# notes on methodology

## Separation of neutral lipids of shark liver by "dry-column" chromatography

ADRIA C. CASEY

*New England Institute for Medical Research, Ridgefield, Connecticut 06877*

SUMMARY "Dry-column" chromatography in mixed solvents has been successfully used to separate gram quantities of neutral lipids from shark liver oil into simpler fractions.

SUPPLEMENTARY KEY WORDS mixed solvents · thin-layer chromatography

THIN-LAYER chromatography (TLC) has been used by many investigators to separate neutral lipids into classes. However, even with preparative techniques the amount of material that can be separated by TLC is limited. A column chromatographic technique called "*dry-column*" chromatography has been reported recently (1, 2) to afford separations on a preparative scale that are comparable to those obtained by TLC. Since the $R_f$ values obtained are similar to those measured by TLC, this technique allows the separation conditions to be determined on TLC and then applied directly to the preparative system.

The "dry-column" method (2) involves loading a column (either glass, quartz, or nylon) with dry adsorbent, depositing the mixture to be separated on the top of the adsorbent, and allowing added solvent to move down the column. When the solvent front reaches the bottom of the column, chromatography is stopped. The method used for identifying the position of the bands depends on the nature of the mixture to be separated. If the compounds are colored, visual observations will give the position of the bands. If the mixture is colorless or slightly colored, trial and error or knowledge of the $R_f$ values of the expected bands is necessary. The column is extruded or cut at the desired places and the materials are extracted with appropriate solvents.

In connection with other studies being carried out in these laboratories, it was desirable to prepare large amounts of certain fractions of the liver lipids of Lemon shark (*Negaprion brevirostris*).

This communication describes the separation of neutral lipids from shark liver extract into simpler fractions by means of "dry-column" chromatography. A mixed solvent system was employed for this separation. An essential feature of using mixed solvents is the equilibration of the adsorbent with the solvent mixture prior to use.

*Solvents.* All solvents were freshly distilled and peroxide-free.

*Adsorbent.* 1000 g of silica gel No. 3405 (J. T. Baker Chemical Co.) was washed with 3 liters of hexane and 3 liters of methanol to remove impurities from the adsorbent, and then dried in a vacuum oven at 160°C for 8 hr in order to produce an adsorbent with an activity of II or III on the Brockmann scale. The silica gel activity was found to be extremely important and was measured for each batch of adsorbent by the capillary method described by Loev and Goodman (2). Activity of II or III was necessary in order to obtain $R_f$ values comparable to those obtained on silica gel TLC plates. The same range of activity is obtained on silica gel plates by heating for 1 hr at 110°C.

*Column.* Nylon tubing, 1 inch diameter flat (M and Q Plastics Co., Freehold, N. J.).

*Standards.* "TLC No. 1" standard lipid mixture, obtained from The Hormel Institute, Austin, Minn., was used (containing cholesterol, cholesteryl oleate, triolein, oleic acid, and methyl oleate). Batyl alcohol, a gift from Western Chemical Industries Ltd., Vancouver, Canada, was purified before use. Batyl distearate was prepared from batyl alcohol and stearic acid.

*Lipid Sample.* Neutral lipids from shark liver were obtained by extracting the livers with twice their weight of hexane (bp 66–67°C) followed by centrifugation at 0°C and evaporation of the solvent in a rotary evaporator at 30°C. This crude hexane extract was used to charge the column.

In some species of shark, the oil content of the liver constitutes about 90% of the weight of the liver (3). The constituents of these oils are: hydrocarbons (such as squalene), triglycerides, alkoxyglyceryl diesters, higher alcohols, fatty acids and vitamin A and its esters, and other lipids (3, 4). Because of this high lipid content, the livers have been extracted in different ways (5–8), depending on the materials under investigation, but in these laboratories the method described gave the highest yield of neutral lipids; it is mild enough to be considered nondestructive.

TLC of the total shark liver extract is shown in Fig. 1, lane 2. Analysis: 0.01% N, <0.01% P. The IR spectrum of the total extract is similar to that of a simple mixture of triglycerides (major bands: C=C, 3020; $CH_2$ and $CH_3$, 2945 and 2880; C=O, 1750 $cm^{-1}$). Unsaponifiable material: 2.3% containing no triglyceride, steryl ester, or alkoxyglyceryl diesters as judged by TLC. Glass plates, 20 × 20 cm were coated with 0.25 mm of silica

Abbreviation: TLC, thin-layer chromatography.

