# notes on methodology

## Detection and isolation of minor lipid constituents

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SUMMARY The use of thick layers of adsorbent for the concentration and subsequent isolation of neutral lipid constituents is described. Lipids not perceptible by conventional methods are demonstrated in concentrates of bovine heart extracts and identified as fatty aldehydes, *O*-alk-1-enyl diglycerides (neutral plasmalogens), and *O*-alkyl diglycerides (alkoxydiglycerides).

KEY WORDS detection · isolation · thick adsorbent layers · preparative TLC · minor lipid classes · free aldehydes · neutral plasmalogens · *O*-alk-1-enyl diglycerides · *O*-alkyl diglycerides · bovine heart

CHROMATOGRAPHY ON adsorbent layers is used widely for the fractionation of natural mixtures and the preparative isolation of their constituents (1–6). However, lipid classes that occur only in trace amounts are difficult to detect and isolate, especially if these compounds resemble a major component closely in structure and physical properties. In the present communication, we describe a simple method for the preparation of adsorbent layers of high capacity and their use for the concentration and isolation of classes of minor neutral lipids. The bulk of the major constituents is removed by chromatography on such layers (procedure A). The concentrate, in which the minor components are present in a ratio more favorable for efficient fractionation, is then further resolved by preparative TLC (procedure B). The efficacy of this technique is demonstrated by the isolation of minor lipid classes from an extract of bovine heart from which most of the phospholipids had been removed by selective adsorption onto silicic acid (7).

*A. Concentration of Minor Lipid Classes.* An adhesive label tape, 1 inch wide (e.g., Adhere Corporation, Cincinnati, Ohio), is applied to the edges of a glass plate (20 × 20 cm) to form a corral about 1 cm high. The plate is coated by pouring a slurry of 30 g of Silica Gel H (E. Merck A.G., Darmstadt, Germany) in 60 ml of water onto it. The adsorbent slurry is distributed evenly by tilting the plate in various directions and tapping it gently. The plate is then allowed to stand on a horizontal support for several hours until the layer of silica gel has lost its transparent appearance. After the tape has been removed, the adsorbent "cake" is activated in an oven for 3 hr at 120°C. The dry activated layer is about 2 mm thick.

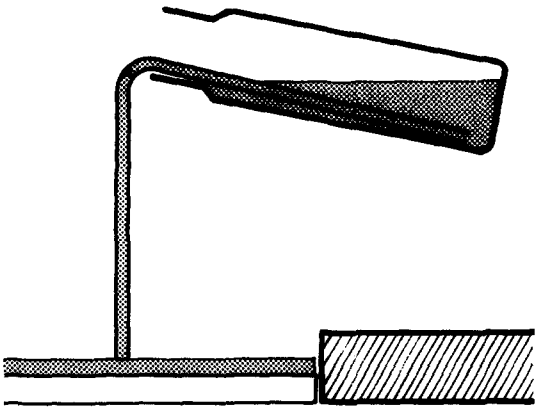

FIG. 1 Device for applying sample solution to the adsorbent layer.

The sample, in an amount not exceeding 750 mg of its major lipid class, is dissolved in hexane to give a 10% solution and applied to the layer in an even streak parallel to the lower edge of the plate. A sample applicator similar to that described by Monteiro (8) was found useful. It consists of a small vial and a glass capillary as illustrated in Fig. 1.

The vial is mounted in such a position that the inserted capillary extends to the surface of the adsorbent layer, 2.5 cm from the lower edge of the plate. The sample solution is transferred to the vial by pipette or syringe. It ascends by capillary action and flows evenly onto the adsorbent layer as the plate is moved along a guiding edge. When a capillary with 1 mm I.D. is used, 2 ml of sample solution can be spread in about 20 sec. Multiple development with the same solvent in unlined tanks is preferred for all preparative fractionations. Lipid classes less polar than triglycerides are fractionated by developing the plate twice with the solvent system hexane–diethyl ether 95:5. After the first run, the solvent is evaporated from the plate under a stream of nitrogen. The major fractions are, in most cases, visible without the use of an indicator, whereas the positions of the minor fractions are deduced relative to the major ones. For example, neutral plasmalogens and alkyl diglycerides are located in and above the leading edge of the triglyceride fraction. Hence, all lipid classes less polar than triglycerides are obtained by scraping off the adsorbent layer between the solvent front and a line about 2 mm below the leading edge of the triglyceride fraction. The lipids are eluted from the adsorbent with diethyl ether on a jacketed, sintered glass funnel at room temperature.

