review

Separations of lipids by silver ion chromatography

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ABSTRACT The possibility of separating lipid materials on the basis of the number, type, and position of the unsaturated centers they contain, by virtue of the complexing of these unsaturated bonds with silver ions, provides a relatively recent but now very important addition to the range of separatory methods available to lipid chemists and biochemists. In this review, the nature of the complexing of silver ions with olefins is considered briefly and the history of the development of separation methods based on argentation is traced. Some practical considerations of argentation chromatography are discussed and separations of fatty acids and aldehydes, substituted fatty acids, neutral lipids, polar lipids, and sterols and other terpenoid compounds, by argentation methods alone and in conjunction with other separation techniques, are then reviewed. Some conclusions are finally presented as to the present and potential utility of argentation methods in studies of the occurrence, metabolism, and function of lipids.

KEY WORDS lipid separations · argentation · silver–olefin complexes · thin-layer · column · paper · chromatography · fatty acids · neutral lipids · polar lipids · sterols · terpenoid compounds

It is only some 4 or 5 years since separations of lipophilic materials according to unsaturation by chromatographic or countercurrent distribution methods involving silver ions were first reported. Already separation methods based on complexing of unsaturated centers with silver ions are in use in most laboratories engaged in lipid research and more than 150 publications have appeared describing separations by such methods or their utilization in lipid research. It is now probably true to say that argentation chromatography is third only to gas–liquid chromatography (GLC) and "normal" thin-layer chromatography in importance as a separatory tool for natural lipid materials. That argentation chromatographic methods have gained such wide acceptance and popularity so quickly indicates that there was a widely felt need for a simple method of separating lipids according to degree of unsaturation. The basic simplicity of the method and the ready availability of such equipment as is required have also encouraged its rapid acceptance.

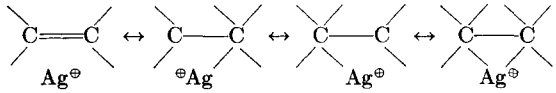
The applications of separation methods involving silver ions for the separation of lipids have been specifically reviewed a number of times (1–5) and have been summarized in several other general reviews of lipid separations (e.g. 6–13). However, few of these reviews were at all comprehensive and since new procedures and applications have been appearing almost monthly most of them are now somewhat out of date. In the present review, the nature of the complexes between silver ions and unsaturated centers is first considered and the development of the various separation methods depending on the formation of such complexes is briefly traced. After consideration of some practical aspects of the various methods, the bulk of the review describes applications of these methods to separations within the various categories of lipids. Separation of sterols and other lipophilic materials is also considered. While no guarantee is made that all publications which have described separations by argentation chromatography are included, it is hoped that most of the important applications to date have been considered and critically assessed.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

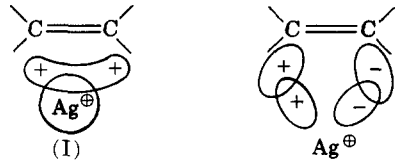
NATURE OF THE SILVER ION COMPLEXES

It has been known for many years that there are weak interactions between silver ions and certain compounds containing ethylenic or acetylenic bonds. This phenomenon was first quantitatively studied in 1938 by Winstein and Lucas (14). They determined equilibrium constants for the reaction of silver ions with a number of acyclic and alicyclic olefinic compounds by measuring the partitioning of the olefin between an aqueous silver nitrate phase and carbon tetrachloride. Many studies of the equilibrium constants of these reactions have been carried out since, using either the original partition method, or a method in which the solubility of the pure unsaturated compound in aqueous solutions of silver salts is determined, or, more recently, chromatographic methods, notably GLC on solutions of silver nitrate in suitable glycols as stationary phase (15,16). These basic chemical studies were done principally by three groups: Lucas and his coworkers (17) dealt chiefly with aliphatic olefins and alkynes, Andrews and Keefer (18) were concerned with aromatic compounds, and Traynham and coworkers (19) have studied alicyclic olefins. Many other groups, of course, have contributed to current knowledge of complexing between centers of unsaturation and silver and other transition metal ions, and the effects of substitution, stereochemistry, ring size, etc. on the stability of such complexes. Many complexes have been isolated in crystalline and relatively stable form, particularly those between silver nitrate, silver perchlorate, and silver fluoroborate and cyclic olefins. This review, however, is primarily concerned with chromatographic separations of lipids that depend on silver ion complexing and although much can be learned from these chemical studies of relatively simple compounds, particularly by those concerned with separations and structure elucidations of sterols and other alicyclic olefins, more detailed consideration here of their findings and implications seems inappropriate. More detailed information may be obtained from the original publications and from a number of reviews (e.g. 20–22).

The nature of the bonding in complexes of unsaturated compounds with silver (and other transition metals with nearly filled *d*-orbitals) has been a matter for some discussion. It was originally suggested (14) that the [olefin Ag]$^+$ species could be represented as a resonance hybrid of three forms, later increased to four forms (17*a*):



This represents a simple π-complex in which only deformation of the π-orbitals of the olefin is involved. An alternative picture, which now seems to be generally accepted, was presented by Dewar (23):



The bonding is considered to involve a σ-bond formed by overlap of the filled π-orbital of the olefin with (in the case of silver) the free *s*-orbital (I) and a π-bond formed by overlap of the vacant antibonding π-orbitals of the olefin with filled *d*-orbitals of the silver (II). The bonding in the complex will be affected by the availability of electrons in the filled orbitals and the ease of overlap of these orbitals, which is determined by steric factors. On the basis of the Dewar concept, complexing with silver should not cause great change in the double bond. That the double bond does remain almost intact in such complexes is indicated by Raman spectra, which show a lowering of only 50–60 cm^{-1} in the C=C stretching frequency (24), and by proton resonance spectra, which do not differ greatly from those of the free olefins (25). From the distribution studies (e.g. 17*b*, 26), evidence has been adduced for the existence of disilver complexes of olefins, i.e. (Ag$_2$ olefins)$^{2+}$, at relatively high silver ion concentrations. However, in the chromatographic systems in which we are interested most of the observed differences resulting in separation may be satisfactorily understood on the assumption of 1:1 complexing.

DEVELOPMENT OF ARGENTATION CHROMATOGRAPHY

Since the first study of silver–olefin complexes by Winstein and Lucas (14), such complexing has been used many times for isolation or purification of olefins by isolation of crystalline complexes or by simple extraction or liquid–liquid distribution procedures (e.g. 17*e*, and references cited therein). However, the first real appreciation of the possibilities of argentation as the basis of sophisticated separatory techniques for lipophilic compounds was by Nichols in 1952 (27). In the first study of silver complexes of lipid components, he measured argentation constants of methyl oleate and methyl elaidate by distribution between isooctane and aqueous methanol mixtures. He suggested that countercurrent distribution or paper chromatography could be adapted to separate these two esters and also to separate saturated and unsaturated esters or olefinic compounds in general.

The first chromatographic application of silver–olefin complexing, however, did not follow Nichols' pre-

diction—this had to wait 9 years for fulfillment—but was in the realm of GLC. Bradford, Harvey, and Chalkley (28) found that a saturated solution of silver nitrate in ethylene glycol, as a stationary phase in GLC, gave excellent separations of traces of ethane in ethylene. Tenny (29) clearly illustrated the selectivity of a silver nitrate–triethylene glycol stationary phase for a series of normal olefins and the applications of "argentation GLC" to separations of volatile olefins were rapidly extended by other workers (e.g. 30, 31). More recently, of course, argentation GLC has been used for determination of argentation constants of large numbers of ethylenic and acetylenic compounds (15, 16) as a simpler alternative to the classical methods of distribution or solubilization.

