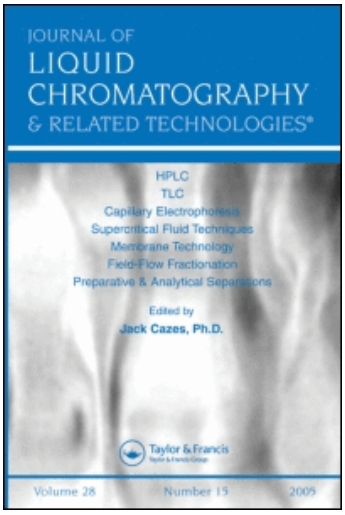

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Jamie Jarusiewicz[a]; Joseph Sherma[a]; Bernard Fried[b]
[a] Department of Chemistry, Lafayette College, Easton, Pennsylvania, USA [b] Department of Biology, Lafayette College, Easton, Pennsylvania, USA

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Separation of Sterols by Reversed Phase and Argentation Thin Layer Chromatography. Their Identification in Snail Bodies

Jamie Jarusiewicz and Joseph Sherma
Department of Chemistry, Lafayette College, Easton, Pennsylvania, USA

Bernard Fried
Department of Biology, Lafayette College, Easton, Pennsylvania, USA

Abstract: Separation of the sterols: cholesterol, cholestanol, β-sitosterol, stigmasterol, ergosterol, campesterol, desmosterol, and brassicasterol was compared using reversed phase, multimodal, and argentation thin layers. Layers tested include C_{18}, $C_{18}W$, NH_2, CN, diol, C_2, C_8, and phenyl bonded layers; hydrocarbon impregnated layers; and commercially prepared silica gel layers precoated with 10% silver nitrate. It was determined that C_{18} layers with acetonitrile–chloroform (40 : 35) or petroleum ether–acetonitrile–methanol (2 : 4 : 4) gave optimal sterol separations. Desmosterol, campesterol, brassicasterol, β-sitosterol, ergosterol, and cholesterol and/or stigmasterol were identified in the bodies of the snails *Biomphalaria glabrata, Helisoma trivolvis*, and *Pomacea bridgesii.*

Keywords: Reversed phase TLC, Argentation TLC, Thin layer chromatography, *Biomphalaria glabrata*, *Helisoma trivolvis*, *Pomacea bridgesii*

INTRODUCTION

In previous studies in our laboratory,[1–10] neutral lipids have been determined using thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) on silica gel layers. It is known that normal

Address correspondence to Joseph Sherma, Department of Chemistry, Lafayette College, Easton, PA 18042, USA. E-mail: shermaj@lafayette.edu

phase silica gel layers cannot separate individual sterols but provide only a single mixed sterol fraction, which was evaluated by comparison to a cholesterol standard zone in this earlier research.

Earlier studies evaluated the separation of individual sterols on chemically bonded C_{18} silica gel TLC layers[11] and by argentation TLC on silica gel layers impregnated by manual dipping into silver nitrate solution.[12–15] In the current study, we have tested the separation of individual sterols on different brands of TLC and HPTLC C_{18} chemically bonded layers; a hydrocarbon-impregnated (nonbonded) layer; and chemically bonded NH_2, CN, diol, C_2, C_8, and phenyl layers. These layers are known to exhibit reversed phase characteristics with mobile phases of different polarities for certain compound types.[16] We also upgraded the argentation TLC by using standardized, commercially prepared silica gel layers precoated with 10% silver nitrate, rather than layers coated in the laboratory. Comparison of these layers, as well as their use in identifying individual sterols in the bodies of the snails *Biomphalaria glabrata, Helisoma trivolvis*, and *Pomacea bridgesii*, are reported.

