesteryl palmitate.

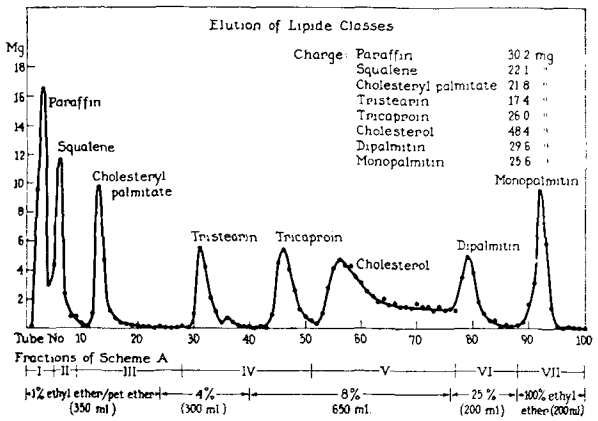

FIG. 1. Elution by steps of seven major classes of lipids from silicic acid (14).

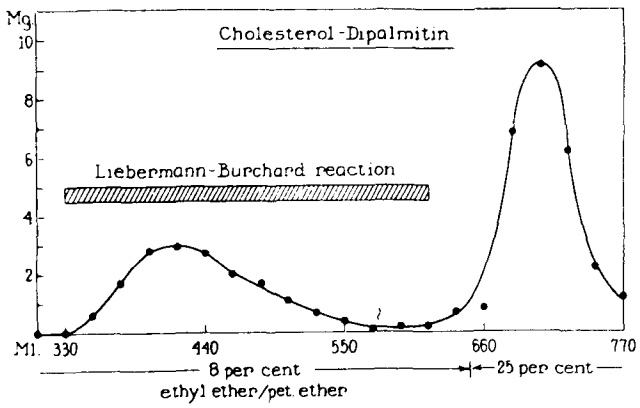

FIG. 2. Separation of highly purified cholesterol from diglyceride by elution from an 18gm column of silicic acid (14).

Application of a gradient elution technique (Table I, Scheme C) to the separation of synthetic mixtures was found to yield separations equal to those of the stepwise scheme, and its value for natural mixtures is demonstrated by the elution diagram in Fig. 4.

While these separations are excellent and applicable over a wide range of lipid structures, the high degree of volatility of ethyl ether makes it necessary to provide the column with a cooling jacket to prevent excess evaporation and possible variation in solvent mixture composition. In this light Horning *et al.* (15) developed a method utilizing a hexane-benzene solvent pair which is more convenient and which gives excellent separations of the major neutral lipid classes. Employ-

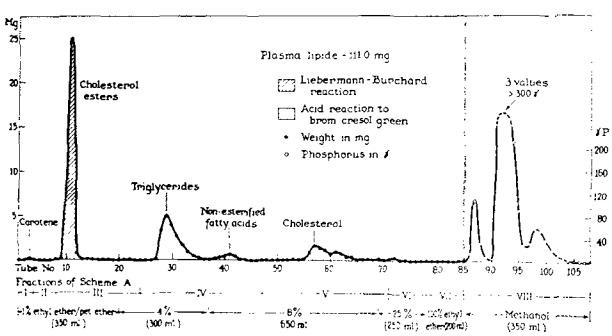

FIG. 3. Separation of plasma lipids by elution by steps from silicic acid (14).

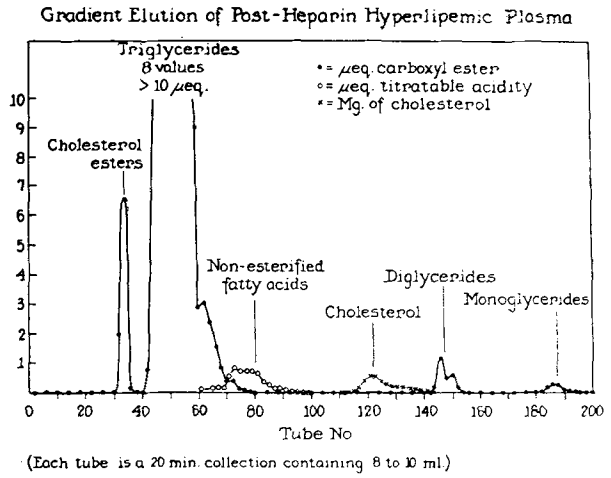

FIG. 4. Separation of the lipid components of a hyperlipemic human plasma by gradient elution from silicic acid (14).