Although very useful for olefins and acetylenes of low molecular weight, argentation GLC has little relevance in lipid separations because of the high temperatures required for GLC of lipid derivatives. Keulemans (30) stated that the temperature for argentation GLC should be kept below 40°C, because above this temperature the adducts do not form and the stationary phase is not stable. This statement was modified by Bednas and Russell (31), who found that $AgNO_3$–ethylene glycol and $AgNO_3$–glycerol stationary phases were stable and could be used satisfactorily at 65° but underwent slow decomposition above that temperature and could be used only for short periods at 85°C. Even this, however, is of little value for lipid work.

Nichols' prediction was first verified by Dutton, Scholfield, and Jones (32), who showed that countercurrent distribution between 0.2 M silver nitrate in 90% methanol and hexane would give excellent separations of oleate and elaidate and of saturated, mono-, and diunsaturated esters. Scholfield and his coworkers have since extended this method and used it in a variety of studies (33–35) and de Vries (36) has reported an alternative countercurrent distribution system for the separation of oleate and elaidate. Countercurrent distribution with argentation, however, has not been at all widely used for lipid separations, presumably because of the tedium of the procedure and because few laboratories have the necessary equipment. It was only the advent of chromatographic methods based on the silver ion–double bond interaction which broadcast the potentialities of argentation in lipid separations.

The first reference to argentation chromatography which this author has been able to trace was the separation of *cis*- and *trans*-5-cyclodecenols by Goering, Closson, and Olson (37). They used a column of silica gel impregnated with a stationary phase of aqueous silver nitrate and eluted with benzene–light petroleum and benzene–ether mixtures. This was, of course, a partition chromatographic system but similar in effect to the more recent adsorption systems employing silver nitrate. At about the same time, reversed-phase partition chromatographic methods based on silver complexing and developed by Wickberg (38) were being used for separations of various natural terpenoid materials (39–42). These methods employed not silver nitrate but silver fluoroborate in aqueous methanol as mobile phase in conjunction either with glass papers impregnated with hexadecane as stationary phase, or with columns of polyvinyl chloride powder. Besides the obvious disadvantage of using the expensive and dangerous silver fluoroborate, these procedures resulted in elution of silver salt with the separated components, which thus required further purification. However, as separation methods they were effective and resulted in the detection and isolation of a number of novel terpenoid compounds (e.g. 38).

True argentation adsorption chromatography, particularly as applied to lipids, was first described simultaneously by Morris (43), who used TLC, and by de Vries (36), who used columns. Both authors demonstrated the clear separation of oleate and elaidate, and of these from saturated and polyunsaturated esters. Morris, in addition, showed separations of saturated, ethylenic, and acetylenic hydroxy and epoxy esters and, by double impregnation with silver nitrate and boric acid, achieved simultaneous separation of *threo* and *erythro*, saturated and unsaturated dihydroxy esters (43). De Vries (36), as well as separating methyl esters, achieved virtually quantitative separations of triglycerides according to total number of double bonds, even of elaidodipalmitin from oleodipalmitin. He also claimed quantitative separation of cholesterol from cholestanol. At the same time Barrett, Dallas, and Padley (44) described separations of glyceride mixtures on silver nitrate-impregnated thin layers. These first three short notes (36, 43, 44) between them indicated the potentialities of argentation chromatography in virtually all important classes of lipid compounds, other than phospholipids and glycolipids, which are only recently being fractionated by this method.

Only one other significantly different procedure need be mentioned in this short history of the development of argentation methods, namely the fractionation of large amounts of triglycerides by low temperature crystallization from acetone–methanol solutions of silver nitrate described by Gunstone and coworkers (45, 46).

SOME PRACTICAL ASPECTS

The practical techniques of argentation chromatography are, in general, very little different from more conventional chromatographic procedures. Relatively large samples may be fractionated on columns but analytical

separations are best performed by TLC and, in many cases, preparative argentation TLC is the best and most convenient means of isolating material sufficient for further analysis and (or) structure determination. Argentation column chromatography is less satisfactory than argentation TLC for separations of highly unsaturated components, such as triglycerides with more than four double bonds (47), and of compounds with fairly similar migration behaviour, such as monoacetylenes and *cis*-monoenes (48).

The adsorbent most commonly impregnated with silver nitrate has been silicic acid. The disadvantage of low flow rates of many silicic acid columns may be obviated by using acid-washed Florisil impregnated with silver nitrate (49, 50). Columns of ion-exchange resins containing silver ion have also been used (51, 52); they offer the advantage that polar solvents may be used without leaching any of the silver ions from the column. Column chromatographic procedures, of course, tend to be tedious, hence the much wider use of TLC and preparative TLC methods, but automatic detectors such as the one described by James, Ravenhill, and Scott (53) in conjunction with argentation column chromatography relieve the tedium to some extent.

In argentation TLC the most common adsorbent by far is silicic acid. However, alumina impregnated with silver nitrate has been used a number of times, e.g. for separations of dinitrophenylhydrazones of various classes of aliphatic aldehydes and ketones (54, 55) and for resin acid methyl esters (56), some of which were unstable on silicic acid. Badings and Wassink showed that kieselguhr–silver nitrate was also effective for separation of carbonyl dinitrophenylhydrazone derivatives (57) and, in this excellent paper, they concluded that besides complexing with C=C bonds the silver ions also complexed with the C=N bonds of the dinitrophenylhydrazones.

Impregnation of thin layers with silver nitrate may be effected in a number of ways, most commonly by using an aqueous silver nitrate solution of appropriate concentration instead of water for preparing the adsorbent slurry with which to spread the layers (e.g. 44, 58). One serious difficulty in preparing plates in this way is caused by the interaction of the silver nitrate solution with the metal of conventional plated spreaders. This interaction has two effects; metallic silver is precipitated onto the layers being prepared and the spreader itself becomes rapidly pitted and corroded until, eventually, it is useless. At least one manufacturer (Desaga, Heidelberg, Germany) supplies silver-plated spreaders to counteract this problem or, alternatively, a spreader made of anodized aluminum, such as the excellent spreader produced by Quickfit and Quartz Ltd., Stone, Staffs., England that we use, is impervious to silver nitrate. A third possibility, which we use for small plates ($3^1/_4$ inches square), is to construct a spreader from some suitable plastic sheet. As an alternative to this direct method of making impregnated layers, thin-layer plates already prepared may be wholly or partly impregnated by spraying with an aqueous or methanolic solution of silver nitrate (43) or by development with an aqueous solution of silver nitrate (59). These last two procedures permit comparisons of migration behavior on normal and impregnated adsorbents on a single plate.

The level of silver nitrate impregnation recommended has varied from 30–40% (60) down to 3% (61). Using sample mixtures of saturated, monoenoic, and dienoic fatty acid methyl esters, wax esters, and cholesterol esters on a whole series of plates of 0.5–30% silver nitrate content (i.e. silver nitrate:silicic acid ranged from 0.5:99.5 through 30:70, w/w), we found that there was no improvement in separations above 2% silver nitrate (58, 1). A similar conclusion was reached by Klein, Knight, and Szczepanik (62) for steroids and by Stahl and Vollman (61) for terpenoid alcohols. The last authors arrived at their conclusion very elegantly by means of a layer having a linear gradient of impregnation with silver nitrate, prepared with Stahl's "GM-spreader" (63), as illustrated in Fig. 1. In this illustration a level of about 1.5% silver nitrate is seen to be necessary for separation of nerolidol from geraniol, but at this level of impregnation guaiol and borneol are inseparable. At about 2% impregnation all components are separated and there is no further improvement of separation with increasing silver nitrate concentration. Note the crossover of guaiol and borneol, the former being less mobile than the latter at low levels of impregnation and more mobile at higher levels. Stahl considered that 3% impregnation was the optimum and most economical level of impregnation, whereas we normally use 5%. The only case where we have found higher levels of impregnation (10% or more) to be advantageous is for separations of positional isomers of monoenoic fatty acid esters (64). We do not know why this should be, but it may be that disilver–olefin complexes have some role in these separations. One disadvantage of higher levels of impregnation for TLC, apart from the expense, is that detection by corrosive reagents becomes progressively more difficult.

