ELSEVIER

Journal of Chromatography B, 671 (1995) 197–222

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Silver ion chromatography of lipids and fatty acids

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Abstract

Silver ion chromatography as applied to the analysis of lipids is reviewed. Thin-layer, column, high-performance liquid and supercritical fluid chromatography in the silver ion mode are included. The lipid types covered are fatty acids, triacylglycerols and complex lipids. Separations are divided into those according to number, geometry and position of double bonds, as well as acyl positional isomers for triacylglycerols. The mechanism of silver ion chromatography is discussed in relation to recent studies using silver ion high-performance liquid chromatographic methodology.

Contents

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0378-4347/95/$29.00
SSDI 0378-4347(95)00157-3

List of abbreviations

FAME	Fatty acid methyl ester
PUFA	Polyunsaturated fatty acid
SFC	Supercritical fluid chromatography.

For triacylglycerols:

Cr	Crepenynoyl
D	Dienoic
E	Elaidate
H	Hexaenoic
L	Linoleate
M	Monoenoic
O	Oleate
P	Pentaenoic
Pe	Petroselinate
S	Saturated
T	Trienoic
Te	Tetraenoic
U	Unsaturated

1. Introduction

Silver ion (or "argentation") chromatography is a technique that utilises the property that silver ions form polar complexes reversibly with unsaturated centres in organic molecules such as lipids. It enables separation according to the number, geometrical configuration and position of double bonds in molecules, for example. In practise, it is carried out in conjunction with one of the established chromatographic procedures: TLC in much of the earlier work but in recent years with HPLC, and to a lesser extent with low-pressure column chromatography and SFC. The system usually involves incorporation of silver ions into the solid support although RP chromatography together with silver ions dissolved in the mobile phase has sometimes been employed. The other components of the basic practical arrangement are similar to those for conventional chromatography.

If an attempt was made to include every paper which had some silver ion chromatography content, the review and list of references would be enormous and the information would be repetitious. In this review it is hoped that all key papers have been included while less attention has been given to those publications which have not added to the knowledge of silver ion chromatography per se. Particular attention has been given to recent literature and, as well as advances in the methodology and the separations that can be achieved by the various types of silver ion chromatography, space is devoted to developments in the mechanism of the technique at the molecular level, an area which is being keenly pursued. For the early literature, readers are referred to the reviews of Morris [1] and Morris and Nichols [2], to a more general TLC review [3] and the book of Litchfield on Analysis of Triglycerides [4]. Some overlap with a comprehensive review [5] by one of the present authors could not be avoided and valuable information on practical considerations may be found here. Two books by Christie [6,7] contain additional information. Two recent reviews [8,9] include sections on silver ion HPLC, and a section on lipids, including prostaglandins, leukotrienes and steroids as well as the classes included in this publication, can be found in another review [10].

Many of the silver ion chromatographic methods are of general applicability to a variety of samples, whereas others are more specific. Numerous studies have involved samples which belong to categories, such as seed oils, which would not usually be considered as coming under the umbrella of Biomedical Sciences. However, they have not been excluded from the present review if they add to the knowledge of the types of separations possible and indeed may be adapted for biomedical samples. Studies which can be classified under Biomedical Sciences include those on brain lipids [11,12], rat microsomes [12], plasma cholesterol esters [13], skin fibroblasts [14], human [15] and bovine [16–18] milk fat, cyclic fatty acids in frying oils [19], hydroperoxides [20,21], and several aimed at the *trans* monoenoic acid content [22–31] and partially hydrogenated triacylglycerols of dietary fats [32,33]. When choosing a method for a particular separation it must be taken into account that some publications have only been

concerned with standard lipids. Achieving the same separation on real samples, which may be far more complex, may be a different matter.

Silver ion chromatography is an especially useful technique when used in combination with other methods of separation. The following sections give many examples and the area has been covered in previous reviews [5,34]. Essentially, fatty acid methyl ester fractions obtained by silver ion chromatography may be analysed by GC or GC–MS, and fractions of triacylglycerols and phospholipids, after hydrolysis to diacylglycerols, may be separated by high-temperature GC. A combination of silver ion TLC or HPLC with RP-TLC or RP-HPLC has proved invaluable for the analysis of complex triacylglycerol mixtures culminating in a comprehensive if still incomplete separation of fish triacylglycerols using HPLC sequentially in both modes [35,36]. The basis for all these approaches is that whereas GC and RP-chromatography separate according to both chain length and unsaturation, silver ion chromatography discriminates only on unsaturation. The result is that components, which might overlap if any one of the techniques was used alone, are resolved. Indeed silver ion chromatographic profiles are simpler and easier to interpret and as a consequence are ideally suited for a preliminary simplification of complex mixtures or for concentration of minor components.

2. Silver ion thin-layer chromatography

2.1. Introduction

In silver ion TLC, both precoated and homemade plates can be impregnated with silver ions to give satisfactory separations. Homemade plates are more versatile but precoated plates are more convenient and are often the choice. The adsorbent is commonly silica gel G for fatty acids and triacylglycerols, and silica gel H for complex lipids. Accurate control of the silver ion content can only be achieved by incorporation of silver nitrate into the slurry of the adsorbent during preparation of homemade plates. However, the silver content is often not critical and more often the plates are immersed in, or less satisfactorily sprayed with, a solution of silver nitrate.

Other practical aspects of performing silver ion TLC have been reviewed elsewhere [5]. Suffice to say that many factors including the thickness of the adsorbent layer, the concentration of silver ions, the nature of the anion, humidity, type of developing tank, temperature and of course the mobile phase can be varied and affect the chromatography to some degree.

2.2. Fatty acids

Fatty acids, usually as the methyl esters, can be separated according to the number and configuration of the double bonds. Some success with separating positional isomers has also been achieved.

