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IMPROVED SEPARATION OF LIPID ESTERS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Methods for the separation of ethyl- and 2-chloroethyl esters of fatty acids using argentation thin-layer chromatography (TLC) and linear and circular reversed-phase TLC are described. An argentation TLC method for the separation of cholesterol esters is also described. The separation is based on the degree of unsaturation in the fatty acid moiety. In all instances good and reproducible resolution was achieved by unidirectional single developments at room temperature with reduced analysis times (9 min for argentation TLC, 7 min for circular reversed-phase TLC).

INTRODUCTION

It is often difficult to separate lipids which differ from each other only in the degree of unsaturation by conventional thin-layer chromatographic (TLC) methods. Such methods are either time consuming or the resolution is sometimes poor. This difficulty has been overcome by the use of argentation TLC. Since its conception in the early 1960s, numerous applications of argentation TLC have been reported[1–9]. These include the separation of neutral lipids, phospholipids, cholesterol esters and fatty acid esters. In several instances the separations have been achieved using multiple developments, in either the same or different solvent systems[1,6,10–13] or at relatively low temperatures (2 to −25°C)[6,14]. Usually the separation takes 60–120 min and may result in sample degradation during the intermediate drying step, between successive developments.

Recently, high-performance thin-layer chromatographic (HPTLC) methods including normal-phase (NP)[15–19], reversed-phase (RP)[20,21] and over-pressured (OP) TLC[12,22] have been used for the separation of lipids. The high resolving power and homogeneity of the sorbent ensures excellent and reproducible separation of the lipids. The total material applied to HPTLC plates is generally low and the analysis is fast. We have used silver nitrate-impregnated HPTLC plates for the separation of cholesterol esters. For the separation of ethyl- and 2-chloroethyl esters of fatty acids, in addition to argentation TLC we have also developed linear and circular reversed-phase HPTLC methods. These methods are fast, reproducible and do not require multiple developments or low temperatures.

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Fatty acid ethyl esters have recently been described as enzymic products of non-oxidative metabolism of ethanol in the heart[23] and brain[24], and these esters may represent mediators and/or markers of ethanol-induced organ pathology such as alcoholic cardiomyopathy[25]. Fatty acid chloroethyl esters (FACEs) were first reported in foods[26,27] resulting from ethylene oxide fumigation[28] and have been found in high levels in foods by the U.S. Food and Drug Administration[29,30]. 2-Chloroethyl stearate has recently been isolated from rat liver after feeding the animal with 2-chloroethanol[31]. FACEs have been shown to release ethylene chlorohydrin (ECH) in digestive systems and this is the focus of toxicological concern associated with FACE residues in foods[32].

The TLC methods described in this paper were developed with the aim of analyzing different tissue samples for the formation of ethyl- and 2-chloroethyl esters of fatty acids and these methods can be applied in the analysis of food samples.

EXPERIMENTAL

Solvents and materials

Analytical-reagent grade solvents and materials were used. *n*-Hexane, methanol and acetonitrile were purchased from Fisher Scientific (Houston, TX, U.S.A.), diethyl ether and acetic acid from Mallinckrodt (Paris, KY, U.S.A.), isopropanol from Curtin Matheson Scientific (Houston, TX, U.S.A.) and chloroform from J. T. Baker (Phillipsburg, NJ, U.S.A.). Fatty acids such as stearic, oleic, linoleic and linolenic and their corresponding ethyl esters and cholesterol esters were bought from Sigma (St. Louis, MO, U.S.A.) and 2-chloroethanol from Aldrich (Milwaukee, WI, U.S.A.).

HPTLC plates (glass), 10 × 10 cm, coated with silica gel and inorganic binder (HPTLC-GHLF) and high-performance reversed-phase (HPTLC-RP 18F) plates were obtained from Analtech (Newark, DE, U.S.A.).

