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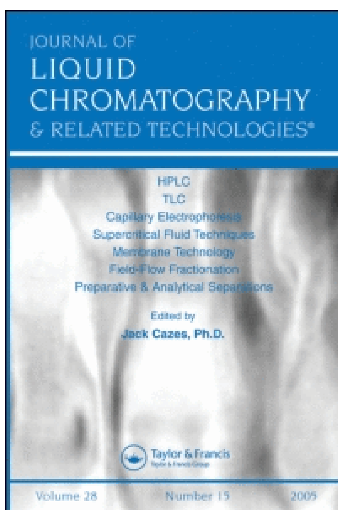

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TWO-STAGE, ONE-DIMENSIONAL THIN LAYER CHROMATOGRAPHIC METHOD FOR SEPARATION OF LIPID CLASSES

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ABSTRACT

A thin-layer chromatographic (TLC) technique was developed for routine analysis of lipid classes of blood, milk, tissue and egg yolk. This procedure provided rapid and reproducible separations suitable for in situ quantitation by densitometry

The spotted TLC plate was subjected to two developments in one dimension to separate, in ascending order from the origin: phospholipids, monoglycerides, free fatty acids, cholesterol, 1,2-diglycerides, 1,3-diglycerides, triglycerides, and cholesterol esters. Development 1: chloroform:methanol:acetic acid (98:2:1) to 17.0 cm. Development 2: hexane:ethyl ether:acetic acid (94:6:0.2) to the top of the plate. After air drying, the plate was dipped into a solution of 3% cupric acetate in 8% phosphoric acid for 3 seconds, and heated at 130°C for 30 minutes to char the separated lipid classes. The chromatograms were scanned at 350 nm.

Overloading of TLC plates with sample using the spotter resulted in the appearance of distorted kidney-shaped spots. A mathematical model and general explanation for the shape of these distorted spots was described.

INTRODUCTION

Numerous reports describing TLC techniques for lipid class separation have appeared in the literature (1, 2, 3, 4). Several of these procedures were studied, but, in our hands, did not provide satisfactory separations. Thus, the objective of the present work was to develop a rapid, sensitive, specific, and reproducible TLC procedure for measuring lipid classes of blood, milk,

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tissue and egg yolk. An additional requirement of this method was that the separated zones would be sufficiently discrete and non-diffuse to be suitable for in situ quantitation by densitometry.

EXPERIMENTAL PROCEDURES

Thin Layer Chromatographic Plates

Readily available standard commercial silica gel plates (Type K5 20x20 cm plates, 250μ thick, without ultraviolet indicators and without preadsorbent zone manufactured by Whatman, Inc., Clifton, New Jersey) were used. The plates were uniform in thickness and surface and reproducible results from package to package were obtained. This silica gel formulation is designed for the separation of moderately polar to strongly polar substances. The K5 plate has a recommended temperature limitation for charring of 130°C.

The K5 plate contains an organic polymer binder which presented a problem in quantitation of the lipids. The solvents used in separating the lipids caused the organic binder to move at or near the solvent front. The organic binder gave rise to color when phosphomolybdic acid, cupric acetate or dichlorofluorescein were used to visualize the lipids. This interfered with quantitative estimation of the cholesterol ester fraction which also moved near the solvent front. This problem was eliminated by a preliminary development with ethyl ether of all K5 plates in a non-saturated tank, to the top of the plate, thus moving the interfering organic binder ahead of the cholesterol esters.

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Sample Application

The Kontes Chromaflex 12-position spotter was used for routine application of lipid samples and standards onto the thin layer plates. The entire spotter was placed on a Thermolyne Type 2200 hot plate with a plate temperature maintained at 150°C. Because of heat loss to the bed of the Chromaflex spotter and to the glass TLC plate, temperatures of the plate did not exceed 50-60°C.

A mixed standard containing 7 μg of each of the lipid classes was spotted at position 6 of the 12-position spotter. The composition of the standard mixture was designed to approximate a lipid of biological relevance to our experimental samples. Accordingly, the standard mixture was composed of lipids containing equal amounts of C_{18} monounsaturated (oleic) and

polyunsaturated (linoleic) fatty acids in the lipid moiety (Table 1). The phospholipid standards were purchased from Supelco, Inc., Bellefonte, PA. All other lipid standards were purchased from Nu Chek Prep, Inc., Elysian, MN.