The choice of mobile phase depends on the separation required. As in a recent study of the monoenoic fatty acids in brain lipids [11], this may simply be separation of a monoene fraction from saturated (top of plate) and polyenoic (bottom of plate) fatty acids as their methyl esters using appropriate proportions of hexane–diethyl ether as mobile phase. Resolution of methylene-interrupted unsaturated fatty acids with up to three double bonds can be achieved using either this mixture or benzene–hexane as developing solvent [37–44]. Separation of fatty acids with up to six double bonds is possible with a single development of benzene–ethyl acetate [41], while other methods involve double developments with hexane–diethyl ether–acetic acid mixtures [45] or a two-stage development, firstly with a polar solvent (chloroform–methanol–water or hexane–diethyl ether mixtures) to separate fatty acids with three to six double bonds, followed by a development suitable for fatty acids with fewer double bonds [37,39,41,45–48].

These methods have been applied to a diverse range of samples including brain fatty acids [49]. Very-long-chain (C_{24} to C_{36}) PUFAs have been analysed [49–51], including fatty acids with four to six double bonds from bovine retina [51]. Recently, silver ion TLC was used to concentrate

isoprenoid PUFA fractions from freshwater sponges [52].

Usually silver ion TLC fractions of PUFAs of similar degrees of unsaturation have been separated subsequently according to chain length by GC (e.g. Ref. [53]). In some circumstances fatty acids can be separated according to degree of unsaturation and chain length by silver ion TLC [14,41]. In one study with high-performance TLC plates and toluene–acetonitrile mixtures as mobile phase [14], components with two to six double bonds and eighteen, twenty and twenty-two carbons were resolved into zones according to degree of unsaturation, and within each zone there was separation of fatty acids apparently according to chain length, although the position of the first double bond in each ester may be the important factor (Fig. 1). All common ($n-6$) PUFAs were separated, as were ($n-3$) PUFAs, although components of similar unsaturation and chain length but different biosynthetic class were less well resolved. The utility of the technique for metabolic studies on chain elongation and desaturation was noted and the metabolic products from skin fibroblasts were examined. Interestingly under similar conditions, and probably because of the presence of toluene in the solvent mixture, PUFAs were shown to be self-staining and the method was suggested to be potentially useful when radio-labelled bands were to be detected by autoradiography and quantified by liquid scintillation counting [54].

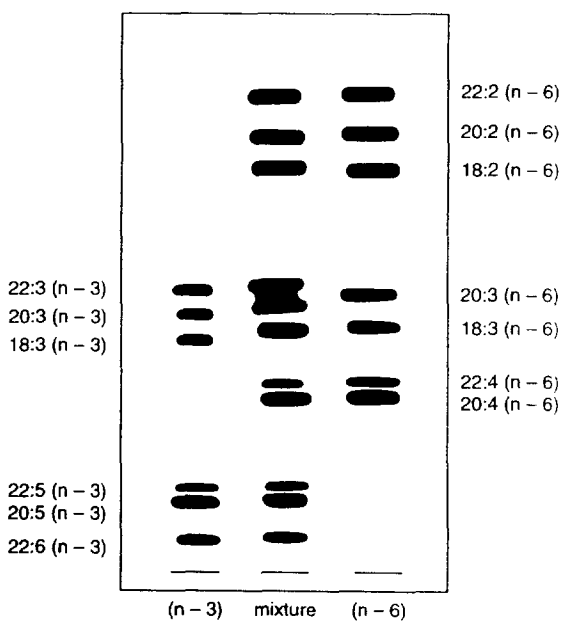

Fig. 1. Diagrammatic silver ion TLC separation of ($n-3$) and ($n-6$) PUFA methyl esters [14]. The silica gel 60 TLC plate was prepared by spraying with a 10% (w/v) solution of silver nitrate in acetonitrile. The developing solvent was toluene–acetonitrile (97:3, v/v) and bands were visualized by spraying with 3% (w/v) copper acetate–8% (v/v) orthophosphoric acid in water and charring at 180°C. (Reproduced by kind permission of the authors and *J. Chromatogr.*, and redrawn from the original.)

Geometrical isomers with *trans* double bonds are readily separated from the corresponding *cis* isomers, the former having greater mobility [55]. This simple separation has been applied in numerous investigations, one of the most recent being the isolation of unusual minor *trans*-3-monoenoic acids from seaweeds [56]. In conjunction with GC, the method is an effective way of determining the *trans*-monoenoic acid content of dietary fats, separating well from *cis*-monoenes as their methyl esters (e.g. Refs. [23,26–28]) and isopropyl esters [31], the latter giving quantitative recovery of short-chain fatty acids. A comprehensive comparison of GC–silver ion TLC and IR methods for measuring *trans* fatty acids has not been performed but considerable discrepancies between the methods has been noted [57,58]. Separation is not as clear if the fatty acids cover a wide range of chain lengths and there are several double bond positional isomers [55,59]. In a recent study of the monoenoic fatty acids of butter, several positional isomers of *trans* 16:1 and 18:1 fatty acids, as their butyl esters, were not resolved from a short-chain-rich saturated fatty acid fraction [60].

Resolution of mixtures containing fatty acids with more than one double bond with all possible combinations of geometrical isomers provides more of a challenge. For mixtures of C_{18} fatty acid isomers with zero to two double bonds, *cis*-monoenes overlapped with *trans,trans*-dienes under standard conditions [61]. They could be resolved, however, by using silver benzenesulphonate-impregnated plates and by modifying

the mobile phase. A similar mixture with the addition of conjugated dienes (*trans/trans*, *cis/trans*, *cis/cis*) could be resolved completely only by using two plates of differing silver nitrate concentrations with large volumes of different developing solvents (petroleum ether–acetone and chloroform–methanol) in open tanks [62]. Coupled with densitometry the method was successfully applied to the quantification of isomerised fatty acids in selenium- and alkali-isomerised sunflower oil as well as hydrogenated sunflower oil, despite the presence of positional isomers of monoenes in the latter. Conjugated octadecatrienoates have been resolved with toluene as developing solvent at −20°C [63].

