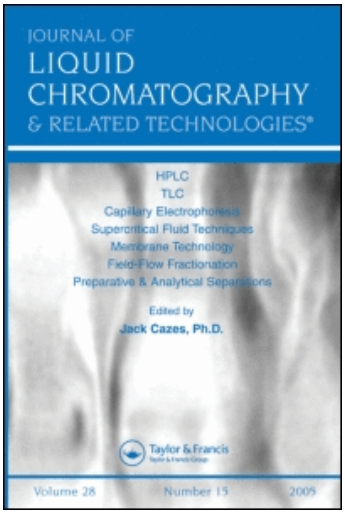


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# SILVER ION THIN-LAYER CHROMATOGRAPHY OF FATTY ACIDS. A SURVEY

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# SILVER ION THIN-LAYER CHROMATOGRAPHY OF FATTY ACIDS. A SURVEY

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## ABSTRACT

Silver ion thin-layer chromatography (Ag-TLC) as used for the separation of fatty acids according to the number, configuration, and position of double bonds, is surveyed in this paper. The efficiency of Ag-TLC/gas chromatography for determination of *trans*-fatty acids in food is emphasized. Described is the possibility for *in situ* quantification of fatty acids by photodensitometry. Some aspects of the possible interaction mechanism with silver ions in relation to recent studies are also discussed.

## INTRODUCTION

For many years, silver ion thin-layer chromatography (Ag-TLC) has been one of the basic separation techniques employed in lipid analysis. TLC is rapid, simple, and versatile and does not require expensive instrumentation. The information obtained reflects the whole sample, thus, helping the analyst to make rapid and correct judgments. Determination of fatty acids (FA) is mandatory for

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lipid analysis and, therefore, the resolution of FA mixtures has always been one of the main tasks of Ag-TLC. FA is separated on the basis of the number, the configuration, and to some extent, the position of the double bonds.[1,2]

Since 1962, when Ag-TLC was first introduced in lipid analysis, it has been invaluable in providing information about fatty acid structure and content of natural and modified lipids of terrestrial and marine origin. While gas chromatography (GC) has always been the basic method in FA analysis, the specific separation features of Ag-TLC make this technique indispensable in solving certain analytical tasks. The complementary employment of GC or/and gas chromatography/mass-spectrometry (GC-MS), together with Ag-TLC, is probably the most powerful tool for elucidation of fatty acid composition in complex lipid samples.

An effort is made here to present some of the basic features of the Ag-TLC technique, and some of its most important achievements in the analysis of FA. The presentation is limited to the "classical" Ag-TLC performed on glass plates. The TLC technique based on thin layer applied on a quartz rod, Chromarod™, is not discussed here as it has, in general limited application, while the separation principles are the same. A detailed description of this technique was made by Ackman[3] and the achievements up to 1990 were reviewed by Ackman *et al*.[4]

Additional information on Ag-TLC of fatty acids can be found in several books and reviews.[5-8]



## SILVER ION COMPLEXATION WITH DOUBLE BONDS

The Ag-TLC of fatty acids is based on the ability of Ag(I) to form weak, reversible charge transfer complexes with olefinic double bonds. It is now considered that a σ-type bond is formed between the occupied 2pπ orbitals of the olefinic bond and the free 5s and 5p orbitals of Ag(I), and a weaker π-acceptor backbond is formed between the occupied 4d orbitals of Ag(I) and the free antibonding 2pπ* orbitals of the olefinic bond.[9-13] Quantitative data on equilibrium constant exists for some short chain mono- and diolefines only. The retention of longer chain unsaturated compounds, like FA, is described on the basis of data collected by different silver ion separation techniques (GC and Ag-TLC mostly) and is supposed to depend on the strength of complexation with Ag(I). The latter, in its turn, depends on the number, configuration, and the distance between double bonds. Thus, the general migration rules in Ag-TLC can be summarized as follows:

FA are held stronger the higher is the number of double bonds in the chain.

FA with *trans* double bonds are held less strongly than FA with *cis* double bonds.

The retention of FA with more than one double bond depends on the distance between the bonds, the order of decreasing retention being: separated double bonds>methylene interrupted double bonds>conjugated double bonds.

Longer chain FA are held less strongly than are shorter chain FA of the same unsaturation.

FA with an olefinic double bond are held stronger than are FA with an acetylenic bond.

Deuterated FA are held stronger than are hydrogen analogues.

The retention of FA in Ag-TLC is expressed by the respective $R_f$ values, which decrease when retention increases. Only a few attempts have been made to present the complexation (or retention) in quantitative terms and they show that it is not proportional to the increase in the number of double bonds.[14-16] Gunstone and Padley[15] ascribed arbitrary values to what they called "complexating power" of 18:0, 18:1, 18:2, and 18:3 (number of carbon atoms:number of double bonds), namely 0, 1, 2+2a, 4+4a, where a<1. More precise empirical equations have been proposed later (see for example),[16] but they generally led to similar values. This phenomenon is probably due to the ability of Ag(I) to complex simultaneously with two double bonds from different or the same FA molecule.

