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Note

Activation of thin adsorbent layers by an anhydrous organic solvent

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Earlier, by using adsorption thin-layer chromatography (TLC), we have shown that the qualitative composition of polar lipids from soy bean seeds is highly complex[1]. Therefore, to determine it completely an highly efficient and selective TLC procedure is necessary.

To achieve maximum efficiency and selectivity while solving this separation problem, the activity of the adsorbent employed must be optimal[2,3]. However, when the relative humidity (r.h.) of ambient air is above a certain level, almost all of the conventional TLC techniques do not ensure the attainment of this goal (see below). Therefore, we have adapted a procedure for the activation of a thin layer[4]. According to this procedure, after applying lipids on the plate, physically absorbed water is removed from its surface by treating the adsorbent with anhydrous diethyl ether; until now, to the best of our knowledge, organic solvents with various water contents have been used for the conditioning of silica gel activity only in column chromatography[5,6].

EXPERIMENTAL

Diethyl ether (reagent grade) was dried and distilled[7]. Methanol (reagent grade) and chloroform (pharmaceutical grade) were purified only by distillation. Aqueous solutions of NH_3 (26% w/v, commercial grade) and phosphomolybdic acid (analytical grade) were used without further purification. Silufol TLC plates (Kavalier, Votice, Czechoslovakia) were predeveloped with chloroform–methanol (3:1, v/v). The r.h. was determined using a BM-2 hygrometer.

The mixture of seed lipids (20 μg)[1] was applied on the plate not activated by heating. The various TLC conditions are shown in Table I. Those used for the control experiments (variants A and B) were the same as described previously (continuous flow TLC in the chamber described by Van den Eijnden[8]), whereas a specially designed TLC apparatus was used in variant C (Fig. 1). After assembling the apparatus (fumehood!), 3 ml of diethyl ether were introduced into chamber 1 through the tube 8. The ether together with water dissolved therein migrated through the adsorbent layer and a sheet of paper, then passed out of chamber 1 and evaporated. There was no desorption of polar lipids from the starting point on the plate. Just after the disappearance of ether from the thin layer surface and chamber bottom, *i.e.*, after about 20

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TABLE I

CONDITIONS FOR TLC SEPARATION OF POLAR LIPIDS[a]

Variant	*r.h. (%)*	*Type of TLC chamber*	*Chloroform–methanol–ammonia mobile phase (v/v/v)*
A	40	Continuous flow	26:7:0.7
B	70	Continuous flow	32:7:0.7
C	70	See Fig. 1	32:7:0.7

[a] See text for A, B, C and r.h.

min, 20 ml of mobile phase were introduced into the chamber in the same way (Table I) and the plate was developed for 2 h. Vaskovsky and Dragendorff colour reagents as well as ninhydrin and phosphomolybdic acid were applied to visualize and identify the lipids[9]. They were also identified by using TLC standards and by comparing found R_F values with those obtained by other workers[1].

RESULTS AND DISCUSSION

The results of TLC of the lipid mixture are presented in Fig. 2. At r.h. = 40% (A), sufficiently high selectivity was achieved, but at r.h. = 70% (B) there was no separation of phosphatidylcholines from phosphatidylethanolamines although in the latter case the mobile phase was less polar. Finally, the variant C (r.h. = 70%), due to the activation of the layer by the removal of water, was quite similar to variant A as regards the selectivity of lipid fractionation. The same results were obtained over the range of r.h. 50–90%.