Abbreviation: TLC, thin-layer chromatography.

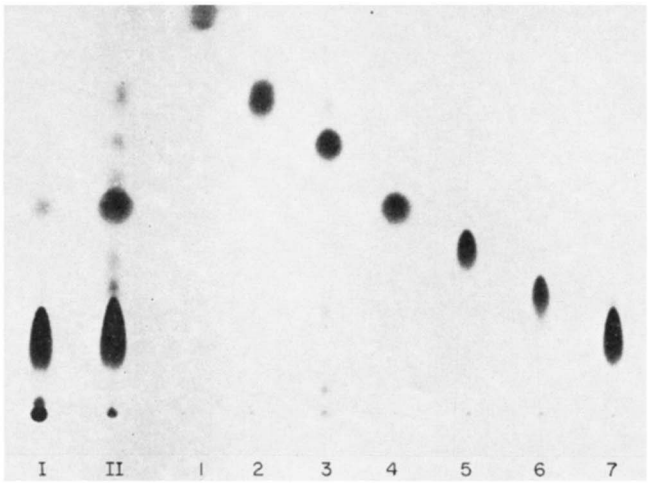


FIG. 2 Thin-layer (analytical) chromatogram of neutral lipids from stored extracts of bovine heart. Chromatography took place on Silica Gel G, 0.25 mm, in hexane–diethyl ether, developed twice. *I*, stored extract; *II*, concentrate of stored extract obtained by procedure A. *1–7* are analytical runs of pure lipid fractions obtained by procedure B. *1*, unidentified least polar fraction; *2*, steryl esters; *3*, unidentified; *4*, aldehydes; *5*, neutral plasmalogens; *6*, alkyl diglycerides; *7*, triglycerides. Indicator: charring with chromic–sulfuric acid mixture.

*B. Isolation of Minor Lipid Classes.* The amounts that have to be fractionated are reduced drastically by the removal of much of the major lipid class. The minor lipid fractions are then isolated from the concentrate in the conventional manner by repeated chromatography on layers of Silica Gel H, 0.5 mm thick. 75 mg of the concentrated sample is applied to a 20 × 20 cm plate. Chromatography and elution are carried out as described above (A).

*Results.* The method described here was applied to extracts of bovine heart that had been stored at −40°C for about a year. This extract was chosen because it contained several minor constituents. Several fractions that could not be detected in the total lipid extract became apparent (Fig. 2) in the concentrate. The spot due to aldehyde, which is much increased in the stored extract, is prominent. All the fractions were isolated by preparative TLC and are shown as pure or almost pure compound classes in Fig. 2. Fractions *1* and *3* were not identified. The others—steryl esters (fraction *2*), free aldehydes (fraction *4*), neutral plasmalogens (fraction *5*), alkyl diglycerides (fraction *6*), and triglycerides (fraction *7*)—were characterized by their IR spectra and chemical reactions and by gas–liquid chromatography of derivatives (9, 10). The small fraction in the chromatogram of the concentrate that migrated above the aldehydes was identified as methyl esters. Free long-chain aldehydes have been found in bovine heart previously (11, 12), but the presence of neutral plasmalogens and alkyl diglycerides could be demonstrated only in an indirect way (11).

*Discussion.* In column chromatography on silicic acid, the minor constituents of a lipid extract are not satisfactorily resolved from the major constituents having similar physical properties, and even repeated chromatography of successively enriched fractions fails to produce them as pure lipid classes. Chromatography on thin layers of the same adsorbent effects a more satisfactory separation of closely related substances, but the amounts that can be fractionated efficiently are too small to permit the isolation of minor lipid classes in quantities sufficient for their identification and further analysis. The method of removing the bulk of the major constituents of a lipid mixture before isolating the minor lipid classes permits the preparation of the latter compounds in substantial amounts.

In each fraction, the saturated constituents are enriched in the leading portion, whereas the highly unsaturated compounds appear in the trailing portion. Therefore, if the long-chain moieties of these lipids are to be analyzed, every effort must be made to avoid preferential losses. Each fraction must not only be pure as a class but must also contain all constituents of that class in true proportion. To insure this, all fractions of the same compound class have to be combined, and controls by TLC, after each preparative fractionation, are essential.

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