Impregnated plates are activated in the usual way; we heat at 110°C for 30 min for thin layers or for 60 min for thicker layers (1 mm). Plates should then be stored in sealed glass containers because laboratory fumes frequently cause rapid deterioration in the selectivity of impregnated layers. We store the plates over saturated calcium chloride solution (relative humidity ca. 30%), thereby ensuring reproducible results even after several weeks.

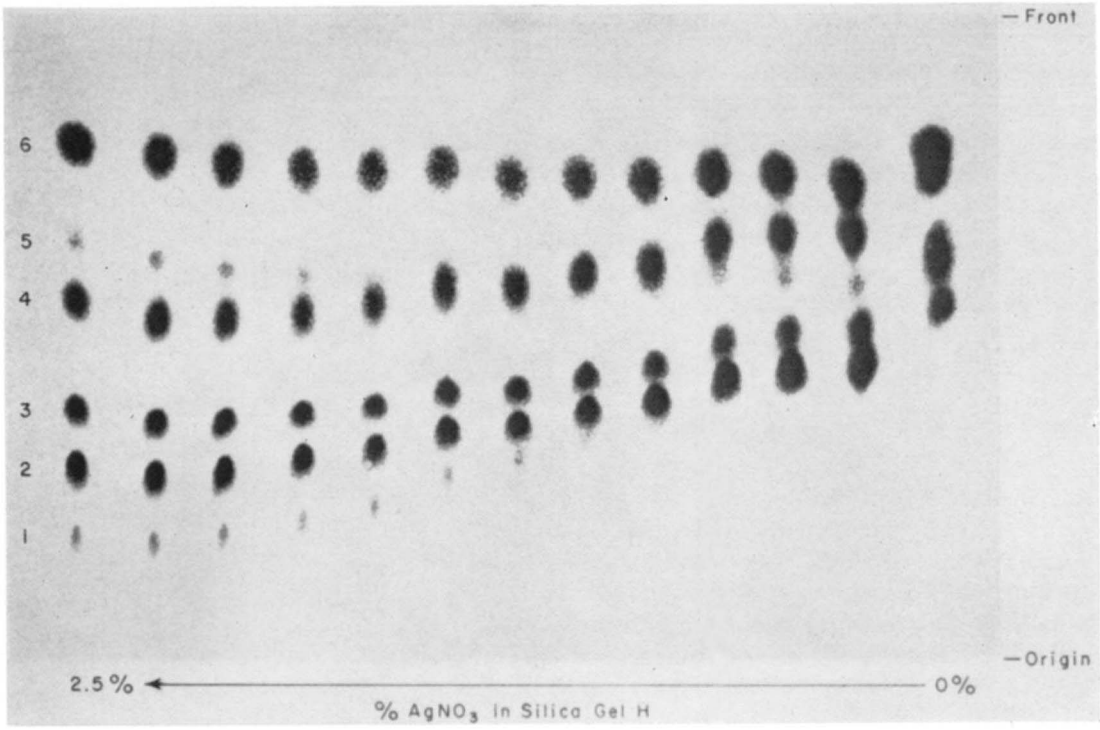


FIG. 1. Gradient layer thin-layer chromatogram of mono- and sesquiterpenic alcohols demonstrating the effect of increasing levels of impregnation with silver nitrate. The adsorbent was Silica Gel H (Merck) with a linear gradient of silver nitrate from 0 to 2.5% and the solvent was methylene chloride–chloroform–ethyl acetate–*n*-propanol 50:50:5:5. Samples: *1*, nerolidol; *2*, geraniol; *3*, nerol; *4*, guaiol; *5*, borneol; *6*, cedrol.
Reproduced from Stahl and Vollmann (61) with the permission of the authors and Pergamon Press Ltd.

The choice of visualization reagents is somewhat restricted by the presence of silver nitrate; for example iodine vapors are no longer suitable. However, corrosive agents such as aqueous sulfuric acid (43), phosphomolybdic acid in ethanol (65), chlorosulfonic acid–acetic acid (66), or phosphoric acid (67), all followed by heating at a suitable temperature, are satisfactory as general detection reagents. Visualization under ultraviolet light after spraying with dichlorofluorescein (43, 58) or dibromofluorescein (44, 67), or in daylight after spraying with water (65) is also suitable for general detection and, of course, for preparative work. Quantitative analysis of separated components may be effected by any of the procedures normally used in conjunction with TLC.

Several other analytical methods for lipids deserve mention in this section. Wood and Snyder (68) very recently described a modified argentation TLC system wherein the plates were prepared with a solution of silver nitrate in ammonia instead of in water, and the active species in complexing was considered to be the diamminesilver ion $Ag(NH_3)_2^+$. These plates were reported to give better separations of fatty acid methyl esters, to retain their resolving power much longer, and to involve less corrosion of conventional spreaders, claims with which we concur.

A number of reversed-phase partition methods involving silver ions have also been described. Apart from the methods developed by Wickberg (38) employing silver fluoroborate in the mobile phase, which have been mentioned already, Vereshchagin (69) and Paulose (70) have described separations of lipids by paper chromatography and TLC respectively with a nonpolar stationary phase and silver nitrate in the mobile phase. These procedures separate according to chain length and degree of unsaturation simultaneously and avoid the "critical" groups which occur in conventional reversed-phase chromatography. Paulose (70) impregnated his layers with silicone oil and then again with silver nitrate by spraying, but this latter step was almost certainly unnecessary because it was clearly the silver ions in the mobile phase which effected the separations.

One thing that has surprised this author is that no one, apparently, has utilized the tremendous solubility of silver nitrate in acetonitrile as the basis of a partition chromatography or countercurrent distribution procedure.

SEPARATIONS BY ARGENTATION METHODS

In the following sections, classified according to compound types, the various possibilities of lipid separations

by argentation chromatography are briefly reviewed. There is some discussion of suitable combinations of argentation methods with other separatory and analytical methods and of some of the ways in which these methods have already been employed in research on lipids.

Fatty Acids

The most obvious, and probably most common application of argentation chromatography is in separation of a mixture into fractions of differing numbers of double bonds (usually *cis*), and many such fractionations of fatty acid mixtures have been reported. Separations of fatty acid methyl ester mixtures in this way have been carried out, on a relatively large scale, by column chromatography on silicic acid (e.g. 36, 48, 60, 71), Florisil (49, 50), or ion-exchange resins (51, 52) impregnated with silver nitrate. On an analytical or smaller preparative scale, TLC on silicic acid impregnated with silver nitrate (e.g. 43, 72, 73) or with ammoniacal silver nitrate (68) is most convenient and the degree of separation obtained is illustrated in Fig. 2. These separations have been carried out generally with methyl ester mixtures, but free acids may be similarly separated if a little formic or acetic acid is added to the developing solvent to suppress dissociation. Alcohols and other simple aliphatic compounds are also easily separated. Some excellent separations of aldehydes and ketones as their dinitrophenylhydrazone derivatives have also been carried out by TLC on silver nitrate-impregnated alumina (54, 55) and kieselguhr (57).

As with all forms of chromatography, argentation chromatography is most effectively employed in conjunction with other types of chromatography. The fact that it separates basically according to degree and type of unsaturation, with little if any separation of differing chain lengths, makes it particularly suitable for combination with liquid–liquid and gas–liquid methods. Bergelson, Dyatlovitskaya, and Voronkova (74) described two-dimensional TLC of esters, reversed-phase TLC in the first and argentation TLC in the second dimensions, for complete separation according to both chain length and degree of unsaturation. The reverse sequence was used by Badings and Wassink (57) for carbonyl dinitrophenylhydrazone derivatives. Vereshchagin (69) and Paulose (70) obtained the same type of complete separation in one dimension, on paper and by TLC respectively, by reversed-phase chromatography employing a mobile phase containing silver nitrate. This same type of separation, by chain length and unsaturation, is of course the result of countercurrent distribution with silver ions in the polar phase (32, 34).

