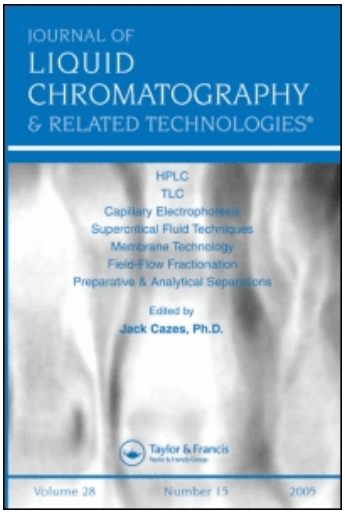


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# Comparison of Mobile Phases for Separation and Quantification of Lipids by One-Dimensional TLC on Preadsorbent High Performance Silica Gel Plates

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# COMPARISON OF MOBILE PHASES FOR SEPARATION AND QUANTIFICATION OF LIPIDS BY ONE-DIMENSIONAL TLC ON PREADSORBENT HIGH PERFORMANCE SILICA GEL PLATES

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## ABSTRACT

Twenty-four solvent systems reported in the literature for the one-dimensional TLC separation of lipids and phospholipids were compared under identical conditions using high performance preadsorbent silica gel plates. The best overall separation of mixtures of neutral lipid and phospholipid standards and compounds extracted from the digestive gland-gonad complex of Biomphalaria glabrata snails was obtained with a system utilizing consecutive development with chloroform-methanol-water (65:25:4), chloroform-hexane (3:1), and carbon tetrachloride. The best system for quantification of neutral lipids was hexane-diethyl ether-formic acid (80:20:2). $R_f$ data are tabulated and results discussed for all systems tested.

## INTRODUCTION

TLC is the most widely used method for separation of neutral and polar lipid classes. Because of its importance and the great

amount of research in the field, a bewildering number of chromatographic systems, involving from one to three developments, have been reported in the literature for separation of neutral and polar lipid classes by one-dimensional silica gel TLC. In order to ascertain the relative merit of these different mobile phases, 24 of the most promising systems from the literature were evaluated under identical conditions, involving TLC of constant amounts of neutral and polar lipid standards and extract of the digestive gland-gonad (DGG) complex of Biomphalaria glabrata snails on a single brand of preadsorbent high performance silica gel layers and detection with phosphomolybdic acid (PMA) reagent. Two types of systems were tested: those resolving only neutral lipids and leaving polar lipids at the origin, and those separating both neutral and polar lipids. $R_f$ values are tabulated, and the systems are ranked by their ability to visually separate the most lipids from standard mixtures and snail DGG extract, to give the best resolved densitogram, and to provide the best separation for each individual lipid class.



## EXPERIMENTAL

Whatman 20 x 10 cm LHP-KDF silica gel plates were developed with chloroform-methanol (1:1) and dried with a hair dryer for 10 min before use. Using a 10 ul Drummond digital microdispenser, plates were spotted in the preadsorbent area with 5 ul of lipid standard 18-1A (Nu-Check Prep) (containing 0.25 ug/ul each of monolein, diolein, triolein, and methyl oleate); 5 ul of lipid standard 18-5A [containing 0.20 ug/ul of cholesterol, cholesteryl oleate, triolein, oleic acid, and phosphatidylcholine (PC,

lecithin)]; 5 ul of a mixed standard with equal amounts of standards 18-1A and 18-5A; 5 ul of Polar Lipid Mix B (Matreya) [containing 0.20 ug/ul of cholesterol, phosphatidylethanolamine (PE), PC, and lysophosphatidylcholine (LPC)]; 5 ul of lipid extract of the DGG from lettuce-fed Biomphalaria glabrata snails; and 5 ul each of 1 ug/ul solutions of individual lipid standards to unequivocally identify the order of migration in the mixtures. Plates were air dried for 10 min after spotting.

The solvent systems tested and development distances are listed in Table 1. ACS reagent or HPLC grade solvents and deionized water were used in all cases to prepare these mobile phases. Developments were carried out in a rectangular glass chamber designed for 20 x 10 cm plates (Analtech catalog #70-21), which was lined with filter paper and presaturated with 30 ml of mobile phase for 25 min before inserting the spotted plate. Plates were dried with cool air from a hair drier for 10 minutes between multiple developments and after the final development. Lipids were detected by spraying with 5% PMA in 95% ethanol until a uniform yellow color was achieved, and then heating in a gravity convection oven at 100°C for 15 min.

