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DRY COLUMN CHROMATOGRAPHY OF PHOSPHOLIPIDS

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SUMMARY

Separation of common phospholipids can be effected by dry column chromatography on silica gel. The method involves packing the column with dry gel and developing it in solvent mixtures used for thin-layer chromatography of the same lipids. Solvent is allowed to migrate only to the end of the column; access to the bands of separated material is obtained by using columns with a removable glass front. R_F values of lipids on development columns and those on thin-layer plates are nearly identical when the column is packed with thin-layer chromatography gel. Such columns, however, develop very slowly. Columns packed with fine silica gel designed for elution column chromatography develop very rapidly and yield separations that are still quite comparable to those obtainable from thin-layer plates. Such columns are convenient for the purification of phospholipids in amounts of 10 mg to about 10 g. Column design and construction are described in detail.

INTRODUCTION

The first serious examination of column chromatography as a technique for separating complex mixtures was made by Tswett[1] in the early 1900's. Tswett packed columns with dry adsorbent, usually cellulose, applied samples in solution and developed the column with solvent. When the developing solvent reached the bottom of the column, the adsorbent was extruded and cut into sections corresponding to the bands that separated. Subsequent development of chromatography lead to emphasis on elution methods, culminating, in the case of lipid chromatography, with the introduction of silica gel columns by Börgstrom[2] in 1952. Subsequently, thin-layer chromatography (TLC) was developed[3]. For separation of lipids, this technique also utilized silica gel, but the development procedure is much like that of Tswett, except that the adsorbent is formed as a layer on a plate rather than being packed into a column. The great resolving power of chromatography on layers of silica gel has prompted a re-investigation of Tswett's method with silica gel as the adsorbent[4]. This method is now commonly termed "dry-column" chromatography[5]. According to one investigation, there is good agreement between R_F values on thin-layer plates and dry columns provided the adsorbent in the latter is appropriately treated[6].

The procedures developed during the investigation described below are also

based upon modifications of the Tswett method. Our purpose was to develop method for rapid and simple isolation of complex lipids on a preparative scale. The major innovation we have made is to utilize columns that can be opened from the front. In addition to allowing easy access to the undisturbed adsorbent, an important advantage of these columns is that optimization of separation conditions is quick and simple The procedures we have found to be useful for phospholipids should also be applicable to the purification of other substances.

CHEMICALS

Lysolecithin (LPC), phosphatidylinositol (PI) and phosphatidylserine (PS standards were obtained from Supelco (Bellefonte, Pa., U.S.A.). Sphingomyelin (SM was obtained from Sigma (St. Louis, Mo., U.S.A.). A chloroform–methanol extrac of egg yolk was the source of phosphatidylcholine (PC) and phosphatidylethanol amine (PE) standards. Soybean phosphatides were obtained from several biochemical supply houses. An alcohol-insoluble fraction of soy lipids (AI) was obtained by adding 50 ml of absolute ethanol to a solution of 5 g of soy phosphatides in 10 ml of chloroform. The resulting precipitate was collected by centrifugation and the procedure was repeated twice. Sources of silica gel are given in the text below.

METHODS AND RESULTS

Columns

The method of chromatography described here involves filling a column with silica gel, adding a chloroform solution of the lipids to be separated to the top of the column, and then developing the column with one of a variety of solvent mixtures until the solvent front reaches the bottom of the column. The region(s) of adsorbent containing the desired component(s) is located, the adsorbent removed, and the component eluted therefrom.

It was concluded that access to the adsorbent was most simply obtained with columns having a removable glass front. The column designs found to be most suitable in this respect are shown in cross section in Fig. 1. The column illustrated in 1A consists of two lengths of heavy-wall glass tubing, one side of each of which has been ground off to produce a semicircular cross section. The two halves are clamped together with sturdy spring clamps to form a cylinder. After development, the column is split into two halves with a fine wire.

The columns illustrated in B, C, D, E and F differ from that in A in that one side of the column is removable so that the adsorbent is left intact. In these cases, the front of the column consists of glass and the back and sides of either PTFE, polypropylene, or PTFE-covered metal or plastic.