Geometrical isomers of linoleic [64–66] and α-linolenic [66] acids were resolved according to the numbers of *trans* and *cis* double bonds but a mixture of the two series was not totally resolved [66]. In a study on the geometrical isomers formed in γ-linolenic acid in heat-treated borage oil, silver ion TLC resolution into all-*trans*, di-*trans*, mono-*trans* and all-*cis* triene fractions was a prerequisite to complete separation of all components prior to GC [67]. An analogous separation of the geometrical isomers of α-linolenic and arachidonic acid into four and five bands, respectively, has also been achieved [68]. GC resolution of all geometrical isomers of α-linolenate in heat-treated rapeseed and soybean oils was possible, however, without prior separation by silver ion TLC [69]. It should be noted that, unlike GC or indeed silver ion HPLC (see Section 3.2), silver ion TLC does not discriminate between geometrical isomers which differ only in the order of *cis* and *trans* double bonds.

As part of a multi-analytical approach to studying the positional and geometrical isomers of linoleic acid in a partially hydrogenated canola oil-based spread and several margarines, five bands, resolved by silver ion TLC, corresponded to geometrical isomers of the usual methylene-interrupted dienes as well as the less mobile non-methylene-interrupted dienes (2 to 4 methylene groups between double bonds) [70]. *Trans/trans*-non-methylene-interrupted dienes and *cis/trans*-methylene-interrupted dienes overlapped and the non-methylene-interrupted diene fractions contained several positional isomers as shown by GC.

All positional isomers of the *cis*- and *trans*-octadecenoates [55], methylene-interrupted *cis*-octadecadienoates [71], dimethylene-interrupted *cis*- and *trans*-octadecadienoates [72] and octadecynoates [73] as well as some octadecadiynoates [72] have been analysed. For each series, the fatty acid with a double or triple bond in the 2-position had by far the greatest mobility, so that for the monoenes the *cis*-isomer migrated ahead of all the *trans* positional isomers [55]. The migration of each series on a plate in sequence of increasing double bond position approximated a sinusoidal curve. For the monoenes, the 6-isomers had the lowest mobilities reaching a second maxima for the 13-isomer (*cis*) or 16-isomer (*trans*). Although a mixture of all the isomers of any one series would give an unresolved streak the method has found practical uses when the range of isomers is limited and their mobilities are sufficiently different.

Three *cis*-octadecenoates with double bonds in the 6- (petroselinate), 9- (oleate) and 11- (vaccenate) positions can occur together in tissues and natural oils and have been separated at low temperatures as their methyl esters [74–77], although this has proved difficult to repeat [5]. Resolution of these fatty acids together with *trans*-9-18:1 (elaidate) and *trans* (brassidate) and *cis* (erucate) isomers of 13-22:1 was also achieved [74]. Recently the greater resolving ability of phenacyl esters compared to methyl esters (demonstrated earlier by HPLC (Section 3.2)) was confirmed when petroselinate, oleate and vaccenate as well as 5-, 8-, 11- and 13-eicosenoates were resolved completely at ambient temperature on plates containing relatively low levels of silver nitrate [78]. Limited studies on other longer-chain fatty acids, which usually migrate faster than corresponding shorter-chain compounds, have included positional and geometrical isomers of docosamonoenoic [74,79,80], docosadienoic [80] and eicosadienoic acids [79].

Silver ion TLC has been applied to a variety of substituted fatty acids including unsaturated epoxy, hydroxy, dihydroxy and halohydroxy fatty acids. The earlier literature has been

reviewed [2]. One interesting separation was that of methyl 11(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoate from the unresolved pair of methyl 9-hydroxy-10(*E*),12(*Z*)- and 13-hydroxy-9(*Z*),11(*E*)-octadecadienoates in a study of the oxidation products from linoleic acid treated with a red algal enzyme preparation [81]. Recently, hydroperoxides of methyl oleate were converted to hydroxides and separated into *cis* and *trans* isomers by silver ion TLC prior to separation of the hydroxy positional isomers by HPLC and identification by GC–ion trap–MS [20,21]. A solid-phase silver ion column method was tried as an alternative to TLC but the separation was not complete [21].

2.3. Triacylglycerols

Since the first separations of triacylglycerols by silver ion TLC [82] the technique has been used, either on its own or as a preliminary simplification step, to elucidate the structures of many fats and oils, including cocoa butter [83,84], cod liver oil [85], cottonseed oil [86–88], evening primrose oil [89], grape seed oil [90], lard [83,84,91], maize oil [92,93], milk fat [17,18], olive oil [84,88], orange seed oil [94] (Fig. 2), pepper seed oil [90], soybean oil [95,96], sunflower oil [84,88,93] and tomato seed oil [90], as well as for studying the process of interesterification [97] and the changes produced by heating [98]. Many earlier examples have been reviewed [4].

Triacylglycerols are separated according to the overall degree of saturation [95,99]. Of species with the same total number of double bonds, that in which the double bonds are more concentrated in individual acyl residues has the slower mobility. For example, SMM elutes just ahead of SSD. Triacylglycerols containing linolenate (e.g. SMT) are usually retained more strongly than those containing acyl groups with up to two double bonds but with one more double bond in total (e.g. MDD), although the order of species with trienoic acids can vary according to the nature of the mobile phase. The twenty possible species containing fatty acids with zero to three double bonds (i.e. trisaturated to trilinolenate) can be totally resolved [94,95,99,100]. The technique has been less useful with samples such as fish oils which in addition contain more highly unsaturated species [85].