Synthesis of fatty acid chloroethyl esters (FACEs)

To 1 g of stearic acid were added 10 ml of 2-chloroethanol and three drops of concentrated hydrochloric acid and the reaction mixture was warmed on a water-bath (50°C) for 4 h. The progress of the reaction was monitored by TLC. After the reaction was complete, 30 ml of methanol were added to the reaction mixture and cooled. The crystals formed were filtered under vacuum, dried (yield 90%) and characterized by proton nuclear magnetic resonance and chemical ionization mass spectrometry (data not shown). FACEs of oleic, linoleic and linolenic acids were synthesized in a similar manner but the crystallization step was omitted and these esters were purified on preparative silica gel TLC plates (500 μm) using *n*-hexane–diethyl ether–methanol–acetic acid (20:4:1:0.4, v/v) as the mobile phase. The TLC-purified samples were further purified by reversed-phase high-performance liquid chromatography (HPLC) on an Ultrasphere ODS column (5-μm particle size, 25 cm × 4.6 mm I.D.) using methanol–water (23:2, v/v) as the mobile phase at a flow-rate of 1 ml/min.

Standards

The standards were prepared in *n*-hexane at a concentration of 10 μg/μl, except for ethyl stearate (20 μg/μl). The standard mixture contained final concentrations of 20 μg/μl ethyl stearate, 10 μg/μl ethyl oleate, 10 μg/μl ethyl linoleate and 10 μg/μl ethyl

linolenate. 2-Chloroethyl ester and cholesterol ester standards were prepared at the same concentration.

Preparation of silver nitrate-impregnated plates

Silica gel HPTLC plates were dipped in 10% aqueous silver nitrate solution for 10 min, the plates were removed, the excess of solution allowed to drain and the plates were dried at 50°C for 15 min and activated by heating at 110°C for 1 h. The plates were stored in dark dry-boxes containing anhydrous calcium chloride.

Chromatographic procedures

Linear TLC developments were carried out in a glass chamber (inside dimensions height 23 cm × diameter 10.5 cm) at 25°C. The chromatographic chamber was presaturated for 30 min with the solvent used for subsequent development. Authentic standards were applied 5 mm from the origin of the TLC plates using 1-μl disposable pipettes (Drummond Microcaps). All the standards were applied at a concentration of 10 μg/μl (total applied volume 1 μl), except for the esters of stearic acid (20 μg/μl). The solvent was allowed to run up to 2 mm below the top edge of the plate. Circular chromatography was conducted using equipment purchased from Analtech. The sample was applied in the center of a 10 × 10 cm high-performance reversed-phase plate. In the circular developing system, the solvent moves from the solvent reservoir to the center of the TLC plate through a porous wick. The solvent then moves from the center of the plate to the periphery in a circular manner. The chromatography is stopped by lifting the TLC plate along with the wick from the solvent reservoir. A 3-ml volume of the solvent was sufficient for the circular chromatographic separations.

Detection

NP-HPTLC plates were sprayed with copper (II) sulfate reagent [10% (w/v) copper (II) sulfate in 8% (v/v) phosphoric acid][12] and detected by heating at 100–120°C for 5 min. Lipids on high-performance reversed-phase plates were revealed by exposure to iodine vapor. Esters of saturated fatty acids do not develop an intense yellow color on exposure to iodine and may not be seen clearly in the photographs of TLC plates.

RESULTS AND DISCUSSION

Separation of fatty acid ethyl esters (FAEEs) and fatty acid 2-chloroethyl esters (FACEs)

Separation of ethyl stearate (I), ethyl oleate (II), ethyl linoleate (III) and ethyl linolenate (IV) was accomplished on HPTLC plates impregnated with silver nitrate (Fig. 1) using *n*-hexane–diethyl ether–methanol (18.5:1.5:0.5, v/v/v) as the mobile phase. Table I shows the effect of variations in the proportion of methanol on the R_F values of FAEEs and cholesterol esters. As is evident, all the FAEEs can be separated from each other even if methanol is omitted from the mobile phase, but the esters with a higher degree of unsaturation (III and IV) are not resolved well from each other and their R_F values are very low; ethyl linolenate barely moves from the baseline (R_F 0.04). In contrast, if the proportion of methanol is increased to 1.0 ml, the four