EXPERIMENTAL

Maintenance of Snails and Preparation of Samples

P. bridgesii snails ranging from 34 to 40 mm in shell length were obtained from Carolina Biological Supply Company (Burlington, NC). Laboratory-raised *B. glabrata* (NMRI strain) snails ranging from 9 to 12 mm in shell diameter and *H. trivolvis* (Colorado strain) snails ranging from 10 to 11 mm in shell diameter were also used for analysis. All snails were maintained at $23 \pm 1^{\circ}C$ in aerated glass jars. *P. bridgesii* snails were maintained with 1 to 3 snails per 800 mL of artificial spring water (ASW).[17] *B. glabrata* and *H. trivolvis* snails were maintained with 10 to 20 snails per 800 mL of ASW. All snails were kept under diffuse overhead fluorescent light for 12 h day^{-1}. All snails were fed *ad libitum* on a boiled Romaine lettuce leaf diet, and food and water were changed three times a week.

For TLC analysis, whole snail bodies were prepared. The snails were gently crushed with a hammer, and the shells were removed with forceps. The opercula of *P. bridgesii* were dissected free of the body and discarded. The whole body samples were prepared by grinding them in a glass homogenizer using a volume of chloroform-methanol (2 : 1) 20 times greater than the sample's mass. After homogenization, the supernatant of each sample was passed through a glass wool filter and treated with *Folch* wash (0.88% KCl) in a volume ¼ that of the chloroform-methanol (2 : 1) used for lipid extraction. The upper hydrophilic layer was discarded using a Pasteur pipet, and the lipophilic layer was dried under nitrogen gas in a water bath at 40°C. *P. bridgesii* samples were reconstituted with 2500–4000 μL of

chloroform-methanol (2 : 1). *B. glabrata* and *H. trivolvis* samples were reconstituted with 500–700 μL of chloroform–methanol (2 : 1).

TLC Analysis

Cholesterol, cholestanol, β-sitosterol, and stigmasterol standards were obtained from Supelco (Bellefonte, PA); campesterol, desmosterol, and brassicasterol standards were obtained from Steraloids, Inc. (Newport, RI); and the ergosterol standard was obtained from Matreya Inc. (Pleasant Gap, PA). The standard solution of β-sitosterol was prepared at a concentration of 0.10 mg mL^{-1} in chloroform-methanol (2 : 1), and all other standard solutions of the sterols were prepared at a concentration of 1.0 mg mL^{-1} in the same solvent. TLC Reference Standard 18-4A (Nu-Chek-Prep, Inc., Elysian, MN), which is composed of equal amounts of cholesterol, oleic acid, triolein, methyl oleate and cholesteryl oleate to provide markers for free sterols, free fatty acids, triacylglycerols, methyl and steryl esters, respectively, was dissolved in chloroform-methanol (2 : 1) to prepare a standard containing 0.20 mg $mL^{-1}$of each compound.

Precoated Nano-SIL C_{18}-100 UV_{254} 10 × 10 cm plates were obtained from Macherey-Nagel (Easton, PA). Precoated reversed phase LKC18 20 × 20 cm, and diphenyl reversed phase 20 × 20 cm plates were obtained from Whatman (Clifton, NJ). High performance RP-18 F_{254S} 10 × 20 cm, RP-18 WF_{254S} 10 × 10 cm, RP-2 F_{254S} 10 × 10 cm, RP-8 F_{254S} 10 × 10 cm, diol F_{254S} 10 × 10 cm, CN F_{254S} 10 × 10 cm, and NH_2 F_{254S} 10 × 10 cm plates were obtained from EMD Chemicals, Inc. (Gibbstown, NJ; an affiliate of Merck KGaA, Darmstadt, Germany). Precoated 10% $AgNO_3$ on silica gel 20 × 20 cm and RPS-F hydrocarbon impregnated silica gel 20 × 20 cm plates were obtained from Analtech (Newark, DE). The 10% $AgNO_3$ and hydrocarbon impregnated plates were used as received from the manufacturer. All other plates were prewashed by development to the top with dichloromethane-methanol (1 : 1) and then air-dried in a fume hood before use.

Standards and samples were applied to the thin layer plates in aliquots ranging from 1.0–8.0 μL with a 10-μL Drummond (Broomall, PA) digital microdispenser. Initial zones were applied as round spots 2 cm up from the bottom edge and 1 cm apart on the plates without a preadsorbent area. They were applied in diffuse, downward streaks on lanes of plates with a preadsorbent area, and band-shaped analyte zones formed automatically at the preadsorbent-sorbent interface during development.