Thus, the increase in r.h. substantially diminished the selectivity of separation although in other cases the opposite was observed[3]. Apparently, in our work this was due to the decrease in the activity of the adsorbent layer, which in turn resulted from

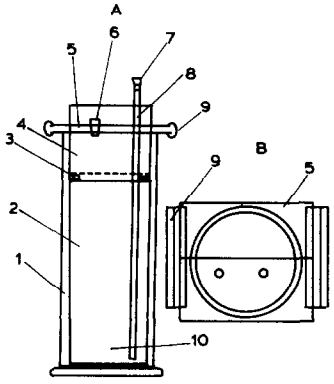

Fig. 1. Apparatus for adsorbent activation and TLC analysis. (A) Front view, (B) top view. 1 = Chamber, 180 mm × 60 mm; 2 = TLC plate, 150 mm × 54 mm; 3 = metal clamps; 4 = filter-paper; 5 = chamber cover consisting of two glass plates ground to the upper edge of the chamber and to each other (all ground surfaces are lubricated with glycerol); 6 and 7 = rubber stoppers; 8 = PTFE tube; 9 = spring clamps; 10 = starting zone of TLC plate.

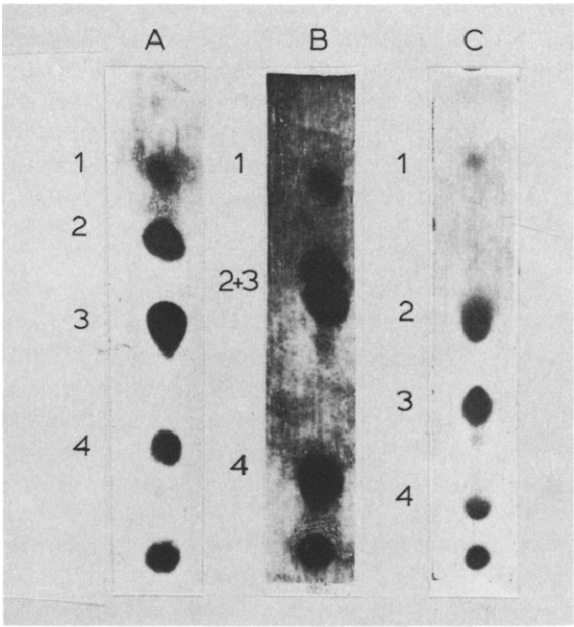

Fig. 2. Chromatograms of a mixture of polar lipids. 1 = N-acylphosphatidylethanolamine; 2 = phosphatidylethanolamine; 3 = phosphatidylcholine; 4 = phosphatidylinositol. See Table I for TLC separation conditions for the variants A, B and C.

the absorption of ambient moisture by this layer[2]. The extent of the latter process is known to rise sharply at r.h $\geqslant$55%: thus, at r.h. = 45.6 and 74.5%, moisture makes up 13.3 and 38.8% respectively of the layer weight[10], and at r.h. $\geqslant$60% an equilibrium between the water content of the adsorbent and of the surrounding atmosphere was reached within the first few minutes of their contact[2,10]. Thus, the extent of moisture absorption by the adsorbent layer depends on the r.h. of the ambient air. At the same time, it is inversely proportional to the initial humidity of the layer[3].

In our experiments another factor responsible for the decrease in selectivity with increasing r.h. is the extent of mobile phase demixing under these conditions[11,12]. This phenomenon consists in the migration of one or more secondary mobile phase fronts on the plate in addition to the major one. In our experiments it brought about a significant distortion of the normal shape of chromatographic zones and a drastic change in their usual mobility which resulted in a partial or even complete loss of separation selectivity of polar lipids. Most investigators also consider demixing to be harmful for TLC separation[2,3,12].

Demixing is usually observed when the mobile phase consists of solvents which differ considerably in their polarities, the more hydrophobic and more polar components being concentrated above and below the secondary front, respectively. In our experiments, demixing appeared only at higher r.h.; however, under other separation conditions it was observed even at low ambient humidity[3]. Therefore, the possibility of its appearance should be taken into account in each TLC separation, including cases where the migration of the secondary front on the plate is not obvious[11].

Within a certain r.h. range, demixing can be prevented by preliminary saturation of the layer with the mobile phase vapour[3,12]. This, however, considerably

increases the duration of the analysis, and therefore a special technique has been developed making it possible to perform the TLC analysis after the appearance of demixing. To this end, continuous flow TLC in a sandwich chamber is used[3,13]; the sample is applied through the special holes in the cover below the secondary solvent front[12]. TLC after the demixing had appeared is again subject to considerable difficulties. First, the chamber required is not produced commercially. Moreover, the attainment of equilibrium between the mobile and stationary phases requires much time. Finally, because the sample is applied directly into the continuous flow of solvent the chromatographic zones become very diffuse[6].