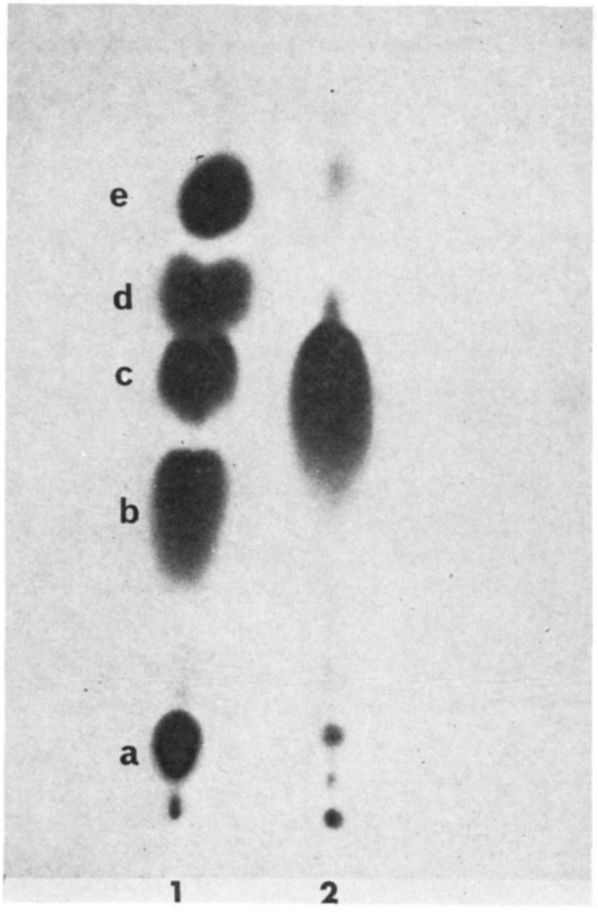


FIG. 1. TLC of total neutral lipids from shark liver. Lane 1, standard mixture: *a*, cholesterol; *b*, oleic acid; *c*, triolein; *d*, methyl oleate; *e*, cholesteryl oleate. Lane 2, total neutral lipid extract from shark liver. Adsorbent: Silica Gel G. Solvent: petroleum ether–ethyl ether–acetic acid 90:10:1. Detection of spots: 50% $H_2SO_4$ spray. Amounts: 400 μg.

gel according to the procedure of Stahl (9). Each plate was washed in chloroform–methanol 4:1, activated at 110°C for 1 hr, and used immediately after activation. The solvent used for development was petroleum ether–ethyl ether–acetic acid 90:10:1.

*Procedure.* Silica gel of the indicated activity (300 g) was equilibrated with 30 ml of petroleum ether–ethyl ether 90:10 for 1 hr in a rotating flask. This equilibration treatment produces a free-flowing adsorbent that can be handled in the same manner as dry silica gel and does not change the Brockmann activity of the adsorbent. However, the activity of the adsorbent should be measured in each batch after equilibration. The solvents used for equilibration are not visible during development of the column.

Silica gel treated in this way was poured into the type of nylon column described in Reference 2. The crude shark liver extract, 1 g, was dissolved in 10-ml of hexane in a round-bottomed flask; 10 g of silica gel was added, and the mixture was evaporated to dryness in a rotary evaporator. This mixture of extract and adsorbent was then poured evenly onto the surface of the column and covered with a layer of washed sand. For 1 g of hexane extract a tube of 1 × 34 inches, filled to a bed depth of 28 inches was used, and development time was 45 min; for 3 g of extract a tube 3 × 50 inches, filled to a bed depth of 46 inches was used and the time was 1.75 hr. The column was developed with a petroleum ether–ethyl ether mixture 9:1 until the solvent front reached the bottom of the column.

The columns were then cut at the desired places with a sharp knife, the adsorbent was extracted with ethyl ether–methanol 7:3, and the solvents were evaporated at reduced pressure and at room temperature. The residue was applied to TLC plates along with the standard mixture.

*Results and Discussion.* In experiments in which non-equilibrated silica gel was used as adsorbent, the distribution of lipid classes in the column did not correspond with that obtained on TLC using the same solvent mixture as eluent. Moreover, much trailing was observed. It was then recognized that the two-component solvent mixture was being separated as it penetrated the "dry" adsorbent. This phenomenon is well-known (10) and forms the basis of the "frontal analysis" technique originated by Tiselius (11).