Plates were developed in a rectangular Camag (Wilmington, NC) twin-trough TLC (for 20 × 20 cm plates) or HPTLC (for 10 × 10 cm or 10 × 20 cm plates) chamber to a distance 1 cm from the top of the plate. The plate and mobile phase systems used are listed in Tables 1–3. The volume of mobile phase used was ca. 25 mL in each side of the twin-trough

Table 1. RF values of sterols in various chromatographic systems

Plate	Mobile phase[a]	R_F values							
		Cholesterol	Cholestanol	β-Sitosterol	Stigmasterol	Ergosterol	Campesterol	Desmosterol	Brassicasterol
Whatman C_{18}	1	0.24	0.24, 0.39	0.24	0.25	0.22	0.22	0.22	0.22
Whatman C_{18}	2	0.19	0.18, 0.58	0.18	0.18	0.17	0.12	0.12	0.12
Whatman C_{18}	3	0.36	0.31, 0.43	0.35	0.36	0.41	0.29, 0.32	0.34	0.30
Whatman C_{18}	4	0.21	0.17, 0.29	0.19	0.21	0.26	0.21, 0.24	0.26	0.23
Whatman C_{18}	5	0.31	0.27	0.27	0.30	0.38	0.30, 0.37	0.38	0.33
Merck C_{18}	1	0.27	0.27, 0.49	0.31	0.31	0.26	0.26, 0.34	0.31	0.32
Merck C_{18}	2	0.31	0.31, 0.49	0.36	0.36	0.28	0.29, 0.35	0.32	0.37
Merck C_{18}	3	0.18	0.17, 0.32	0.20	0.21	0.24	0.18, 0.24	0.23	0.19
Merck C_{18}	4	0.085	0.070, 0.16	0.085	0.10	0.12	0.095, 0.14	0.13	0.11
Macherey-Nagel C_{18}	1	0.20	0.17, 0.32	0.17	0.13	0.14	0.21	0.20	0.21
Macherey-Nagel C_{18}	2	0.15	0.13, 0.22	0.090	0.13	0.12	0.27	0.26	0.26
Macherey-Nagel C_{18}	3	0.25	0.24, 0.37	0.19	0.22	0.26	0.28	0.34	0.28
Macherey-Nagel C_{18}	4	0.18	0.11, 0.24	0.095	0.14	0.21	0.22, 0.25	0.29	0.25
10% Silver nitrate	6	0.10	0.12	ND[b]	0.11	0.012	0.12	0.086	0.10
10% Silver nitrate	7	0.65	0.68	ND	0.67	0.57	0.67	0.67	0.68
10% Silver nitrate	8	0.33	0.33	ND	0.33	0.15	0.33	0.32	0.32
10% Silver nitrate	9	0.34	0.33	ND	0.34	0.24	0.33	0.31	0.31
10% Silver nitrate	10	0.22	0.22	ND	0.21	0.065	0.20	0.19	0.19
10% Silver nitrate	11	0.28	0.30	ND	0.30	0.13	0.28	0.26	0.27
10% Silver nitrate	12	0.42	0.42	ND	0.43	0.34	0.43	0.41	0.42
10% Silver nitrate	13	0.44	0.44	ND	0.43	0.22	0.42	0.41	0.40
10% Silver nitrate	14	0.67	0.67	ND	0.70	0.65	0.70	0.67	0.67