Chromatograms of mixtures were scanned at 700 nm with a Shimadzu CS 930 densitometer in the single lane/single wavelength, reflection mode, with a light beam 6 mm high and 0.4 mm wide.

## RESULTS AND DISCUSSION

Standards representing most important lipid classes were included in the study: monolein (monacylglycerols), diolein (diacylglycerols), triolein (triacylglycerols), methyl oleate

TABLE 1 SOLVENT SYSTEMS

| Solvent number | System components | Ratio of components | Solvent front (cm from origin) | Ref |
|---|---|---|---|---|
| 1 | Petroleum ether-diethyl ether-acetic acid | 80:20:1 | 7.0 | 1 |
| 2 | Petroleum ether-diethyl ether-acetic acid | 90:10:1 | 7.0 | 2 |
| 3 | Isopropyl ether-acetic acid | 96:4 | 4.8 | |
| | Petroleum ether-diethyl ether-acetic acid | 90:10:1 | 7.3 | 3 |
| 4 | Toluene-diethyl ether-ethyl acetate-acetic acid | 80:10:10:0.2 | 7.0 | 4 |
| 5 | Heptane-isopropyl ether-acetic acid | 60:40:4 | 7.0 | 5 |
| 6 | Hexane-diethyl ether-formic acid | 80:20:2 | 7.5 | 6 |
| 7 | Petroleum ether-diethyl ether-acetic acid | 70:30:1 | 7.0 | 7 |
| 8 | Petroleum ether-diethyl ether-acetic acid | 85:15:1 | 7.0 | 8 |
| 9 | Hexane-heptane-diethyl ether-acetic acid | 63:18.5:18.5:1 | 7.0 | 9 |
| 10 | Hexane-diethyl ether-acetic acid | 30:70:1 | 7.0 | 10 |
| 11 | Chloroform-methanol-acetic acid | 98:2:1 | 6.3 | |
| | Hexane-diethyl ether-acetic acid | 96:6:0.2 | 7.8 | 11 |
| 12 | Benzene-2-propanol-ethyl acetate-acetic acid | 72.5:3.5:22:2 | 7.5 | |
| | Carbon tetrachloride-benzene | 30:70 | 6.3 | 12 |
| 13 | Petroleum ether-diethyl ether-acetic acid | 70:30:1 | 5.8 | |
| | Diethyl ether-petroleum ether-acetic acid | 70:30:1 | 3.3 | 13 |
| 14 | Hexane | 30 ml | 7.5 | |
| | Toluene | 30 ml | 7.5 | |
| | Hexane-diethyl ether-acetic acid (Twice) | 70:30:1 | 4.5 | 14 |
| 15 | Benzene-diethyl ether-95% EtoH-acetic acid | 50:40:2:0.2 | 4.0 | |
| | Hexane-diethyl ether-acetic acid | 94:6:1 | 6.0 | 15 |
| 16 | Chloroform-methyl acetate-ethyl acetate-hexane | 2000:15:30:30:15 | 7.5 | 16 |
| 17 | Chloroform-methanol-$H_2O$ (Twice) | 95:20:1 | 6.0 | |
| | Petroleum ether-diethyl ether-acetic acid | 80:20:1 | 7.5 | |
| | Petroleum ether | 30 ml | 7.5 | 17 |
| 18 | Chloroform-methanol-$H_2O$ | 65:25:4 | 3.5 | |
| | Hexane-diethyl ether | 4:1 | 5.25 | 18 |
| 19 | Chloroform-methanol-$H_2O$ | 65:30:5 | 2.0 | |
| | Hexane-diethyl ether-formic acid | 80:20:1.5 | 7.0 | 19 |
| 20 | Chloroform-methanol-$H_2O$ | 65:25:4 | 2.8 | |
| | Chloroform-hexane | 3:1 | 4.7 | |
| | Carbon tetrachloride | 30 ml | 7.5 | 20 |
| 21 | Chloroform-methanol-$H_2O$ | 65:25:4 | 4.0 | |
| | Hexane-acetone | 100:1 | 7.5 | 21 |
| 22 | Diethyl ether-benzene-ethanol-triethylamine | 40:50:2:1 | 5.6 | |
| | Diethyl ether-hexane-triethylamine | 10:90:1 | 6.65 | |
| | Diethyl ether-hexane-acetic acid | 74:25:2 | 2.10 | 22 |
| 23 | Chloroform-methanol-0.25% NaCl water solution-isopropanol-triethylamine | 40:10:6:25:18 | 3.0 | |
| | Chloroform-methanol-isopropanol-ethyl acetate-triethylamine | 30:10:25:7:35 | 7.0 | |
| | Hexane-diethyl ether-formic acid | 80:20:2 | 7.5 | 23 |
| 24 | Methyl acetate-1-propanol-chloroform-methanol- 0.25% KCl | 25:25:25:10:9 | 4.5 | |
| | Hexane-diethyl ether-acetic acid | 75:23:2 | 7.0 | |
| | Hexane | 30 ml | 7.0 | 24 |