ing relatively large changes in benzene concentration, these workers found that quantitative class separations could be carried out more rapidly than in previously described methods. Phospholipids were eluted with methanol as described earlier (14). An elution diagram of this method applied to the separation of plasma lipids is shown in Fig. 5. With this solvent system, 6% benzene elutes hydrocarbons, 18% elutes cholesterol esters, 60% removes triglycerides, and cholesterol is removed with benzene. Although the authors made no attempt to study the separation of the mono- and diglycerides, our work indicates that they can be eluted as one fraction by chloroform. In this procedure the uniformity of the cholesterol ester, triglyceride, and cholesterol peaks was confirmed by comparison of the mass data with colorimetric sterol (16) and triglyceride (17) determinations. The data for both sterol fractions, and triglycerides, are seen in Fig. 6 and 7. In these studies only the mass value for cholesterol did not correspond to the colorimetric data. Subsequent examination of this fraction indicated this to be caused by the presence of free fatty acids which were eluting as a band on the leading edge of the cholesterol peak. Presumably the free acids could be eluted by a solvent mixture intermediate in polarity between 60% benzene and 100% benzene. Apparently the order of displacement is not changed in benzene-hexane elution as the elution of unesterified fatty acid as a peak precedes cholesterol (14). See Fig. 3.

In the two procedures described above, 18gm

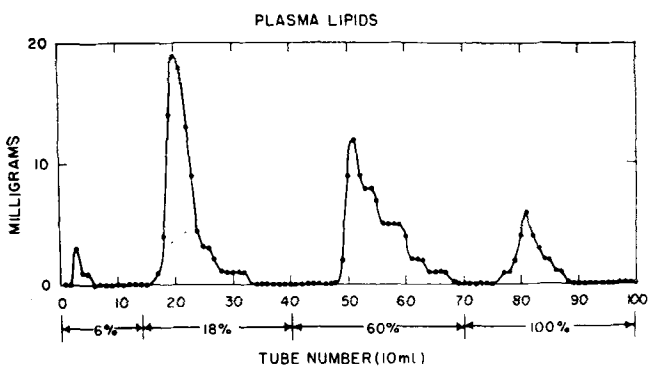

FIG. 5. Separation of human plasma neutral lipids by stepwise elution from silicic acid with varying concentrations (v/v) of benzene in hexane (15).

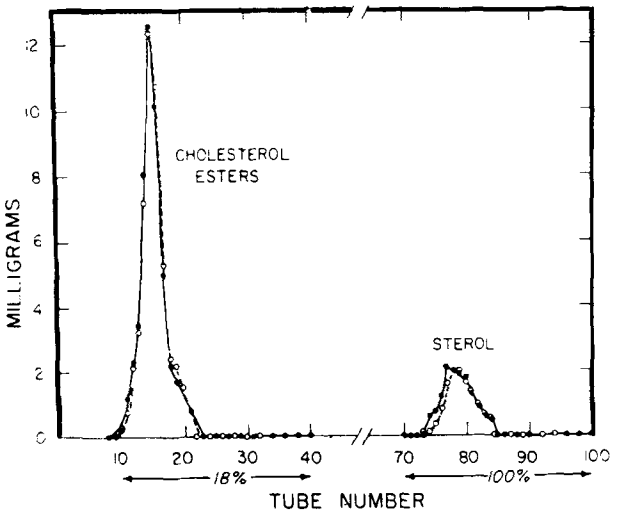

FIG. 6. Chromatographic separations of cholesterol esters and sterol fractions of human plasma; • = gravimetric determination; ○ = colorimetric determination (16). The total values are 48.7mg (gravimetric) and 48.4mg (colorimetric) for cholesterol esters, and 13.6mg (gravimetric) and 11.5mg (colorimetric) for sterol. A band impurity is evident in the sterol peak (15).