Positionally isomeric FA have a specific behavior in Ag-TLC. When applied in a sequence on a single plate, the series of all *cis*- and *trans*-octadecenoates,[17] all the methylene-interrupted *cis*-octadecadienoates,[18] all the *cis*- and *trans*-dimethylene-interrupted octadecadienoates,[19] all the octadecynoates,[20] and many octadecadiynoates[19] migrate in a form of a more or less well expressed sinusoidal curve (an example of the migration pattern of *cis*- and *trans*-octadecenoates is shown in Figure 1). The weak retention of components with a double bond close to the ester moiety was ascribed to the delocalization of electron density at the double bond, resulting in the formation of either very weak or no complex with Ag(I) (as with *trans*-2-18:1 which runs ahead of 18:0, for example).[17]

The specific migration of the other positional isomers was first explained with the separate interaction of double bond(s) with Ag(I) and of the ester moiety with the free silanol groups of the adsorbent (silica gel). Thus, these isomers are held more strongly when the distance between the double bond and ester moiety matches the distance between Ag(I) and a silanol group in the supporting material.[21] Later, Gunstone and Lie Ken Jie ascribed the specific migration pattern of some isomeric *cis*-octadecenyl hydrocarbons, aldehydes, alcohols, and acetates to the presence of two centers in the molecule which can interact with the adsorbent.[22]

Finally, practically the same elution pattern was observed for *cis* and *trans* octadecenoates and *cis* octadecadienoates in silver ion high-performance liquid chromatography (Ag-HPLC).[23] Ag-HPLC was performed on columns where the surface of the supporting material and the introduction of Ag(I) differ very much from those in Ag-TLC.

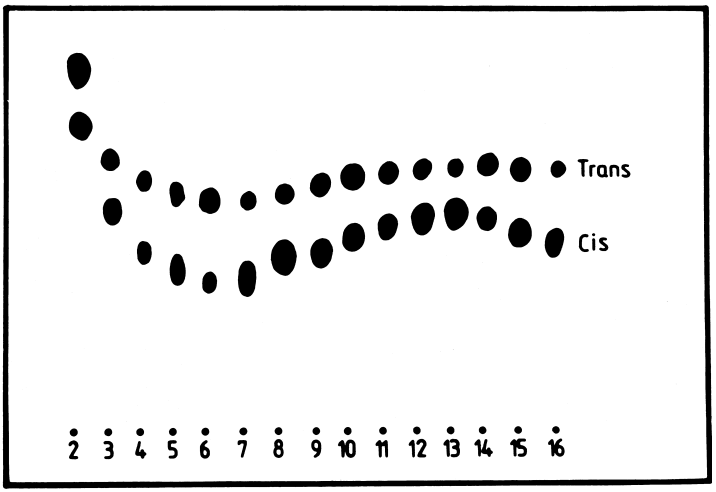


***Figure 1.*** Ag-TLC of methyl *cis*- and *trans*-octadecenoates.[17] The plate was impregnated with 15% (w/w) silver nitrate and developed with hexane-dibutyl ether (60:40, v/v); spots were detected by heating with a glass-blowers torch. The numbers indicate the position of the double bond in the fatty acid molecule. (Reproduced by kind permission of the authors and of *Chemistry and Physics of Lipids*, and redrawn from the original.)



Both sets of results are consistent with the assumption that one silver ion interacts simultaneously with one double bond (on the acyl chain) and a free electron pair on the carbonyl oxygen in the ester moiety.[24,25] Obviously, only components with double bonds in a suitable position in the acyl chain (positions 6-8 and 15-16) are able to form stable chelate-type complexes. If this is true, the electron density of the ester moiety should effect the strength of complexation. It should increase in the presence of electron-donating and decrease in the presence of electron-withdrawing groups in the ester moiety. Indeed, particularly good resolution results were obtained in Ag-HPLC for FA converted into aromatic derivatives with electron-donating substituents, especially if these bear an additional carbonyl oxygen.[25] As will be shown later, the same derivatives ensure excellent resolution of positionally isomeric octadecenoic and eicosenoic FA by Ag-TLC.

Although there is evidence that complexation with silver ions is the governing interaction in Ag-TLC, other factors should be considered as well. Thus, silica gel, which is the most widely used supporting material, possesses appreciable polarity and adsorption activity. In many cases, therefore, an impact of mixed retention mechanism on migration, geometry of spots, and selectivity of resolution is to be expected. Also, the mobile phase solvents are active elements of the

chromatographic system and interactions both with the supporting material and FA is possible, and this may have also a serious effect on the whole separation process.

## SOME PRACTICAL CONSIDERATIONS

Both home-made and pre-coated glass plates are used in Ag-TLC. Silica gel G (with calcium sulfate as binder) is usually the supporting material. Pre-coated plates with alumina layers[26] were also tested with good results, but did not find a wide application. Layer thickness varied between 0.2-0.3 mm for analytical plates to 0.5-1.0 mm for preparative plates. Fully automated spreaders are now available but simple spreaders are also effective. Some practice is needed to prepare the layer in the laboratory, thus; pre-coated plates are often preferred. Our experience, however, has shown that while pre-coated plates are suitable for qualitative or preparative work, home-made plates are more versatile, easier to impregnate with silver salt, and provide better results during photodensitometric quantification.