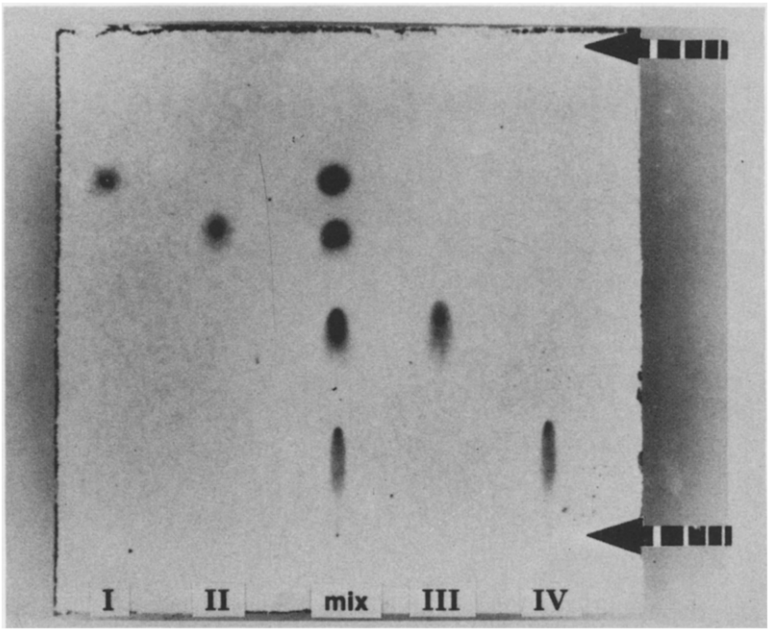

Fig. 1. Separation of fatty acid ethyl esters on silver nitrate-impregnated HPTLC plates. Separation time, 9 min (mix = mixture of I, II, III and IV); 20 μg of I and 10 μg each of II, III and IV were applied on the plate. The solvent system used and the names of the compounds are given in the text.

FAEEs are still separated from each other, the R_F value of compound I decreases while those of II, III and IV remain unchanged in comparison with when 0.5 ml of methanol is used. If the plates are not impregnated with silver nitrate, compounds I–IV cannot be resolved.

Fatty acid chloroethyl esters were separated (Fig. 2) with the same solvent system as used for the separation of FAEEs. The R_F values of FACEs in this solvent system are 2-chloroethyl stearate (V) 0.65, 2-chloroethyl oleate (VI) 0.52, 2-chloroethyl linoleate (VII) 0.34 and 2-chloroethyl linolenate (VIII) 0.12. These R_F values

TABLE I

EFFECT OF VARIATION IN THE PROPORTION OF METHANOL ON THE R_F VALUES OF FATTY ACID ETHYL ESTERS AND CHOLESTEROL ESTERS, USING 10% $AgNO_3$-IMPREGNATED HPTLC (NORMAL-PHASE) PLATES

Mobile phase composition (n-hexane–diethyl ether–methanol, v/v/v)	R_F							
	I	*II*	*III*	*IV*	*IX*	*X*	*XI*	*XII*
18.5:1.5:0	0.45	0.22	0.10	0.04	0.63	0.31	0.11	0.03
18.5:1.5:0.5	0.70	0.59	0.40	0.14	0.85	0.78	0.64	0.24
18.5:1.5:1.0	0.66	0.58	0.40	0.13	0.79	0.67	0.40	0.11

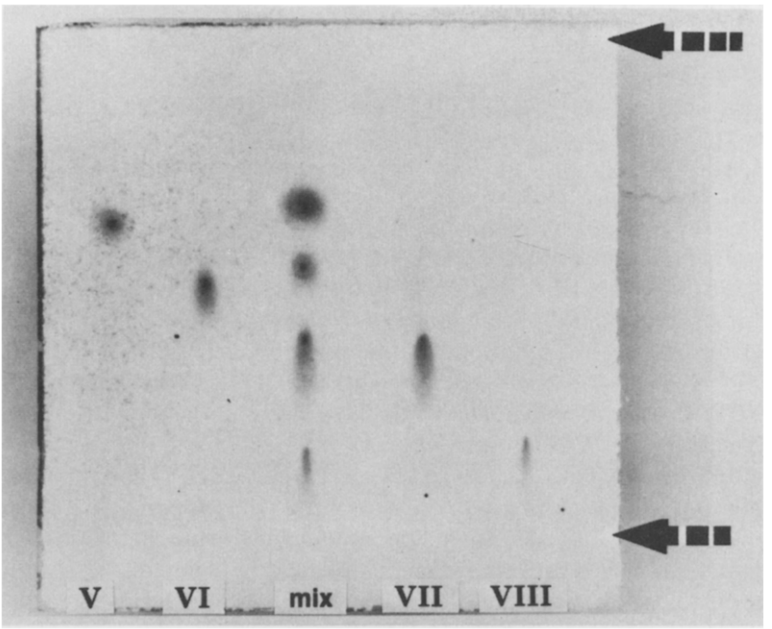

Fig. 2. Separation of fatty acid chloroethyl esters on silver nitrate-impregnated HPTLC plates. Separation time, 9 min (mix = mixture of V, VI, VII and VIII); 20 μg of V and 10 μg each of VI, VII and VIII were applied on the plate.