columns were used with maximum loads of about 300–350mg of lipid. When mixtures of naturally occurring lipids of this magnitude are separated, the individual fractions contain adequate amounts for future study by gas chromatography or other techniques. However caution should be exercised with respect to the load size since even small overloads may destroy an otherwise excellent separation. From these data and our experience with serum lipids, the maximum permissible load is approximately 15mg of mixed lipid per gram of silicic acid. Even at this level an overload of one or more components is possible since their individual adsorption isotherms may differ greatly.

In any procedure involving silicic acid, extreme care must be taken in the preparation of the adsorbent to obtain reproducible results. The best method of preparation may vary with the individual chromatographic technique. For example, Horning (15) recommends a treatment with hydrochloric acid for removal of in-

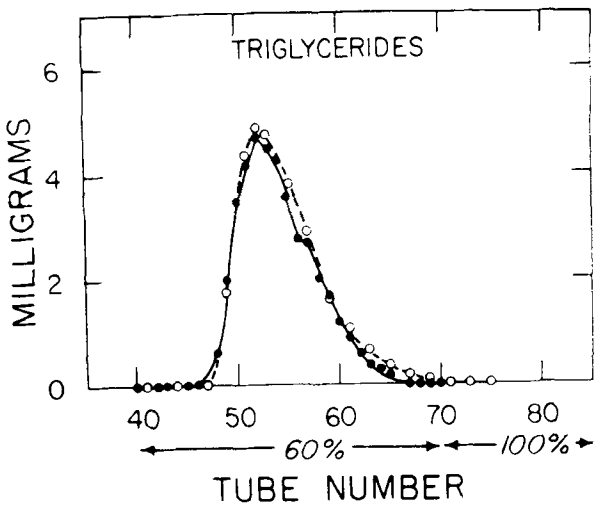

FIG. 7. Comparison of gravimetric (•–•) and colorimetric (○–○) triglyceride (17) determinations on a fraction isolated by chromatography on silicic acid (15).

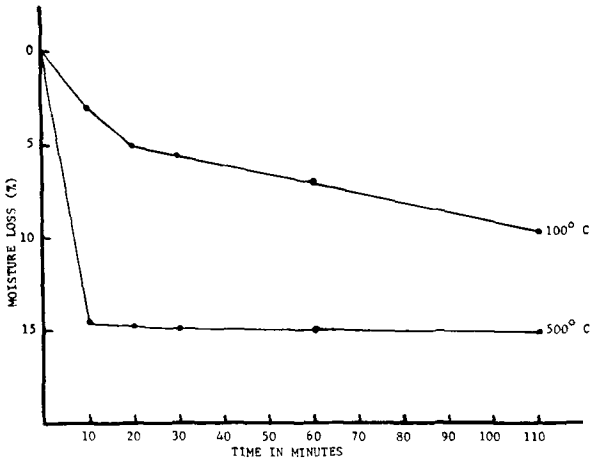

FIG. 8. Silicic acid dehydration curves at surface temperatures of 100°C. and 500°C. The material was Mallinckrodt, #2847, 100–200 mesh and contained 20% water.

organic contaminants while Hirsch and Ahrens (14) make no mention of this. It seem likely that the specific details of the techniques are determined by the mode of application and solvent system used. Our experience has shown that the acid treatment is a necessity in the benzene-hexane system and that the recommended preparation should be followed carefully if one is to obtain reproducible separations. As with all experimental procedures, it is best to follow the instructions of the authors explicitly unless experimentation indicates that alteration is advantageous.

A difficult problem which is common to all silicic acid separations, is that of degree of activation. The adsorbent contains a variable amount of water which affects its absorptive properties. At about 5% water activation becomes maximal and the effect approaches adsorption on a solid surface (18). A method which has been developed with solvent mixtures which displace a given solute at 10% water will behave differently at values above or below 10%. In practice a variation of about ± 0.5% is maximal. When the column is too wet the materials are usually very easily displaced and have a tendency to overlap. If it is too dry they are more strongly adsorbed and have a tendency to form tails. In a mixture of lipids the most critical point of this effect is between triglyceride and cholesterol.

