An Improved Reversed-Phase Thin-Layer Chromatography Method for Separation of Fatty Acid Methyl Esters

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ABSTRACT: Resolution of fatty acid methyl esters (FAME) by thin-layer chromatography often is complicated by co-migration of certain acyl-isomers in heterogeneous mixtures. However, a novel reversed-phase thin-layer chromatography method which employs 10% (wt/vol) silver nitrate in a mobile phase containing acetonitrile/1,4-dioxane/acetic acid (80:20:1, vol/vol/vol) allows one-dimensional resolution of a wide range of acyl-methyl esters. This innovation enables improved separation of saturated FAME ranging from C_{12} to C_{22}, and geometric isomers of C_{14} to C_{22} unsaturated FAME by thin-layer chromatography.
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KEY WORDS: Argentation chromatography, fatty acid methyl esters, reversed-phase thin-layer chromatography, separation.

Investigation of lipid composition in plant tissues frequently involves preparation of fatty acid methyl esters (FAME) derived from glycerolipids. FAME analysis and separation typically are achieved by a variety of chromatography methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) (1–7). The method of choice depends on the objectives of the investigation and available resources. When TLC is practical, a two-dimensional approach typically is required to obtain acceptable resolution of certain FAME. As an example, silica gel impregnated with silver nitrate ($AgNO_3$) in a normal phase system may be used to separate FAME by degree of unsaturation. In this system, saturated FAME advance farthest, then monounsaturated, and finally polyunsaturated acyl methyl esters. Resolution of saturated FAME may then be enhanced by employing C_{18} reversed-phase gels (RPTLC) in a second dimension where separation depends on carbon number. On RPTLC, FAME interact with a polar solvent on a nonpolar stationary phase in a manner that allows more rapid migration of molecules with greater polarity and lower mass. Both techniques usually are necessary because different FAME containing the same degree of unsaturation are not resolved well by $AgNO_3$-TLC, and FAME like 16:0 and Δ9*c*-18:1 tend to co-migrate in RPTLC systems. Thus, $AgNO_3$-TLC may be used to separate FAME by number of unsaturated bonds in one dimension, and reversed-phase TLC may be used to separate FAME by carbon number in a second dimension. FAME may then be visualized by a number of detection methods (4,5) and subsequently quantified by GC or scraped into scintillation vials if the sample is radiolabeled.

Given the desirable attributes of each method in the traditional two-dimensional approach, an attempt was made to improve the logistical efficiency of FAME resolution by TLC. A one-dimensional TLC system was developed using silver ions within the mobile phase and a reversed-phase C_{18} (*n*-octadecylsilyl) silica stationary phase. Although addition of silver ions in the mobile phase of reversed-phase TLC has been reported (8), this innovation enabled an improved single-dimensional TLC method with the capability of resolving a wide range of FAME standards, including geometric isomers of unsaturated acyl methyl esters.

MATERIALS AND METHODS

Twenty-four FAME standards with greater than 99% purity were purchased from Sigma-Aldrich (St. Louis, MO) or Alltech Associates, Inc. (Deerfield, IL). Saturated acyl methyl ester standards included lauric acid (12:0), tridecyclic acid (13:0), myristic acid (14:0), pentadecylic acid (15:0), palmitic acid (16:0), margaric acid (17:0), stearic acid (18:0), nondecyclic acid (19:0), arachidic acid (20:0), heneicosanoic acid (21:0), and behenic acid (22:0). Monounsaturated acyl methyl ester standards included myristoleic acid (9*c*-14:1), myristelaidic acid (9*t*-14:1), palmitoleic acid (9*c*-16:1), palmitelaidic acid (9*t*-16:1), oleic acid (9*c*-18:1), elaidic acid (9*t*-18:1), erucic acid (13*c*-22:1), and brassidic acid (13*t*-22:1). Diunsaturated acyl methyl ester standards included: linoleic acid (9*c*,12*c*-18:2), linoelaidic acid (9*t*,12*t*-18:2), and eicosadienoic acid (11*c*,14*c*-20:2). Triunsaturated acyl methyl ester standards included linolenic acid (9*c*,12*c*,15*c*-18:3) and eicosatrienoic acid (11*c*,14*c*,17*c*-20:3). All standards were made to 10 mg/mL in solvents specified by the manufacturer. A composite sample containing 20% (w/w) each of 16:0, 18:0, 9*c*-18:1, 9*c*,12*c*-18:2, and 9*c*,12*c*,15*c*-18:3 was prepared to simulate the major FAME derived from soybean oil. FAME

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derived from palm, soybean, peanut, and olive oils also were compared at their natural concentration.