The impregnation of the layer with silver ions is performed by either incorporating the silver salt into the silica gel slurry or by immersing or spraying the plate with water, ethanol, methanol, ammonia, or acetonitrile solutions of the salt. Silver nitrate is normally used, although silver sulphamate[27] or silver benzenesulphonate[28] were also tested with good separation results. The only method that affords proper control of the Ag(I) content in the layer is to add silver nitrate in the slurry. This is, however, inconvenient and messy and is less used now. Since it is evident, from analytical practice,[7] that the content of silver ions in the layer is not critical in rather broad limits, immersion and spraying are considered equally as good.

Immersion procedures can be standardized sufficiently well to provide satisfactory results and can be applied both to home-made and pre-coated plates. We soak pre-coated plates for 5 min in 0.5% methanolic silver nitrate in order to obtain reliable analytical separation.[29] Spraying procedures are also often used in FA analysis, although they are less easily standardized and messier. Spraying may have to be repeated from two to six times until the layer is properly wetted. This is especially important for pre-coated plates.

The concentrations of impregnating solutions vary depending on the purpose. Immersion, or dipping is carried out most often with 5 to 20% solutions of silver nitrate (see for example ref. 30), and spraying - with 10 to 40% solutions (see for example ref. 31). On the other hand, we and our colleagues achieved excellent separations of complex fatty acid samples by dipping in only 0.5% (analytical plates) to 2% methanolic silver nitrate (preparative plates).

After impregnation, the plates are air-dried, preserved in a dark place, and activated (between 5 min[32] and 30 min,[30,33] at 110-120°C in an oven) prior to sample application. The necessity of long activation is questionable as it might make the plates even more sensitive to atmospheric humidity. The latter strongly effects the separation of highly unsaturated FA.[34] In spite of all precautions, humidity is not easy to control and is one of the main reasons for the relatively poor overall reproducibility of separations in Ag-TLC.

FA are subjected to Ag-TLC usually in the form of methyl esters. The methods for methylation and transmethylation were reviewed by Christie.[5,6] They are simple, easy to perform, and with practically 100% yield. Methyl esters are particularly suitable when Ag-TLC is used as a complementary method with GC. Ag-TLC is not "wedded" to methyl esters, however. Butyl[35] and isopropyl[36] esters were employed for the fractionation of butter fat FA by Ag-TLC, as these derivatives provided better resolution in GC. Conversion of positionally isomeric 18:1 and 20:1 FA into phenacyl esters ensured complete resolution of the components by Ag-TLC, while this is not possible when using methyl esters.[37,38]

Mobile phases will be discussed below, but generally they consist of two, rarely three component mixtures. Hexane or petroleum ether (b.p. 40-60°C), chloroform, benzene, and toluene are most often the major components, while smaller proportions of diethyl ether, acetone, methanol, ethanol, or acetic acid may be added to these.

The conventional approach is to perform the development in closed standard rectangular tanks lined with filter paper to saturate the atmosphere with the mobile phase vapors (see for example refs. 30 and 31). Good and reproducible resolution and well formed zones are expected under these conditions. On the other hand, poor separation and tailing were also reported.[39] As described in a previous paper on Ag-TLC of triacylglycerols,[40] we use "open" cylindrical containers where a fixed volume of the mobile phase is added, and after passing through the plate is permitted to evaporate from the upper edge of the plate. Although this system is quite sensitive to the laboratory environment, it operates well in trained hands and provides good separation of complex fatty acid samples.

Detection of separated zones depends on the analytical task. Destructive procedures are used for qualitative analysis and for quantification by photodensitometry. They consist in carbonization of the FA by heating at 180-200°C after treating the plate with charring reagents. These can be introduced by spraying, by treatment with the respective vapors, or by incorporation of the reagent into the layer. Up to 50% ethanolic sulphuric[41] or phosphomolybdic[19] acids and copper acetate-phosphoric acid[33] have been used as spraying reagents. Reliable results have been obtained by saturating the silica gel layer with vapors of sulphuryl chloride.[32]

Non-destructive procedures are used in preparative Ag-TLC. It is performed by spraying the plate with fluorescent indicator, mostly 2,7-dichlorofluo-

rescein in ethanol, and viewing under UV light. The bands are then scraped from the plate, and extracted with diethyl ether or hexane-methanol (in appropriate proportions). The excess silver ions and indicator are removed by passing the extract through a small silica column, or by washing with bicarbonate, ammonia, or sodium chloride solutions.[5]

Briefly, the practice of Ag-TLC shows that most analysts rely on their own experience in choosing the experimental conditions. The separation of FA is affected by the adsorbent type and layer thickness, by the mobile phase composition, the size and geometry of the developing tank, the developing mode, and the laboratory environment.

## SEPARATION OF FA BY Ag-TLC

### Separation According to the Number of Double Bonds

The separation of common FA methyl esters (FAME), i.e., FAME with 16-18 carbon atoms in the acyl chain and zero to three methylene-interrupted double bonds, is now considered routine.[1,27,39,42-44] Mixtures of hexane-diethyl ether and benzene-hexane in proportions between 90:10 and 80:20 by volume, and light petroleum ether-acetone, 100:3.5, are most often the mobile phases. The resolution of a FA mixture with zero to six double bonds is more difficult and is usually attempted in two stages.