Hirsch and Ahrens (14) recommend the use of dehydrating washes to obtain the desired degree of activation. This method, and heat dehydration have been used with equal success. The difficulty is to determine the best moisture level for the separations desired. This can vary from one laboratory to another depending on conditions of humidity, degree of solvent purity, and other factors. After much investigation we have established a moisture content of 14.5% to be optimal under our conditions with the method of Horning (15). When the silicic acid is prepared as directed and the moisture content reproduced, elution diagrams of the same sample are identical. Reproduction of the desired moisture level is achieved by determination of the water content, after five washes with acetone, on a moisture balance [1] and subsequent heating at about 100°C. to the desired level. With this apparatus the moisture percentage is read directly

[1] O'Haus Moisture Determination Balance, Model 6000, O'Haus Scale Corp., Union, N.J.

TABLE III

Elution Schedules for Florisil Chromatograms *

Solute	30 g. Column (2.0 cm. x 17.0 cm.)	Eluting solvent	12 g. Column (1.2 cm. x 15.0 cm.
	ml.		*ml.*
Hydrocarbons	50	Hexane**	20
Cholesterol esters	120	5% ether in hexane	50
Triglycerides	150	15% ether in hexane	75
Cholesterol	150	25% ether in hexane	60†
Diglycerides	150	50% ether in hexane	60†
Monoglycerides	150	2% methanol in ether	75
Free fatty acids	150	4% acetic acid in ether	75

* Reprinted from (10).
** Purified Skellysolve B.
† It may be found more convenient to elute both cholesterol and diglyceride fractions with 140 ml. of 25% ether in hexane.

and loss may be halted at the desired level. Then the material is cooled and placed in hexane for column introduction as a slurry. A dehydration curve for silicic acid at 100°C. and 500°C. is presented in Fig. 8. When silicic acid is dried at about 500°C. it loses water rapidly at first, but stabilizes at approximately 5% within thirty minutes. The remaining moisture is lost very slowly, even at this temperature. With freshly prepared silicic acid, the loss was determined after thirty minutes at 500°C.; total content was then calculated by the addition of this value to a standard value of 5% known to be slowly lost. Comparison of these calculated values with moisture contents determined by ignition to constant weight at 500°C. showed close agreement. The loss at 100°C. (Fig. 8) is seen to be a gradual one allowing the reproduction of any desired level within small limits.

Regardless of the method used to accomplish moisture content reproduction, much of the difficulty encountered in work with silicic acid can be attributed to this problem.

Florisil

A recent addition to column chromatographic methods available for neutral lipid separations is the method of Carroll (10). This technique utilizes Florisil [2] as the adsorbent with an ether-hexane solvent pair applied in a stepwise elution scheme (see Table III). Under the conditions of these separations it was necessary to add methanol to ether to obtain sufficient solvent polarity to displace monoglycerides, and acetic acid to displace free fatty acids. For an elution diagram carried out with model compounds see Fig. 9. In this experiment monoglyceride was eluted with 10% methanol in ether which caused elution of extraneous material shown as an extra peak at the end of that fraction. This was eliminated in later studies by the use of 2% methanol in ether. The method gave excellent separation of the synthetic mixture with either 12 or 30g columns.

The degree of activation appears to be as important with Florisil as with silicic acid. The separation seen in Fig. 9 was carried out with Florisil which had been in the laboratory for several years and was used without preliminary treatment. When separations were attempted with a new supply of adsorbent, activated at 600°C., good separations were not obtained (Fig. 10). At this state of activation, cholesteryl palmitate was seen to tail considerably, and tripalmitin adsorbed

[2] Florisil is available from the Floridin Co., Tallahassee, Fla.