A rather exotic variant of combined chromatographic techniques was recently described (75), wherein the

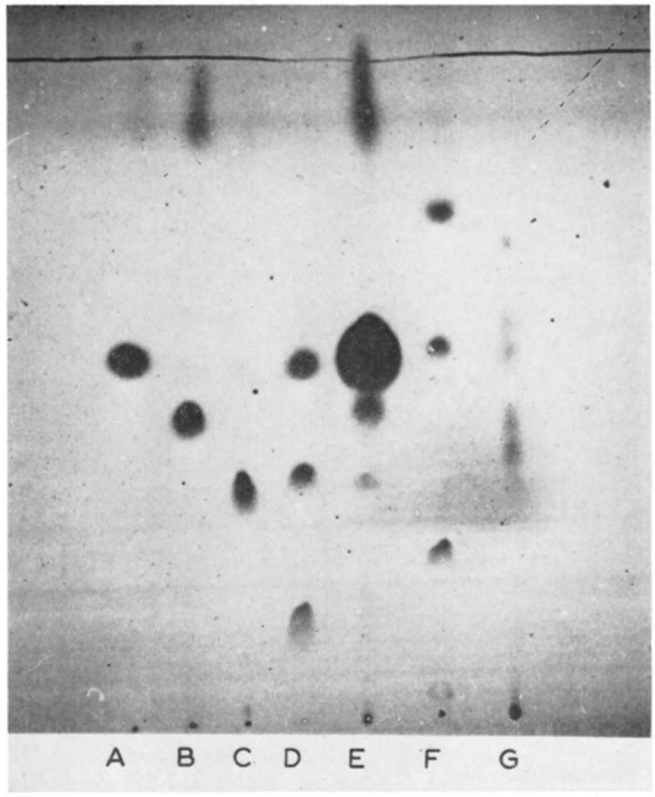


FIG. 2. Thin-layer chromatogram of fatty acid methyl esters and simple lipids on Silica Gel G impregnated with silver nitrate (5%, w/w). Developing solvent was diethyl ether–light petroleum 5:95 and spots were located by spraying with 50% H_2SO_4 and charring. Samples: *A*, methyl stearate; *B*, methyl elaidate; *C*, methyl oleate; *D*, mixture of stearate, oleate, and linoleate; *E*, methyl esters from fecalith lipids; *F*, mixture of cholesteryl stearate, cholesteryl oleate, and cholesteryl linoleate; *G*, sperm oil, i.e., a wax ester mixture.

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"first dimension" of chromatography was GLC, the components emerging from the column being eluted on to the edge of a logarithmically travelling thin-layer plate impregnated with silver nitrate, which was developed to provide the second dimension. While admiring the elegance of this method, this author has some doubts on the returns in practical utility which may be expected for the investment in the rather elaborate equipment and procedures necessary. The much simpler system of preliminary fractionation by preparative argentation TLC followed by GLC of the individual unsaturation classes is perfectly adequate for identification of most compounds and is more suitable for detection and identification of minor components and for quantitative analysis of complex mixtures. This latter approach has been used by many workers (e.g. 1, 48, 76, 78) and is exemplified in Fig. 3.

After argentation chromatography, each unsaturation class may be fractionated according to chain length by

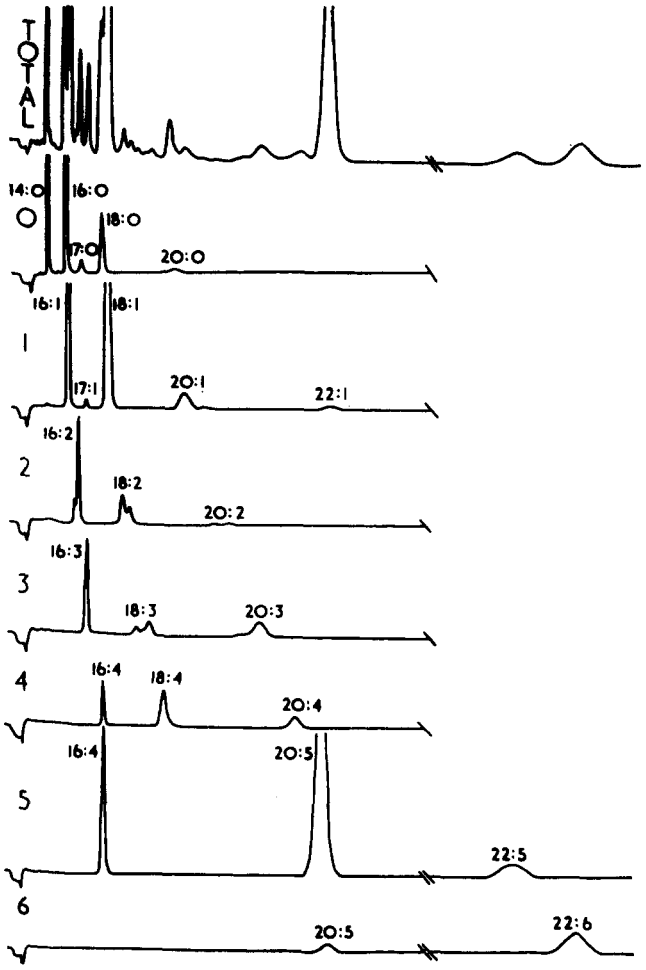

FIG. 3. Reproduction of the GLC curves (obtained on an ethylene glycol adipate polyester stationary phase) of sardine oil total mixed esters (*Total*), and the saturated (*0*), monoene (*1*), diene (*2*), triene (*3*), tetraene (*4*), pentaene (*5*), and hexaene (*6*) ester fractions. These fractions were isolated by preparative TLC on Silica Gel G impregnated with silver nitrate (5%, w/w) developed with diethyl ether–light petroleum 5:95 for the 0, 1, and 2 fractions, and methanol–diethyl ether 1:9 for the 3, 4, 5, and 6 fractions. Note the separations of positional isomers of dienoic and trienoic esters.

Reproduced from Morris (1).

preparative GLC and the structure of each compound or mixture so obtained determined by permanganate–periodate oxidation (e.g. 78) or by reductive ozonolysis (e.g. 73).

These and other procedures have assisted in the detection and characterization of a whole range of novel *cis*-ethylenic and acetylenic acids from natural sources (a number of *trans*-ethylenic acids have also been discovered but will be described later). Thus 4,7,10,13-eicosatetraenoic acid was detected in rat liver phospholipids (73) and several odd-number and isomeric acids were shown to be minor components of a number of seed oils (76, 77). Alpha- and γ-linolenic acids and 6,9,-12,15-octadecatetraenoic acid were shown to occur in several Boraginaceae seed oils (79) and all-*cis* 5,9,12-octadecatrienoic acid was demonstrated in *Xeranthemum annum* seed oil (80). Stearolic acid was shown to be present in a number of Santalaceae seed oils (81, 82) and more complex acetylenic acid mixtures from the seeds of *Onguekoa gore* (83, 84) and *Acanthosyris spinescens* (85) were separated into fractions with and without terminal ethylenic bonds and the individual components of these fractions then identified.

