The Nature of the Technique

The technique of silver ion chromatography, which is sometimes termed ‘argentation chromatography’, was first introduced for the separation of fatty acid derivatives in two papers which appeared almost simultaneously in Chemistry and Industry in 1962. In its earliest manifestations, it was adapted to thin-layer chromatography (TLC) and to low pressure column chromatography. The former is still in widespread use today, but high performance liquid chromatography (HPLC) has more recently been adapted to the purpose and may be expected to slowly supplant TLC. Gas chromatography with stationary phases containing silver ions has been used to separate mixtures of hydrocarbons containing low molecular weight olefins, but is not practical for analytes of higher molecular weight. While lipid chemists, especially those concerned with fatty acid derivatives, have made most use of silver ion chromatography, it has also been used for a wide range of aliphatic and alicyclic compounds, including terpenes, sterols, carotenoids, insect pheromones, etc. However, applications only to separation of more range of aliphatic and alicyclic compounds, including terpenes, sterols, carotenoids, insect pheromones, etc. However, applications only to separation of more molecular weight olefins, but is not practical for analytes of higher molecular weight. While lipid chemists, especially those concerned with fatty acid derivatives, have made most use of silver ion chromatography, it has also been used for a wide range of aliphatic and alicyclic compounds, including terpenes, sterols, carotenoids, insect pheromones, etc. However, applications only to separation of more.

Liquid Chromatography

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Silver ion chromatography is based on a distinctive property of unsaturated organic compounds, that is the capacity to complex with transition metals in general, and with silver ions in particular. The complexes are of the charge-transfer type in which the unsaturated compound acts as an electron donor and the silver ion as an electron acceptor. In the accepted model, it is believed that there is formation of a \(\sigma\)-type bond between the occupied 2p orbitals of an olefinic double bond and the free 5s and 5p orbitals of the silver ion, and a (probably weaker) \(\pi\)-acceptor backbond between the occupied 4d orbitals of the silver ion and the free antibonding 2p \(\pi^*\) orbitals of the olefinic bond. In chromatographic systems, complexes are only formed transiently and are in kinetic equilibrium with the free olefin. The coordination forces are weak, and IR spectra show very little shift in frequencies from those of free double bonds, for example. Stable silver ion–olefin complexes have been isolated in some circumstances, and then X-ray crystallography has demonstrated that each silver ion can interact with two double bonds simultaneously. Other metals can form such complexes but none other than silver has the correct combination of properties for general chromatographic use.

Until recently, the technique was very much an \textit{ad hoc} one that worked, but without a sound theoretical basis. Silver ion TLC cannot be used to generate reproducible chromatographic data, for example. However, some useful qualitative data are available from studies with simple model compounds and in particular it is apparent that:

- Unsaturated aliphatic and alicyclic compounds form more stable complexes than do aromatics.
- The stability decreases with increasing chain length of the aliphatic substrate.
- The stability decreases with increasing numbers of substituents of a double bond in the order \(RCH=CH_2 > R_2C=CH_2 > \text{cis} \ RCH=CHR > \text{trans} \ RCH=CHR > R_2C=CHR > R_2C=CR_2\). The greater stability of the \(\text{cis}\)-isomer may be due either to the relief of strain when the complex is formed or to steric hindrance by the two alkyl moieties when they are in a transposition to each other.
- Conjugated polyynes form less stable complexes than those with methylene-interrupted double bonds, and the greatest stability is when two methylene groups separate double bonds, perhaps because a chelate complex can then be formed.
- The stability of a silver ion complex increases when a hydrogen atom from a molecule of the \(RCH=CHR\) type, for example, is replaced with deuterium or tritium.
- Monoenes form stronger complexes than monoynes (one acetylenic bond).
- The strength of complexation increases as the temperature is lowered.

As far as has been ascertained, these rules also apply to larger molecules such as simply fatty acid...
derivatives, and to all forms of silver ion chromatography. However, there are few quantitative experimental or theoretical data on the mechanism of complex formation between silver ions and complicated unsaturated molecules like glycerolipids, which have up to three unsaturated fatty acids per molecule, although again, there is a substantial body of qualitative information.