Lipids were extracted with chloroform:methanol (2:1) by the method of Folch et al. (5) from cow, calf, rabbit, chicken, lamb, and rat plasma, chicken, cow, and rat liver, human and bovine milk, corn oil, and egg yolk. Aliquots of these experimental lipid samples and standards are applied as dilute solutions in chloroform 2.3 cm from the bottom edge of the plate. Usually about 1.0 ml of solution was spotted and complete transfer required 10-15 minutes. Air flow velocity was approximately 27 liters/minute. Under

TABLE 1
Composition of Lipid Class Standard Mixture

Code	Lipid Class	Compound	RF value after development in solvent 1	2
CE	Cholesterol ester	50% chol. oleate 50% chol. linoleate	0.71	0.80
TG	Triglyceride	50% triolein 50% trilinolein	0.71	0.70
1,3 DG	1,3-diglyceride	50% 1,3-diolein 50% 1,3-dilinolein	0.66	0.62
1,2 DG	1,2-diglyceride	50% 1,2-diolein 50% 1,2-dilinolein	0.60	0.57
C	Cholesterol	cholesterol	0.52	0.50
FFA	Free fatty acid	50% oleic acid 50% linoleic acid	0.40	0.39
MG	Monoglyceride	50% 2-monoolein 50% 2-monolinolein	0.22	0.22
PL	Phospholipid	50% phosphatidyl choline 50% phosphatidyl ethanolamine	0.00	0.00

Solvent 1: Chloroform:methanol:acetic acid 98:2:1. Development distance (from bottom of plate): 17.0 cm. Time: 40 min.

Solvent 2: Hexane:ethyl ether:acetic acid 94:6:0.2. Development distance (from bottom of plate): 20.0 cm. Time: 38 min.

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these conditions and a plate temperature of 50-60°C, both sample and standard spots were compact, with a diameter of 4 mm.

Mobile Phases and Development

The chromatogram was developed in the ascending mode in a Brinkmann rectangular developing tank (30 x 10 x 23 cm) containing 200 ml of chloroform:methanol:acetic acid (98:2:1) under saturated conditions (tank lined with filter paper and equilibrated for 30 min) at 25°C. The plate was removed when the solvent front had advanced to a height of 17 cm. After air drying for 1-2 min, the plate was placed in a second tank (saturated conditions) containing 200 ml of hexane:ethyl ether:acetic acid (94:6:0.2). The plate was removed when the solvent front had advanced to the top of the plate and air dried for 10 min. Mobile phases were prepared fresh each day and discarded after use.

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Visualization

The separated lipids were visualized by use of a cupric acetate-phosphoric acid reagent with subsequent heating to produce charred spots (6). After development and air drying, the TLC plate was dipped for 3 seconds in a solution of 3% cupric acetate in 8% aqueous phosphoric acid contained in a Kontes Chromaflex dipping tank (5 x 170 x 475 mm). The plate was drained for 2 min, the back of the plate was wiped, and heated in an oven at 130°C for 30 min. After removal from the oven, some fading of charred spots occurred (6). Spot densities were greatest immediately after heating, attained constancy after several hours, and were relatively stable for several days. For routine analysis, plates were scanned 18 hours after heating.

Quantitation

The amount of lipid in the charred spots was quantified by densitometry in situ. Plates were scanned with a Shimadzu Dual-wavelength TLC Scanner Model CS-910 using the deuterium lamp at a single wavelength (350 nm) in transmission or reflectance modes. Plates were scanned linearly with a beam width of 0.3-1.0 mm and length of 10 mm or by a zigzag method using a 1.25 x 1.25 mm light beam.

Standard calibration curves were constructed every two days from a TLC plate containing 1, 2, 4, 7, 10, 25 and 35 µg of the standard lipid mixture. The densitometric areas were fitted by computer to a linear regression model. The coefficient of determination, r^2, was always 0.98-1.00, indicating that

the quality of fit achieved by the regression was good. A 7 μg standard sample was included on every experimental plate to establish an adjustment factor to correct for any deviations from the standard calibration curve.