**TABLE 2**

***NEUTRAL LIPID SYSTEMS***

| Solvent System # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rf Values (cm x 100) | | | | | | | | | | | | | | | | | |
| Cholesterol oleate | 68 | 88 | 90 | 90 | 81 | 77 | 91 | 94 | 74 | 87 | 85 | 100 | 91 | 90 | 76 | 88 | 93 |
| Methyl oleate | 62 | 74 | 82 | 90 | 81 | 62 | 82 | 82 | 58 | 81 | 78 | 100 | 83 | 66 | 65 | 88 | 90 |
| Triolein | 60 | 61 | 73 | 80 | 68 | 48 | 82 | 64 | 41 | 87 | 78 | 95 | 83 | 41 | 53 | 82 | 89 |
| Oleic acid | 32 | 28 | 58 | 41 | 40 | 24 | 48 | 30 | 17 | 65 | 55 | 76 | 53 | 14 | 45 | 24 | 77 |
| Cholesterol | 24 | 18 | 41 | 38 | 24 | 11 | 27 | 20 | 12 | 47 | 55 | 68 | 37 | 14 | 36 | 46 | 77 |
| Diolein | 24 | 18 | 49 | 57 | 24 | 8 | 30 | 18 | 10 | 65 | 71 | 88 | 48 | 9 | 45 | 70 | 76 |
| Monolein | 2 | 1 | 17 | 7 | 5 | 0 | 4 | 0 | 0 | 17 | 44 | 36 | 8 | 0 | 22 | 9 | 74 |

TABLE 3

NEUTRAL LIPID AND PHOSPHOLIPID SYSTEMS

| Solvent System # | 18 | 19 | 20 | 21 |
|---|---|---|---|---|
| Rf Values (cm x 100) | | | | |
| Cholesterol oleate | 87 | 77 | 66 | 67 |
| Methyl oleate | 80 | 68 | 57 | 61 |
| Triolein | 77 | 48 | 52 | 53 |
| Oleic acid | 66 | 42 | 32 | 45 |
| Cholesterol | 60 | 35 | 37 | 48 |
| Diolein | 58 | 34 | 40 | 51 |
| Monolein | 53 | 30 | 29 | 42 |
| Phosphatidyl-ethanolamine | 38 | 30 | 22 | 32 |
| Phosphatidyl-choline | 23 | 26 | 13 | 22 |



(fatty acid methyl esters), cholesterol (free sterols), cholesteryl oleate (cholesterol esters), oleic acid (free fatty acids), and PE, PC, and LPC (phospholipids). Table 1 contains the mobile phases tested, including development distances from the origin (the silica gel/preadsorbent interface). Some of the mobile phases included in this study were designed for use with regular silica gel and some for HPTLC. Because of their recognized advantages (25), HPTLC plates were used exclusively in this research. In order to adjust for the 10 cm height of HPTLC plates compared to 20 cm for TLC plates, migration distances reported in the literature for TLC were halved in this study. PMA detected all neutral lipids and phospholipids as dark blue zones on a yellow background, except for LPC. PC was detectable from the Polar Lipid Mix but not from the 18-5A standard.