Type B is a multiple column that is most useful for preliminary evaluation of developing solvents. This column is most conveniently milled from a 1/2-in. PTFE slab. Useful sizes for the channels are 1/8–1/4 in. in cross section by about 10 cm long. The column array is covered by a piece of double-strength glass which, in turn, is covered by a piece of 1/2-in. plexiglas. The back side of the columns (bottom in figure) is supported by a piece of 1/2-in. aluminum. The array is clamped together with about four evenly spaced C-clamps.

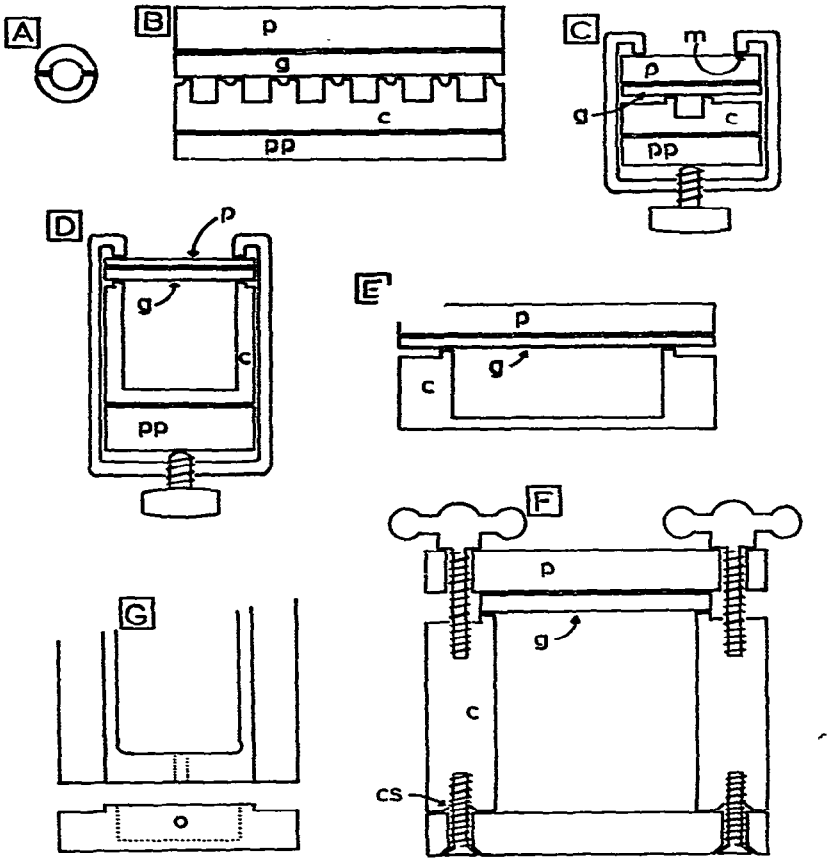

Fig. 1. Column designs. A through F represent column cross sections. With the exception of the all-glass column A, a complete column consists of a column back (c) and a glass cover (g). Above the glass cover is a plexiglas sheet (p) and in some cases there is a metal plate (pp) under the column back. G is a detail of the bottom of column backs of the type shown in D and E; a front view (upper) and a bottom view (lower) are shown.

Type C represents a convenient arrangement of clamping columns up to about 1 in.^{2} in cross section. The clamping frame of C consists of a section of one 5/8 in. × 1 5/8 in., P1000 "unistrut" (Unistrut Corporation, Wayne, Mich., U.S.A.), the back of which is drilled and tapped at 2–3-in. intervals for knobs with 1/4–20 threaded studs. The ends of the studs are rounded to minimize friction. The two parts of the column are pressed together between a pressure plate of 3/8-in. metal (pp) and a 1/4- or 3/8-in. sheet of plexiglas (p). The purpose of the latter is to spread the pressure along the glass front of the column so that it will not crack. The clamping arrangement illustrated in D is similar to that of C except a larger piece of unistrut (P5500, 1 5/8 in. × 2 7/16 in.) is used to accomodate a larger column. Since here the pressure on the glass cover is applied from both sides along the same line, it needs to be covered with only a thin piece of plexiglas. It may be necessary to mill the pressure-bearing surfaces (m) of the unistrut so that they are smooth and parallel. The design shown in E is useful for intermediate-sized columns. The column is milled from a 3/4-in. or

1-in. sheet of PTFE or polypropylene. Appropriate channel dimensions are 1/2–3/4 in. × 2–4 in. A lower pressure plate may or may not be necessary, depending upon the thickness of the side walls of the column. A 1/2-in. sheet of plexiglas is placed above the glass cover. C-clamps placed at intervals of about two or three inches along each side hold the column together.