So far in this section, we have only discussed separations on the basis of degree of unsaturation. One of the most useful attributes of argentation methods is that they can separate compounds with nonconjugated unsaturation according to the geometry of the double bonds. In this they have an advantage over methods involving mercuric salt adduction, which do not separate *cis*- and *trans*-ethylenic isomers. Separations of *cis*- and *trans*-monoenoic esters are readily achieved by argentation adsorption chromatography in columns (e.g. 36, 60) or on thin layers (e.g. 43, 64), by partition methods on thin layers (70, 74), and by countercurrent distribution (32, 34, 36). The separation of elaidate from oleate and stearate by TLC is shown in Figs. 2 and 4 and an example of one application of this type of separation, the separation of the *cis* and *trans* monoenes from fecalith mixed esters, which were then analyzed separately by GLC and shown to be very different in chain-length distribution (86), is also illustrated in Fig. 2 (sample E). Geometrical isomers of unsaturated aldehyde dinitrophenylhydrazones are similarly easily resolved by TLC (57, 87). Separations of the various geometric isomers of polyenoic esters are also readily achieved by countercurrent distribution (e.g. 34) or TLC (e.g. 34, 88, 89).

The ability to fractionate ester mixtures according to the geometry as well as the degree of their unsaturation has been particularly useful in studies of the mechanism and intermediate products of hydrogenation. Both heterogeneous (e.g. 33, 35, 90) and homogeneous (e.g. 91, 92) catalytic hydrogenation of unsaturated esters have been studied, and Jardine and McQuillin (93) have recently compared rates of catalytic hydrogenation of various ethylenes and acetylenes (not fatty acids, however) with their retention on silicic acid–silver nitrate thin layers and have found some parallelism. Partial reduction of polyunsaturated esters with hydrazine, which causes no positional or geometrical isomerization of unreduced double bonds, has also been studied with the help of argentation TLC (e.g. 94). Indeed, partial reduction with hydrazine, isolation of the *cis* or *trans* monoenoic products by argentation TLC, and determination of their structures by GLC after oxidation or ozonolysis comprise the most powerful combination of techniques yet devised for unequivocal elucidation of the structures of polyunsaturated fatty acids. This approach has already

been used a number of times on novel acids from various seed oils; for example all-*cis* 5,9,12-octadecatrienoic acid (80), *cis*-9, *trans*-11, *trans*-13, *cis*-15-octadecatetraenoic acid (95), *cis*-9, *trans*-12-octadecadienoic acid (96), and *trans*-3, *cis*-9, *cis*-12-octadecatrienoic acid (97); and has very recently been written up as a defined and general procedure by Privett and Nickell (98). In some cases, for example *cis*, *trans*-linoleate (96) and a series of *trans*-Δ^3-acids (97), the novel acids were detected in the first place and were isolated by argentation TLC prior to final elucidation of their structures by the above procedure.

Besides effecting separations according to degree of unsaturation and to geometry of double bonds, argentation chromatography has a third major attribute, namely the ability to separate suitable positional isomers of unsaturated fatty acids. Scholfield, Jones, Butterfield, and Dutton by countercurrent distribution (34) and de Vries and Jurriens by TLC (88) showed that with dienoic esters the effect of silver complexing increased with increasing separation of the two double bonds. Thus, 9,11-, 9,12-, and 9,15-octadecadienoates could be readily separated and, on this basis, a number of positional isomers of linoleic acid were detected in butter fat by argentation TLC (99). De Vries and Jurriens also demonstrated (88) the separation of the 6-, 9-, and 12-octadecenoate isomers by argentation TLC using a benzene–light petroleum mixture as solvent. Similar separations of the 7-, 9-, and 11-octadecenoates and of the 9- and 11-isomers were reported by Bergelson et al. (74), and by Lees and Korn (89), respectively, using mixtures of ethers and light petroleum fractions, and Vereshchagin (69) separated the 6- and 9-octadecenoates by his reversed-phase paper system. We have been unable to obtain adequate, reproducible separations of isomeric monoenoic esters by argentation TLC with the solvents and conditions described by these workers. We have devised a procedure (64) involving double or triple development with toluene at −15°C or −25°C, which consistently gives quantitative separation of suitable mixtures of isomeric monoenoic esters, as illustrated in Fig. 4. This system, incidentally, gives much better separations of mixtures containing acetylenic acids than are obtained with the more conventional ether–light petroleum systems (81, 100).

Although all of the octadecenoate isomers have not been examined, we consider (64) that both the *cis* and the *trans* series will conform to a sinusoidal type of curve similar to that produced by normal TLC of positionally isomeric substituted esters (101, 102); i.e. decreasing mobilities from Δ^2 to about Δ^5, then progressively increasing mobilities to about Δ^{12}, and decreasing mobilities again to Δ^{17}. Positionally isomeric unsaturated aldehyde

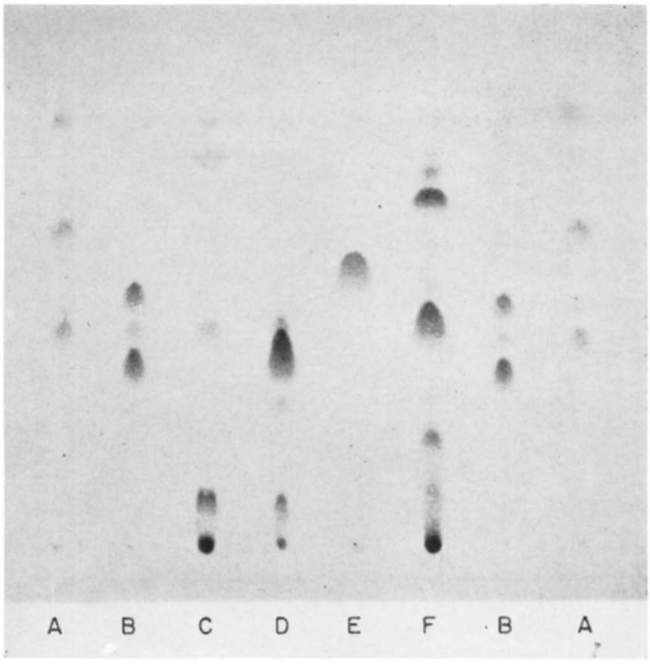

FIG. 4. Thin-layer chromatogram of some fatty acid methyl esters on Silica Gel G impregnated with silver nitrate (10%, w/w). The plate was developed three times with toluene at −15°C and spots were located by spraying with chlorosulfonic acid–acetic acid 1:2 and charring. Samples: *A*, methyl elaidate (upper) and methyl oleate (lower); *B*, methyl *cis*-vaccenate (Δ^{11}, upper), methyl oleate (Δ^9, middle) and methyl petroselinate (Δ^6, lower); *C*, rat liver mixed esters (small proportion of Δ^{11}-18:1 not visible on reproduction); *D*, parsley seed oil mixed methyl esters (separation of Δ^9- and Δ^6-18:1 isomers); *E*, methyl stearolate; *F*, *Exocarpus cupressiformis* seed oil mixed methyl esters. In this last sample (*F*) the components above oleate, the major constituent, are stearolate (a small amount), santalbate (octadec-9-yn-11-enoate, the other major constituent), a novel furanoid acid ester (see text), and saturated esters (small amount).

derivatives have also been separated by argentation TLC (e.g. 57, 87).

Substituted Fatty Acids

Argentation chromatography is also very suitable for separation of most substituted fatty acids according to degree and type of unsaturation. This is particularly valuable because the conditions required for mercuric adduct formation degrade some acid-labile compounds, such as epoxy esters, and GLC is also unsuitable for a number of hydroxy esters (e.g. 103, 104).

