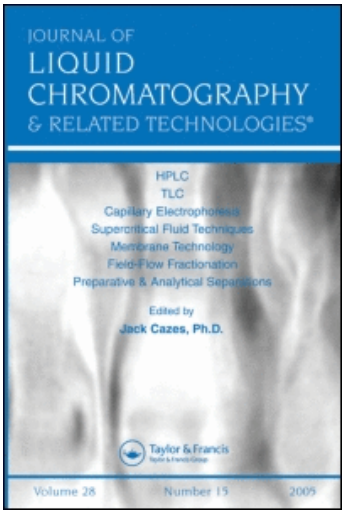

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COMPARISON OF MOBILE PHASES AND DETECTION REAGENTS FOR THE SEPARATION OF TRIACYLGLYCEROLS BY SILICA GEL, ARGENTATION, AND REVERSED-PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Triacylglycerol standards and TGs from lard, egg yolk, and the planorbid snail Biomphalaria glabrata were examined using TLC with various layers, solvent systems, and detection reagents. The three types of thin-layer chromatography plates used were plain silica gel, silica gel plates impregnated with $AgNO_3$, and reversed-phase C-18 plates. The best solvents found were isopropyl alcohol-chloroform (1.5:98.5) for plain and silver-loaded silica gel TLC, and acetonitrile-methyl ethyl ketone-chloroform (50:35:15) for reversed-phase TLC. The best detection reagents were found to be phosphomolybdic acid for plain silica gel TLC and reversed-phase TLC, and 50% sulfuric acid for argentation TLC.

INTRODUCTION

Silica gel thin-layer chromatography (TLC) is the most widely used method for separation of neutral and polar lipid mixtures into classes of compounds, and a recent study (1)

compared the utility of 24 solvent systems for the fractionation of lipid classes. We have also reported TLC separations of compounds within a single lipid class in the case of phospholipids (2), cholesterol esters (3), and sterols (4), and in this paper we report the results of a comparative study of separation and detection systems for triacylglycerol (TG) TLC.

The major techniques reported in the literature for separating TGs are TLC on silver-impregnated silica gel plates (argentation TLC) (5) and reversed phase (RP) TLC (6). Separations of TGs by argentation TLC are based on the degree of saturation (5); compounds theoretically migrate in the order SSS> SSM> SMM> SSD> MMM> SMD> MMD> SDD> SST, where S, M, D and T denote saturated, monoenoic, dienoic and trienoic fatty acyl groups, respectively (7). Separation occurs when the silver ions complex with double bonds in the TGs, which has the effect of retarding migration of compounds proportionately to the number of double bonds (8).

RP-TLC separates TGs according to their partition number (PN), sometimes called equivalent carbon number: PN = CN - 2m, where CN is the carbon number and m is the number of double bonds in the TG molecule (9). In general, PN values are inversely related to R_f values (10), but, depending on the particular mixture of TG components and their exact PN values, the polarity of the mobile phase has to be varied to achieve resolution (6).

The combination of argentation and RP-TLC provides separations based on differences in both saturation and

partition number, and allows reliable classification of the components in a complex TG mixture. In order to ascertain which systems are optimal for TG analysis, various layer types, mobile phases, and detection methods suggested in the literature were tested under identical conditions using a variety of TG standards. Along with the standards, three actual samples were analyzed in order to presumptively classify the components found in their TG mixtures. Neutral and polar lipid standards were also spotted to determine how well TGs separated from the other lipid components in the sample extracts.

EXPERIMENTAL

Layers

Three different types of layers were compared by chromatographing standards and extracts from hen's egg yolk, lard, and snails: plain (unimpregnated) TLC and HPTLC silica gel; silica gel layers impregnated by the manufacturer or by us in the laboratory with $AgNO_3$; and silica gel layers chemically bonded with C-18 groups.

Baker-flex (20 x 20 cm) IB2 silica gel sheets (J.T. Baker, Phillipsburg, NJ) and Whatman (20 x 10 cm) LHP-KDF channeled high performance silica gel plates with a pre-adsorbent spotting area (Whatman, Clifton, NJ) were used unimpregnated for silica gel TLC as well as after impregnation with $AgNO_3$ for argentation TLC.

Argentation plates were prepared by hand-dipping the silica gel plates into a glass Desaga dipping chamber

(Bodman, Aston, PA) containing a 2.5% solution of $AgNO_3$ in methanol, followed by activation for one hour in an oven at 110°C just prior to use (11). Commercially prepared Uniplates (20 x 20 cm) impregnated with 10% $AgNO_3$ were purchased from Analtech (Newark, DE).

Standards

Standards were chosen based on both saturation class (S3, S2M, SM2, M3, and D3) and partition number. These two classifications, along with the designations of the TG groups (St=stearate, P=palmitate, O=oleate), follow each compound in parentheses below. All standard solutions were prepared at a 1.0 ug/uL concentration in chloroform.