In order to obtain $R_f$ values similar to those resulting from TLC, where the chambers are saturated with solvent vapors, I found it necessary to equilibrate the dry silica gel with 10% v/w of the solvent mixture used (petroleum ether–ethyl ether 9:1). Several runs were carried out using different solvents:adsorbent ratios (e.g., 2, 5, 10, and 15% v/w) and 10% was found to give the best results. In all probability, the ratio of solvent mixture-to-adsorbent necessary for equilibration will depend on the nature and composition of the solvent mixture as well as on the nature and activity of the adsorbent, and should be determined in each case.

The position of the different lipid classes was determined by slicing and extracting the column in 2-inch sections in the case of the 28-inch column and 3-inch sections in the case of the 46-inch column. Fig. 2 shows TLC of the fractions of a 28-inch column. The class composition of these fractions was determined by physical and (or) chemical means and by TLC comparison with compounds belonging to the different expected classes. For example, fraction 1 from the column (Fig. 2, lane 2), had an IR spectrum and $R_f$ value very similar to that of squalene. Fraction 6 (lane 7) had an IR spectrum similar to that of a mixture of batyl distearate and tripalmitin; its unsaponifiable fraction gave only one spot on TLC, which had the same $R_f$ value as batyl alcohol.

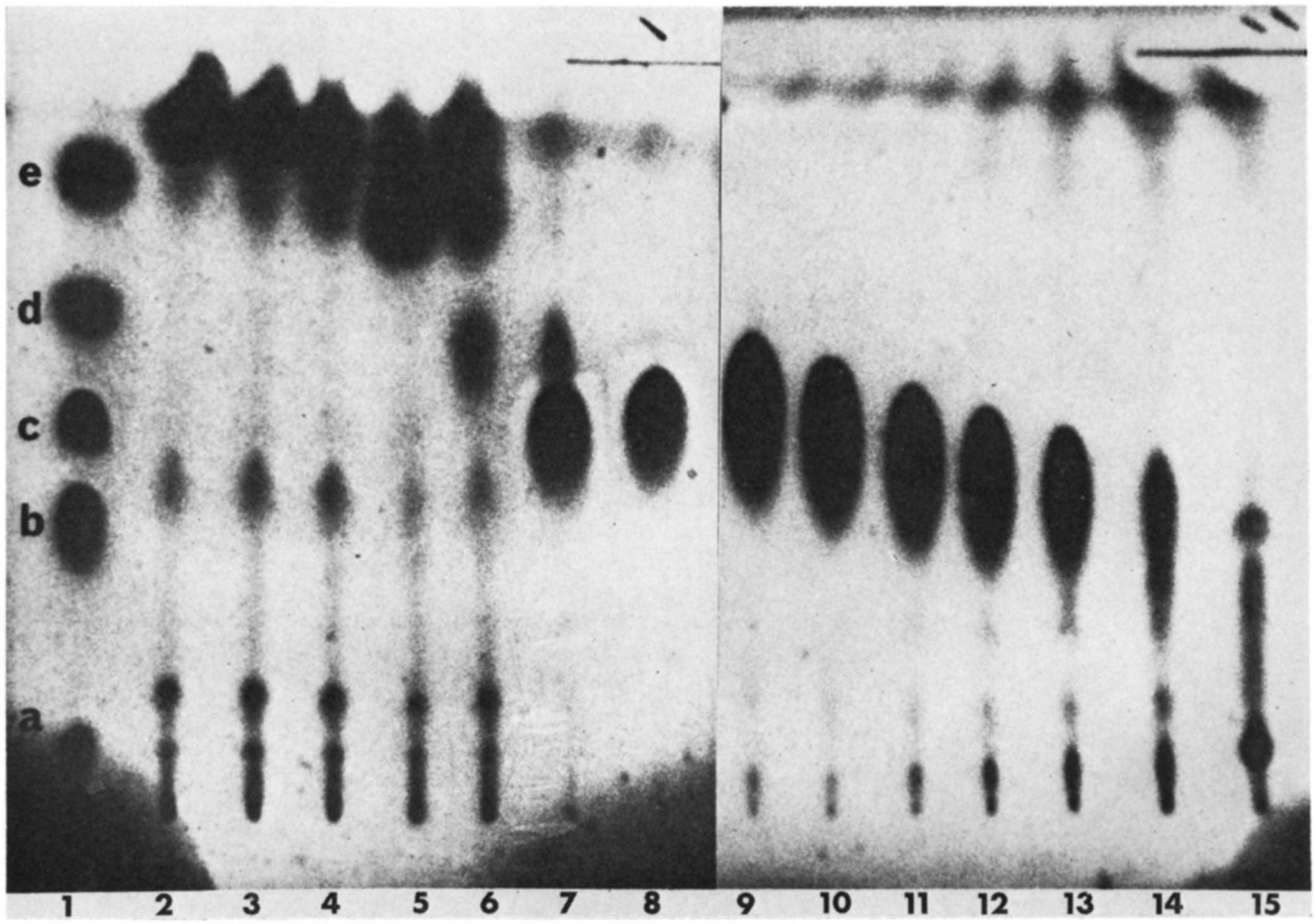


FIG. 2. TLC (conditions as in Fig. 1) of 2-inch fractions of a 28-inch "dry-column" chromatogram of shark liver neutral lipids. *1*, standard mixture as in Fig. 1. *2–5*, fractions contain hydrocarbons, sterol esters, and unidentified compounds (steroids, carotenoids?). *6*, contains alkoxy-glycerol diesters in addition. *7*, shows the beginning of the triglyceride band, which continues through fraction *13*, with increasing amounts of more polar compounds, which are contained chiefly in *14* and *15*. These may include cholesterol and free fatty acids.

The lower spots in lanes 2–6 (Fig. 2) gave a variety of colors with hot 50% sulfuric acid (yellow, blue, and red), whereas the lower spots in lanes 12–15 did not. The former may be steroids or carotenoids. Cholesterol, cholesteryl esters, vitamin A and provitamin A and their esters are known to be present in shark liver oil and are also known to give colors on spraying with sulfuric-acetic acid or chromic-sulfuric acid mixtures (12, 13). In the standard Hormel mixture, for example, spots *a* (cholesterol) and *e* (cholesteryl oleate) were colored red-brown. Although the manufacturers of the nylon tubing claim that the polymer contains no plasticizers, stabilizers, or other additives extractable with organic solvents, I tested this by extracting a piece of nylon tubing, 3 × 50 inches, for 1.75 hr with 250 ml of the solvent mixture used to develop the column. Evaporation of the solvents gave no residue detectable by TLC. This indicates that the spots that give colors with sulfuric acid are not artifacts but occur naturally in the shark extract, and that they travel along the column with the hydrocarbon components of the extract (possibly as molecular complexes, since they are evidently more polar than hydrocarbons).

As shown on Fig. 2, the separation into simpler fractions obtained with this "*dry-column*" technique is good. Poorer resolution is obtained for classes whose $R_f$ values differ by less than about 0.1 (e.g., alkoxyglyceryl diesters and triglycerides, Fig. 2, lane 7). Among the advantages of the method are its speed, reproducibility, and ability to handle several grams of sample.

The author is indebted to Dr. Bernard Loev of Smith, Kline and French Laboratories for helpful discussions during the course of this investigation. The capable assistance of Mrs. Sallie Reynolds and Miss Marcia Racki is greatly appreciated.

This investigation was supported by the John A. Hartford Foundation, T. Mellon and Son, Fannie E. Rippel Foundation, Reynolds Bagley, Verney Foundation, and the Whitehall Foundation.

*Manuscript received 7 August 1968; accepted 3 March 1969.*

## References


1. Loev, B., and K. M. Snader. 1965. *Chem. Ind.* (*London*). 15.
2. Loev, B., and M. M. Goodman. 1967. *Chem. Ind.* (*London*). 2026.
3. Tsujimoto, M. 1932. *J. Soc. Chem. Ind.* **51:** 317T.
4. Andre, E., and A. Bloch. 1935. *Bull. Soc. Chim. Série 5 Tome 2*: 789.
5. Hallgren, B., and S. Larsson. 1962. *J. Lipid Res.* **3:** 31.
6. Blumer, M. 1967. *Science.* **156:** 390.
7. Cain, J. C., and R. A. Morton. 1955. *Biochem. J.* **60:** 274.
8. Ali, S. M., S. Abdul Haq, and S. Mahdihassan. 1958. *Pakistan J. Sci. Ind. Res.* **1:** 70.
9. Stahl, E. 1956. *Pharmazie.* **11:** 633.
10. Boman, H. G. 1952. *Nature.* **170:** 703.
11. Tiselius, A. 1940. *Ark. Kemi Mineral Geol.* **14B:** No. 22.
12. Mangold, H. 1965. *In* Thin-Layer Chromatography. E. Stahl, editor. Academic Press, Inc., New York. 150–151.
13. Waldi, D. 1965. *In* Thin-Layer Chromatography. E. Stahl, editor. Academic Press, Inc., New York. 268.