Columns of 2–4 in.^{2} can be made inexpensively according to design E. The back and sides of the column are 1/2-in. and 3/4-in. polypropylene or PTFE, respectively. In our experience the less expensive polypropylene is quite suitable for use with solvents consisting of mixtures of chloroform and methanol. In a column such as E, which is built up from three pieces, it is important that the butt joints are tight all along their surfaces of contact. The contacting surfaces must therefore be smooth and flat and the threaded holes are countersunk (cs) so that deformation of the walls as the screws are tightened does not cause separation of the joint. The cover is 1/4-in. plate glass. The bottom can be a plate of the same material as the channel with a small hole in it. It need not fit tightly against the glass cover.

The only critical aspect of these designs is the seal between the back and the cover. A good seal is easily made if surfaces of the column back which bear against the glass cover are sanded very smooth. This is done with a wet piece of 400-grit "wetordry" silicon carbide paper on a flat bench top. Fewer problems with irregularities will be encountered if part of the front surfaces of the column is recessed, leaving a 1/8-in. strip at the inside edge of the column to bear against the glass.

Inexpensive but inert column backs may be constructed by covering extruded aluminum channel (a large variety of sizes and shapes is available) with adhesive-backed PTFE sheet. Polypropylene column backs may similarly be covered with PTFE.

Packing columns

All columns are packed in much the same way; a plug of glass wool is placed in the bottom of the column and adsorbent is poured into it until it is full. An electric vibrating massager or a small hammer is applied to the bottom of the column until the adsorbent settles. Silica gel settles by about 30%, leaving an appropriate volume above the gel for a solvent reservoir.

Column testing

It may be advisable to test new columns or new designs for evenness of development with a mixture that can be visually monitored during separation. A mixture of acid fuchsin, rhodamine G, and sudan black, dissolved in and developed with solvent 1 (see Table I) is suitable. On silica gel, this mixture gives 5 fairly evenly distributed bands (three are from commercial acid fuchsin).

Loading and developing columns for phospholipid fractionation

Lipid mixtures are dissolved in chloroform at concentrations of about 0.1–0.3 g/ml. Higher concentrations may be used if the top 1/4–1/2 in. of the column is moistened with chloroform to prevent the heat of adsorption from causing the applied solution to float on a layer of vaporized solvent. When columns larger than about 1 in.^{2} are used, it is advisable to place a piece of thick, solvent-extracted felt on the top of the column to facilitate even distribution of the solution.

TABLE I

SOLVENTS

Solvent No.	*Composition*
1	Chloroform–methanol–7% ammonia (60:20:3)
2	Chloroform–methanol–7% ammonia (30:15:3)
3	Chloroform–methanol–water (60:20:3)
4	Chloroform–methanol–water (60:40:3)
5	Chloroform–methanol–water (5:8:1)
6	Chloroform–methanol–30% ammonia (9:5:1)
7	Chloroform–acetone–methanol–acetic acid–water (10:4:2:2:1)

After the sample has been adsorbed by the column packing, the developing solvent is added, initially in small portions, each being absorbed successively into the column. When the solvent front has moved 10–20% of the way down the column, the reservoir space may be filled with developing solvent. Columns containing adsorbents of 200 or smaller mesh do not "run dry" although, if a column is very long, the solvent level may drop a short distance below the top of the adsorbent. When the solvent front reaches the bottom of the column, excess developing solvent is removed and the column allowed to stand for a minute or two until migration of the solvent in the column stops. The clamping apparatus is then removed and the glass cover is slid off the column in a direction perpendicular to the length of the column. As a precaution against disrupting a separation by solvent redistribution, the column may be kept in a near upright position for this and subsequent operations. Once wet by developing solvent, silica gel remains very tightly packed. When the desired band(s) is located (see below), it is removed with a spatula or spoon, or, with a thin sheet of stainless steel of the same dimensions of the column, appropriate sections of adsorbent are cut and slid off the end of the column.

Detection of separated bands and assessment of purity

After the column front is removed, a strip of laboratory label tape is pressed onto the surface of the silica gel. A thin layer of silica gel adheres to the tape and phospholipids adsorbed thereon are detected by spraying the tape with molybdate reagent[7] or, as appropriate, with ninhydrin.