For example,[45] a plate is first developed with a polar solvent mixture, e.g., chloroform-methanol-water (80:20:2 by volume), when fatty acid methyl esters (FAME) with three to six double bonds are resolved; saturated, monoenoic and dienoic components, which formed one or two zones, can either be scraped from the plate and resolved on their own,[45] or the separation can be continued on the same plate with a less-polar mobile phase (see above).[31,34,39,42,46,47]

Similar separations are accomplished by a double development with hexane-diethyl ether-acetic acid (94:4:2 by volume) on a plate containing 9% silver nitrate.[34] Inomata *et al*.[31] resolved FAME on pre-coated silica plates, impregnated by spraying with 40% aqueous silver nitrate, and given a single development with benzene-ethyl acetate (9:1, v/v). These conditions are suitable for preparative separation. We separated a reference mixture of FAME with zero to six double bonds on a single home-made analytical plate impregnated by dipping with 0.5% methanolic silver nitrate and developed with 5 mL of light petroleum ether-acetone-formic acid, 97:2:1, by volume (Fig. 2).[48] Note the clear separation of 20:4 and 18:4 (migrating in this order).

Among many examples, these general methods have been applied in the analysis of FA in marine organisms[49,50] and in brain.[51,52]

### Separation According to the Configuration of Double Bonds

Undoubtedly, one of the most important achievements of Ag-TLC is the clear separation of *cis*- and *trans*-isomeric FA. Ag-TLC is probably the easiest and cheapest way to determine the *trans*-monoene content of dietary fats.[32,53-56] Usually, the *trans*-monoenes are completely separated from the saturated and the *cis*-monoenoic FA under the chromatographic conditions established for resolution of saturated, monoenoic, and dienoic components (see also Figure 2).[1,27,32] An excellent separation, for example, can be achieved on home-made plates (4.5 x 19 cm) by developing with petroleum ether-acetone (100:4, v/v; 3.0-3.5 mL) in open cylindrical tanks. These conditions are employed in our laboratory for the quantification of *trans*-monoenes in margarine and other dietary fats by photodensitometry[32] (see below for more details). The analytical separation worsens,

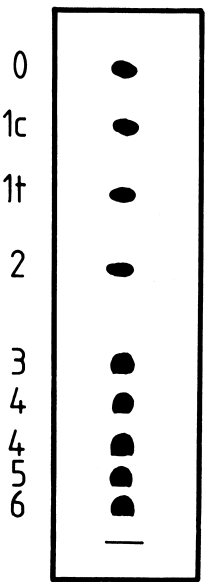


***Figure 2.*** Ag-TLC of reference mixture of fatty acid methyl esters with zero to six double bonds. The plate was impregnated by dipping with 0.5% methanolic silver nitrate (w/v) and developed with 5 mL of light petroleum ether-acetone-formic acid, 97:2:1 (v/v/v); spots were detected by treating the plate in sequence with bromine and sulphuryl chloride vapors, followed by heating at 180-200°C. Numbers alongside denote the number of double bonds; c - *cis*, t - *trans* double bond. (Reproduced from the *Encyclopedia of Chromatography*, J. Cazes, ed., with the publisher's permission.)

when the sample contains a variety of FA differing in chain-length and position of the double bonds.[57]

On the other hand, conditions were found for preparative Ag-TLC isolation of *trans*- and *cis*-isomers in complex FAME mixtures. For example, Molkentin and Precht[30] used pre-coated silica gel 60 G plates, impregnated by immersion in 20 % (w/v) aqueous silver nitrate for 20 min, and a single development with n-heptane-diethyl ether, 90:10, by volume, in a lined chamber. Other authors use 0.75% ethanol in chloroform,[58] toluene-hexane, 50:50 (v/v),[59] or toluene as a single solvent (at –20°C)[55] for the same separation. These methods are extensively exploited in conjunction with capillary GC for complete and more accurate analysis of the *cis*- and *trans*-components in dietary fats,[30,55,58-62] milk,[63,64] human milk,[65-67] and animal depot fat.[68]

Ag-TLC provides good resolution of configurational isomers derived by stereoisomerization of linoleic (*cis*-9,*cis*-12-18:2)[69,70] and linolenic (*cis*-9,*cis*-12,*cis*-15-18:3)[2] The migration order was tt > ct > cc and ttt > ctt > cct > ccc, respectively (c, *cis*-, t, *trans*-). However, the complex mixture of all possible isomers has not been resolved on a single plate, and formation of a number of mixed zones should be expected. Indeed, the migration order of C18 FAME with zero to two double bonds is saturated > *trans*-monoenes > *cis*-monoenes plus *trans*,*trans*-dienes > *trans*,*cis*- / *cis*,*trans*-dienes > *cis*,*cis*-dienes.[28] The critical pair, *cis*-monoenes and *trans*,*trans*-dienes, was resolved at ambient temperature on plates impregnated with silver benzenesulphamate and developed with hexane-pentane-diethyl ether-acetic acid (100:30:6:3 by volume).[28]