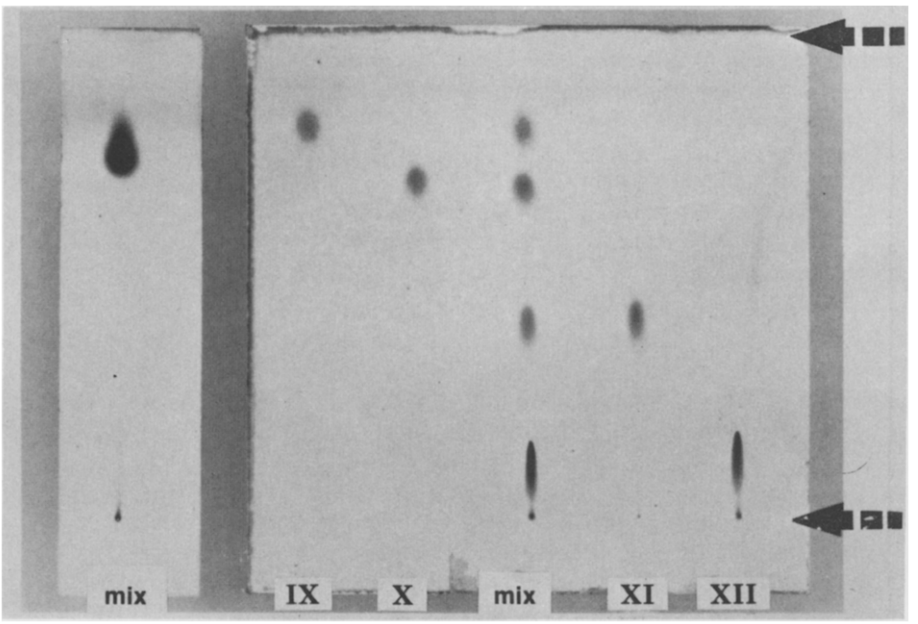

Fig. 3. Separation of cholesterol esters on silver nitrate-impregnated HPTLC plates. Separation time, 9 min (mix = mixture of IX, X, XI and XII); 10 μg each of X, XI and XII and 20 μg of IX were applied on the plate. The panel on the left shows that the mixture could not be resolved without silver nitrate impregnation.

are slightly lower than that of the corresponding FAEEs. This observed decrease may be due to the increase in the polarity of the compounds when hydrogen is replaced with the polar chlorine atom.

Separation of cholesterol esters

Cholesterol stearate (IX), oleate (X), linoleate (XI) and linolenate (XII) were also resolved from each other (Fig. 3) using *n*-hexane–diethyl ether–methanol (18.5:1.5:1, v/v/v) as the mobile phase on silver nitrate-impregnated HPTLC plates. As is evident from Table I, cholesterol esters could also be resolved with the same solvent systems that resolved FAEEs. This shows that the separation is mostly on the basis of the unsaturation in the fatty acid moiety of cholesterol esters. There was no separation if silver nitrate was not used for the impregnation of plates.

Reversed-phase HPTLC separation of FAEEs and FACEs

FAEEs I–IV and FACEs V–VIII were separated on HPTLC (RP) plates (Figs. 4 and 5) using acetonitrile–methanol–acetic acid–water (15:5:2:0.5, v/v) as the mobile phase. The R_F values of FAEEs using different solvent systems for reversed-phase chromatography are shown in Table II. There does not seem to be much difference in separation if methanol is replaced with isopropanol and the proportion of acetonitrile is reduced from 15 to 10%. In the solvent system acetonitrile–methanol–acetic acid–water (15:5:2:0.5, v/v) the R_F values of different FACEs were V 0.29, VI 0.41, VII 0.50 and VIII 0.59.