RESULTS AND DISCUSSION

Non-uniform Distribution of Solute in Sample Application

Although spot diameters were compact using the Kontes Chromaflex spotter as described above the separated lipids sometimes assumed kidney-like shapes as the chromatogram developed. Fig. 1 shows the separation of 1, 2, 4, 7, 10,

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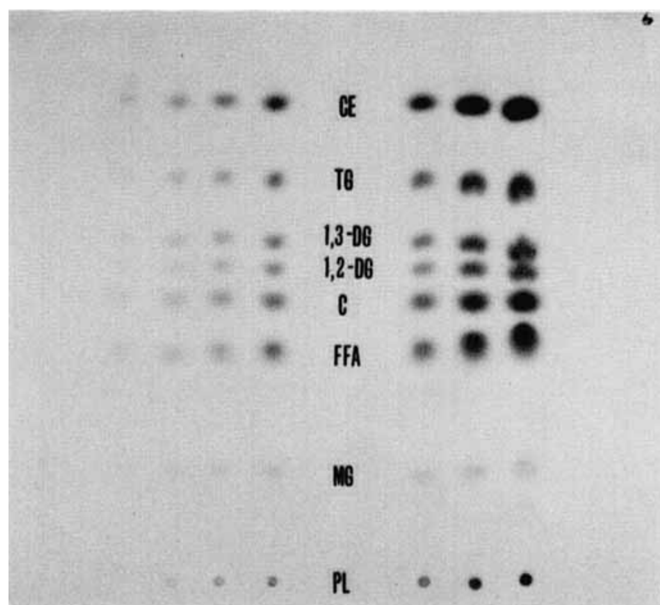

FIGURE 1. Separation of lipid class standards on a Whatman K5 TLC plate by the two-stage, one-dimensional technique. 1st solvent:chloroform: methanol:acetic acid (98:2:1) to 17.0 cm. 2nd solvent:hexane:ethyl ether:acetic acid (94:6:0.2) to top of plate. Detection was with cupric acetate-phosphoric acid charring reagent. From left to right: 1, 2, 4, 7, 10, 25 and 35 μg of each lipid in each vertical lane.

25, and 35 μg of the standard lipid mixture. The kidney shape appearance of some of the separated lipids, particularly at higher concentrations, suggested that overloading was one of the causes of this phenomenon.

Under ideal conditions, each separated lipid should migrate in a symmetrical fashion, and remain as a circular spot. Deviations from ideal behavior could arise because of overloading with solute, uneven distribution of the sorbent, changes in temperature, demixing of a multicomponent phase, change in flow rate, or uneven solute distribution (7).

Uneven solute distribution and overloading appeared to be the predominant factors giving rise to these characteristic shapes. Touchstone and Dobbins (8) described the appearance of "rings" caused by overspotting, i.e., solvent washing of sample to the outer edges of the application area when applied too rapidly. A hole in the layer caused by excessive pricking of the surface by the applicator needle also results in distorted spots (9). The kidney-shaped phenomenon was not due to interactions between constituent components of the lipid mixture. This was verified by spotting a large triglyceride sample on a TLC plate and developing with a single solvent, ethyl ether. Fig. 2

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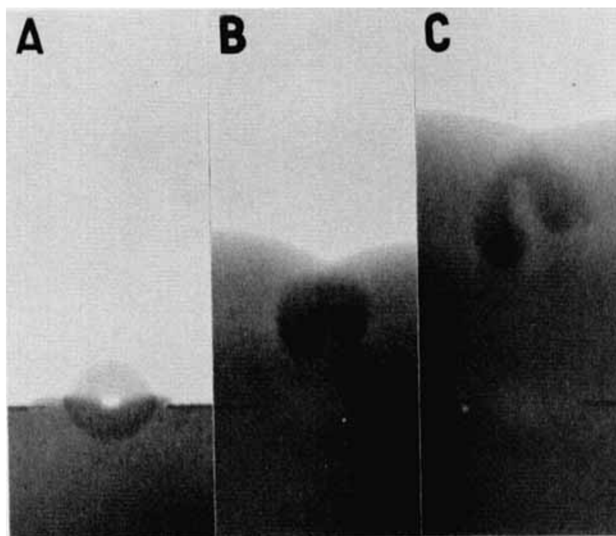

FIGURE 2. Successive stages in migration of a mixed triglyceride sample on a Whatman K5 silica gel plate developed with ethyl ether. A. Solvent front has reached origin. B. Sample has moved from origin with the solvent front and assumed the kidney shape. C. Solvent front has advanced up the plate, further emphasizing the kidney shape.

demonstrates that the lipid moved from the origin as a single spot and assumed the characteristic kidney shape.