10% Silver nitrate	15	0.15	0.17	0.17	0.17	0.012, 0.055	0.18	0.16	0.17
10% Silver nitrate	16	0.20	0.20	0.21	0.20	0.032, 0.065	0.19	0.18	0.18
10% Silver nitrate	17	0.031	0.031	0	0.031	0.019	0.037	0.025	0.031
CN	20	0.81	0.81	ND	0.65, 0.81	0.69, 0.81	0.81	0.81	0.81
CN	21	0.53	0.53	0.53	0.53	0.56	0.53	0.61	0.53
CN	19	0.73	0.73	0.73	0.73	0.71	0.73	0.71	0.73
Hydrocarbon	5	0.52	0.48	ND	0.51	0.58	0.50	0.50	0.55
C_8	5	0.25	0.24	0.22	0.24	0.31	0.24, 0.31	0.33	0.25
Diol	20	0.68	0.68	0.68	0.65	0.65	0.68	0.65	0.65
Diol	22	0.14	0.13, 0.18	0.13	0.13	0.13	0.13	0.13	0.13
RP-18 WF	18	0.046	0.046	0.046	0.046	0.062	0.046	0.046	0.046
RP-18 WF	23	0.17	0.17	0.15	0.15	0.20, 0.35	0.15, 0.21	0.20, 0.35	0.17
RP-18 WF	24	0	0	0	0.036	0.036	0.036	0.036	0.036
Diphenyl	5	0.86	0.86	0.86	0.85	0.85	0.86	0.86	0.86
Diphenyl	25	0.72	0.71	0.72	0.72	0.57, 0.72	0.72	0.72	0.72

[a]Mobile phases.

[b]ND = not detected.

1. Hexane–ethyl acetate (9 : 1).[11] 2. Petroleum ether–diethyl ether–acetic acid (90 : 10 : 1).[11] 3. Acetonitrile–chloroform (40 : 35).[11] 4. Acetonitrile–chloroform–ethyl acetate (55 : 23 : 15).[11] 5. Petroleum ether–acetonitrile–methanol (2 : 4 : 4).[20] 6. Hexane–methylene chloride (7 : 13).[21] 7. Chloroform–methanol (95 : 5).[22] 8. Chloroform–ether–ethyl acetate (97 : 2.5 : 0.5).[22] 9. Petroleum ether–ethyl acetate–toluene (80 : 15 : 5).[22] 10. Chloroform–ether (49 : 1).[23] 11. Chloroform–ether (97 : 3).[23] 12. Hexane–ethyl acetate (7 : 3).[26] 13. Chloroform–acetone (95 : 5).[15] 14. Diethyl ether–hexane (9 : 1).[24] 15. Methylene chloride. 16. Chloroform.[25] 17. Hexane–benzene (1 : 1).[22] 18. Acetonitrile–water (75 : 25).[20] 19. Petroleum ether–acetone (80 : 20). 20. Petroleum ether–acetone (7 : 3).[20] 21. Chloroform–hexane–methanol (25 : 70 : 5).[20] 22. Hexane–acetone–acetic acid (90 : 10 : 1).[20] 23. Methanol–acetone–water (20 : 4 : 3).[20] 24. Methanol–water (80 : 20).[20] 25. Toluene–hexane–acetone (7 : 2 : 1).[20]

Table 2. R_F values of sterols in additional chromatographic systems

Plate	Mobile phase	R_F values for all sterols
NH_2	Chloroform–methanol (93 : 7)[20]	0.69
NH_2	Acetonitrile–water (75 : 25)[20]	0.75
NH_2	Petroleum ether–acetonitrile–methanol (2 : 4 : 4)[20]	0.88
NH_2	Petroleum ether–acetone (80 : 20)	0.67
CN	Chloroform–hexane–methanol (65 : 25 : 10)[20]	0.80
C_8	Petroleum ether–acetonitrile–methanol (30 : 40 : 30)[20]	0.88
Diol	Isooctane–acetone–diethyl ether (3 : 1 : 1)[20]	0.37
C_2	Petroleum ether–acetone (7 : 3)[20]	0.79
C_2	Acetone–hexane (3 : 7)[20]	0.67
RP-18 WF	Water–tetrahydrofuran (75 : 25)[20]	0.046
Diphenyl	Toluene–methanol (6 : 4)[20]	0.82
Diphenyl	Petroleum ether–acetonitrile–methanol (3 : 3 : 4)[20]	0.86
Diphenyl	Toluene–methanol (5 : 5)[20]	0.82

TLC chamber and ca. 13 mL in each side of the HPTLC chamber. The side opposite to that in which the plate was developed contained a saturation pad (Analtech), and the chamber was equilibrated with the mobile phase for 15 min prior to inserting the plate for development. Development times ranged from 10 to 45 min.