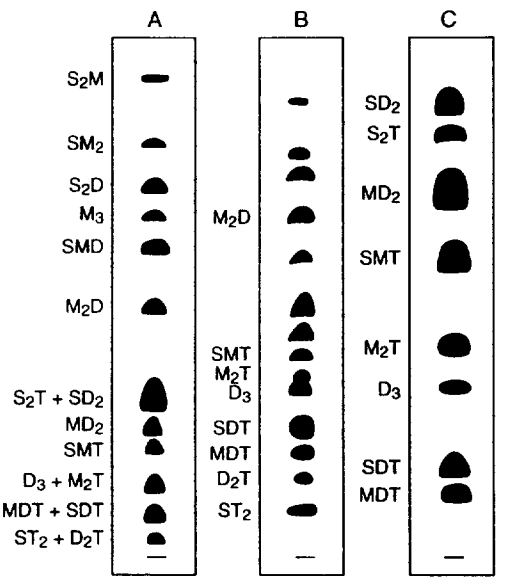

Fig. 2. Diagrammatic silver ion TLC separation of triacylglycerols from orange seed oil [94]. The silica gel G layer was impregnated with 0.5% methanolic silver nitrate and plates were developed in open cylindrical tanks. Plate A, mobile phase of petroleum ether–acetone (8 ml, 100:6, v/v); plate B, petroleum ether–acetone (5 ml, 100:8, v/v) followed by petroleum ether–acetone (8 ml, 100:5, v/v); plate C, petroleum ether–acetone (4 ml, 100:7, v/v) followed by petroleum ether–acetone (15 ml, 100:4, v/v). Bands were visualised by exposing plates sequentially to bromine and sulphuryl vapours followed by heating to 180–200°C. (Reproduced by kind permission of the authors and *La Rivista Italiana delle Sostanze Grasse*, and redrawn from the original.)

A range of developing solvents have been used for resolving triacylglycerols. These include benzene with cyclohexane [101], cyclooctane [101], diethyl ether [88,95] or methanol [89], petroleum ether–acetone [84,94], tetrachloromethane–acetic acid [75], isopropyl alcohol–chloroform [102] and toluene with chloroform [103], diethyl ether [104] or hexane [105]. Methanol (1%) in chloroform can be recommended for resolving components with up to six double bonds, increasing the amount of methanol to 5% for separation of more highly unsaturated species on a separate TLC plate [5]. Nikolova-Damyanova [5] believes that the volume of mobile phase and

duration of development, which are seldom mentioned, are important. Continuous development in open tanks has proved to be beneficial for many separations [77,84,94,100,106–108]. Solvent conditions may have to be altered not only according to the types of species present but also according to the relative amounts of neighbouring zones [90].

Triacylglycerol isomers differing only in the position of the acyl moieties (at the *sn*-2 and *sn*-1/3 positions) can be resolved also, allowing the identification of species with specific fatty acids at the *sn*-2 position [106] without the need for techniques such as enzyme hydrolysis. SSM–SMS, SSD–SDS and MSM–MMS pairs have been separated [82,83,104,106] with the former member of each pair migrating the least. All these pairs and others (DDS–DSD and DDM–DMD) were resolved by continuous development with chloroform–methanol in open tanks [106,107] (Fig. 3). The proportions of methanol and silver ions were increased for pairs of increasing unsaturation. Silver ion TLC can be used to determine whether or not dietary oils [104] and fats [103] are pure natural products, since some triacylglycerol species (SSM, MSM and SSD) are only present in significant amounts after interesterification.

Few examples have been reported of separations of triacylglycerols differing in the geometry and position of the double bonds. Standards of species containing saturated fatty acids together with either oleate or elaidate (SSS > SSE > SEE > SSO > EEE > SOO > OOO [66,88,105]) and those comprised of oleate and petroselinate (OOO > PeOO > PePeO > PePePe [88]) can be separated. The separation by silver ion TLC of SSS and SSE fractions from the bulk of triacylglycerols, prior to elucidating their composition and quantifying by GC, was utilised as part of a scheme to determine the composition of a hydrogenated palm oil fraction [27]. In a study of the short-chain triacylglycerols from milk fat, *cis*-monoenes were partially separated from *trans*-monoenes although the latter contained some *cis* isomers [18].

For the detailed qualitative and quantitative analysis of complex natural triacylglycerol mixtures, such as vegetable oils, silver ion TLC has been applied in combination with RP-TLC [109–111]. The results are, to date, the most impressive separations of triacylglycerols by these techniques and are comparable to those obtained for the same samples by RP-HPLC.

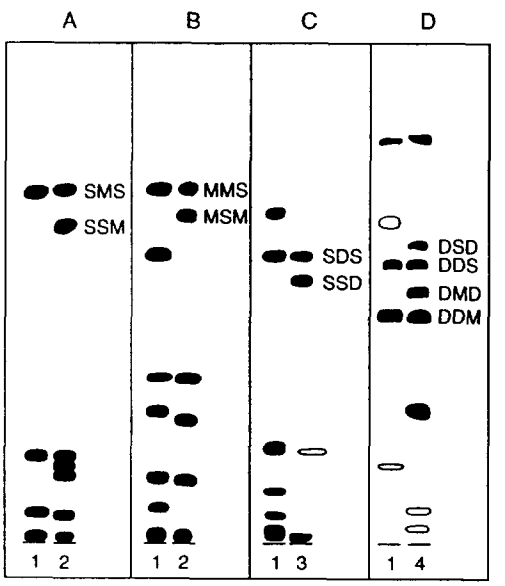

Fig. 3. Diagrammatic silver ion TLC separation of positional isomers of triacylglycerols [106]. Plates A–C and D were impregnated with 1% and 2% methanolic silver nitrate, respectively, and developed in open cylindrical tanks. Plate A, mobile phase of chloroform–methanol (12 ml, 99.5:0.5, v/v); plate B, chloroform–methanol (14 ml, 99:1, v/v); plate C, chloroform–methanol (16 ml, 99:1, v/v); plate D, chloroform–methanol (6 ml, 97.5:2.5, v/v) followed by chloroform–methanol (15 ml, 98.5:1.5, v/v). Sample 1 = grapefruit seed oil; sample 2 = randomized lard; sample 3 = randomized mixture of sunflower oil and tristearin; sample 4 = randomized sunflower oil.