With the more complex mixture of saturated, *trans*-monoene, *trans*,*trans*-conjugated diene, *cis*,*cis*-conjugated diene plus *cis*,*trans*-conjugated diene, *cis*-monoene, *trans*,*trans*-diene, *cis*,*trans*-diene, and *cis*,*cis*-diene (migrating in this order), complete resolution was achieved on two different plates[32] (Figure 3). On the first plate, impregnated with 0.5% silver nitrate and developed in sequence with petroleum ether-acetone (100:2, v/v; 2 mL) and petroleum ether-acetone (100:0.7, v/v; 3 mL), only the *cis*,*trans*-conjugated diene, *cis*-monoene and *trans*,*trans*-diene remained as a mixed zone. To resolve this, the whole sample was applied to a second plate, impregnated with 1% silver nitrate, and this was developed with either chloroform (8 mL; with the stabilizing ethanol removed) or with 0.4% methanol in chloroform (v/v, 5 mL). The separations were performed in the open cylindrical tanks. Some conjugated octadecatrienoates were resolved also (as distinct fractions) at –20°C on a plate impregnated with 30% silver nitrate and developed with toluene.[71]

While these separations illustrate the potential of the technique, complex mixtures of FAME, which contain geometrical isomers of dienoic and trienoic FA, cannot be fully resolved by applying only Ag-TLC, and a combination of preparative Ag-TLC with GC on capillary columns is usually applied.[72-74]

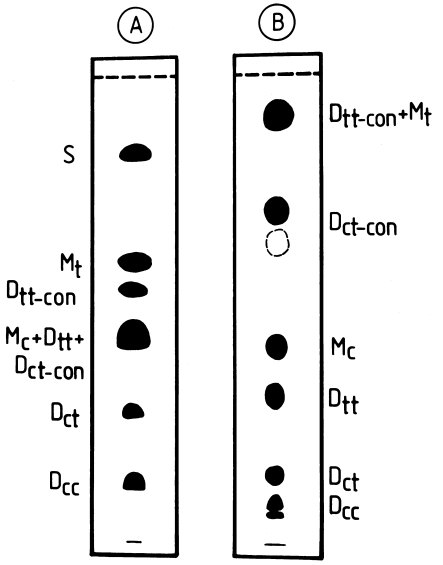


***Figure 3.*** Ag-TLC of reference mixture of isomeric fatty acid methyl esters.[32] Plate A, the layer was impregnated by dipping with 0.5% methanolic silver nitrate (w/v) and developed with 2 mL light petroleum ether-acetone, 100:2 (v/v) followed by 3 mL light petroleum ether-acetone, 100:0.7 (v/v). Plate B, the layer was impregnated with 1% silver nitrate (w/v) and developed with 8 mL chloroform. Development was carried out in open cylindrical tanks. Spots were detected by treating the plates in sequence with bromine and sulphuryl chloride vapors, followed by heating at 180-200°C. S, M and D denote saturated, monoenoic and dienoic fatty acid methyl esters, c - *cis*, t - *trans* and con - conjugated double bonds. (Reproduced by kind permission of the *Journal of Planar Chromatography-Modern TLC*.)



## Separation According to the Position of the Double Bond

The systematic study of the behavior of positionally isomeric octadecenoates[17] and methylene-interrupted octadecadienoates[18] has shown that the practical outcome is of limited value, as only few isomers possess different enough mobility in Ag-TLC. So far, only the three naturally occurring isomeric octadecenoates: 6-18:1 (petroselinic acid), 9-18:1 (oleic acid) and 11-18:1 (*cis*-vaccenic acid) have been resolved (retention decreases in this order). The first partial separation was carried out on a layer with high silver nitrate content and at temperatures of –20°C. While the low temperature seems to be crucial, the amount of Ag(I) is not.

Thus, acceptable resolution of 6- from 9-18:1 was achieved on plates pre-coated with alumina, impregnated by dipping (for 30 sec) in 10% silver nitrate, and given a single development with toluene (temperature –20°C).[26,75] Similarly, the same isomeric pair was partially resolved on home-made silica gel plates, impregnated by dipping in 1% methanolic silver nitrate and given a continuous development with 5 mL petroleum ether-ethyl ether, 100:5 (temperature –18°C).[76]

A new solution to this problem was found, when prior to analysis, FA were converted in phenacyl instead of methyl esters. Base-line resolution of 6-, 9- and 11-18:1 and of 5-, 8-, 11- and 13-20:1[37] (in order of decreasing retention) was achieved and for the first time in Ag-TLC of positionally isomeric FA, the separation was performed at ambient temperature (Figure 4 and Figure 5). The resolution was equally successful on both home-made and pre-coated plates, and the concentration of silver nitrate in the impregnating solution was either 0.5% or 1%. The beneficial effect of the derivatization is ascribed to the participation of the carbonyl oxygen in the phenacyl moiety in the complexation with Ag(I). Formation of a chelate type complex of higher stability than those formed by methyl esters, is assumed for FA with double bonds in favorable positions, resulting in a substantial difference in the migration of isomers.