The following standards were obtained from Sigma (St. Louis, MO USA): tripalmitin (S3, 48), trilinolein (D3, 42), triolein (M3, 48), tristearin (S3, 54). All other individual standards were obtained from Matreya Co. (Pleasant Gap, PA): rac-glyceryl-1,3-palmitate-2-oleate (POP-S2M, 48); rac-glyceryl-1-palmitate-2-oleate-3-stearate (POSt- S2M, 50); rac-glyceryl-1,2-oleate-3-stearate (OOSt-SM2, 50); rac-glyceryl-2,3-stearate-1-oleate (OStSt-S2M, 52); rac-glyceryl-1-palmitate-2-stearate-3-oleate (PStO-S2M, 50).

The neutral lipid standard 18-4A (Nu-Check Prep, Elysian, MN), containing 0.20 ug/ul each of cholesterol, cholesteryl oleate, oleic acid, triolein, and methyl oleate, and individual standards of these lipids (obtained from Matreya) were also chromatographed to determine if these compounds had the same R_f in the solvent systems used as any of the TGs.

Samples

Biomphalaria glabrata snails were maintained following Masterson et al. (12). The snails were fed either a high fat diet consisting of hen's egg yolk (2.30 +/- 0.04 g) supplemented with leaf lettuce (0.356 +\- 0.005 g), or a control diet of leaf lettuce (4.21 +\- 0.04 g) supplemented with Tetramin fish food (0.051 +\- 0.001 g). Snail bodies were extracted with 4-5 ml of chloroform-methanol (2:1), the extract dried under a stream of nitrogen, and the residue reconstituted with 1 ul of chloroform-methanol (1:1) per mg of body weight. Extracts of a 1% hen's egg yolk solution (1 g hen's egg yolk in 100 mL chloroform-methanol) and a 1% lard solution (1 g lard in 100 mL chloroform-methanol) were prepared in the same way.

TLC analysis

A 25 ul Drummond (Broomall, PA) digital microdispenser was used to spot the plates. Standards and extracts from hen's egg yolk, lard, and B. glabrata fed either yolk or lettuce diets were applied to an origin line 2 cm from the bottom of the plate for the plain silica gel and argentation layers, or to the preadsorbent area of the RP plate. Aliquots of samples and standards were spotted in volumes ranging from 0.5 uL to 10 uL. The solvent systems tested are listed in Table 1. ACS reagent or HPLC grade solvents and deionized water were used in all cases to prepare these mobile phases. Development was carried out for a distance of 10 cm past the origin in a rectangular glass chamber

(Analtech) that was lined with filter paper. Plates were air dried before and after development.

The detection reagents tested for plain silica gel plates were 50 % aqueous sulfuric acid, iodine vapors, phosphomolybdic acid (PMA) (5% in ethanol), 2,7-dichlorofluoroscein (0.1% in methanol), Sudan Black B (0.1% in methanol), and Oil Red O (3.5% in isopropyl alcohol). Reagents tested for detection of TGs on silica gel/Ag^+ layers were based on successful detection on silica gel plates without silver, and included 50 % aqueous sulfuric acid, iodine vapors, and PMA. Detection reagents used for RP plates were PMA and 2,7-dichlorofluoroscein. After the detection reagent was applied, the plates were heated at 110°C for 15 min, except for iodine vapors or 2,7-dichlorofluoroscein, which did not require heating.

RESULTS

Attempts to use Analtech plates impregnated with silver nitrate were not successful because the layers turned black soon after delivery, despite storage in the dark. In-house preparation of impregnated silica gel plates by dipping into silver nitrate solution just before use was determined to be the best method to avoid this discoloration of the plates. It was found that 2.5% $AgNO_3$ was the highest concentration that would dissolve in methanol when preparing the dip solution. Both Baker-flex and Whatman silica gel plates were impregnated with silver and preliminarily tested for TG

separations; the Baker-flex plates provided better separations and were used for the remainder of the study.

The mobile phase isopropyl alcohol: chloroform (1.5:98.5) proved to be the best of those tested for the separation of TGs and other lipids both on plain and silver-loaded silica gel (Table 1). Light petroleum ether-acetone efficiently separated TGs on the argentation plate, but it made the standards run with very high R_f values on the plain silica gel TLC plate.

The solvent systems that separated TGs and lipids most efficiently in reversed-phase TLC was acetonitrile-methyl ethyl ketone-chloroform (50:35:15). Other systems gave problems with detection (THF-acetonitrile), or resolution of compounds was not as good (acetone-acetonitrile-water). Only three of the nine individual TG standards were detected with PMA on the plate developed with THF-acetonitrile, because the plate background became dark blue. The acetone-acetonitrile-water mobile phase separated the standards into poorly-resolved bands with very low R_f values.