2.4. Complex lipids

Silver ion TLC has been applied to complex lipids, often as a preparative step, in the analysis of the species of a particular phospholipid class. The literature up to the early 1980s has been reviewed [112] and in recent years TLC has been largely superseded by RP-HPLC especially. The subject will therefore only be briefly considered

here. Lipids may either be studied intact with or without masking the polar moiety or after hydrolysing to phosphatidic acid (which may be derivatised) or diacylglycerols. The advantage of retaining the phosphate group is that the metabolism of isotopically labelled phospholipids can be studied. However, diacylglycerols are more suitable for other analytical techniques such as RP-HPLC and high-temperature GC, which may give additional valuable information.

Intact underivatised phosphatidylcholine and phosphatidylethanolamine were resolved into species with up to six double bonds [113,114]. The conditions employed for plate activation were critical with less activation required for separation of most species, but prolonged activation was a prerequisite for resolution of saturated and monoenoic species. Derivatisation of the polar group, e.g. methylation of the phosphate group to render the molecule less polar, aids separation and this and related methods have been applied to the analysis of phosphatidylethanolamine [115–117], phosphatidylinositol [118,119], phosphatidylserine [117,120], ceramides [121], mono- and digalactosyldiacylglycerols [122] and sulphoquinovosyl diacylglycerols [123].

Hydrolysis of phospholipids has been carried out enzymatically with various phospholipases. Phospholipase D transforms phospholipids to phosphatidic acid, which after conversion to the non-polar dimethylphosphatide can be separated by silver ion TLC into species containing up to six double bonds [115,118,124]. More often, hydrolysis to diacylglycerols is performed with phospholipase C [125] or by acetolysis, although the latter is not suitable for plasmalogens [126,127]. The diacylglycerols have been converted to ^{32}P-labelled phosphatidic acid prior to resolution by silver ion and reversed-phase TLC and quantification of the label [128]. Indeed this procedure was used to study the metabolism of diacylglycerols per se in mast cells [129]. Usually the diacylglycerols are converted to non-polar derivatives such as acetates [112,130–132], *tert.*-butyldimethylsilyl ethers [133,134] and benzoyl esters [135]. Species with up to twelve double bonds have been separated as acetates according to degree of unsaturation [131]. The diacylglycerol acetates from phosphatidylethanolamine of *Escherichia coli*, grown on elaidate, contained palmitate and *cis*- and *trans*-hexadecenoate and octadecenoate, and were resolved not only according to number and configuration of the double bonds but also on the basis of chain length of the monoenes (shorter chain length held strongest) [130].

With a model mixture of 1,2-*rac*-diacylglycerols derived from palmitate, stearate, oleate, linoleate and linolenate, only eight, seven and twelve of the possible fifteen species were resolved by adsorption silver ion TLC, RP-TLC and silver ion RP-TLC, respectively [136,137]. Adsorption silver ion TLC did not resolve certain species differing only in the saturated (palmitate, stearate) residue [138], whereas silver ion RP-TLC was unable to separate other species such as dioleate and stearate/linoleate [139,140]. All species were resolved only when the silver ion adsorption and silver ion RP-TLC step were incorporated into a two-dimensional TLC system [139,140]. The separations were claimed to be superior to RP-HPLC and equal to GC.

2.5. Quantification

Methods for quantification of silver ion TLC fractions have been reviewed elsewhere [5] and, not surprisingly, are essentially those used for TLC in general. A common procedure is to extract fractions in the presence of an internal standard followed by GC either directly or following transmethylation. An alternative method is to quantify components directly on the plate following charring and densitometry, using suitable calibration curves. A requirement for the procedure is that all aspects of the charring step are standardised. The responses to fatty acids and triacylglycerols vary according to degree of unsaturation, but the effect can be minimised by forming bromo derivatives of double bonds prior to charring [141].

3. Liquid column chromatography in the silver ion mode

3.1. Low-pressure chromatography

As early as 1962 [142,143], silver ion column chromatography was used with petroleum ether–benzene mixtures to separate FAMEs and triacylglycerols with up to three double bonds and containing both *cis* and *trans* isomers. FAMEs with up to six double bonds were resolved with petroleum ether–diethyl ether mixtures [144] and small-scale separations of triacylglycerols of animal and plant origin were achieved [145]. Replacing the silica adsorbent with acid-washed Florisil allowed greater sample capacity [146,147], but mixtures containing geometrical and positional isomers of FAMEs were not resolved [148].

Silver ion columns packed with macroreticular sulphonic acid ion-exchange resins have some value for large-scale preparative separations but the resolution tends to be poor. Amberlyst 1005 was used with methanol as the mobile phase to separate saturated and *cis*- and *trans*-monounsaturated FAMEs in a reasonably pure state together with partial separation of positional isomers [149]. Cleaner separations of this type were achieved with another resin, Amberlite XE284, and by using a temperature gradient, geometrical isomers of linolenic acid could be separated [150]. FAMEs with more than one double bond adhered strongly to the resins, but inclusion of acetonitrile in the mobile phase allowed separation of FAMEs with up to four double bonds [151]. Phosphatidylcholines from egg and soybean were fractionated using a solvent gradient of increasing amounts of acetonitrile in methanol, although some fractions were mixtures of species having three to six double bonds [152]. Using similar solvents, the geometrical isomers of arachidonic acid, sixteen in all, were separated on a preparative scale into five bands according to the relative number, but not position, of *cis* and *trans* double bonds [153].

Solid-phase extraction columns (ion exchange) with benzenesulphonic acid residues bound to silica (Bond Elut SCX, Chromabond SA) can be quickly converted to the silver ion form just prior to use by eluting with a solution of silver nitrate, followed by washing with organic solvents of decreasing polarity [154,155]. This type of column has essentially superseded other types for small-scale separations (ca. 0.5 mg). They are inexpensive in terms of initial outlay and are especially useful when a preliminary simplification of a complex fatty acid mixture is required prior to GC or GC–MS analysis.