A complicating factor, when considering silver ion complexation in the context of a chromatographic system, is the role of the support material. The most widely used support for TLC, silica gel, possesses appreciable polarity and absorptive activity. Therefore, the elution order of lipids cannot always be ascribed to the complexation reaction with silver ions and double bonds only, although this is usually the most important factor. A separate but related problem is the topology of silver ions on the surface of the adsorbent. For example, in silver ion TLC it is apparent that part of the silver nitrate remained in crystalline form filling the pores of the silica gel while a further proportion remains dissolved in the water which is always bound to silica gel. The aqueous silver nitrate is assumed to be responsible for complex formation, and some experience seems to confirm this observation. For example, Nikolova-Damyanova considers that there is no need for incorporation of excessive amounts of silver nitrate (1–2%) into TLC systems.

When discussing mixed retention mechanisms in chromatography, it is also necessary to consider the mobile phase. A proper choice of solvents determines the selectivity of a separation to an appreciable extent. Again, there are no systematic data available, but it has often been noted that better resolution is achieved by using chlorinated solvents as major components of the mobile phase in silver ion chromatography.

The more recent marriage of silver ion chromatography with HPLC has given us a better understanding of the mechanism of silver ion chromatography and this is discussed below.

**Silver Ion Thin-Layer Chromatography**

Silver ion TLC uses simple equipment and can afford excellent results in practice. Precoated TLC plates are available commercially, although it is not difficult to prepare one’s own (but wear gloves!). Thus, silver nitrate is simply incorporated into the aqueous slurry used to suspend the silica gel and the plates are spread and activated in the usual way, though some care is necessary to minimize exposure to light. Sometimes 10–20% of silver nitrate relative to silica gel is recommended by authors, but 1–2% is generally sufficient. Alternatively, plates can be impregnated with silver nitrate by careful immersion in a bath of a solution of silver nitrate in methanol or acetonitrile, and this option is often favoured with precoated TLC plates. After the plates have been activated, they should be stored in a desiccator in the dark.

Lipids are spotted on to the TLC plate and this is usually developed in a closed tank (in the dark) containing an appropriate mobile phase. However, Nikolova-Damyanova recommends using open tanks and carefully regulated volumes of solvent. Chromatography is most often carried out at ambient temperature, although temperatures as low as −20°C have on occasion been recommended to increase the strength of complexation and improve the separation. When the mobile phase nears the top of the plate, the latter is removed from the tank, and dried in a stream of air or nitrogen.

Various methods of detection and quantification are available. For example, one popular method consists in spraying the plate with concentrated sulfuric acid, and heating it at 180°C in an oven. The separated components are charred (converted to carbon) and can be quantified directly by scanning densitometry. A procedure of this kind is of course destructive to the sample, and has to be carried out with great care to avoid hazard to the operator.

Alternatively, the developed plate can be sprayed with a solution of 2,7-dichlorofluorescein in methanol (0.1% w/v). After evaporation of the solvent, the plate is viewed under a UV lamp; lipids appear as yellow spots against a dark purple background. The lipid/silica gel spots are scraped from the plate, and lipids are recovered by extraction with an appropriate solvent, though the extracts may have to be washed with a solution of dilute buffer (pH 9) to eliminate any silver nitrate and dye that co-elute. Commonly components are identified and quantified by gas chromatography of the fatty acid methyl esters, following transesterification, in the presence of an added internal standard, such as an odd-chain fatty acid.

As an example, Figure 1 illustrates the separation of fatty acid methyl esters by silver ion TLC. Saturated fatty acids do not form complexes with silver ions, so migrate ahead of the other components on the plate. *Trans*-monoenes form less stable complexes than *cis*-monoenes, so the former migrate faster. Dienoic fatty acids come next followed by polyenes, which under these conditions remain near the origin. A separation of this kind is the standard method for reliable quantification of fatty acids with *trans* double bonds. If the polarity of the mobile phase is increased, polyenoic fatty acid derivatives can be resolved into fractions with three, four, five and six double bonds, but saturated, and mono- and...
benches, equipment and the fingers of the analysts. HPLC methods do not suffer from these difficulties.