The approach was applied for the Ag-TLC/densitometric determination of saturated, 6-, 9-, 11- 18:1 and 9,12-18:2 in some *Umbelliferae* seed oils on a single home-made silica gel plate.[38] The total fatty acid mixture was applied on the plate, impregnated by dipping in 1% methanolic silver nitrate, and developed twice in closed cylindrical tank (without saturation of the atmosphere) with a mobile phase of chloroform-acetone, 100:0.25 (v/v) at ambient temperature (Figure 6).[38]

## Miscellaneous

In some cases, FA of the same unsaturation are separated according to the chain-length, with longer chain FA migrating ahead of shorter chain FA. So far, differentiation on the basis of chain-length has been achieved only for FAME with two or more double bonds. Indeed, as shown above (Figure 2), 20:4 and 18:4 FAME are clearly separated by Ag-TLC. Wilson and Sargent[33] reported on the resolution of FAME of the biologically important series (n-3) and (n-6) with two to five double bonds and acyl chains of 18, 20, and 22 carbon atoms (in order of increasing $R_f$). The separation was performed on pre-coated silica gel 60 plates, impregnated by spraying with 10% $AgNO_3$ in acetonitrile, and given a single development with toluene-acetonitrile, 97:3 (v/v) in a lined chamber. A closer look at these results reveals that chain length is hardly the only reason for the good separation.

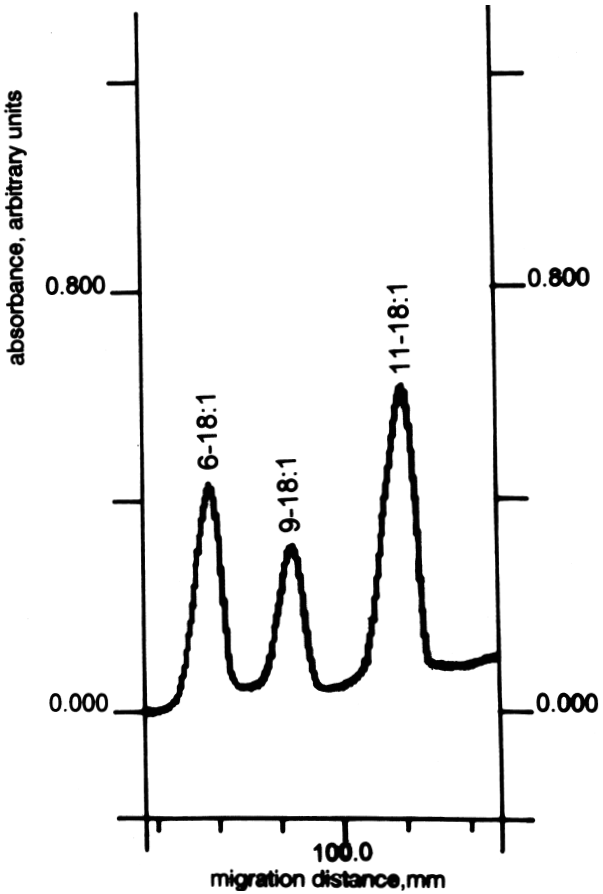


***Figure 4.*** Densitogram of positionally isomeric phenacyl octadecenoates.[37] Separation was performed on pre-coated silica gel 60 analytical plate (aluminum-backed, strips of 4x20 cm) impregnated by immersion for 5 min in 0.5% methanolic silver nitrate. The plate was developed three times to a solvent front of 17.5 cm, each time with fresh 3 mL dichloromethane, in closed cylindrical tank without saturation of the atmosphere. Spots were detected by treatment the plate with sulphuryl chloride vapors, followed by heating at 180-200°C. The plate was scanned by a Shimadzu CS-930 densitometer in zig-zag reflection mode at 450 nm (beam-slit 1.2x12 mm). (Reproduced by kind permission of the *Journal of Planar Chromatography-Modern TLC*.)