$AgNO_3$ RP TLC. Typically 10 µg of each FAME standard was applied in 2-cm lanes on Whatman (Maidstone, United Kingdom) LKC_{18} Preabsorbent reversed-phase TLC plates (20 cm × 20 cm × 200 µm). A maximum of 300 µg total FAME could be applied per lane. Plates were developed in one of the following mobile phases: solvent A, acetonitrile (4); solvent B, acetonitrile/acetic acid/water [70:10:10, vol/vol/vol; (9)], or solvent C, 10% $AgNO_3$ in acetonitrile/1,4-dioxane/acetic acid (80:20:1, vol/vol/vol; determined experimentally). Data are presented for plates developed for 1 h at ambient conditions or for 3 h at 2°C. FAME were visualized with 0.03% 1,6-diphenyl-2,3,5-hexatriene (DPH) in chloroform (Sigma-Aldrich) on air-dried plates. All FAME standards appeared as blue-white spots on a violet background under ultraviolet light (365 nm). Relative migration (R_f) was determined and each FAME was extracted from the *n*-octadecylsilyl silica stationary phase sequentially with: 2.5 mL chloroform/methanol (2:1, vol/vol); 2.5 mL chloroform/methanol (1:2, vol/vol), and 2.5 mL methanol. Eluates were collected under vacuum, dried under N_2, and dissolved in 50 µL chloroform/methanol (2:1, vol/vol). Identity and purity of each extract were determined by GC (10) using a Hewlett-Packard (Palo Alto, CA) 5890-II equipped with a model 7673 auto sampler, dual flame-ionization detectors, and a 0.53 mm × 30 m AT-Silar capillary column (Alltech). Operating conditions were: carrier, He (3 mL/min); 25:1 (vol/vol) split injection; injection temperature, 250°C; detector temperature, 275°C; column temperature, 190°C. Images of RPTLC plates were made with a Stratagene Eagle-Eye Developing/Processing system (La Jolla, CA). All data were reported as the mean of at least three replications.

RESULTS AND DISCUSSION

Performance of one experimental and two published solvents used with single-dimensional RPTLC systems were compared using a mixture of FAME standards representing the major constituents of soybean oil. As shown in Table 1, all three solvents resolved methyl esters of 18:0, Δ9*c*,12*c*-18:2, and Δ9*c*,12*c*,15*c*-18:3 in mixture, but 16:0 and Δ9*c*-18:1 co-migrated on plates developed in solvent A and solvent B. This problem was overcome with solvent C.

Solvent C differed from other published mobile phases for one-dimensional RPTLC systems in that $AgNO_3$ was added to the developing solvent instead of being impregnated in the stationary phase. Coupling the properties of $AgNO_3$ and reversed-phase TLC in this manner facilitated separation of individual FAME by degree of unsaturation as well as by carbon number. In theory, relatively free association of silver ions with the sample on a reversed-phase gel should enhance resolution among unsaturated and saturated FAME plus accentuate migration of unsaturated FAME varying in double-bond number and isomeric configuration. In this RPTLC system, 1,4-dioxane was used to change the absorptive properties of the *n*-octadecylsilyl stationary surface in a manner that facilitated either interaction of proton-donor solutes with oxygen atoms in the solvent film (co-adsorption) or displacement of solvent molecules from the absorbent surface (11–13). Overall, properties of 1,4-dioxane further enabled the resolution of saturated FAME differing by only one carbon residue.