A considerable range of epoxy, halohydroxy, hydroxy, and dihydroxy esters have been separated by argentation TLC according to degree, type, and position of unsaturation by Morris (43) and Morris and Wharry (101). Dihydroxy esters were separated not only on the basis of unsaturation but also according to the *threo* or *erythro* configuration of the glycol group by double impregnation with silver nitrate and boric acid (43). In combination with normal adsorption TLC and GLC, which

effect remarkable separations of positionally isomeric substituted esters (101, 102, 104), argentation chromatography is a great aid in elucidation of structures of novel oxygenated acids. By these means the structures of the acetylenic hydroxy acids of isano oil (83, 84), an epoxy acid in *Aster alpinus* seed oil (97), and a whole series of previously uncharacterized α-hydroxy acids from brain cerebrosides (48, 105) have been determined. The study of that currently very exciting class of oxygenated fatty acid derivatives, the prostaglandins, has also been greatly facilitated by a combination of TLC and argentation TLC (e.g. 65, 106–109). The separation of all of the known prostglandins described by Gréen and Samuelsson (65) provides an excellent example of the scope and selectivity of this combination of methods. We have very recently detected and isolated, by argentation TLC, a highly unusual oxygenated fatty acid ester from the mixed esters derived from *Exocarpus cupressiformis* seed oil (Fig. 4, sample *F*) and proved it to be methyl 8-(5-hexylfuryl-2)-octanoate (100).

Nonoxygenated substituted acids are also separable by these methods. A whole range of branched-chain acids from skin lipids were characterized by GLC after preliminary fractionation of the mixed esters according to unsaturation on a silicic acid–silver nitrate column (110). Cyclopropanoid esters may be separated from unsaturated esters (e.g. 111), but an early claim that cyclopropenoid esters could be separated from normal saturated and unsaturated esters by argentation TLC (112) seems to be generally refuted by others (e.g. 5). The breakdown of cyclopropenoid esters is presumably due to the ready reaction of such compounds with silver nitrate (cf. 113). By formation of methyl mercaptan derivatives of cyclopropenoid esters, however, these compounds are readily separated from unsubstituted and cyclopropanoid esters by argentation TLC (114).

Neutral Lipids

Argentation chromatography of intact lipid classes, particularly of glycerides, has been applied much more widely and usefully in the short time since its inception than has reversed-phase partition chromatography. This is because it is more convenient, more definitive, and more suitable for isolation of sufficient quantities of individual fractions for further study, e.g. by lipolysis.

Cholesterol esters from blood serum have been quantitatively separated by argentation TLC into seven fractions having zero to six double bonds (58) and the less complex sterol esters from skin have been fractionated by column and thin-layer argentation chromatography (115). Separation of a simple cholesterol ester mixture is illustrated in Fig. 2 and the fractionation of a wax ester mixture, sperm oil, is also shown in the same figure. The obviously composite spots of the monoenoic and dienoic components of sperm oil was shown by GLC to be due, largely, to chain-length variations (1, 2). The wax esters from skin have also been resolved in this way (115) and the isolation of several homologous series of hydrocarbons from various plant waxes by argentation chromatography (116) should also be mentioned here.

In one of the first publications on argentation TLC (44) separations of diglycerides and monoglycerides according to unsaturation were described and this procedure has very recently been extended to the equally easy fractionation of glycerol ethers (117). However, the vast majority of neutral lipid separations by silver ion complexing have been of triglycerides.

Of the first three papers on argentation chromatography of lipids, two described separations of triglycerides, on columns (36) and on thin layers (44). Columns of silicic acid impregnated with silver nitrate have been used on many occasions (e.g. 45–47, 118–120) for preparative fractionation of natural triglyceride mixtures but are not very satisfactory with glycerides containing more than four *cis* double bonds (47). Gunstone and coworkers (45, 46), however, partially circumvented this difficulty by preliminary fractionation of relatively large amounts of triglyceride mixtures by low temperature crystallization in the presence of silver ions. The simpler mixtures thus obtained were then separable by column chromatography. Argentation TLC, however, has proved more popular even for preparative work because of its greater convenience and the superior separations obtained, and it has been used by many authors in studies of natural triglyceride compositions (e.g. 67, 121–132). Quantification has been achieved in a variety of ways: by densitometry after charring (67), by glycerol determination after hydrolysis (122, 123), by colorimetry after reaction with hydroxamic acid and ferric ions (124) or with chromotropic acid (129), or by GLC of the component fatty acids after addition of a suitable internal standard (125, 131). Lipase hydrolysis of the separated triglyceride fractions was used in most cases to obtain still more information on glyceride compositions.

An example (not particularly good) of argentation TLC of some natural triglyceride mixtures is illustrated in Fig. 5. It is clear from this that some triglycerides having the same total number of double bonds are clearly separated from each other. Gunstone and Padley (125) determined the order of complexing powers of triglycerides, with from nine to zero double bonds and without considering positional isomers, to be as follows (3 = linolenic, 2 = linoleic, 1 = oleic, and 0 = palmitic and/or stearic): 333, 332, 331, 330, 322, 321, 320, 311, 222, 310, 221, 300, 220, 211, 210, 110, 100, 000. They have found it useful to assign arbitrary values for the complexing power of each acyl chain, viz. saturated = 0,

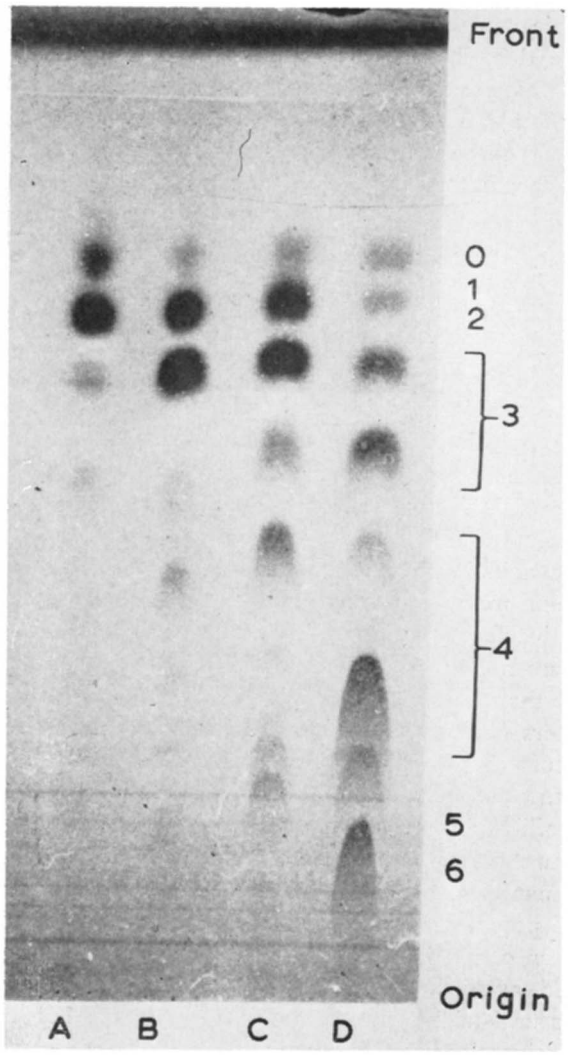

FIG. 5. Thin-layer chromatogram of natural triglyceride mixtures on Silica Gel G impregnated with silver nitrate (5%, w/w). The solvent was isopropanol–chloroform 1.5:98.5 and spots were located by spraying with 50% H_2SO_4 and charring. Samples: *A*, palm oil; *B*, olive oil; *C*, groundnut oil; *D*, cottonseed oil. The numbers represent total numbers of double bonds in each triglyceride molecule.

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oleic = 1, linoleic = $(2 + a)$, and linolenic = $(4 + 4a)$, where a is some fraction less than unity. Thus the triglyceride 330, with six double bonds, has a complexing power of $(8 + 8a)$ which is greater than the value of $(8 + 6a)$ for the triglyceride 322, which has seven double bonds. Positional isomerism may result in further separation of some of the classes listed above (44).