FAMEs with up to six double bonds were separated on Bond Elut SCX columns in the silver ion form using dichloromethane, acetone and acetonitrile in varying proportions [154]. Good resolution, particularly of the least unsaturated fractions (0–3 double bonds) was achieved. The methodology has been applied to the separation of saturated, monounsaturated and polyunsaturated fatty acids from soil phospholipids of bacterial origin [156,157] and fatty acids with up to three double bonds to differentiate between yeast strains and species [158]. Separation of methyl esters of cyclopropyl and unsaturated hydroxy fatty acids isolated from soil was achieved by using a dichloromethane–ethyl acetate mixture [156].

Plasma cholesterol esters with up to six double bonds were resolved under identical conditions to those of methyl esters, as described above [154], since the double bond in the sterol moiety did not influence the chromatography significantly [13]. Highly saturated triacylglycerols from palm oil, cocoa butter and sheep adipose tissue were resolved, with mixtures of dichloromethane, methyl acetate, acetone and acetonitrile, into SSS, SSM and SMM–SSD fractions, with more unsaturated species eluting together [159]. The method was adapted for the fractionation of lipase-modified butter oil according to degree of unsaturation up to trienes [160]. For this sample, a dichloromethane–*n*-pentane mixture improved separation of the saturated triacylglycerols, but although the purity of fractions was acceptable, there was significant overlap of saturated and monoene fractions probably because of the presence of short-chain (butyrate) fatty acids.

3.2. High-performance liquid chromatography

3.2.1. General

Two main types of columns have been used for silver ion HPLC: those containing silica gel impregnated with silver nitrate and those based on silver ions attached to cation exchangers. Hammond and Irwin impregnated HPLC grade silica with silver nitrate prior to packing into columns (reviewed in Ref. [161]). The grade of silica and method of impregnation were critical and the life of the column was limited by elution of silver ions in the mobile phase but could be prolonged by incorporating a silver-saturation pre-column into the solvent line. Leached silver ions are corrosive, and may therefore damage the detector, as well as contaminating fractions from preparative separations. Another disadvantage is that this type of column is not available commercially.

Two types of cation exchangers have been used: macroreticular sulphonic acid resins [162,163] and benzenesulphonic acid chemically bonded to silica [164]. The advantage of these materials is that leaching of silver ions does not occur because the silver is held by ionic bonds. Columns prepared using the ion-exchange resin Amberlyst XN1010, ground down to a mesh size of 270–350, can be used for long periods without loss of resolution or sample capacity and are well suited to larger-scale preparative applications because of the high capacity for silver ions. However, the range of useful solvents is limited since the resin expands and contracts with changes in mobile phase. Impregnation of the silica-based ion-exchange columns with silver ions is simple. A pre-packed column (Nucleosil 5SA) is washed with ammonium nitrate, then aqueous silver nitrate is injected via the Rheodyne valve at regular intervals while pumping water through the column. After eluting the column with organic solvents the column is ready for use. The silver content of the stationary phase is small (up to 80 mg), but many separations, including small-scale preparative ones, can be achieved. Columns may last for up to a year and although retention times and resolution gradually diminish they may be restored by washing procedures or re-silvering. A commercial silver ion column of this type is now available (Chrompack, Middelburg, Netherlands) and has been reported to show only small deviations in retention times after three months continuous use [165].

A third more-limited approach has been the use of a RP column with silver ions in the mobile phase [33,166–169]. Such a system can be troublesome because of the corrosive nature of the solvent. However, one group of workers [33] have reported that they have used a system of this kind over several years without serious problems, by taking precautions to minimise both reduction of silver ions by light and contact of silver ions with the detector and pump. The contamination of samples with silver ions is a major disadvantage in preparative applications.

The choice of solvent systems is very important. Some early studies with columns of the cation-exchange type (resin) were disappointing because methanol was incorporated into the mobile phase and caused lipid transesterification catalysed by free sulphonic acid residues [170]. Chlorinated solvents together with acetonitrile, which complexes strongly with silver ions displacing unsaturated lipids, are suitable for many applications.

Ultraviolet detectors are those most commonly used in HPLC, but in the silver ion mode they are usually restricted to specific derivatives of lipids which absorb above 235 nm, because of restrictions on the choice of solvents for the mobile phase. The incompatibility of refractive index detection with gradient elution, essential for many silver ion HPLC applications, is well known. Evaporative light-scattering detectors have often been utilised in recent years and have the advantage of being universal in application without requiring lipid derivatisation, permitting the use of complex gradients of any solvent which can be volatilized. As newer models have come on to the market the sensitivity has greatly increased and, although they are destructive, preparative separations can be achieved by including a stream-splitter just before the detector. Disadvantages are the non-linearity of the response and the need for calibration if precise

quantitative information is required. In this respect, transport flame ionisation detectors have potential.

3.2.2. Fatty acids

Eighteen carbon FAMEs with up to two double bonds were separated on a silver nitrate-impregnated silica gel column (Partisil 20) using a mobile phase of 1% tetrahydrofuran in hexane [22]. Cod liver oil FAMEs were separated into fractions containing three to six double bonds using 0.4% acetonitrile in hexane, but more saturated fatty acids were poorly resolved [96].

Much better separation into fractions with zero to six double bonds was achieved on cation-exchange-type columns using simple gradients [164,171]. This procedure has been applied extensively to many complex natural fatty acid mixtures, as their methyl esters, as a simplification step prior to structural analysis by GC–MS. Sources include algae [172], animal tissues [173], marine invertebrates [171,174,175], plasma [7] (Fig. 4) and seed oils [176–179]. In studies of sponges [180,181], ten fractions were collected (Fig. 5) and subsequent analysis by GC–MS revealed over one hundred fatty acids including

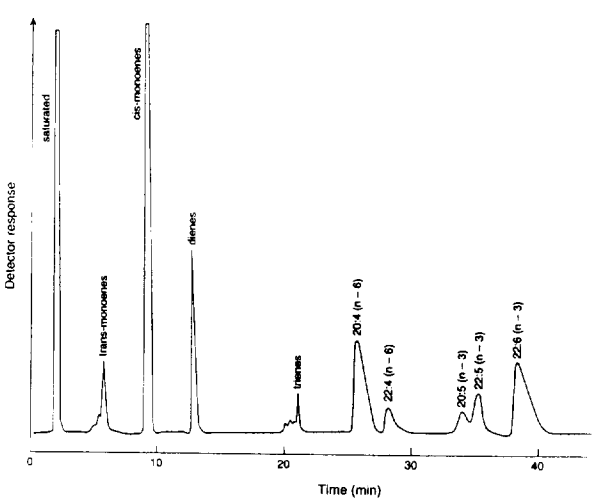

Fig. 4. Separation of fatty acid methyl esters from plasma lipids by HPLC on a Nucleosil 5SA column in the silver ion form with evaporative light-scattering detection [7]. The mobile phase was a linear gradient of 1,2-dichloroethane–dichloromethane (1:1, v/v) to 1,2-dichloroethane–dichloromethane–methanol–acetonitrile (45:45:5:5, v/v) over 40 min at a flow-rate of 1.5 ml/min.