**Preparative Scale Column Chromatography**

By analogy with silver ion TLC it has proved possible to impregnate silica adsorbents (or better, acid-washed Florisil™) with silver nitrate and pack into columns to enable separation of fatty acids by degree of unsaturation. However, the technique suffers from many of the problems associated with silver ion TLC.

As an alternative, macroreticular sulfonic acid ion exchange resins have been utilized as adsorbents for silver ion column chromatography. The resin is loaded with silver ions by passing an aqueous solution of silver nitrate through a column of resin until excess silver ions start to elute. The column is then washed with water and methanol, and methanol is used further as the mobile phase. Recently, Amberlyst XE 284™ has been shown to give the best results, but the separations improved significantly only when the mobile phase of methanol was modified with.

Figure 1 Silver ion TLC (Kieselgel G™ containing 2% silver nitrate) of fatty acid methyl esters, with hexane/diethyl ether (9 : 1, v/v) as mobile phase.

Dienoic fatty acids will then run together near the solvent front.

Figure 2 is a schematic representation of a silver ion TLC separation of triacylglycerols (which can include all the common oils and fats of commerce), which contain three fatty acids per molecule. In this instance, trisaturated species elute first followed by disaturated monoenoic; the latter separating into two fractions (more or less completely) according to whether the unsaturated fatty acid is on position 2 of the glycerol moiety or in one of the outer positions. Then, the monosaturated dimonoenoic species is eluted followed by a fraction containing one dienoic fatty acid with two saturated, i.e. a dienoic fatty acid is retained more strongly than two monoenoes, and so on.

Phospholipid molecular species have been resolved in this way also, both in intact form (technically difficult) or after enzymatic conversion to non-polar diacylglycerol derivatives.

Although the equipment is simple and inexpensive, there are many drawbacks to silver ion TLC procedures, not least that silver ions are eluted from TLC plates and contaminate fractions in preparative applications, as do silica gel and dyes used for detection purposes. Silver nitrate leaves indelible stains on

Figure 2 Silver ion TLC (Kieselgel G™ containing 10% silver nitrate) of triacylglycerols, with chloroform/methanol (99 : 1 v/v) as mobile phase. Abbreviations: S = saturated, M = cis-monoenoic and D = dienoic fatty acyl residues.
acetonitrile, which enabled a good resolution of monoenes, dienes, trienes and tetraines (DeJarlais et al., 1983). Further improvements were obtained by grinding the resins first to 270–350 mesh.

This technique permits fractionation of mixtures of unsaturated fatty acids by degree of unsaturation on the 10 to 20 gram scale. As the silver ions are held by ionic bonds, they are not leached from the column, while rigidity of the silica matrix prevents compaction of the packing material during gradient elution. Preparation of the column involves merely taking a standard prepacked column with the appropriate stationary phase (Nucleosil™ 5SA) and introducing the silver ions via an injector while pumping water through the column. Finally, the aqueous phase is replaced with organic solvents. Only 50 to 80 mg of silver ions are bound to the stationary phase, but this is quite sufficient for very many useful separations.

Lipids lack chromophores that facilitate UV detection, although it is possible to use UV detection with appropriate fatty acid derivatives. Therefore, for much of the work with silver ion HPLC columns, evaporative light-scattering detectors have been employed as they permit the use of complex gradients and mobile phases containing such solvents as dichloromethane and acetone. Although the detector is destructive in that the sample is lost in the current of air, it is possible to insert a stream splitter between the end of the HPLC column and the detector to divert much of the sample for collection.

Chlorinated solvents, such as dichloromethane or dichloroethane, form the basis of the more useful mobile phases, and the polarity can be increased to elute highly unsaturated components by adding acetone or especially acetonitrile, which has a high affinity for silver ions. Presumably the high dielectric constant of the chlorinated solvents facilitates the interaction between silver ions and the double bonds. However, acceptable results can also be obtained with hexane as the main component of the mobile phase if a little acetonitrile is present.