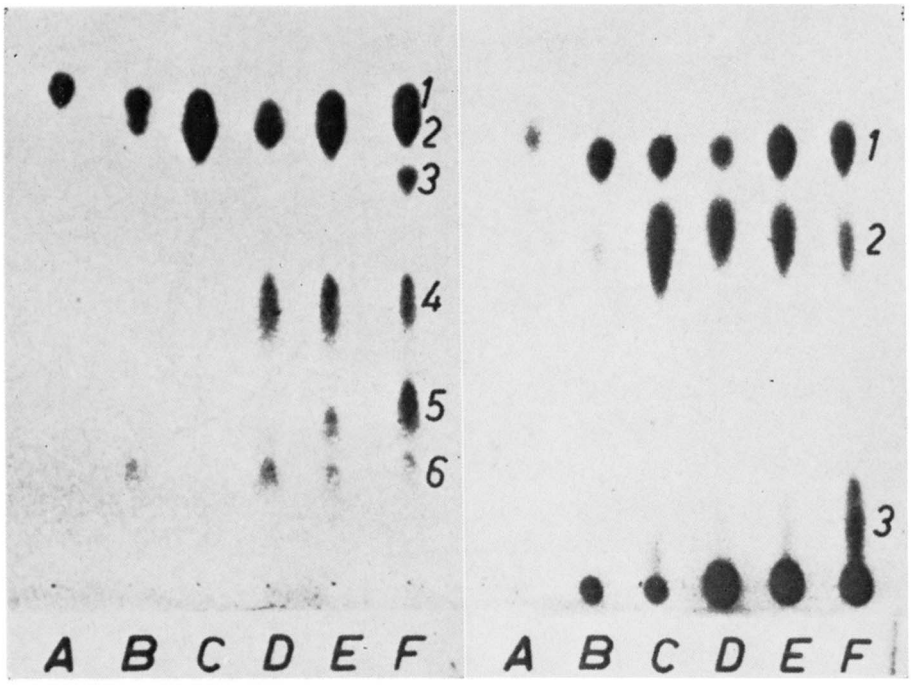
Even greater selectivity of separation is, of course, obtained by complementary use of argentation chromatography with some other chromatographic method. Thus, Cubero and Mangold (59) have combined it with normal adsorption TLC to separate triglycerides, free acids, and sterols as classes and then to resolve them according to unsaturation in the second dimension. Blank and Privett (128) also combined it with normal adsorption TLC to determine the composition of milk fat triglycerides. Kaufmann and Wessels, on the other hand, combined it with reversed-phase partition TLC to separate, in two dimensions, virtually all the glycerides of sunflower oil (127), while Vereschagin used the same combination simultaneously on paper (69). Perhaps the most promising combination for triglyceride separations, however, is of argentation chromatography with GLC of the intact glyceride fractions so obtained (129, 130) or of the azelao-glycerides produced from them by oxidative cleavage (47).

The much greater precision of analysis now possible by these methods has led Privett and coworkers (131, 132) to call into question one of the basic assumptions of Vander Wal's 1,3-random, 2-random hypothesis of glyceride distribution, namely that the fatty acids in the 2-position are randomly distributed relative to the 1,3-positions. Morris (133) has devised a procedure, based on separation by argentation TLC of derivatives of the diglycerides produced from pure triglycerides by lipolysis, to demonstrate optical asymmetry in natural triglycerides for the first time. This, of course, is counter to the other assumption of the 1,3-random, 2-random theory that the 1- and 3-positions are equivalent.

Polar Lipids

Phospholipids and glycolipids are more difficult to separate according to unsaturation by argentation chromatography than are neutral lipids and, so far at least, relatively little work in this direction has been done. Kaufmann, Wessels, and Bondopadhyaya (134) claimed to have separated soya lecithin and egg lecithin into nine and seven fractions, respectively, by argentation TLC. However, using their solvent system (chloroform–diethyl ether–acetic acid 97:2.3:0.5), we have been unable to reproduce their separations or even to effect migration of a pure saturated lecithin from the origin. More convincing separations of a number of natural lecithins have recently been reported by Arvidson (135) and are illustrated in Fig. 6. Less complete fractionations of lecithins from a number of animal tissues (136, 137) and of phosphatidyl glycerols from photosynthetic tissues (138, 139) have been described by van Deenen and his collaborators.

A more complete fractionation and analysis of the molecular species of various phospholipid classes is possible if recovery of the intact phospholipid fractions is not required (140). This is effected by first converting the relevant polar lipid class to the corresponding diglycerides; these, or derivatives of them, being neutral lipids, are easily separable into individual unsaturation classes by argentation TLC. This approach was pioneered



a *b*

FIG. 6. Thin-layer chromatograms of lecithins on Silica Gel H impregnated with silver nitrate (ca. 20%, w/w). The solvent was chloroform–methanol–water 65:25:4, plate *a* having been activated at 175°C for 5 hr and plate *b* at 180°C for 24 hr. Detection was by charring after spraying with 50% H_2SO_4. Samples: *A*, hydrogenated egg lecithin; *B*, egg lecithin; *C*, rabbit liver lecithin; *D*, rat liver lecithin; *E*, pig liver lecithin; *F*, bovine liver lecithin. The numbers represent the total numbers of double bonds in each lecithin molecule.

Reproduced from Arvidson (135) with the permission of the author.

by Renkonen (140, 141) and has been applied successfully by him and by van Deenen and his school (136–139) for the analysis of a number of polar lipids from various sources. Whereas Renkonen initially produced acetoxy diglycerides from the phospholipids by acetolysis (141), van Deenen (136–139) utilized enzymatic cleavage with phospholipase C. Very recently, Renkonen (142) has reported that the acetolysis procedure induces some intramolecular rearrangement of the fatty acids on the diglycerides and, therefore, he has also recommended the use of phospholipase C for this cleavage.

Sterols and Other Terpenoid Compounds

In his first paper on argentation column chromatography (36), de Vries claimed quantitative separation of cholesterol from cholestanol. Avigan, Goodman, and Steinberg (143) showed that silver nitrate-impregnated plates gave excellent separation of 5,7-dienic sterols from saturated and monounsaturated sterols. Morris (1–3) also noted the strong complexing of 5,7-dienes on argentation TLC and showed that whereas Δ^5-stenols were retained strongly enough to be clearly separated from cholestanol, as illustrated in Fig. 7, Δ^7-stenols were not (cf. 144). Free sterols differing only in side-chain unsaturation have very similar mobilities on silver nitrate-impregnated plates but if less polar derivatives, such as acetates, are made separation on the basis of side-chain unsaturation is possible (e.g. 4, 143).

Several similar types of separations of small numbers of sterols by argentation TLC have been reported (e.g. 145–147) but the largest ranges of sterols so far examined, mostly as their acetates, were described by Copius Peereboom (148, 149) and by Klein et al. (62). These three excellent papers show clearly the great influence of the molecular environment around centers of unsaturation on the degree to which they participate in complexing. They also, incidentally, demonstrate the considerable potential of argentation TLC, particularly in conjunction with reversed-phase partition (148, 149) or adsorption (62) chromatography, for analytical and structural studies of sterols. Whereas most of the authors cited above have assumed that silver ion–double bond complexing provides the selectivity whereby these separations of various sterols are attained, Klein et al. (62) have provided fairly convincing evidence to suggest that silver–olefin complexing may be less effective in TLC of sterols than are geometrical changes in the adsorbent pore diameter induced by the impregnation. Clearly there are more complex and structurally specific effects in argentation TLC of sterols and other alicyclic