An alternative method for locating band positions is to press the gel side of a TLC plate against the surface of the exposed column packing until sufficient solvent migrates from the column to wet the thin layer. The plate is then sprayed with an appropriate detection reagent. Transferring bands to TLC plates has the advantage that it allows detection of compounds by charring or other reactions requiring heat.

If the only purpose of developing a column is to determine optimum conditions for a particular separation, there is no need to avoid contamination of the packing, and the exposed surface of the adsorbent may be sprayed directly with molybdate reagent. Fig. 2 shows the appearance of bands of soybean phosphatides on a column that has been sprayed directly with molybdate reagent.

TLC is used to assess the purity or identity of questionable bands. Bands may be transferred directly onto TLC plates by pressing the latter against the surface of the column at an angle of about 45° (thin layer facing column gel) until solvent

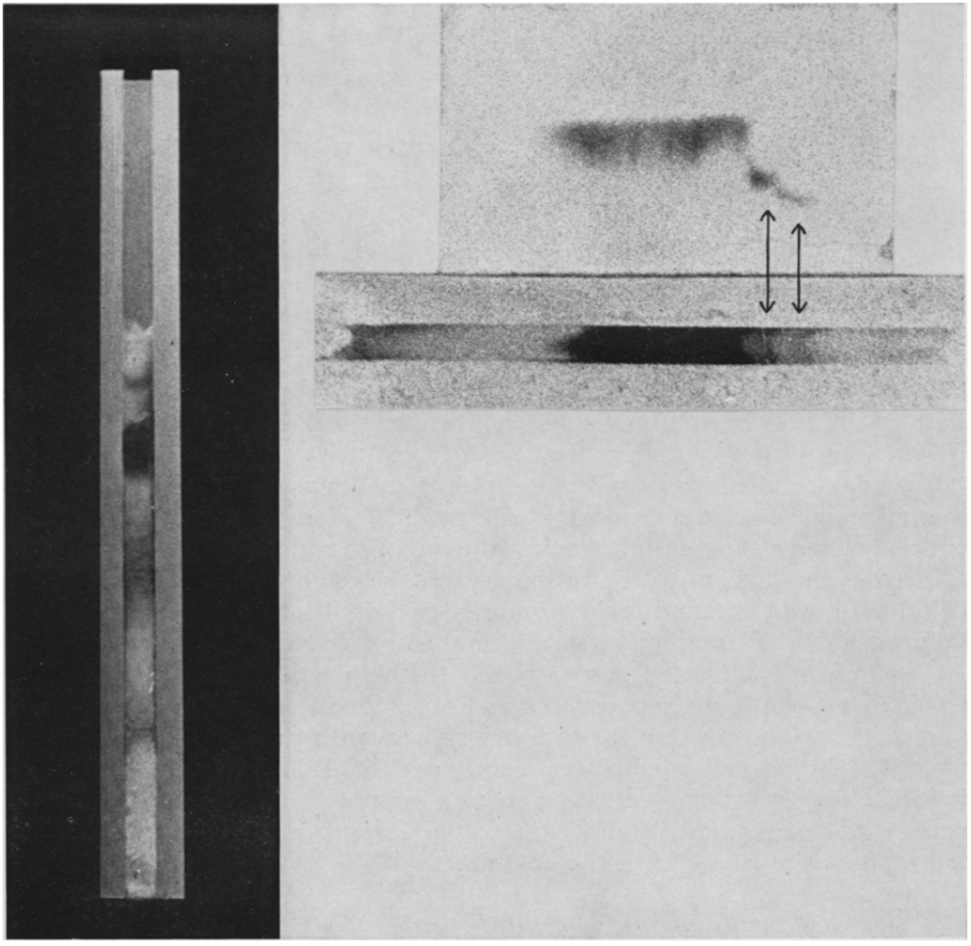

Fig. 2. Direct visualization of lipids on column gel. Soy phosphatides were applied to the column and it was developed with solvent 2. The column was then sprayed with molybdate reagent. The top and bottom bands were not identified but may be phosphatidic acid and cardiolipin, respectively. The second band is a mixture of PC and PI that are unresolved in this solvent system and the third band is PE.