Silver Ion High Performance Liquid Chromatography

The approach to silver ion HPLC adopted by Christie (1987) is to load a silica-based ion exchange medium (chemically bonded phenyl sulfonic acid groups) with silver ions. Again, the silver ions are held by ionic bonds and are not leached from the column, while rigidity of the silica matrix prevents compaction of the packing material during gradient elution. Preparation of the column involves merely taking a standard prepacked column with the appropriate stationary phase (Nucleosil™ 5SA) and introducing the silver ions via an injector while pumping water through the column. Finally, the aqueous phase is replaced with organic solvents. Only 50 to 80 mg of silver ions are bound to the stationary phase, but this is quite sufficient for very many useful separations.

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Methyl esters are the most widely used fatty acid derivative for chromatography, because of the ease of preparation and their relatively low molecular weight. One useful application of silver ion HPLC is the separation of such derivatives from animal or fish lipids, into fractions with zero to six double bonds. Simplification of complex mixtures by this means makes the task of identification by other chromatographic means or by mass spectrometry much easier.

However, by an appropriate choice of solvents, it is possible to separate positional and geometrical isomers of unsaturated fatty acids on a micro-preparative scale, a feat not readily achieved by other chromatographic procedures. For example, the separation obtained with phenacyl esters of the three main naturally occurring octadecenoic acid isomers is illustrated in Figure 3; all are clearly resolved to baseline. It has become evident that the distance of the double bond from the carboxyl group is more important in governing the separation of positional isomers than is the terminal region of the molecule. Phenacyl derivatives of fatty acids were prepared at first so that quantification with UV detection was possible, but fortuitously, it has now become apparent that such derivatives give especially favourable separations (and this is also true for silver ion TLC). This technique can be used with equal facility for the separation of simple fatty acid derivatives with trans double bonds, and will undoubtedly supplant TLC for the purpose. Positional isomers of polyunsaturated fatty acid derivatives can be resolved similarly by increasing the polarity of the mobile phase.

Silver ion HPLC is also of great value for separation of molecular species of triacylglycerols. The simplest elution scheme is a gradient of acetone into dichloroethane–dichloromethane, which serves for fats with low levels of linoleic acid, such as sheep adipose tissue or bovine milk fat. This

![Figure 3](396x732)  
**Figure 3** Separation of phenacyl esters of the isomeric octadecenoic acids, petroselinic (6-18 : 1), oleic (9-18 : 1) and vaccenic (11-18 : 1), by HPLC on a Nucleosil™ SSA column in the silver ion form eluted with dichloromethane/dichloroethane/acetonitrile (50 : 50 : 0.25 by volume), with evaporative light-scattering detection.
gives resolution of the main components with zero to three double bonds in total in the fatty acyl chains, including separation of fractions with trans- from those with cis-monoenoic residues. Most triacylglycerol samples are likely to contain appreciable proportions of linoleic acid, and resolution into molecular species is then accomplished by ternary gradient elution, simply by introducing acetonitrile into acetone after the first fractions are recovered. Such a separation of adipose tissue triacylglycerols is illustrated in Figure 4. One dienoic acyl moiety is retained more than twice as strongly as a monoene, and one triene (18 : 3 (n-3)) is retained by the same amount as two dienoic residues in a molecule, so there is some overlap of dienoic and trienoic species when z-linolenic acid is present in a sample. Otherwise, the basis of the separation is similar to that described earlier for TLC applications, in that trisaturated species elute first, followed by disaturated monoenoic and so forth.

Such highly unsaturated triacylglycerols as linseed oil and fish oils have been resolved satisfactorily. With the former, trilinolenin is the most abundant single fraction, and a simple progression of fractions with increasing numbers of double bonds are eluted until this species is reached. When the more saturated molecules of fish oils are eluted, resolution is excellent and it is perhaps surprising to find appreciable amounts of trisaturated and disaturated monoene species. Baseline resolution is no longer possible when molecules containing polysaturated fatty acids begin to elute, because the wide range of positional isomers causes similar components to overlap. Nevertheless, valuable separations of species containing two saturated and/or monoenoic fatty acids and one polyenoic fatty acid especially can be achieved.