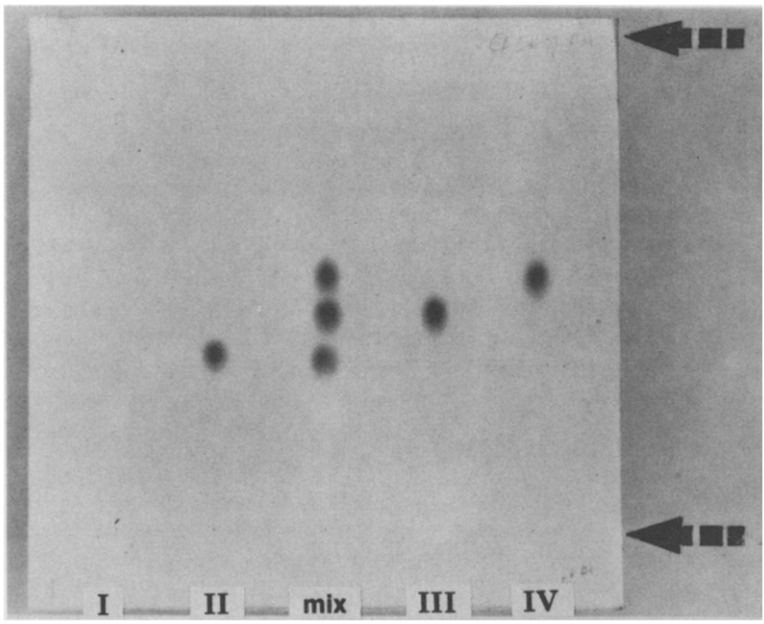

Fig. 4. Separation of fatty acid ethyl esters on reversed-phase HPTLC plates. Separation time, 15 min. Other conditions as in Fig. 1. Ethyl stearate appears as a white spot on exposure to iodine.

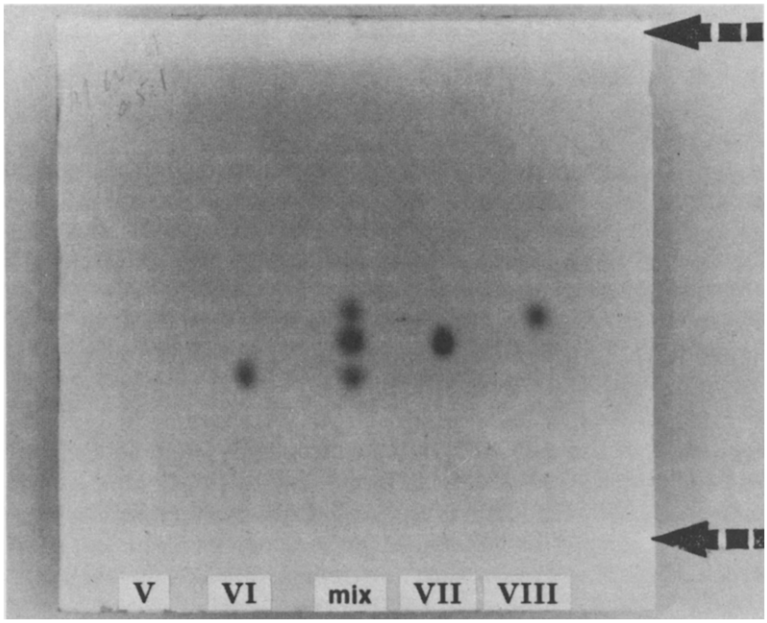

Fig. 5. Separation of fatty acid chloroethyl esters on reversed-phase HPTLC plates. Conditions as in Fig. 2.

Circular reversed-phase HPTLC for the separation of FAEEs and FACEs

FAEEs and FACEs can be separated by circular reversed-phase HPTLC using acetonitrile–methanol–acetic acid–water (10:2:2:1, v/v) (Fig. 6) or methanol–water (9:1, v/v) (Fig. 7) as the mobile phase. When these solvent systems were used for linear reversed-phase HPTLC, both FAEEs and FACEs were poorly resolved. The R_F values of FAEEs and FACEs on circular RP-HPTLC using acetonitrile–methanol–acetic acid–water were I 0.37, II 0.44, III 0.53, IV 0.60, V 0.40, VI 0.50, VII 0.58 and VIII 0.63 and those using methanol–water were I 0.25, II 0.29, III 0.35, IV 0.40, V 0.29, VI 0.34, VII 0.40 and VIII 0.45.