Fig. 3. Comparison of two methods of band location. Partially purified egg PC, containing some LPC, SM, and PE, was applied to the column. Development was initiated with solvent 4 and completed with solvent 3. Bands were located by the tape method (tape is in lower part of photograph) and by the TLC method. The TLC plate was developed with solvent 3. Both plate and tape were sprayed with molybdate reagent.

migrates a few millimeters up the plate. To avoid washing off the thin strip of lipid at the bottom edge of such plates, they are developed in a chamber having a sheet of stiff felt or porous polypropylene on the bottom. The latter acts as a wick to conduct the developing solvent onto the plate but prevents the latter from being immersed in solvent to above the height of the strip of applied sample. The plates are inclined at about 45° to insure contact between the solvent and the gel surface. Positions and

identities of lipids on the TLC plates are determined in the usual way and from this information the column gel is sectioned to isolate the desired compound(s).

The glass cover is replaced on the column to prevent evaporation of solvents while the TLC plate is being developed (micro TLC plates are preferred to save time). The location of the bands on the column should be checked by the tape method if identification procedures take a long time.

TLC monitoring is advisable when two or more bands in the column may be so close that they appear as a single band on tape. An example is shown in Fig. 3. A large amount of egg lecithin and much smaller amounts of SM, PE, and LPC were applied to this column. The stained tape (bottom of figure) shows only slight indication that the trailing edge of the major band is not homogeneous. The TLC plate, however, shows very clearly that the PC band is directly abutted by bands of SM and LPC. With this information, the column gel can be cut to isolate pure PC in almost quantitative yield. In this instance, PE ran to the end of the column and is seen on the left of the tape.

Elution of separated lipids from adsorbent

The lipid is most conveniently removed from silica gel by placing the latter in a glass Buchner funnel having a "fine" fritted disc and eluting with methanol. The volume of methanol required is normally 5–10 times the volume of the silica gel containing the band of interest. Addition of sufficient methanol to diethyl ether–water (5:2) to give a one-phase system yields an effective eluting agent which, however, evaporates more slowly than methanol. Depending upon the lipid, some of the mixtures given in Table I are also effective.

Silica gel

Most of the silica gels available in the United States that are designed for either column or thin-layer chromatography were tested in columns using solvent mixtures similar to those that are useful for TLC. The most suitable were Merck silica gel H (Brinkmann, Westbury, N.Y., U.S.A.; for TLC), Unisil 200–325 mesh (Clarkson, Williamsport, Pa., U.S.A.) and Bio-Sil A, 200–325 mesh (Bio-Rad Labs., Richmond, Calif., U.S.A.). Other commercial products may also be satisfactory with developing solvents other than those used in this work or after various pretreatments.

Thin-layer gel gives slightly greater resolution than the gels intended for elution column chromatography, but this advantage is, for purposes of preparative isolation of major lipid classes, more than offset by the very long times required for development. It is noteworthy, however, that R_F values of TLC plates and R_F values of columns made with the same gel and developed with the same solvent are proportional and nearly identical. Fig. 4A presents a comparison of such R_F values; column values are all very close to 80% of the TLC values. Such a linear correspondence is only obtained if both the plate and the column gel are activated for at least 1 h at 120° prior to use.

R_F values on TLC plates and those of columns prepared with either Biosil A or Unisil do not exhibit a linear correspondence although they are similar. Fig. 4B shows such a comparison. For gel that is taken from a freshly opened jar, the correspondence is nearly linear for the lower 80% of the column. This relationship is lost

