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A simplified technique for thin-layer microchromatography of lipids

In the course of a comparative study of polar lipids of marine organisms[1,2], we felt the need of chromatographic techniques that would allow us to make analyses in shorter times, using lesser quantities of material. However, PEIFER's[3] and other micromethods recently described in the literature[4,5] proved ineffective.

BELENKY *et al.* showed the theoretical[6] and practical[7] possibilities of effective separation of mixtures on microplates covered with adsorbents whose particle sizes were smaller than usual.

This work describes the use of BELENKY's technique for analysing complex lipid mixtures.

Materials and methods

Extraction of lipids and preparation of standards for microchromatography. Lipids were extracted in accordance with the methods of FOLCH *et al.*[8]. Individual lipids were isolated from hen egg, rat brain and rat heart by means of column chromatography on alumina and silica gel or two-dimensional chromatography on plates of 20 × 20 cm, the layer thickness being 0.5 mm.

Preparation of plates. Plates (6 × 6 cm) were thoroughly washed with a detergent, rinsed with water and pretreated for 10 min in a heated alkaline solution (50 g/l NaOH). After that, the plates were again washed, first with water, then with distilled water and finally dried in a cabinet drier.

A silica gel with the required size of particles was prepared from Silica Gel KCK by grinding in a ball mill and sedimenting water suspensions in glass jars[7]. The suspensions should be thoroughly rolled to break down the particle aggregates. To this end, a fraction sedimenting in 40–120 min was used. The moist silica gel was then collected and placed in a tightly closed flask. The absorbent content was determined by weighing the aliquot dried at 120°. The silica gel slurry was prepared to contain 210 mg/ml of the adsorbent.

Prior to being spread on to the plates, the slurry was thoroughly shaken; then 10–15% of gypsum (relative to silica gel) was added to a homogeneous weighed portion with subsequent mixing in a magnetic agitator. The weighed portion aliquot (1 ml) was then thoroughly smeared over the plates (60 × 60 mm) and the plates were dried in air for 20 min. They were activated at 110° for 5 min immediately before use.

From 5 to 10 μl of the lipid solution, containing 25–50 μg of the complex mixture or 1–2 μg of the individual compound, were spotted on the starting line in the form of dots or bands situated at a distance of 7 mm from the lower edge of the plate. It is possible to put 5 or 6 dots on one plate. For two-dimensional chromatography, the lipid solution was placed in the corner of the plate at a distance of 10 mm from both edges. The solvent run was 50 mm for both one- and two-dimensional chromatography. Weighing bottles (diameter 90 mm, height 50 mm), or any other vessels of suitable size with glass lids, were used as chromatographic chambers. After developing the plate in one direction, it was dried in an air flow for 5 min and then placed in a second chamber with an appropriate solvent.

Ordinary solvent systems were used for one- and two-dimensional chromatography[9–11]. The quantity of solvent required to develop a plate in one direction was 3–5 ml.

Phospholipids were detected with a newly-modified molybdate reagent[12]. Glycolipids were detected with a modified preparation of Honneger's[2] orcin reagent. Non-specific detection was achieved with the ZIMINSKY-BOROVSKY reagent[13] and with 10% sulphuric acid in methanol subjected to subsequent heating at 180° for 5 min.

Results and discussion

Various methods exist for preparing adsorbents with definite particle sizes involving the use of special devices[14]. Inasmuch as we needed a small quantity of silica gel, we used fractionation in beakers. Although the above chromatography was performed on a silica gel with various particle sizes, we chose 2–7 μ in accord with BELENKY[6], who suggested this range from theoretical considerations.

Fractionated silica gel was kept under a water layer, since on drying it agglutinated into cakes, thereby impeding the formation of an even adsorbent layer.

No special devices are required to prepare the plates, since, when spreading the sorbent slurry by hand, one can obtain a total of 40–60 plates an hour. The layer dries in 20 min, and 5 min is sufficient to activate it.

Spotting the samples presents no difficulty. However, it is advisable to spot the lipid solutions in chloroform.

Weighing bottles were used as chromatography chambers. However, development may be carried out in any other vessel of suitable size with a tight lid. Chromatography was run in usual systems. Spraying was carried out with conventional high-efficiency sprayers. After having detected the lipids, the positions of the spots on

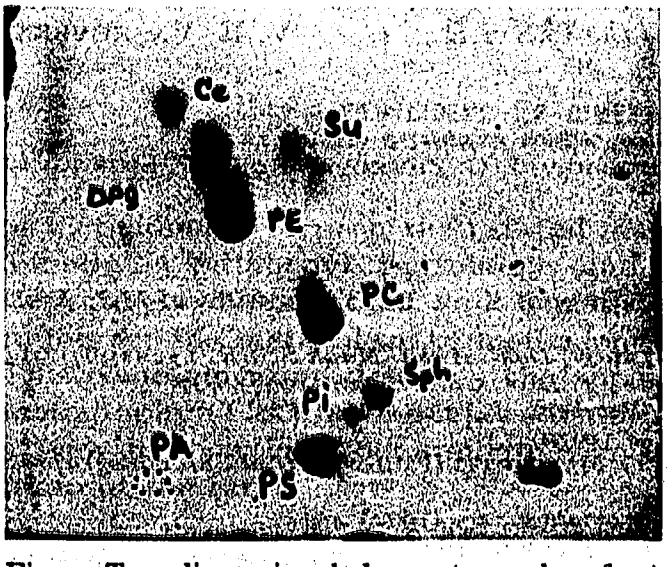

Fig. 1. Two-dimensional chromatography of rat brain lipids. Mobile phase: first direction, chloroform–methanol–28% aqueous ammonia (65:35:5); second direction, chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5). Abbreviations: PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PI = phosphatidyl inositol; PS = phosphatidyl serine; PA = phosphatidic acid; DPG = cardiolipin; Sph = sphingomyelin; Ce = cerebrosides; Su = sulfatides. Spots were located by spraying with 10% H_2SO_4 in methanol and charring.

the plates repeat the pattern obtained on usual chromatograms in similar systems (Fig. 1).

The proposed chromatography technique promotes successful work with lipid quantities of from 0.1 μg for individual substances to 25–50 μg for complex lipid mixtures. It also contributes to effective separation of the principal phospholipids and glycolipids by means of one-dimensional chromatography. Furthermore, the proposed technique makes it possible to quickly obtain two-dimensional chromatograms. On the other hand, the use of PEIFER's[3] procedure does not ensure satisfactory results with complex mixtures. For this reason, in such cases, chromatography on plates of 20 × 20 cm is usual. However, this entails the use of large amounts of materials, time and solvents. Furthermore, we realize that MARIAN's method[4] is more labour-consuming, while EDGAR's procedure[5] involves microscopic observations of the separation results and does not permit one to obtain two-dimensional chromatograms. PIEL[15] described the separation of dyes on a silica gel with particles of less than 1 μ, but made no further advances. Recently, RIBI *et al.* published a new work[16] involving the use of a micro-silica gel, but this procedure proved to be too cumbersome for everyday runs.

KLEINIG AND LEMPERT[17] have recently published a paper on phospholipid analysis on a micro scale, in which they suggested a microtechnique for qualitatively and quantitatively determining lipids using a conventional silica gel produced by Merck (Darmstadt, G.F.R.).

However, the use of still finer silica gel in accord with BELENKY's procedure[6] permits, if need be, reduction of the plate sizes to 3 × 3 cm.

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