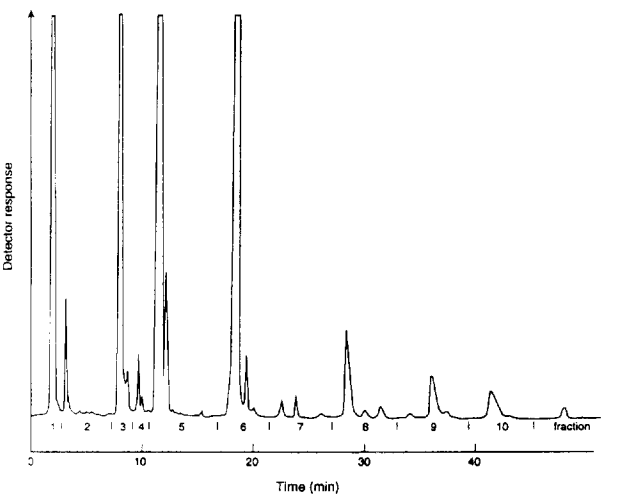

Fig. 5. Silver ion HPLC of methyl esters of fatty acids from the sponge, *Dysidea fragilis*, on a Chromspher Lipids column with evaporative light-scattering detection [180]. Two solvent mixtures [A, 1,2-dichloroethane–dichloromethane (1:1, v/v); B, 1,2-dichloroethane–dichloromethane–methanol–acetonitrile (45:45:5:5, v/v)] were used at a flow-rate of 1 ml/min. There was a linear gradient from 100% A to 95% A–5% B over 15 min, then to 80% A–20% B over a further 25 min and finally to 50% A–50% B over another 10 min. Fraction 1 = saturated; fraction 2 = *trans*-monoenes; fraction 3 = *cis*-monoenes; fraction 4 = non-methylene-interrupted dienes; fraction 5 = dienes; fraction 6 = non-methylene-interrupted trienes; fraction 7 = methylene-interrupted trienes; fractions 8, 9 and 10 = tetra-, penta- and hexaenoic components, respectively. (Reproduced by kind permission of *Lipids* and redrawn from the original.)

cyclic and mono- and multimethyl branched isomers as well as methylene- and multi-methylene-interrupted unsaturated components of several different biosynthetic families.

Simplification of other unusual fatty acids has been performed. For example, saturated and unsaturated oxo fatty acids from cheese [182,183], cyclopentenyl fatty acids from seed oils [176] and potentially toxic monoenoic [19] and dienoic [184] cyclic (five- and six-membered rings) fatty acids from heated vegetable frying oils were fractionated. The unsaturated oxo fatty acids differed according to both the positions of the double bond and the oxo group but there was no obvious pattern for their elution [182]. The cyclic dienes from frying oils were separated as their phenacyl esters on the basis of the size of the rings (containing double bonds) as well as the

position and configuration of the double bonds in the aliphatic moieties [184]. Very-long-chain polyunsaturated fatty acids were partially resolved into tetra-, penta-, hexa- and heptaenoic fractions as their methyl esters [185] and picolinyl esters [186] on columns of the cation-exchange type. The present authors have been unable to achieve separations of picolinyl esters, however. Further investigation may be worthwhile since picolinyl esters are extremely useful for structural determination of fatty acids by GC–MS and indeed were used in the above studies [19,171–184].

The geometrical isomers oleate and elaidate have been separated on a silver nitrate-impregnated silica gel column [22,150,187] and have also been resolved on a reversed-phase column with silver ions in the mobile phase [168]. The geometrical isomers of linoleate were resolved into four bands according to the number of *cis* and *trans* isomers, but no separation according to the order of the *cis*/*trans* double bonds was achieved [150]. A variety of *cis* and *trans* positional isomers of (2-, 9-, 15- and 17-) octadecenoic and (5,12-, 6,10-, 6,11-, 6,12-, 7,12-, 8,12-, 9,11-, 9,12-, 9,15- and 12,15-) octadecadienoic fatty acids were examined, but since only retention volumes were given it is unclear how well they were resolved [150,187].

Much better separation of positional and geometrical isomers of fatty acids is possible with silver bound to cation-exchange columns. Thus, by isocratic elution [dichloromethane–dichloroethane (1:1, v/v) with the addition of small amounts of acetonitrile for dienes/trienes], oleic and vaccenic acids and their *trans* analogues, three of the four possible linoleate geometrical isomers and six out of eight possible linolenate isomers were separated as phenacyl esters [24]. Recently, all eight geometrical isomers of linolenic acid were resolved by two different groups [68,188]. One [188] used a gradient of dichloromethane and methanol and a low temperature to separate the fatty acids as their phenacyl esters and each peak was identified by comparison to published GC data (Fig. 6). The method was used to quantify the isomers formed in heated linseed oil and commercial corn oil