Figures 3-5 represent schematically our concept of the factors causing the kidney shaped spots. Figure 3A shows a cross-section of a drop of sample on the TLC plate. The high air flow impinging on the drop perimeter and heated sorbent surface cause rapid evaporation of solvent and rapid deposition of solute at the perimeter. Adsorption of a compound from a solution onto a solid surface takes place at the air-liquid-sorbent interface. As solvent evaporates (Fig. 3B), deposition of solute molecules occurs at the perimeter or circumference of the drop. More of the solution then moves onto the perimeter and the process is continually repeated, resulting in severe oversaturation of the sorbent at the perimeter.

Mathematical Model of Uneven Solute Distribution

Mathematical consideration of uneven solute distribution is based upon the schematic model represented in Fig. 4. In the graph only one quadrant of the spot will be considered but similar relationships hold for all quadrants. In the most extreme case, according to the model concepts just outlined for solute distribution, the assumption can be made that all of the solute is distributed at the perimeter of the spot. The direction of flow of the solvent is from the bottom to the top of the spot of Fig. 4. The solvent can

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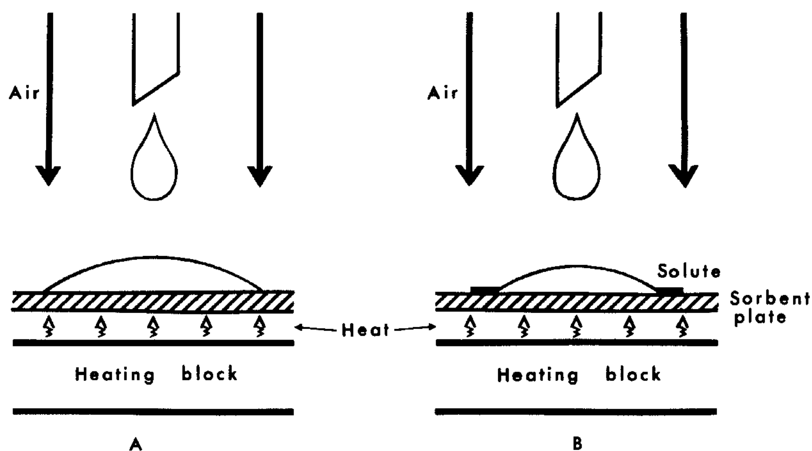

FIGURE 3. Cross-section of drop of sample on TLC plate during evaporation of solvent.

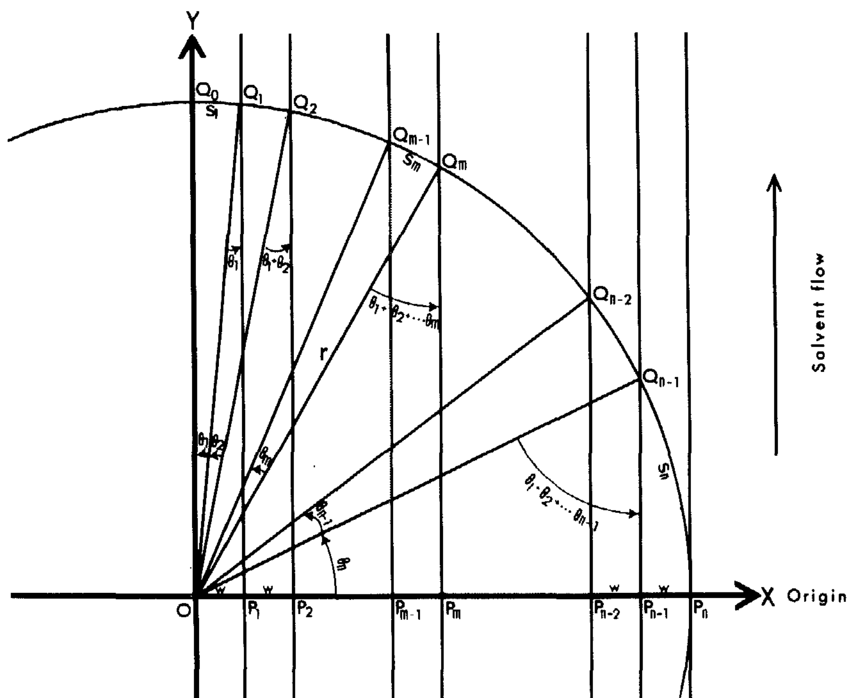

FIGURE 4. Schematic mathematical model of sample spot illustrating uneven solute distribution in perimeter of spot.