Table 2 demonstrates the separation achieved with 19 different FAME standards at ambient conditions. This system gave exceptional resolution of C_{12} to C_{22} saturated FAME, and geometric isomers of C_{14} to C_{22} monounsaturated FAME. Potential co-migration events may be anticipated between: (12:0, Δ9*c*-14:1, and Δ9*c*,12*c*,15*c*-18:3), (13:0 and Δ9*t*-14:1), (Δ9*c*-16:1, Δ9*c*,12*c*-18:2, and Δ11*c*,14*c*,17*c*-20:3), (14:0, Δ9*t*-16:1, and Δ9*t*,12*t*-18:2), (15:0 and Δ9*c*-18:1), (16:0 and Δ9*t*-18:1), or (19:0 and Δ13*c*-22:1). However, as shown in Table 3, the primary problems observed in application of this system to selected oilseeds appears to be limited to detection of FAME at concentrations less than 0.2% (w/w), separation of 16:0 from low concentrations of Δ11*c*-20:1, and co-migration of Δ9*c*-16:1 and Δ9*c*,12*c*-18:2 (olive oil). Although variation in temperature and humidity during plate preparation and development often influence FAME migration, such factors did not appear to be a significant problem. Indeed, plate develop-

TABLE 1
Comparison of Reversed-Phase Thin-Layer Chromatographic (RPTLC) Systems for Resolution of Fatty Acid Methyl Esters

Methyl ester[a]	Solvent A[b]	Solvent B[c]	Solvent C[d]
		R_f	
18:0	0.30	0.08	0.28
16:0	0.40	0.15	0.39
18:1*c*	0.40	0.15	0.46
18:2*c*	0.52	0.23	0.58
18:3*c*	0.62	0.32	0.70

[a]18:1*c*, Methyl oleate; 18:2*c*, methyl linoleate; 18:3*c*, methyl linolenate.
[b]100% Acetonitrile.
[c]Acetonitrile/acetic acid/water (70:10:10, vol/vol/vol).
[d]10% silver nitrate in acetonitrile/dioxane/acetic acid (80:20:1, vol/vol/vol). $LSD_{0.05}$, 0.06 among and between treatments; at ambient conditions.

TABLE 2
Resolution of Other Fatty Acid Methyl Ester (FAME) Species and Isomers by the Proposed Ag-RPTLC System[a]

Saturates	R_f	Monoene	R_f	Diene/triene	R_f
12:0	0.72	14:1*c*	0.70	18:2*t*	0.54
13:0	0.62	14:1*t*	0.63	20:2*c*	0.48
14:0	0.52	16:1*c*	0.57	20:3*c*	0.58
15:0	0.45	16:1*t*	0.52		
17:0	0.33	18:1*t*	0.41		
19:0	0.24	22:1*c*	0.32		
20:0	0.20	22:1*t*	0.27		
21:0	0.18				
22:0	0.15				

[a]Solvent, 10% silver nitrate in acetonitrile/1,4-dioxane/acetic acid (80:20:1, vol/vol/vol) at ambient conditions. Methyl esters: 14:1*c*, myristoleic, 14:1*t*, myristelaidic, 16:1*c*, palmitoleic; 16:1*t*, palmitelaidic, 18:1*t*, elaidic; 22:1*c*, erucic; 22:1*t*, brassidic; 18:2*t*, linoelaidic; 20:2*c*, eicosadienoic; 20:3*c*, eicosatrienoic. Concentration of each methyl ester was 10 mg·mL^{-1}. $LSD_{0.05}$, 0.04 among and between R_f values. For abbreviation see Table 1.