Table 2 lists $R_f$ values for those systems that separate only neutral lipids and Table 3 for those that resolve both neutral

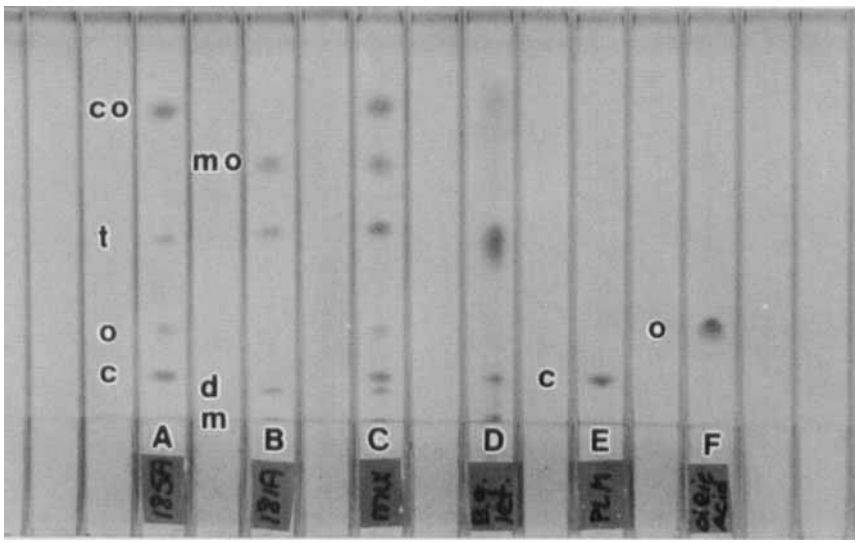


Figure 1. Photograph of System 6 chromatogram of neutral lipid standards and DGG extract of Biomphalaria glabrata snails. Lane A, neutral lipid standard 18-5A; B, neutral lipid standard 18-1A; C, equal mixture of standards 18-5A and 18-1A; D, DGG extract of B. glabrata snails; E, Polar Lipid Mix B; F, oleic acid. Compound abbreviations: cholesterol (c), cholesteryl oleate (co), diolein (d), lysophosphatidylcholine (lpc), monolein (m), methyl oleate (mo), oleic acid (o), phosphatidylethanolamine (pe), and triolein (t). See text for chromatographic details.

lipids and phospholipids. The $R_f$ values for phospholipids were zero in all of the systems in Table 2. As a general rule, the solvents listed in Table 2 produced dense, oval zones that had diameters in the 3-4 mm range, while solvents in Table 3 gave 1-2 mm flat bands that are more typical of preadsorbent plates.

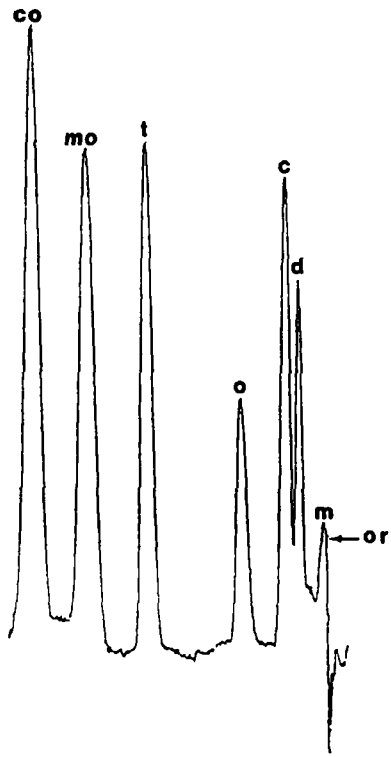


Figure 2. Densitogram of System 6 chromatogram showing representative peaks for lipid zones contained in lane C of Fig. 1. For compound abbreviations, see Fig. 1.

The best system for overall neutral lipid separation was System 6, and the best for lipids and phospholipids was System 20. The chromatogram produced with System 6 (Fig. 1) yielded a densitogram with close to baseline separation of peaks (Fig. 2). The bands formed with System 20 were visually completely separated (Fig. 3). However, resolution of the densitometer peaks of the neutral lipids with System 20 was not as great as with solvent 6, but was adequate for quantification of all zones; the two phospholipid zones showed baseline separation (Fig. 4).