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be considered to be ascending vertically along an extremely large number of strips or columns of adsorbent. Derivation of the amount of material in the perimeter can be calculated from the relationships outlined in Fig. 4. Let n = the number of strips of equal width w through one quadrant of the circle, as shown in Fig. 4.

$$r = nw \tag{1}$$

In general, the line P_mQ_m, where m is any of the subscripts from 1 to n, is parallel to the y-axis, so that the angle $(\theta_1 + \theta_2 + . . . + \theta_m)$ or Q_mOQ_o, is equal to the angle OQ_mP_m. Therefore

$$\sin (\theta_1 + \theta_2 + . . . + \theta_m) = \frac{mw}{r} = \frac{mw}{nw}$$

$$\sin (\theta_1 + \theta_2 + . . . + \theta_m) = \frac{m}{n} \tag{2}$$

shows that the arc at the outermost edge, S_n, is 4.5 times that of the most central arcs. There is little difference in the arc length of the first six arcs (1-6) from the center, and, consequently, in the amount of solute. Towards the edge of the circle, however, the arc lengths, and amount of material increases greatly. Similar conditions prevail in the other quadrants of the circle, resulting in uneven solute distribution, i.e., accumulation of large amounts of sample at the sides of the spot.

During development, adsorption sites in the sorbent at the edges of the spot become occupied, and the sorbent adsorbs less than proportional quantities of solute (7). The mobile phase solvent flows through points of least resistance (8) and migration rates of the solvent and solute at the center are greater. This behavior results in tailing at lateral edges as compared to the center, and in formation of split bands and the characteristic kidney shape shown in Fig. 1.

Experimental support for this explanation was derived. Macro spots (A, 30 mm diameter; B, 40 mm diameter) were obtained on a K5 plate by rapidly spotting a large volume of lipid standard dropwise from a pipet (1 mm bore) with continual drying (Fig. 5). One quadrant of each spot was analyzed: Spot A radius was subdivided into 4 equal segments, Spot B radius into 5 segments. After the entire quadrant was ruled off into squares of silica gel, individual areas were scraped into test tubes, and the lipid extracted and determined

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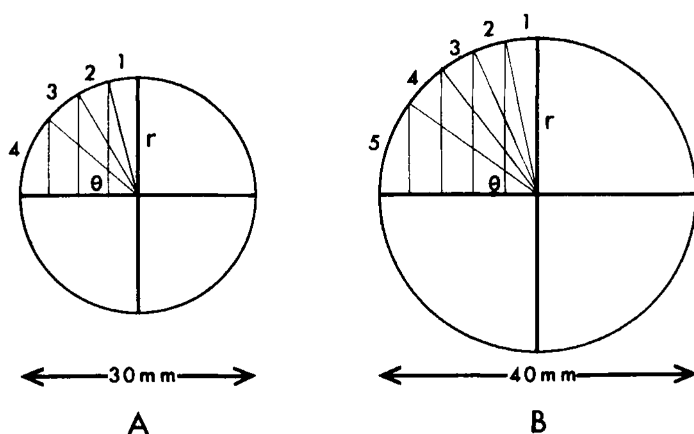

FIGURE 5. Schematic representation of two model macro lipid spots obtained on a K5 plate. Spot A was obtained from 800 ug of lipid standard in 8 ml of solution; Spot B, from 700 ug of standard in 7 ml of solution.