Demixing was observed in almost all our experiments at high r.h. (variant B), but treatment of the layer by diethyl ether completely prevented it.

To assess the activation technique proposed here one must compare it with other similar methods. The major approach employed to this end in TLC comprises heating the plates at 110–115°C for 3–30 min[3,14–16]. In order to prevent subsequent moisture absorption from ambient air, the plate thus treated is immediately covered with a dry glass sheet; the sample to be analyzed can be applied only after this point[2,16]. However, when transferring the plate to the TLC chamber and in the course of subsequent separation (during 30 min or more) the thin layer is permanently in contact with the environment which has practically the same humidity as the laboratory air. Therefore, the layer loses its adsorptive capacity very rapidly[3].

To activate the layer without using an high temperature, the plate with the applied sample was placed in a TLC chamber which, in addition to the mobile phase, contained a vessel with a moisture-absorbing medium (sulphuric acid solution). The plate was kept in the chamber for a long time, up to 12 h, and then brought into contact with the mobile phase while maintaining the chamber hermetically sealed[10,17]. A significant drawback of this approach is the considerable duration of the experiment, which involves also a long-term contact of the sample with atmospheric oxygen. Moreover, the layer absorbs sulphuric acid vapours, which may adversely affect the results of TLC analysis[3]. On the basis of this principle, the twin trough chamber and the Vario-K S chamber (Camag, Birmensdorf, Switzerland) were constructed[3]; both of these chambers share with the original technique the drawbacks mentioned above.

It is possible that high activity of the layer will also be preserved by performing TLC analysis in a closed desk-top cabinet (similar to the "glove-in chamber" used in tracer experiments[6,18]) with a constantly maintained low humidity. However, on a practical scale such an approach would be subject to considerable technical difficulties[19].

As is seen from the preceding two paragraphs, the techniques of adsorbent activation employed presently in TLC are not very efficient. Therefore, we turned our attention to the corresponding methods used in column chromatography. These methods are also usually based on preliminary heating[20]. At the same time, adsorbent dehydration may also be achieved at room temperatures. Thus, the percolation of 2,2-dimethoxypropane (DMP) through a column in the presence of acetic acid or some other acidic catalyst brings about complete disappearance of adsorbed water: $H_2O + CH_3C(OCH_3)_2CH_3 \rightarrow CH_3COCH_3 + 2CH_3OH$[21]. However, DMP cannot be used in planar chromatography including TLC since under these conditions the whole active surface of the adsorbent saturated with DMP would be in contact with

moist air. Besides, the traces of DMP which remained on the plate may promote decomposition of the sample, *e.g.*, the conversion of acyl lipids into fatty acid methyl esters[22].

According to another approach (that used in our work) a non-polar organic solvent with limited hydrophilicity is percolated through a column[4]. In TLC experiments we used diethyl ether as such a solvent because it does not elute polar lipids from the silica gel surface. Ether can dissolve up to 1.2% (w/w) of water[23] and is characterized by high volatility and relatively low chemical activity. Finally, it is readily available.

CONCLUSION

In conclusion it must be stressed that in many geographical regions the laboratory r.h. remains high for up to 5 months or more every year. Consequently, during this prolonged period of time the execution of TLC experiments will be more or less complicated. The technique proposed here may contribute to overcoming these difficulties, because it makes it possible to prepare active TLC plates at any r.h. Such plates are suitable for the separation of both polar lipids and other organic compounds of the same or lower lipophilicity. It is true that this technique cannot be used to analyse ether-soluble compounds such as neutral lipids. However, according to our experience, the separation of these compounds is less dependent on air humidity than the separation of more hydrophilic substances because mixtures of solvents sharply differing in their polarities (see above) are not usually employed for this purpose.

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