The best detection reagents were found to be PMA for reversed-phase plates and plain silica gel plates, and 50% aqueous sulfuric acid for the silica gel plates with silver. The tripalmitin and tristearin standards were not detectable on any of the three plates using any of the reagents, even when spotted up to a level of 40 ug. Occasionally, these compounds could be detected as an inverse white spot on an off-white background at the correct position predicted on the

TABLE 1
Solvent systems.

Solvent number	System components	Volume ratio of components	Ref	Rank* of separation
	Argentation TLC (Silica gel plate impregnated with 2.5% methanolic $AgNO_3$)			
1	Hexane-diethyl ether	80:20	10	P
2	Chloroform-methanol	96:4	10	P
3	Isopropyl alcohol-chloroform	1.5:98.5	11	E
4	light petroleum ether-acetone	10:4.5	6	G
5	toluene-chloroform	50:50	13	P
6	petroleum ether-diethyl ether-acetic acid	80:20:2	14	G
	Plain Silica gel TLC			
7	light petroleum ether-acetone	10:4.5	6	G
8	isopropyl alcohol-chloroform	1.5:98.5	11	E
9	toluene-chloroform	50:50	13	P
10	petroleum ether-diethyl ether-acetic acid	80:20:2	14	P
	Reversed-phase TLC			
11	acetonitrile-methyl ethyl ketone-chloroform	50:35:15	15	E
12	acetone-acetonitrile-water	70:30:12	16	G
13	THF-acetonitrile	3:7	7	P

*E = excellent; G = good; P = poor.
Detection was made with PMA for reversed-phase and silica gel without impregnated silver, and 50% aqueous sulfuric acid for silica gel with silver.

basis of the degree of saturation (argentation TLC) and partition number (reverse-phase TLC).

Table 2 contains R_f values for the standards and TG components of the samples in the best solvent system for each plate. All standards migrated in the proper sequence on the argentation and RP layers as predicted by the theoretical considerations mentioned earlier.

Cholesterol and triolein had the same R_f value (0.34) on the C-18 plate, making it difficult to distinguish between these two compounds in the samples. There was an uexplainable disparity between the individual triolein standard and the triolein contained in the mixed 18-4A standard; the 18-4A triolein component ran lower than the individual triolein standard on the argentation plate and higher on the plain silica gel TLC plate, but the two standards had equal R_f values on the RP plate.

The amount of yolk and lard found to give the best resolution of their components (based on the extracted 1% solutions) was determined to be 0.5 uL on reversed-phase TLC plates, while 5 uL each of standards and reconstituted extracts from the snails fed the two different diets was determined to give the clearest resolution of the lipids. For TLC on plain silica gel, it was found that the amount of lard and yolk (based on the extracted 1% solutions) that provided the best resolution of components was 2 uL. Five uL each of standards gave the best resolution of the components, while 2.5 uL each of the natural products from

TABLE 2
R_f x 100 values

	Solvent System[1]		
	3	8	11
	Rf Values (cm x 100)		
Standards			
OOS	64	75	32
OSS	79	74	28
POP	80	73	34
POS	82	75	30
PSO	78	73	32
Trilinolein	31	73	53
Triolein	62	74	36
Tripalmitin	85	ND	ND
Tristearin	85	ND	ND
18-4A			
Cholesterol	21	22	36
Cholesteryl oleate	64	84	28
Methyl oleate	59	70	70
Oleic acid	05	12	70
TG	40	77	36
Individual Standards:			
Cholesterol	22	23	36
Cholesteryl oleate	71	82	26
Methyl oleate	58	68	70
Oleic acid	07	12	69
Samples:			
Lard	75	90	46
	68		40
			34
Yolk	78	92	39
	65	50	
	48		
	22		
Lettuce-fed snails	76	X	83
	22		70
			44
			35
Yolk-fed snails	77	X	85
	46		69
	21		39

[1] = Refer to Table 1 for solvent composition; ND = did not detect; X = sample not tested in this solvent system

the snails gave the clearest resolution into components. Argentation TLC plates showed that 10 uL of all the standards as well as all of the samples provided the clearest resolution into individual components.

A plain silica gel TLC plate was developed as a control. The following compounds were spotted, and the resulting R_f values follow in parentheses: lard (75), yolk (76,26,0), yolk-fed B. glabrata (74,23,16,0), lettuce-fed B. glabrata (75,23,16,0), triolein (77), cholesterol (24), cholesteryl oleate (80), methyl oleate (67), and oleic acid (15).