Taking the inverse function of each side of equation (2)

$$\Theta_1 + \Theta_2 + \ldots + \Theta_{m-1} + \Theta_m = \arcsin\left(\frac{m}{n}\right) \quad (3)$$

If the summation is stopped at m-1 instead of m, the preceding member of the sequence is obtained:

$$\Theta_1 + \Theta_2 + \ldots + \Theta_{m-1} = \arcsin\left(\frac{m-1}{n}\right) \quad (4)$$

To find Θ_m, equation (4) is subtracted from equation (3):

$$\Theta_m = \arcsin \frac{m}{n} - \arcsin\left(\frac{m-1}{n}\right) \quad (5)$$

Let S_m be the length of arc corresponding to the angle Θ_m. Then

$$S_m = r\,\Theta_m \quad (6)$$

Substitution of the value of Θ_m from equation (5) into equation (6) gives

$$S_m = r\left[\arcsin\left(\frac{m}{n}\right) - \arcsin\left(\frac{m-1}{n}\right)\right] \quad (7)$$

In Fig. 4 the radius was arbitrarily divided into 10 equal segments of width, w, creating 10 parallel columns. The intersection of these equally spaced lines with the circumference creates a series of 10 arcs, the lengths of which are proportional to the amount of solute in the perimeter. Table 2

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TABLE 2
Calculated Arc Lengths of Perimeter of Model Sample Spot

Column number m	$\frac{m}{n}$[a]	Arc sin $\left(\frac{m}{n}\right)$ in radians	Θ_m or $\frac{S_m}{r}$ in radians
1	0.1	0.10017	0.10017
2	0.2	0.20136	0.10119
3	0.3	0.30469	0.10333
4	0.4	0.41152	0.10683
5	0.5	0.52359	0.11207
6	0.6	0.64350	0.11991
7	0.7	0.77540	0.13190
8	0.8	0.92730	0.15190
9	0.9	1.11977	0.19247
10	1.0	1.57080	0.45103

[a] n=10; quadrant radius has been divided into 10 equal vertical strips.

spectrophotometrically using the sulfophosphovanillin reaction (10). The results in Table 3 indicate a close correspondence between the amount of lipid determined and the length of the arc or size of the angle, Θ. Analysis of the more central squares demonstrated amounts that were only 1/3 to 1/4 of the lipid at the edges.

The spot distortions observed occur because of the nature of sample application using the Kontes Chromaflex spotter. The phenomenon, however, is a general one. The kidney shaped spots arise whenever rapid solute adsorption causes overloading at the spot edges, and is followed by normal TLC development in a vertical mode. The mathematical treatment described thus applies generally to distorted spots of this physical shape.

Selection of the Mobile Phase. Two Stage Solvent Development

Separation of the lipids depends upon selection of a suitable mobile phase. In spite of extensive knowledge of the properties of solvents, sorbents, and solutes, the rationale for choice of a mobile phase is still empirical. Table 4 shows lipid class separations obtained with several

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TABLE 3
Lipid in Perimeter of Model Spots

Fraction m	$\frac{m}{n}$	arc sin $\left(\frac{m}{n}\right)$	Θ_m or $\left(\frac{s_m}{r}\right)$	Lipid (OD units)
Spot A (30 mm diameter)				
1	0.25	0.25268	0.25268	148
2	0.50	0.52360	0.27092	172
3	0.75	0.84806	0.32446	206
4	1.00	1.57080	0.72274	440
Ratio 4:1 n = 4	-	-	2.86	2.97
Spot B (40 mm diameter)				
1	0.2	0.20136	0.20136	88
2	0.4	0.41152	0.21016	110
3	0.6	0.64350	0.23198	106
4	0.8	0.92730	0.28380	130
5	1.0	1.57080	0.64350	345
atio 5:1 n = 5	-	-	3.20	3.92

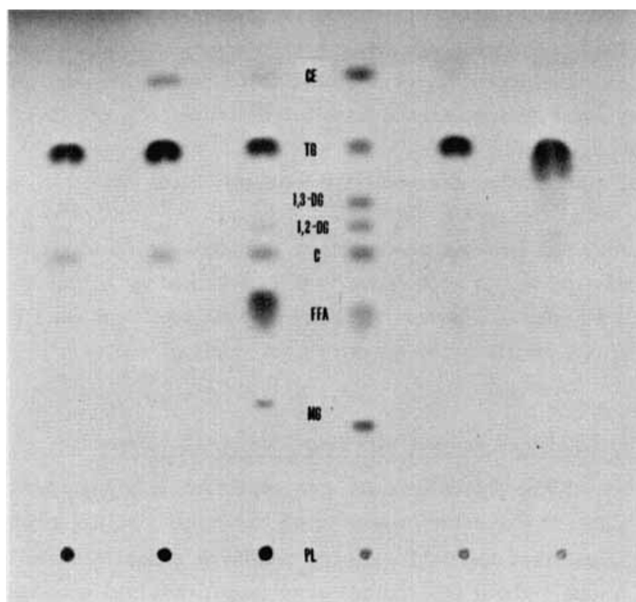

FIGURE 6. TLC separation of lipid classes. Lanes from left to right: 1, egg yolk; 2, chicken plasma; 3, chicken liver; 4, 4 ug lipid standard; 5, human milk; 6, cows milk.