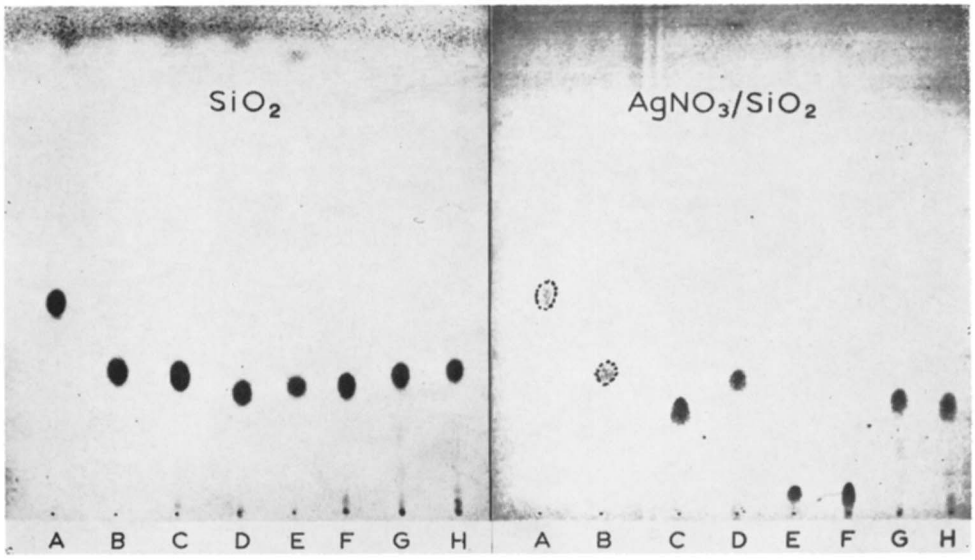


FIG. 7. Thin-layer chromatograms of sterols on Silica Gel G and on Silica Gel G impregnated with silver nitrate (5%, w/w). The solvent was commercial chloroform and spots were located by spraying with 50% H_2SO_4 and charring. Samples: *A*, coprostanol; *B*, cholestanol; *C*, cholesterol; *D*, Δ^7-cholestenol; *E*, 7-dehydrocholesterol; *F*, ergosterol; *G*, β-sitosterol; *H*, stigmasterol.
Reproduced from Morris (2) with the permission of John Wiley & Sons Ltd.

compounds than there are with fatty acids and their derivatives, and more basic comparative work will have to be done before the full potentialities of these methods are realized.

A considerable number of papers have described argentation-chromatographic separations of a wide variety of terpenoid compounds other than sterols. Although these various compounds are not lipids, at least not under this author's classification of lipids, they are certainly lipophilic and deserve a brief summary here. The first argentation separations of terpenoid materials employed the reversed-phase systems with silver fluoroborate in the mobile phase devised by Wickberg (38). These procedures were used for separations of various sesquiterpenic hydrocarbons and resin acid methyl esters (38–42). Sesquiterpenic hydrocarbons were the first of this class to be studied by adsorption TLC on silver nitrate-impregnated silicic acid by Gupta and Dev (66), more recent work having been reported by Czech workers using columns (150) and TLC (151, 152). The range of terpenoid compounds studied was extended to diterpenic hydrocarbons, esters, aldehydes, ketones, and alcohols by Norin and Westfelt (153), who combined argentation TLC with GLC of these compounds. Argentation TLC is particularly suitable for separation and identification of resin acid methyl esters (153, 56), some of which are subject to alteration on GLC. Mono-, sesqui-, and diterpenic alcohols were studied by Stahl and Vollman (61), who used argentation TLC in conjunction with adsorption TLC and reversed-phase partition TLC, and Nano and Martelli (154) described separations of some pairs of allylic–propenylic isomers.

More complex structures have also been separated by argentation TLC, such as tetracyclic triterpenes (155), steroidal alkaloids and sapogenins (156), and ethynyl steroids from other steroids (157). In this last case, however, it is not silver ion complexing which produces the separations but silver–acetylene salt formation.

CONCLUSIONS AND PROSPECTS

That argentation chromatography is an extremely valuable addition to the technical arsenal of the lipid chemist or biochemist should now be abundantly clear. Argentation TLC has the greatest versatility and, therefore, is of most value; but paper and column chromatography and countercurrent distribution employing silver ions all have their uses, and the last two procedures are valuable when relatively large amounts must be fractionated. For the most part, argentation chromatography has superseded the older methods based on mercuric acetate adducts, for example, but there are still occasions when these older methods may be desirable or necessary. Argentation methods, of course, are generally most effective when used in conjunction with other separatory and analytical procedures and this aspect has been emphasized throughout this review.

The applications of argentation chromatography in lipid chemistry and analysis have been summarized fairly comprehensively during the course of this review. Its value in analysis of complex ester mixtures, in de-

tection and structure elucidation of unusual fatty acids and in determination of the fatty acid distribution in triglycerides and polar lipids, for example, should by now be self-evident. However, relatively little has been said of applications of argentation chromatography in biochemical investigations.

Until now, the applications of argentation chromatography in biochemical studies have mostly been very marginal; i.e., simply for isolation or analysis of precursors or products. Thus, it has been valuable in isolating and purifying synthetic labeled precursors such as elaidic acid (158) or the isomeric *cis*- and *trans*-2- and -3-hexadecenoates (159) and -dodecenoates (160) and in isolating and identifying the products of biological reactions (e.g. 159–163). We use argentation TLC routinely in our laboratory, in conjunction with preparative GLC, for isolation of individual acids produced biologically so as to determine the position of the ^{14}C-label by hydrogenation, chemical α-oxidation, and radio GLC (e.g. 162, 163). In this context, it may be pertinent to point out that unsaturated fatty acid esters tritiated at the position of unsaturation are retained slightly less strongly on silver-impregnated adsorbents than the corresponding unlabeled or ^{14}C-labeled esters (164). A similar isotope effect of deuterated olefins had previously been demonstrated by argentation GLC (165) and these findings indicate that some care is required in chromatography of compounds labeled with deuterium or tritium. Other biochemical investigations which have been greatly facilitated by argentation TLC are studies of the biohydrogenation of unsaturated fatty acids in the ovine digestive tract (166) and by rumen microorganisms (167) and the excellent work on biosynthesis of prostaglandins (e.g. 106, 107, 109).

However, in all of these studies summarized above, argentation chromatography has been of only incidental importance as a convenient means of isolating the fatty acid products of biological reactions. In the future it will undoubtedly assume far greater importance in studies of the metabolism and physiological significance of intact lipids by becoming a basic feature of experimental design. It will be particularly useful for studies of absorption, transport, and interconversions of lipids that use double labeling techniques. As an example, the long-standing question of whether or not intact triglycerides are absorbed in the gastrointestinal tract (cf. 168) could be readily resolved by the complementary use of argentation TLC and a doubly labeled substrate. Thus, a mixture of, for example, ($\alpha\alpha$-$^{14}C_2$, β-^{3}H)-triolein with a large excess of unlabeled tripalmitin could be used as substrate for in vitro incubation with intestinal slices (cf. 168). Argentation TLC could then be used to isolate triolein from the absorbed triglycerides. If this isolated triolein had the same ^{3}H:^{14}C ratio as the substrate triolein then absorption of the triglyceride intact would be unequivocally proved.

In the general confusion, at the moment, as to which lipids may be metabolically active and which merely structural, it is beginning to appear likely that certain individual molecular species of a given lipid class are intimately concerned in metabolism while the bulk of that lipid class present in the system may fulfill only a passive structural role. Even a pure lipid such as lecithin or phosphatidyl glycerol is, after all, a complex mixture of compounds of differing physical properties and it is only by studying the behavior of the individual molecular species of such "pure" lipids that this type of problem can be solved. The first steps along this road have already been taken by van Deenen and his school in determining, by argentation TLC and other methods, the individual molecular species of phosphatidyl glycerol in photosynthetic tissues (138, 139) and of lecithin and other phospholipids in animal tissues (136, 137) and in trying to understand the implications of the proportions and compositions of these in membrane structure and function. This type of study will certainly be one of the major avenues of research in lipid biochemistry in the next few years and argentation chromatography will have a major role to play in following it.

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