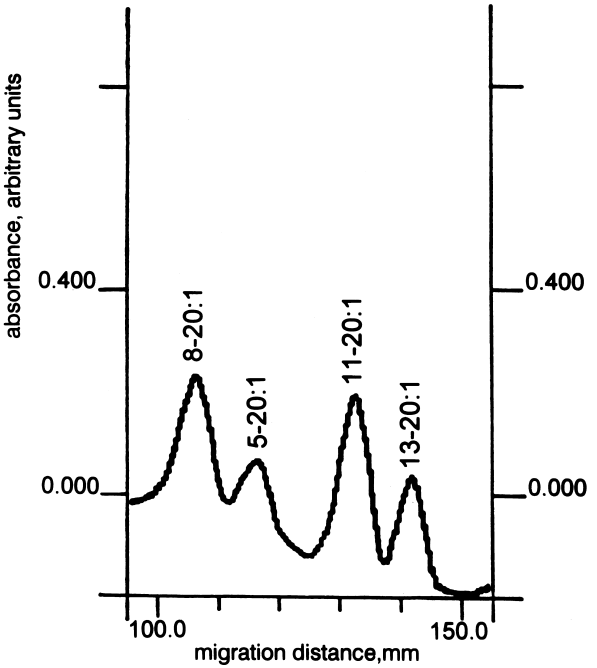


***Figure 5.*** Densitogram of positionally isomeric phenacyl eicosenoates.[37] Separation was performed on pre-coated silica gel 60 analytical plate (aluminum-backed, strips of 4x20 cm) impregnated by immersion for 5 min in 0.5% methanolic silver nitrate. The plate was developed twice to a solvent front of 17.5 cm, each time with fresh 3 mL chloroform-methanol 100:0.1 (v/v) in a closed cylindrical tank without saturation of the atmosphere. Spots were detected by treatment the plate with sulphuryl chloride vapors, followed by heating at 180-200°C. The plate was scanned by a Shimadzu CS-930 densitometer in zig-zag reflection mode at 450 nm (beam-slit 1.2x1.2 mm). (Reproduced by kind permission of the *Journal of Planar Chromatography-Modern TLC*.)

The (n-3) and (n-6) series of FAME comprise components with a different position of the double bonds, which as shown above, is also a factor effecting the separation. Thus, for example, the (n-6) dienoic FA series includes 9,12-18:2, 11,14-20:2, and 13,16-22:2 (migrating in this order). In view of the above discussion, the place of the first double bond in FA affects the separation, and this has been demonstrated and discussed for 18:1, 20:1 in Ag-TLC,[37] and 18:3, 20:3 isomers in Ag-HPLC[77] (after conversion into phenacyl and p-methoxyphenacyl derivatives, respectively).

Evidently, the successful resolution of the (n-3) and (n-6) FA achieved by Wilson and Sargent[33] is due both to the chain length and the favorable double

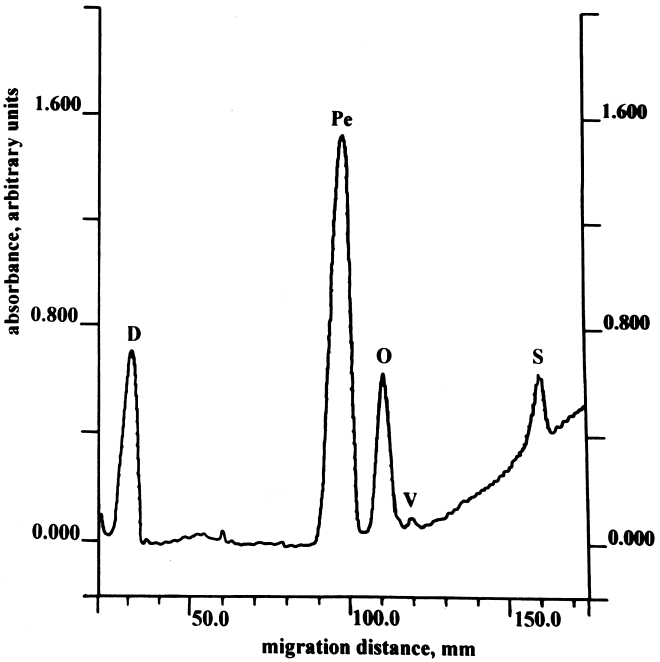


***Figure 6.*** Densitogram of phenacyl esters of fatty acids from *Pimpinella anisum* seed oil.[38] Separation was performed on home-made glass plate, impregnated by dipping with 1% methanolic silver nitrate (w/v), and developed twice with 3 mL chloroform-acetone, 100:0.25 (v/v) in closed cylindrical tank. Spots were detected by treatment the plate with sulphuryl chloride vapors, followed by heating at 180-200°C. The plate was scanned by a Shimadzu CS-930 densitometer in zig-zag reflection mode at 450 nm (beam-slit 0.4x0.4 mm). S, saturated, V, *cis*-vaccenic, O, oleic, Pe, petroselinic and D, dienoic fatty acids. (Reproduced by kind permission of the *Phytochemical Analysis*.)

bond position of the individual components. The procedure was applied in metabolic studies of chain elongation and unsaturation.

An observation that might be of use in studies of radio-labeled FA is the self-staining of polyunsaturated components on silica gel plates when preserved for 2 to 20 days.[78] Silver nitrate (10%, w/w) was incorporated in the slurry and toluene-acetonitrile, 97:3 (v/v) was the mobile phase. Oxidation of the highly unsaturated components by Ag(I) was considered to be the reason.[78]

In an interesting semi-preparative approach, Rezanka[79] applied two-dimensional TLC for the analysis of complex FAME isolated from *Streptomyces avermitilis* and cod liver oil. One quarter of the 20x20 cm glass plate was covered with silica gel containing a 10% urea solution; the rest was covered with silica gel

containing 10% $AgNO_3$. FAME were first fractionated on the urea layer into branched- and normal-chain species and then each fraction was resolved in the other direction according to the unsaturation of the components. A mobile phase of hexane-diethyl ether-methanol, 90:10:1 was used for the Ag-TLC separation. The fractions were further analyzed by GC/MS.