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solvent systems. Hexane/ethyl ether/acetic acid, 80:20:1, or two other ratios, did not separate cholesterol from 1,2-diglycerides. The two-stage development system of Freeman and West (3) did not separate cholesterol from free fatty acids (No. 2A and B, Table 4).

A mixture of chloroform/methanol/acetic acid, 98:2:1, separated 6 of the 8 lipids in a single development (Table 4, No. 3), leaving unresolved only the two least polar solutes, triglycerides and cholesterol esters, which move together near the solvent front. These two compounds, however, had separated well with several other mobile phases (Table 4, No. 1, No. 2A). A combination of two mobile phases, therefore, was able to completely resolve the 8-component mixture into appropriate lipid classes (Table 4, No. 4A and No. 4B). The spotted TLC plate was first developed in chloroform/methanol/acetic acid, 98:2:1, to 17 cm from the bottom of the plate. After air drying, the plate was developed in hexane/ethyl ether/acetic acid, 94:6:0.2, to the top of the plate.

TABLE 4

Separation of 8 Lipids into Classes on Whatman K5 Plate

No.	System	Separation
1.	Hexane/ethyl ether/acetic acid 80:20:1	Did not separate CHOL from 1,2-DG
	70:30:1	"
	60:40:1	"
2.	A. Ethyl ether/benzene/ethanol/ acetic acid 40:50:2:0.2	Did not separate CHOL from FFA
	B. Hexane/ethyl ether 94:6 or B. Hexane/ethyl ether/acetic acid 80:20:1	Did not separate FFA from 1,3-DG
		Did not separate CHOL from 1,2-DG
3.	Chloroform/methanol/acetic acid 98:2:1	Did not separate TG from CE
4.	A. Chloroform/methanol/acetic acid 98:2:1	Develop to 17 cm All lipids except TG/CE separate
	B. Hexane/ethyl ether/acetic acid 94:6:0.2	Air dry. Develop to top of plate. CE separates from TG

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Quantitation of Lipid Classes

The relationship between concentration of the 8 standard lipid classes and response is given in Table 5 for concentrations between 1-35 μg. The wide variation in structure and molecular weight of the lipids in the lipid classes results in differences in yields of carbon for equal amounts of different lipids. Precision and reproducibility, however, have been obtained with the cupric acetate-phosphoric acid charring procedure. Transmittance measurements yielded response areas that were ca 2.8 x higher than reflectance measurements.

TABLE 5

Quantitation of Lipid Classes:
Response vs Concentration

	Response Area (μV sec/10^3)[a]						
	Concentration of Lipid μg						
Class	1	2	4	7	10	25	35
PL	3	15	22	36	46	69	77
MG	4	14	30	47	59	125	158
FFA	18	38	77	120	156	310	407
C	22	46	80	113	140	240	310
1,2-DG	6	19	40	65	80	166	211
1,3-DG	9	22	46	74	91	186	247
TG	11	24	51	79	98	196	264
CE	22	42	69	102	126	276	334

[a] Scanning conditions:

Single λ, 350 nm Reflectance measurement

Zigzag scanning: Beam, 1.25 x 1.25 mm
Scanning speed, 20 mm/min

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Application of the TLC Separation Procedure to Biological Samples

The TLC separation procedure has been applied to a variety of biological samples. Lipids from cow, calf, rabbit, chicken, lamb and rat plasma have been separated. Lipids from chicken, cow and rat livers have been separated and analyzed, as well as human and cow's milk, corn oil and egg yolk. Fig. 6 shows a chromatogram of lipid classes from egg yolk, chicken plasma, chicken liver, human milk and cow's milk. The procedure we have described has proved to give excellent reproducibility. It is relatively simple to perform, it is relatively short, and uses readily available commercial materials.

FOOTNOTES

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar name not mentioned.

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