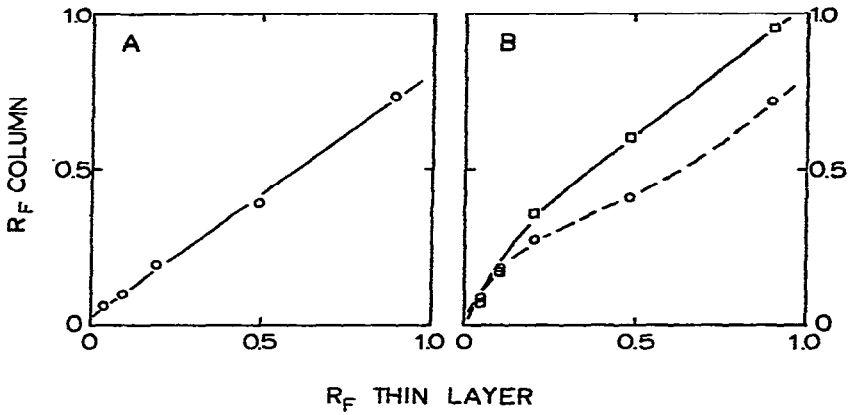

Fig. 4. Comparison of column R_F values with thin-layer R_F values. The lipid mixture was an acetone-precipitated chloroform–methanol extract of dried egg yolk. In order of increasing R_F, the components are LPC, SM, PC, PE, and NL. For the experiment shown in A, the column was packed with Merck silica gel H. Both the column gel and the plates were activated at 120° just prior to use. B is a comparison between thin layer and columns packed with Unisil taken either from a newly opened jar (□), or activated at 120° (○). Thin-layer plates were coated with silica gel H. R_F values correspond to the middle of thin-layer plate spots and column bands. Columns and plates were developed approximately the same distance with solvent 1.

when the gel is activated prior to use. Freshly activated gel of this type is somewhat inferior to that from a newly opened bottle, but we have been unable to completely duplicate the properties of the latter by addition of any amounts of water to the former. Effects of addition of water and methanol to the column gel are considered below. With regard to resolution, Biosil and Unisil are practically identical.

It is noteworthy that although the plate and column of Fig. 4B were of the same length, the time required for development of the column was less than 1/3 that required for the plate. Furthermore, columns develop at an approximately constant rate (about 3 cm/min) whereas TLC plates develop at a rate that diminishes with increasing plate length.

Column length

For the separation of the lipid mixtures considered here, we have used columns that vary from about 2 in. long by 1/8 in^2 to 12 in. long by 2 in.^{2}. Column length is dictated by the difficulty of a particular separation and desired purity. A test of the dependence of R_F on column length was made using the dye mixture mentioned above under *Column testing*, on columns varying from 15 to 60 cm in length. The R_F values of the major bands of this mixture were identical throughout this range. Since the solvent used to separate this mixture is one of the three-component solvents used for lipid separations, constancy of lipid R_F values may also be expected for different column lengths. Column lengths may therefore be increased to increase band separation.

The constancy of R_F values also means that, following development, separated components can be eluted by continuing addition of the same solvent. Knowledge of the void volume of the column and of the R_F values of the desired components (obtained from a small analytical column or, approximately, from thin layer) permits simple calculation of the elution volume, *i.e.*, it is the column volume divided by the

R_F. Such a procedure, a combination of dry column and elution chromatography, could be carried out in normal, tubular glass columns.

If only a slowly moving component is desired, one can economize on adsorbent. For example, if one wishes to isolate a phospholipid from reaction mixtures containing fatty acid, the lower half of the column can be packed with used silica gel. The upper half, where the PC will be found, is packed with the fresh adsorbent. The advantage of packing the lower portion rather than simply washing solvent through a short column is that the position of the desired component can be easily approximated from its R_F when the position of the solvent front is known.

Solvent mixtures

A large variety of solvent mixtures have been tested for their effectiveness in separating components of several different lipid mixtures. As is expected from the general correspondence of R_F values on TLC plates and columns, solvent mixtures that have been most useful in TLC are also most effective in columns, in particular, mixtures of chloroform and methanol plus small amounts of water, acid or base. Solvent compositions are given in Table I.

Components of egg yolk are separated with solvents containing 60 parts chloroform, 20 to 40 parts methanol and 3 to 9 parts of either water or 7% ammonia, although ammonia produces better separation of SM and PC. The major effect of increasing the proportion of the aqueous component within this range is to increase PC–PE separation and decrease the PE–neutral lipid (NL) separation. Soybean phosphatides are more difficult to resolve because of the presence of large amounts of PI. A similar situation occurs with brain phospholipids where PS creates problems (cerebrosides usually run ahead of PE and do not pose any difficulty). PS and PI may be separated from each other and from both PC and PE, but we have not found a method to effect a good separation of all four simultaneously. If all components from soy or brain phosphatides are required, two columns are necessary.

The kinds of separations that are obtained from several useful solvent mixtures are shown in Fig. 5. The profiles shown represent an estimate of band shape made from the intensity of staining. The top of the column is represented at the top of the figure.