Developed plates were air-dried in a fume hood with a steady stream of air from a hair dryer for 5 min, sprayed with a 5% solution of phosphomolybdic acid (PMA) in absolute ethanol, and heated for 10 min at 115°C on a Camag plate heater to detect lipids as brown spots on a yellow background on the 10% $AgNO_3$ plates, and as blue spots on a yellow background on all other plates.

Table 3. Chromatographic systems inadequate for sterol resolution

Plate	Mobile phase
NH_2	Acetone–water (60 : 40)[20]
NH_2	Water–tetrahydrofuran (50 : 50)[20]
NH_2	Water–tetrahydrofuran (75 : 25)[20]
CN	Acetone–water (60 : 40)[20]
Hydrocarbon	Water–tetrahydrofuran (75 : 25)[20]
Diol	Hexane–diethyl ether (99 : 1)[20]
RP-18 WF	Methanol–water (1 : 1)
RP-18 WF	Acetone–water (60 : 40)[20]
RP-18 WF	Water–tetrahydrofuran (75 : 25)[20]
RP-18 WF	Water

RESULTS AND DISCUSSION

The sterols studied were those most likely to be found in the snails based on previous research.[18] It has been proven that thorough homogenization of biological samples with an amount of chloroform–methanol (2 : 1) 20 times the mass of the sample, followed by washing with one-quarter of that volume of 0.88% KCl, leads to complete, consistent recovery of lipids.[19]

Several combinations of thin layer plates and mobile phases allowed for the separation of different sterols. The sterols studied and their R_F values in those chromatographic systems are listed in Table 1. Several combinations of thin layer plates and mobile phases tested did not produce any separation of sterols. The R_F values for all sterols in those systems are listed in Table 2. Additional combinations of thin layer plates and mobile phases were tested, but the sterols either did not move from the origin or migrated with the solvent front. These unsuccessful chromatographic systems are listed in Table 3.

The criteria used to determine the most effective TLC systems for optimal sterol analysis included the degree of separation and compactness of the zones on the layer. The 18-4A standard was used to show that the sterols were separated from other neutral lipid classes, so that they could be reliably identified in the biological samples. It was shown[16] that the polarity of bonded layers decreases in the order silica gel = NH_2 > CN > C_2 > C_8 > C_{18}, and that high polarity mobile phases tended to cause reversed phase conditions and low polarity mobile phases caused normal phase conditions with these layers. It is recognized that certain layers, especially the hydrophilic-bonded NH_2 and CN, can function in reversed phase or normal phase modes depending on the composition of the mobile phase (i.e., they are "multimodal"). A number of the mobile phases we tested were chosen because they gave reversed phase separations of chloroplast pigments from leaves on the different bonded layers in an earlier study; this was indicated by the complete reversal of migration order of the six pigments compared to silica gel layers.[20] However, these mobile phases did not necessarily exhibit reversed phase conditions in the present study with sterols. Normal phase separation of the 18-4A standard is characterized by migration in the order cholesterol (R_F 0.18), oleic acid (0.22), triolein (0.58), methyl oleate (0.70), and cholesteryl oleate (0.83) on silica gel developed with the classic Mangold mobile phase.[10] Some of our tested systems gave the same migration order for the 18-4A standard as silica gel, while others indicated at least a partially reversed phase character [e.g., oleic acid migrated behind cholesterol on a Whatman C_{18} layer developed with petroleum ether–acetonitrile–methanol (2 : 4 : 4)]. We showed earlier that the reversed phase separation of amino acids is a complicated process that cannot be explained by traditional, idealized mechanisms of "reversed" or "normal" phase TLC.[27] The same is true in this study for sterol separations on the polar and nonpolar layers studied. Argentation TLC is based on the charac-

teristic property of unsaturated organic compounds to form transient charge-transfer complexes with transition metals in general, and with silver ions in particular; it is used mainly to improve the selectivity of silica gel for the separation of fatty acid derivatives, triacylglycerols, and sterols among other compound classes.