TABLE 3
Ag-RPTLC[a] Separation of FAME Constituents of Various Oilseeds

	Palm oil		Soybean		Peanut		Olive	
Methyl ester[b]	%	R_f	%	R_f	%	R_f	%	R_f
12:0	0.1	**ND**[c]	ND	ND	ND	ND	ND	ND
14:0	1.2	0.52	ND	ND	ND	ND	ND	ND
16:0	46.8	**0.39**	10.5	**0.39**	11.0	0.39	16.9	0.39
16:1*c*	ND	ND	0.1	**ND**	ND	ND	1.8	**0.57**
18:0	3.8	0.28	3.2	0.28	2.3	0.28	2.7	0.28
18:1*c*	37.6	0.46	22.3	0.46	51.0	0.46	61.9	0.46
18:2*c*	10.0	0.58	54.5	0.58	30.9	0.58	14.8	**0.58**
18:3*c*	ND	ND	8.3	0.70	ND	ND	0.6	0.70
20:0	0.2	0.20	0.2	0.20	0.7	0.20	0.4	0.20
20:1	0.3	**0.38**	0.9	**0.38**	ND	ND	0.1	ND
22:0	ND	ND	ND	ND	2.3	0.15	0.2	0.15
24:0	ND	ND	ND	ND	0.8	**ND**	ND	ND

[a]10% silver nitrate in acetonitrile/dioxane/acetic acid (80:20:1, vol/vol/vol).
[b]Typical fatty acid composition, % total lipid (14).
[c]Not detected or trace levels; co-migration or detection limitations are highlighted in bold print. $LSD_{0.05}$, 0.04 among and between R_f values; at ambient conditions. For abbreviations see Tables 1 and 2.

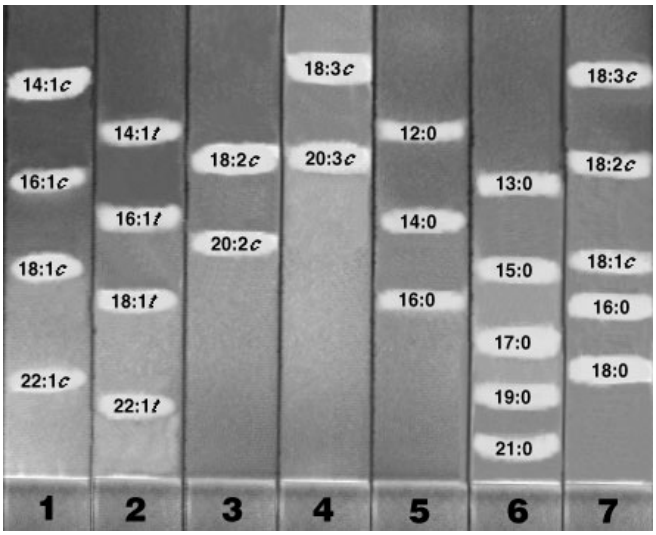

FIG. 1. Separation of fatty acid methyl esters (FAME) species and geometric isomers by a novel one-dimensional argentation reversed-phase thin-layer chromatography (RPTLC) system. FAME standards (10 mg·mL^{-1}) were applied in 2-cm lanes on Whatman (Maidstone, United Kingdom) LKC_{18} Preabsorbent Reverse-Phase TLC plates (20 cm × 20 cm × 200 µm). Plates were developed with 10% $AgNO_3$ in acetonitrile/1,4-dioxane/acetic acid (80:20:1, vol/vol/vol) for 3 h at 2°C. FAME were visualized with 0.03% 1,6-diphenyl-2,3,5-hexatriene (DPH) in chloroform. (Top to Bottom) Lane 1: myristoleic acid (Δ9*t*-14:1), palmitoleic acid Δ9*t*-16:1), oleic acid (Δ9*c*-18:1), erucic acid (Δ13*t*-22:1); Lane 2: myristelaidic acid (Δ9*t*-14:1), palmiteladic acid (Δ9*t*-16:1), elaidic acid (Δ9*t*-18:1), brassidic acid (Δ13*t*-22:1); Lane 3: linoleic acid (Δ9*c*,12*c*-18:2) and eicosadienoic acid (Δ11*c*,14*c*-20:2); Lane 4: linolenic acid (Δ9*c*,12*c*,15*c*-18:3) and eicosatrienoic acid (Δ11*c*,14*c*,17*c*-20:3); Lane 5: lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0); Lane 6: tridecyclic acid (13:0), pentadecylic acid (15:0), margaric acid (17:0), nondecyclic acid (19:0), heneicosanoic acid (21:0); Lane 7: linolenic acid (Δ9*c*,12*c*,15*c*-18:3), linoleic acid (Δ9*c*,12*c*-18:2), oleic acid (Δ9*c*-18:1), palmitic acid (16:0), stearic acid (18:0). Not shown: arachidic acid (20:0), behenic acid (22:0), linoelaidic acid (Δ9*t*,12*t*-18:2).