Separation of the three phospholipids of egg yolk with solvent 1 is shown in A. This solvent mixture does not separate all of the phospholipids of soybean (B), since PI and PC run together in this system. In addition to PI, two other phospholipids not present in egg yolk are seen in the soy bean mixture. One runs behind PI and the other behind NL. Phosphatidic acid and cardiolipin are reported to be present in soy beans and may account for these bands[8]. As shown in C increasing the proportion of polar solvents (methanol and water) increases all R_F values and increases the separation between SM and PC. The strongly basic and polar solvent 6 separates PI from PC (as well as from several other lipids), as is shown in the record of the chromatogram labelled D. The cost of effecting this separation, however, is the loss of separation between LPC, PC, and PE. The alcohol-insoluble fraction of soybean lipids is enriched in acidic phospholipids, as can be seen in E, where the PI is the most prominent band. This band contains a small amount of PS at its leading edge, which can be removed by rechromatography in the acidic solvent 7 (F). Solvent 6 yields a large separation between PE and PC (E).

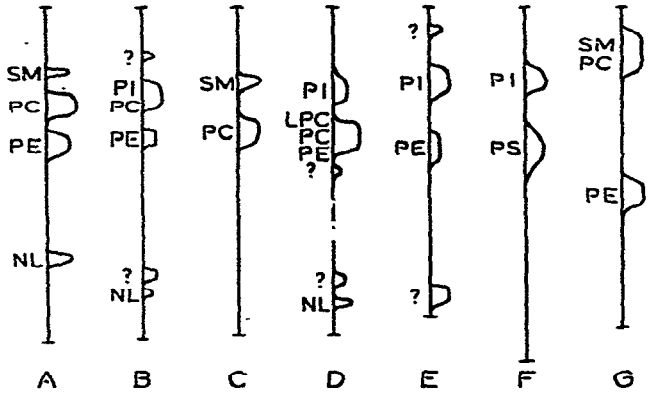

Fig. 5. Comparison of development solvents for several lipid mixtures. The top of the column (origin) is at the top of the figure. Profiles are an approximation of band staining intensity (molybdate spray). The silica gel was Bio-Sil A, 200–325 mesh for all but A, where it was Unisil. Column loadings were 5–20 mg lipid per gram silica gel. A, crude egg yolk phosphatides separated with solvent 1; B, soybean phosphatides separated with solvent 1; C, mixture of SM and PC separated with solvent 2; D, soybean phosphatides separated with solvent 6; E, alcohol-insoluble portion of soybean phosphatides separated with solvent 6; F, mixture of PI and PS separated with solvent 7; G, mixture of SM, PC, and PE separated with solvent 4.

The solvents used for the chromatograms of Fig. 5 may be used alone, in combination with one another, or in combination with a preliminary non-chromatographic separation, to effect the isolation of any of the lipids shown. The choice of procedures depends upon the source of lipid, desired purity, and the number of lipids desired. For the isolation of SM, PC, and PE from egg yolk the solvents 1 or 2 are useful. If only PE or either SM or PC is desired, solvents 2 or 7 may be used with high loadings. For the isolation of PI, the alcohol-insoluble fraction of soy bean may be separated with solvent 6. An alternative method is to isolate the PI–PC band from a column such as B of Fig. 5, dry the gel (with adsorbed lipid) and place it on a second column that is developed with solvents 6 or 7. In the former case, the column is sectioned so that the leading edge, containing PS, is not included for extraction. PS may be obtained in a similar fashion except that the alcohol-insoluble lipid fraction of brain is used. This is prepared in the same way as the corresponding fraction from soy (Chemicals) except that the source is a chloroform–methanol extract of brain and the centrifugation is done in the cold. Although not illustrated, an effective solvent mixture for removing LPC from other phosphatides or for isolating LPC from such a mixture is solvent 5. In this solvent, LPC runs with an R_F of about 0.3 whereas all of the other lipids considered here go nearly to the solvent front.

Gel treatment

Since dry column chromatography[6] of some compounds improves with addition of water to the silica gel, we tested the effects of gel modification on phospholipid chromatography. Fig. 6 presents column band profiles for separations of egg phospholipids on Bio-Sil A equilibrated (for a day or longer) with various proportions of water. The effect of adding up to 5% (v/w) water is to increase the separation of PC and PE.