TABLE II

R_F VALUES OF FATTY ACID ETHYL ESTERS USING REVERSED-PHASE HPTLC PLATES

Mobile phase composition (acetonitrile–isopropanol–methanol–acetic acid–water, v/v)	R_F			
	I	*II*	*III*	*IV*
9:0:0:0:1	0.17	0.25	0.35	0.42
15:0:5:2:0.5	0.22	0.33	0.41	0.48
10:0:2:2:1	0.11	0.19	0.26	0.34
10:0:2:5:2	0.08	0.13	0.19	0.26
10:5:0:2:0.5	0.24	0.34	0.45	0.53

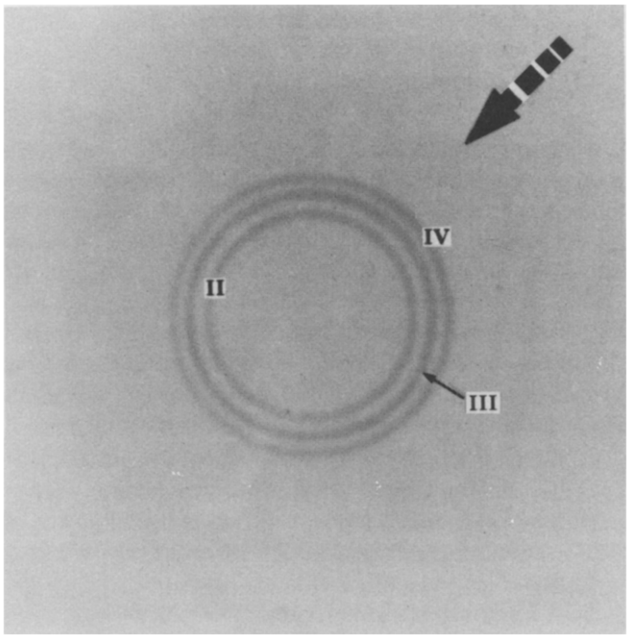

Fig. 6. Separation of fatty acid ethyl esters by circular reversed-phase HPTLC. Separation time, 7 min.

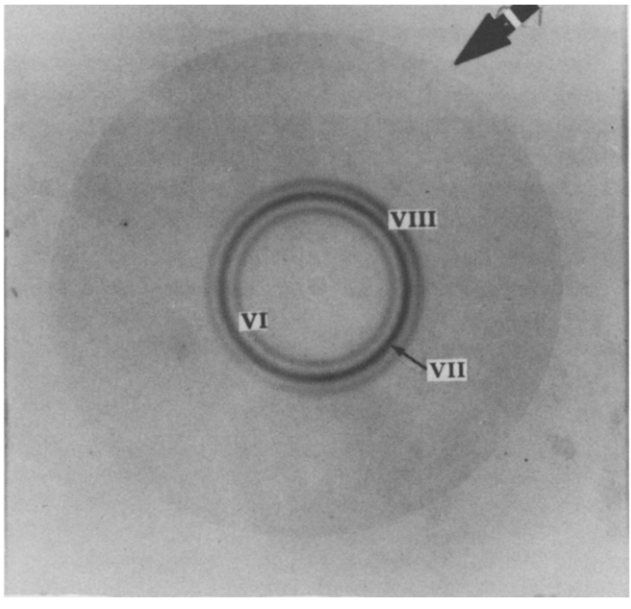

Fig. 7. Separation of fatty acid chloroethyl esters by circular reversed-phase HPTLC. Separation time, 20 min.

CONCLUSIONS

The main disadvantage of the existing argentation TLC methods is the time required for separation; therefore, our aim was to increase the speed of analysis, at the same time maintaining a good resolution. We found that silver nitrate-impregnated HPTLC plates can meet these requirements. Separation is achieved in less than 10 min at room temperature, the resolution is good and the method is sensitive (<3 μg required for detection) and reproducible and does not require multiple developments. The separation of fatty acid chloroethyl esters by argentation TLC and HPTLC are reported for the first time.

We have also developed a reversed-phase circular HPTLC method for the separation of FAEEs and FACEs. It is interesting that the solvent systems [acetonitrile–methanol–acetic acid–water (10:2:2:1, v/v) or methanol–water (9:1, v/v)], which do not give good separations of FAEEs or FACEs using RP-HPTLC plates for linear development, gave excellent resolution when circular chromatography was used. This technique can therefore be applied under circumstances where linear RP-HPTLC may not be successful. Another advantage of circular chromatography is that it requires very small amounts of the solvent and is fast.

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