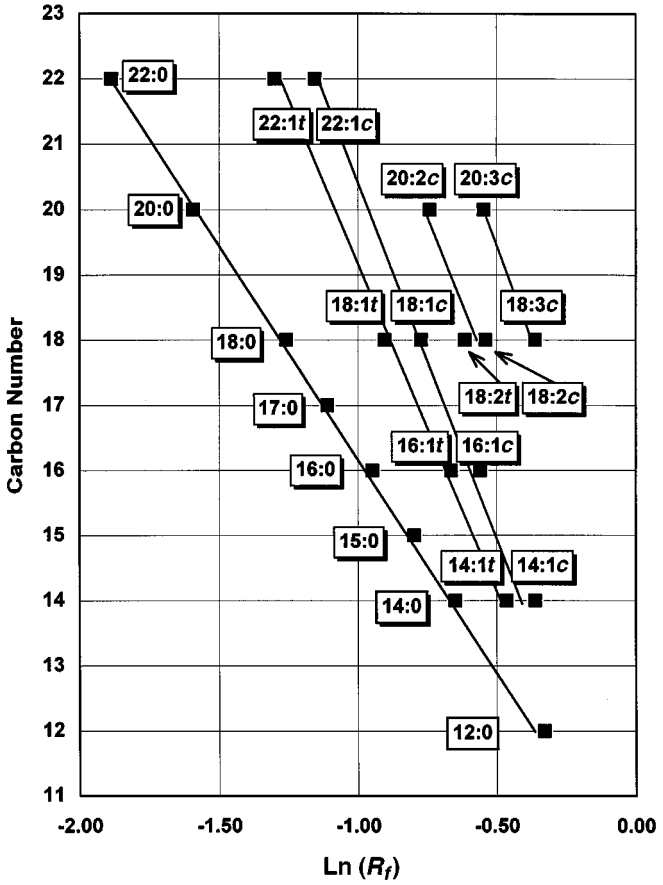

FIG. 2. Linear relations in resolution among FAME species and geometric isomers based on carbon number. Plates were developed with 10% $AgNO_3$ in acetonitrile/1,4-dioxane/acetic acid (80:20:1, vol/vol/vol) for 1 h at ambient conditions. Equations derived from these data were: $y = -6.40\ (x) + 9.89$ (R^2, 0.99) for saturated FAME, $y = -10.05(x) + 10.35$ (R^2, 0.99) for *cis* isomers of monounsaturated FAME, and $y = -9.49(x) + 9.60$ (R^2, 0.99) for *trans* isomers of monounsaturated FAME, where y represents carbon number and x represents $\ln(R_f)$. For abbreviations see Figure 1.

ment at 2 to 3°C generally gave better resolution of the tested FAME (Fig. 1). It should be noted that higher molecular weight saturated FAME may coalesce or precipitate at lower temperatures. Given these considerations, the proposed RPTLC system appears to be an extremely versatile method for separation of FAME.

In addition, the proposed method may be useful for qualitative identification of unknown FAME in heterogeneous mixtures. As shown in Figure 2, a series of independent linear relations were found between saturated and geometric isomers of monounsaturated FAME when carbon number was regressed against logarithmic transformed R_f values. Equations derived from these regressions were: $y = -6.40(x) + 9.89$ (R^2, 0.99) for saturated FAME, $y = -10.05(x) + 10.35$ (R^2, 0.99) for *cis* isomers of monounsaturated FAME, and $y = -9.49(x) + 9.60$ (R^2, 0.99) for *trans* isomers of monounsaturated FAME, where y represents carbon number and x represents $\ln(R_f)$. Similar relations may exist among geometric isomers of di- and triunsaturated FAME.

In conclusion, the proposed RPTLC method achieves separation of 16:0 and Δ9*c*-18:1, which is the primary limitation in analysis of FAME from oilseeds by TLC. To our knowledge, this innovation represents a novel one-dimensional RPTLC system capable of resolving a wide range of FAME standards.

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