DISCUSSION

All plates separated TGs according to expected patterns based on previous studies reported in the literature. No separation of the TGs was expected on plain silica gel TLC, and only small differences in R_f values (+\- 0.02 cm) were obtained for the TG standards (Table 2). The differences did not follow any pattern according to either partition number or degree of saturation: OSS (52, SM2) and POS (50, S2M) both had R_f values of 75, triolein (48, M3) and OOS (52, SM2) had values of 74, and PSO (50, S2M), POP (48, S2M), and trilinolein (42, D3) all had values of 73. The cause of these minor TG separations on unimpregnated silica gel plates is not known.

The argentation TLC plates gave separations based on degree of saturation (5), and all of the standards migrated in the predicted sequence: S3 had the highest R_f value,

followed by S2M> SM2> M3> D3. This separation of TGs was achieved using a Baker-flex silica gel sheet that had been impregnated in a dip-tank containing 2.5% methanolic $AgNO_3$, but was not obtained with Whatman silica gel plates we impregnated or commerically-impregnated Analtech plates.

With reversed-phase TLC, all of the standards migrated as expected (10). The order of migration was PN 42> 48> 50> 52> 54, i.e., PN was inversely related to migration distance. Triolein and cholesterol standards had the same R_f value (34) on the RP layer, making separation of these two standards impossible.

The major lipid fraction of the lard was TG, as established by TLC on plain silica gel. The individual components that made up this TG band were tentatively classified as either triolein or OSS.

The major lipid component of hen's egg yolk and both the lettuce-fed and yolk-fed snails were also TGs, as shown by TLC on all three types of plates. Argentation TLC allowed classification of these TGs as PSO. Lettuce-fed B. glabrata was seen to contain TG, cholesterol, and phospholipid (remained at the origin) fractions based on TLC on the three types of plates. Argentation TLC showed that the TGs could be tentatively classified as PSO or OSS, while the reversed-phase TLC plate gave indication that either methyl oleate or oleic acid may also be present. The major component of yolk-fed B. glabrata was TG, cholesterol, and phospholipids (remained at the origin) in all three systems. Argentation

chromatography again indicated that the main TG fraction may consist of either PSO or OOS, while the RP data indicated that oleic acid or methyl oleate may be present.

The best separation of TGs was achieved by the mobile phase isopropyl alcohol-chloroform (1.5:98.5) for both the silica gel plates without silver and the silver-impregnated silica gel plates, while acetonitrile-methyl ethyl ketone-chloroform (50:35:15) was the best RP-TLC mobile phase. The best overall separation was achieved using reversed-phase TLC rather than argentation TLC. The best detection for reversed-phase and plain silica gel TLC was with PMA. However, the choice of visualization reagents is somewhat restricted by the presence of $AgNO_3$ (10), and the best detection for argentation TLC was with 50% aqueous sulfuric acid.

REFERENCES

1. Aloisi, J.D., Sherma, J., and Fried, B., J. Liq. Chromatogr. 13, 3949 (1990).

2. Aloisi, J., Fried, B., and Sherma, J. J. Liq. Chromatogr. 14, 3269 (1991).

3. Sherma, J., Whitcomb, B., Shane, P., and Fried, B., J. Planar Chromatogr.-Mod. TLC 4, 326 (1991).

4. Morris, K., Sherma, J., and Fried, B., J. Liq. Chromatogr. 10, 1277 (1987)..

5. Mangold, H.K. (ed.), CRC Handbook of Chromatography: Lipids, vol. 1 (Zweig, G. and Sherma, J., editors-in-chief), CRC Press, Boca Raton, FL, 1984, pp. 387- 397.

6. Nikolova-Damyanova, B. and Amidzhin, B., J. Chromatogr. 446, 283, (1988).

7. Christie, W.W. High Performance Liquid Chromatography and Lipids: A Practical Guide, Pergamon Press, Oxford, 1987, pp. 183-208.

8. Ritchie, A.S, and Jee, M.H., in Recent Advances in Thin-layer Chromatography (Dallas, F.A.A., Read, H., Ruane, R.J., and Wilson, I.D., editors), Plenum Press, New York, 1988, pp. 201-205.

9. Nikolova-Damyanova, B. and Amidzhin, B. J. Planar Chromatogr.-Mod. TLC 4, 397 (1991).

10. Christie, W.W. Lipid Analysis, 2nd ed., Pergamon Press, Oxford, UK, 1982, pp. 144-145.

11. Morris, L.J., J. Lipid Res. 7, 726 (1966).

12. Masterson, C., Fried, B., and Sherma, J., Michrochem.J., in press.

13. Jee, M.H. and Ritchie, A.S., J. Chromatogr. 370, 214 (1986).

14. Mangold, H.K., in Thin-layer Chromatography - A Laboratory Handbook (Stahl, E, editor), Springer-Verlag, New York, NY, 1969, p. 363.

15. Sherma, J. TLC Technical Series, 1 , Whatman Inc., Clifton, NJ, 1987, p. 6.

16. Amidzhin, B. and Nikolova-Damyanova, B., J. Chromatogr. 446, 259-266 (1988).