Ag-TLC was also applied for the separation of very long-chain (C24 to C36) polyunsaturated FA.[51,80,81] For example, a sample of bovine retina FA was resolved on silica gel containing 20% $AgNO_3$ (incorporated into the layer), with a mobile phase of chloroform-methanol (95:5, v/v).[81] In this instance, three fractions, i.e. tetra-, penta- and hexaenoic FA, were obtained and each was then further examined by GC. Substituted FA can also be effectively separated on the basis of degree and type of unsaturation. A wide range of unsaturated epoxy, halohydroxy, hydroxy, and dihydroxy FA have been separated by silver ion TLC, and the results were reviewed by Morris and Nichols.[82] Similarly, silver ion TLC was applied in the analysis of cyclopentenyl[83,84] and furanoid FA.[85]



## QUANTIFICATION

According to the most widely applied analytical protocol, quantification of FAME, separated by Ag-TLC, is carried out indirectly by extracting the fraction from the layer in the presence of an internal standard (usually an odd-chain FAME), removing the solvent, re-dissolving in hexane, and subjecting to GC or GC/MS. Information is obtained about the composition of the fraction and the absolute amount of the components.

As an alternative, a densitometric technique has been developed to quantify the separated species directly on the Ag-TLC plate. The basis of the method is the difference in the optical response between the blank part of the plate and the regions where the analytes are present. Nowadays, excellent computerized instrumentation is available,[86] and the problems that arise are rarely a function of the photodensitometer, but depend mainly on the properties of the chromatogram. A clean background and well-resolved, distinct, and evenly-stained zones are required.

In most instances, the staining procedure is the critical step. FA do not possess any chromogenic groups and are usually visualized for direct quantification by charring. Although charring is a sensitive detection procedure, it is not easy to control, and all steps, including treatment of the plate with the charring reagent, and the temperature and duration of heating, must be standardized as far as possible in order to obtain reproducible results. The most common procedure is to spray the plate with a 50 to 70% aqueous methanolic or ethanolic solution of sulphuric acid. However, sulphuric acid is extremely corrosive, and spraying is an inherently inconvenient procedure, so alternative approaches have been pro-

posed. The authors prefer treating the developed plate with vapors of sulphuryl chloride,[32] which saturate the silica layer and decompose to sulphuric acid rapidly when the plate is heated to 180 to 200°C. The procedure is carried out in tightly stoppered containers and in a fume-cupboard, exactly as described in a recent paper on triacylglycerol analysis.[40] Plates with carbonized zones are scanned in the densitometer at 400 to 450 nm.

An important requirement for quantitative purposes, is that the charring reagent should react equally with all components, and in particular, staining should not be influenced by the different degree of unsaturation of the separated FA. If this is not possible, correction factors should be introduced with the densitometric response of one of the components taken as a standard. As an example, FA with zero to four double bonds were determined densitometrically after spraying the plate with 70% sulphuric acid saturated with potassium dichromate and heating at 120°C for one hour. Linear calibration graphs were obtained for each component in the range of 35 to 150 nmol. The molar response of methyl oleate was found to be less than that of methyl stearate, while those of unsaturated FA increased with increasing chain-length and number of double bonds.[31] On the other hand, no correction coefficients were required when the developed plate was first treated with bromine vapor and then with sulphuryl chloride.[32] Bromine vapors are supposed to react with double bonds and give derivatives that are equally carbonized. This is the pre-quantification procedure we and our col-



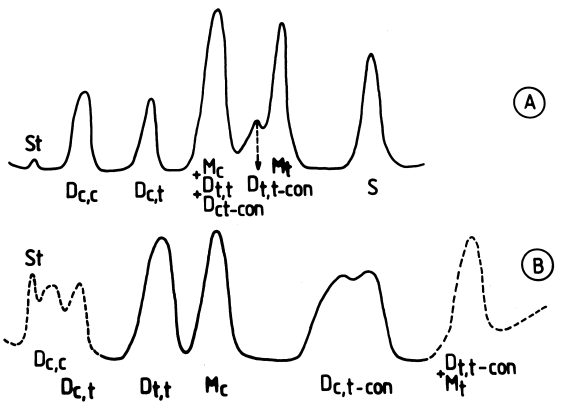


***Figure 7.*** Densitogram of reference mixture of isomeric fatty acid methyl esters.[32] For the chromatographic conditions and spot detection see Fig. 3. S, M and D denote saturated, monoenoic and dienoic fatty acid methyl esters, c - *cis*, t - *trans* and con - conjugated double bonds. The plates were scanned by a Shimadzu CS-930 densitometer in zig-zag reflection mode at 450 nm (beam-slit 1.2x1.2 mm). (Reproduced by kind permission of the *Journal of Planar Chromatography-Modern TLC*.)

leagues presently use in hundreds of analysis of FA of different unsaturation. An example is shown in Figure 7. A comparison with FA composition as determined by GC shows a difference no greater than 5-6% rel. between the two sets of results. We consider the combination Ag-TLC/photodensitometry the most efficient approach for determination of the total *trans* fatty acid content in food.

Lipid analysts have often been skeptical about the possibilities for quantification offered by densitometry; their opinions were generally inferred by earlier experience when instrumentation was quite primitive. Now, the situation has changed considerably, and laboratories with staff that are well-trained in Ag-TLC methods can successfully apply photodensitometry. It can be claimed, that the overall procedure is less costly and more suitable for routine analysis of numerous samples than any alternative technique.



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