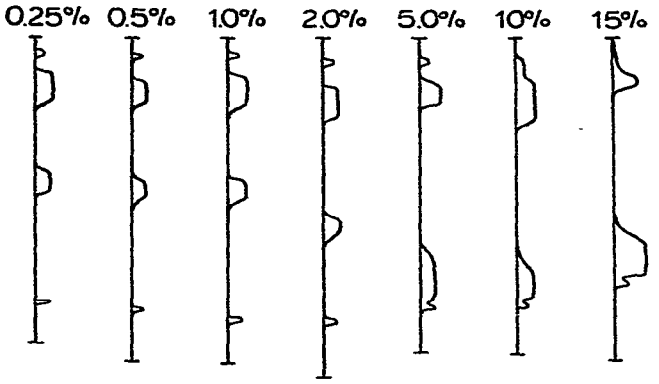

Fig. 6. Effect of equilibrating gel with water. Freshly activated Bio-Sil A was equilibrated with water in amounts shown in the figure. Egg yolk phosphatides were applied at 10 mg per gram silica gel and the columns developed with solvent 1. From top to bottom the bands (if separated) represent SM, PC, PE, and NL.

This may be useful, but the same result is more easily obtained by increasing the amount of water in the solvent mixture. (Upon increasing the proportion of either water or dilute ammonia from 2 parts to 4 parts per 90 parts chloroform–methanol (2:1), the R_F of PE doubles while that of PC does not change.) At water contents much higher than 5%, the separation deteriorates although, curiously, it is better at very high than at intermediate contents.

Fig. 7 shows the effects of adding increasing amounts of methanol to the silica gel. Relative to freshly activated gel, somewhat beneficial effects are seen with small proportions of methanol, but again, much the same effect can be obtained by increasing the proportion of the methanol in the developing solvent.

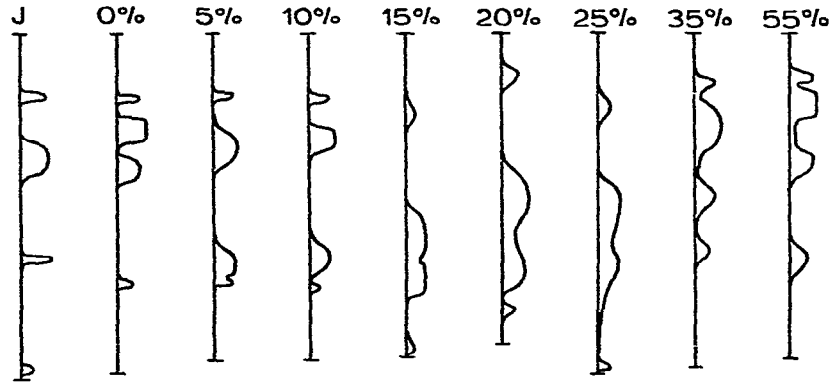

Fig. 7. Effect of equilibrating gel with methanol. Procedures were as described in the legend of Fig. 6 except that the silica gel was Unisil and it was equilibrated with methanol in the amounts shown. J represents silica gel taken from a newly-opened jar.

DISCUSSION AND CONCLUSION

The basic technique of "dry adsorbent" chromatography is, as the remarkable success of TLC has shown, a very powerful separation method. We have circumvented the original difficulties of adapting this method to columns by some simple procedural modifications without appreciably diminishing its resolution. With such modifications, dry column chromatography acquires an advantage over elution chromatography in that it is very much faster, highly reproducible, requires smaller amounts of solvents, and demands neither attention nor monitoring until the column is developed. Since the separations obtained are very similar to those of TLC, advantage can be taken of the extensive literature of the latter technique for establishing conditions for preparative separations of many different substances. In addition, multiple small scale tests can be performed much more quickly with "openable" dry columns than on elution columns. Solvent volumes are fixed relative to adsorbent volumes so that scaling up is particularly straightforward. In addition, modification of the procedures presented above, which could be useful for specific separations, are easily made. For example, the lower portion of a column could be filled with a different adsorbent or one treated in a different way (*e.g.*, equilibrated with acid, base or water) than the upper portion. This would permit a different group of selectivity parameters to operate on substances with large R_F values than on those with small R_F values. Alternatively, a column could be developed part way with one solvent and then completed with another. If the first solvent were relatively non-polar, and the second polar, both neutral and polar lipids could be separated on the same column. Similarly, one solvent could be acidic and the other basic, to permit removal of both acidic and basic impurities from a neutral compound. The ease with which a separation is evaluated makes the optimization of several-step procedures relatively simple and rapid.

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