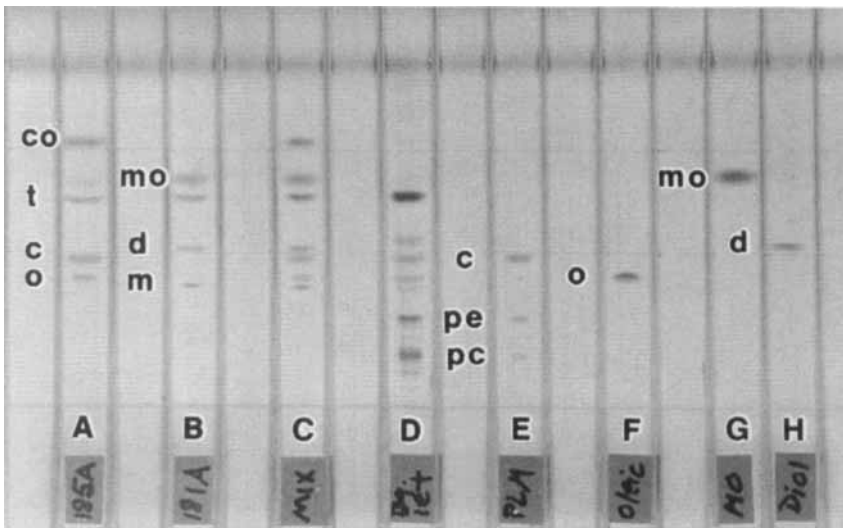


Figure 3. Photograph of System 20 chromatogram of lipid standards and DGG extract of Biomphalaria glabrata snails. Sample locations and compound abbreviations are the same as in Fig. 1. See text for chromatographic details.



Biomphalaria glabrata DGG lipid extract was included in the study with the standard mixtures because it is an example of a real biological sample and is being used in hyperlipidemia studies in our laboratory. Some of the systems that separated the standards well did not resolve the snail DGG lipid extract. For example, System 20 resolved the lipids in the snail extract much better than did System 6 (compare Figs. 1 and 3), although the latter might be perfectly suitable for separation of other real samples. Comparison of the extract chromatograms with standard chromatograms proved that triacylglycerols, free sterols, free fatty acids, PE, and PC were present in the DGG of the lettuce-fed B. glabrata snails.

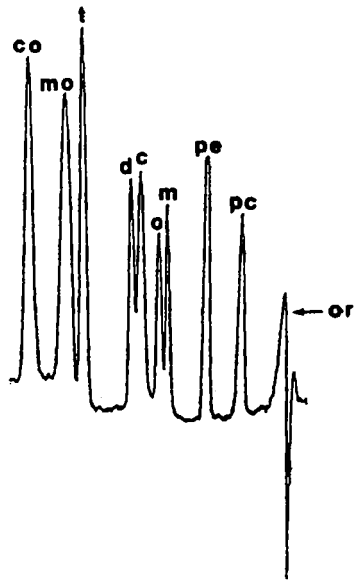


Figure 4. Densitogram of a mixture of neutral lipids and polar lipids separated in System 20. For compound abbreviations, see Fig. 1.



The $R_f$ data in Tables 2 and 3 can be used to evaluate the best system to use for a particular separation or quantification of a single zone or combination of zones. Quantification requires scanning of three or four standards and a sample whose concentration is bracketed by the standards (26), and for this purpose it is best to use a system that gives a dense zone that is well separated from its nearest neighbors and not too close to the origin. System 6 is optimal for scanning of cholesteryl oleate, methyl oleate, triolein, and oleic acid. System 16 is best for cholesterol, 11 for diolein, 20 for monolein and PC, and 21 for PE. These conclusions are based on the TLC of standards

and could be different for particular real samples. System 20 gave the sharpest separation and most compact zones and is the best for visual detection of all individual lipid and phospholipid classes at the lowest concentration.

Data do not appear in Tables 2 or 3 for the last three systems listed in Table 1. Systems 22 and 23 did not show any spots after development and application of PMA detection reagent. Zones were detected in System 24, but resolution was not sufficient to allow meaningful calculation of $R_f$ values. Also tested was the double development system designed earlier in this laboratory (26). This system has been giving higher $R_f$ values and poorer separation than those reported; the reasons for our inability to repeat the earlier results are unknown at this time.

Mobile phases designed for use with impregnated silica gel layers, e.g., layers coated with urea (27), and those reported specifically for polar lipid separations were not included in the study. The latter systems will be compared using a larger number of polar lipids in a separate study to be published later.



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