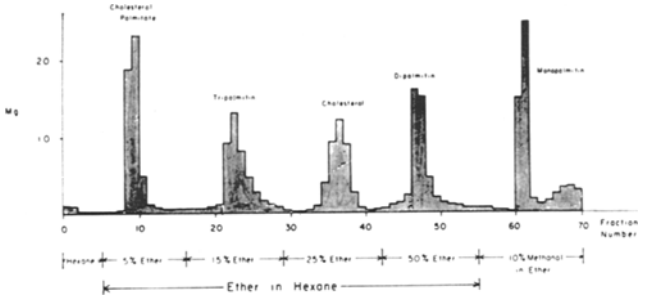

FIG. 9. Separation of lipid classes on a 30gm column of Florisil by stepwise elution. The column load consisted of 40mg of each component (10).

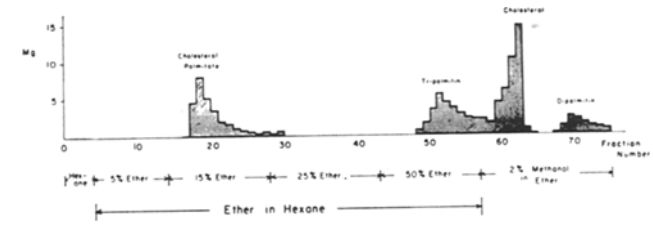

FIG. 10. Separation of lipid classes on a 12gm column of highly active Florisil. The column load consisted of 30mg each of cholesteryl palmitate, tripalmitin and cholesterol, and 15mg each of dipalmitin and monopalmitin (10).

so strongly that it was incompletely separated from cholesterol. When this new material was deactivated with 7% water, good separations were again observed (Fig. 11). Mixtures of naturally occurring lipids of rat blood and liver were well resolved and excellent recoveries were obtained. Studies indicate this method to be an adequate one for separation of human serum and artery lipids.

Florisil has some distinct advantages over silicic acid as an adsorbent for lipids. The rather large particle size (60–100 mesh) permits rapid flow rates without apparent loss of adsorptive surface and the material requires a minimum of preparation for use. An interesting property of this adsorbent is its affinity for free fatty acids. Elution of these lipids is delayed

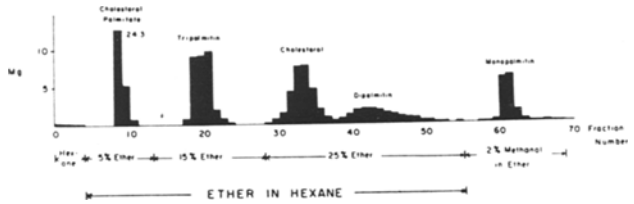

FIG. 11. Separation of lipid classes as in Fig. 10 after deactivation of the Florisil with 7% water (10).

until after monoglyceride, instead of emerging just before cholesterol as previously discussed with respect to silicic acid. This suggests the use of Florisil as a possible selective filter for free acids but presents a problem from the standpoint of recovery, as the addition of acetic acid to the solvent was required for their elution. Although Florisil may have some advantages over silicic acid, the problem of controlling the state of hydration is just as acute.

Whether one chooses Florisil or silicic acid it is obvious that these techniques require rigid control measures. But when the basic fundamentals of good column chromatography are observed, their use for separation of lipids can be a valuable addition to the technical knowledge of a laboratory.

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Quantitative Chromatographic Fractionation of Complex Lipid Mixtures: Brain Lipids[1]

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THE OBJECTIVE of this presentation is to describe methods for the quantitative fractionation of brain lipids by means of column chromatography. It is not possible to give detailed directions for the chromatographic separation of complex lipid mixtures in general, as the types and quantities of lipids in mixtures from different sources may vary widely. Methods that are suitable for brain lipids may require modification for other lipid mixtures. A discussion of this complex subject must be approached in a general way with emphasis upon the variables that are involved in column chromatography, the means by which these variables can be controlled, and the mechanisms involved in the chromatographic processes. When these factors are appreciated, the extension of methods devised for brain lipids should be possible.

Emphasis has been placed on the use of infrared

[1] With comments on the mechanisms of chromatography and the use of infrared spectroscopy.