In silver ion chromatography, the order of elution of triacylglycerol species is easily understood because only one property of the molecules is involved, i.e. degree of unsaturation. The alternative technique used for molecular species separations is reversed-phase chromatography, with octadecysilyl phases, which effects separation both by chain length and degree of unsaturation, each double bond reducing the effective chain length by the equivalent of about two methylene groups. When used in sequence the two techniques make a much more powerful tool. Fish oils give highly complex chromatograms with reversed-phase HPLC, for example, and identification of individual components is impossible. On the other hand, when fractions from silver ion HPLC are collected and then subjected to reversed-phase HPLC, separation is then, in effect, by chain length only and the main peaks are easily identified. Each HPLC fraction can be examined in turn in this way, and much more information obtained in comparison to the use of either technique on its own.

Some Mechanistic Considerations

In silver ion HPLC, we have a reasonable understanding of how the silver ions are bound to the stationary phase via the phenylsulfonic acid groups. There may be some residual silanol groups on the surface of the silica matrix, but these should not influence separations greatly when relatively polar chlorinated solvents are used in the mobile phase. Also in HPLC, we can control both the composition and flow rate of the mobile phase with a high degree of accuracy. Finally, we can control the temperature of the column, an important factor in the complexation reaction between silver ions and double bonds. Accurate chromatographic retention data can thereby be obtained for a variety of lipid analytes of known structure.

It is known that silver ions can interact with two double bonds in a fatty acyl residue at the same time, but can they also react with one double bond and the unpaired electrons on the carboxyl moiety as shown schematically in Figure 5? This might explain how different positional isomers of fatty acids are separable by this technique. For example, electron-rich esters, such as the phenacyl derivatives illustrated, are held much more strongly than are methyl esters when the double bond is within about eight carbons of the carboxyl group, and the elution patterns of series of isomers are very different. From a 9–18 : 1 fatty acid derivative onwards, when the possibility of such a simultaneous interaction would seem to be less likely, there is no significant difference between methyl and phenacyl...
esters. Experiments with esters with a variety of different electron-donating and electron-withdrawing substituents now provide firm evidence for this hypothesis. An interaction between one silver ion and two double bonds at the same time may explain the chromatographic behaviour of fatty derivatives with two or more double bonds in the acyl chain in silver ion HPLC. When the distance between the double bonds is optimum, i.e. with a 1,5-cis,cis-diene system, fatty acids are very strongly retained, and the effect diminishes as the number of methylene groups between the double bonds is varied. If the double bonds interacted singly with silver ions, it might have been anticipated that the kinetics of the system would be such that retention would be comparable in magnitude to the sum of the individual parts, but this is clearly not so. This theory of complexation between silver ions and bis-double bond systems could potentially be applied to polyenoic fatty acid derivatives. It would predict that a triene would be held twice as strongly as a diene, a tetraene three times as strongly and so forth. Such a simple relationship is not found in practice (the degree of complex formation is even greater than anticipated), possibly because interactions with the ester moiety have to be taken into consideration and because the conformations of polyenes may permit some interactions between silver ions and double bonds that are remote from each other, via the formation of pseudo-cyclic structures.

Analogous physicochemical studies of the behaviour of triacylglycerols on silver ion chromatography suggests that a dual interaction is important in this instance also. For example, it has been shown that highly unsaturated triacylglycerols are retained especially strongly; a species with nine double bonds is held 10 000 times as strongly as one with a single double bond. It is the strength of this interaction rather than the efficiency of the column per se that is responsible for the quality of the separations. However, here further work is certainly necessary to confirm the mechanism.


Further Reading


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**Thin-Layer (Planar) Chromatography**

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**Introduction**

Silver ions (Ag⁺), like the ions of many other transition metals, interact specifically with...