Of all the layers tested, C_{18} layers were best for separation of sterols; in particular, C_{18} layers with the mobile phases acetonitrile–chloroform (40 : 35)[11] or petroleum ether–acetonitrile–methanol (2 : 4 : 4)[20] gave optimal sterol separations. While the Merck high performance C_{18} plates provided the most compact standard zones among the three brands of C_{18} plates, the Whatman C_{18} plate did not produce as much streaking of sample aliquots. Also, the Whatman C_{18} plate had a preadsorbent zone, but the Macherey-Nagel C_{18} plate did not. Therefore, the Whatman C_{18} plate was used for identification of sterols in the snail bodies. The C_8 thin layer with petroleum ether–acetonitrile–methanol (2 : 4 : 4)[20] mobile phase also produced separation of β-sitosterol and desmosterol from all other sterol standards tested. Argentation TLC on Analtech precoated plates developed with chloroform–ether (97 : 3)[23] and with chloroform–acetone (95 : 5)[15] produced adequate separation of ergosterol from all other sterol standards. However, these plates became brown in color and lost their resolving capabilities after 3–4 weeks. Chromatographic systems employing NH_2 thin layers were not useful for the separation of any of the sterol standards. Because NH_2 thin layers are similar in polarity to silica gel, this suggests that polar stationary phases are not useful in the separation of sterols. Many of the other chromatographic systems that employed thin layers of intermediate polarity, such as CN thin layer plates with mobile phase 19 in Table 1, produced minor separation between sterols, but were inadequate for identification of sterols in snail bodies.

Qualitative identification of the sterols present in the snail bodies was achieved by comparison of the R_F values of standard and sample zones. In *B. glabrata, H. trivolvis*, and *P. bridgesii* samples, a dark blue spot developed at R_F values comparable with the cholesterol, stigmasterol, β-sitosterol, desmosterol, and campesterol standards on Whatman C_{18} plates using mobile phase 3 from Table 1. Resolution of the cholesterol-stigmasterol pair was not adequate to enable more definite sterol identification in this chromatographic system. A previous study[18] used HPLC to show the presence of cholesterol, stigmasterol, β-sitosterol, desmosterol, and campesterol in hepatopancreas of *B. glabrata.* A very light blue spot was also detected at an R_F comparable to the brassicasterol standard in all three species of snail when this chromatographic system was employed. On Whatman C_{18} plates developed with mobile phase 5, a dark blue spot with the same mobility as the cholesterol, stigmasterol, and campesterol standards was detected in all three snails. Resolution of these sterols in the sample aliquots was not sufficient to enable more definite determination of sterol composition. A lighter blue spot at an R_F comparable with that of the β-sitosterol and choles-

tanol standards was also detected in *B. glabrata* and *H. trivolvis* samples using this chromatographic system, but resolution of this pair of sterols was not sufficient for more definite identification. Using argentation TLC with mobile phase 13, a light brown spot was detected in *B. glabrata, H. trivolvis*, and *P. bridgesii* samples at an R_F value comparable with that of the ergosterol standard. Although ergosterol was not identified in the previous study on *B. glabrata*,[18] that study did not examine whole snail bodies. Ergosterol serves as a precursor of Vitamin D and was determined to be important for the metabolism of another snail species, *Helix aspersa*, in an earlier study.[28] Therefore, ergosterol may be metabolically important in *B. glabrata, H. trivolvis*, and *P. bridgesii* as well.

CONCLUSIONS

This is the first comprehensive study of sterol separations by TLC and HPTLC on a wide selection of commercially prepared impregnated and chemically bonded reversed phase, multimodal, and complexation stationary phases. Twelve different types and brands of precoated plates and a variety of mobile phases chosen from the literature as being most likely to provide conditions allowing separation of eight representative sterol standards were tested and compared. The results were applied to the detection and identification of individual sterols in snail samples in order to complement our earlier studies in which sterols were determined only as a single, mixed fraction. The R_F data tabulated for the sterols will be useful for workers needing to evaluate layer-mobile phase combinations for analyzing other sample types for these and additional sterols.

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