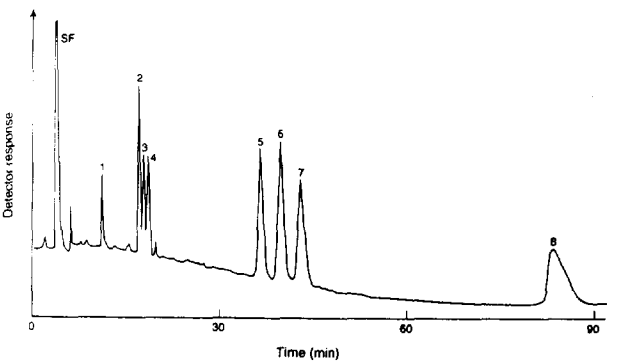

Fig. 6. Separation of phenacyl esters of linolenic acid geometrical isomers by HPLC on a Nucleosil 5SA column in the silver ion form, maintained at 10°C, with UV detection at 238 nm [188]. Two solvent mixtures (A, dichloromethane; B, methanol) were used at a flow-rate of 0.7 ml/min. There was a linear gradient over 30 min from 95% A–5% B to 75% A–25% B, which was held for a further 15 min before changing to 50% A–50% B over another 5 min and finally maintaining this composition for a further 40 min. SF = solvent front; peak 1 = *ttt*; peak 2 = *tct*; peak 3 = *ctt*; peak 4 = *ttc*; peak 5 = *ctc*; peak 6 = *tcc*; peak 7 = *cct*; peak 8 = *ttt* (where *t* = *trans*, *c* = *cis* and, for example, peak 3, *ctt* = 9-*cis*,12-*trans*,15-*trans*-18:3. (Reproduced by kind permission of *J. High Resol. Chromatogr.* and redrawn from the original.)

samples. The other group [68] used isocratic elution with hexane–acetonitrile to resolve the methyl esters, and the same system allowed separation of the four isomers from linoleate and fifteen out of the sixteen isomers from arachidonic acid. Although some of the arachidonic acid isomers were only partially resolved it would appear that the separation cannot be matched by other analytical techniques, including GC. The identities of these isomers remain to be fully determined, although the number of *cis* and *trans* double bonds in each peak was known from silver ion TLC analysis.

A short HPLC column (50 mm × 4.6 mm Spherisorb S5SCX in the silver ion form or Chromspher Lipids) was used as a rapid (5–10 min) means for separating *cis* and *trans* monoenoic isomers in a simple and accurate method for determining the *trans* monoenoic acid content of fats and oils [29] (Fig. 7) (adapted from a silver ion TLC–GC procedure [25,30]). A fraction containing saturates and *trans*-monoenes

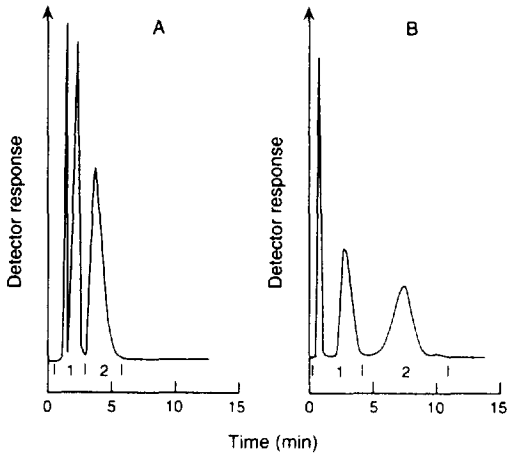

Fig. 7. Silver ion HPLC of methyl esters of partially hydrogenated soybean oil with evaporative light-scattering detection [29]. (A) Chromspher Lipids column; mobile phase, 1,2-dichloroethane–dichloromethane–acetonitrile (50:50:0.0-005, v/v). (B) Spherisorb S5SCX column in the silver ion form; mobile phase, 1,2-dichloroethane–dichloromethane–acetonitrile (50:50:0.001, v/v). The flow-rate was 1 ml/min; fraction 1 = saturated and *trans*-monoenes; fraction 2 = *cis*-monoenes. (Reproduced by kind permission of *J. Sci. Food Agric.* and redrawn from the original.)

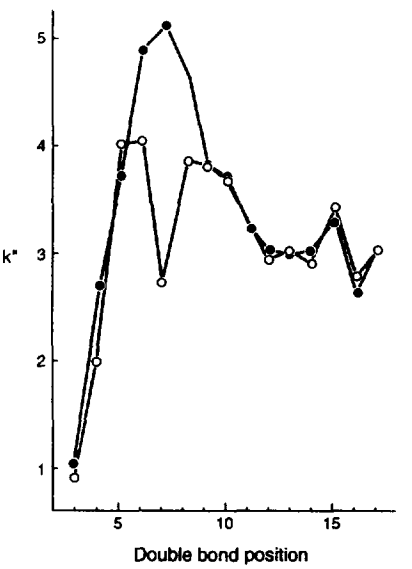

Fig. 8. *k"* values for HPLC of the methyl (○) and phenacyl (●) esters of a series of *cis*-octadecenoates at 10°C on a Nucleosil 5SA column converted to the silver ion form [190]. The mobile phase was 1,2-dichloroethane–dichloromethane–acetonitrile (50:50:0.025, v/v) and the flow-rate was 1.5 ml/min. The *k"* value was defined as the ratio of the capacity factor of the analyte to that of octadec-1-ene. (Reproduced by kind permission of *J. Chromatogr.* and redrawn from part of the original.)

was isolated by HPLC and the relative amount of saturates to total monoenes, determined by GC prior to fractionation, was compared to the relative proportion of *trans* isomers to saturates, also determined by GC. The precision of the method was better than an alternative procedure using Fourier-transform infrared spectroscopy. There is presently no single method that is generally accepted for determining *trans* fatty acids, but in a recent review [189] it was pointed out that only methods based on silver ion chromatography have the potential for high accuracy.

A series of isomers of *cis*-3- to 17-octadecenoic acids have been examined by silver ion HPLC on the ion-exchange-type column [190]. The three common octadecenoic acids (petroselinic, oleic and vaccenic) were resolved to base-line as their phenacyl but not methyl esters. A constant temperature was required for reproducible retention times. For methyl esters, the 3-isomer had the lowest retention time, rising to a maximum at the 5- and 6-isomers (Fig. 8). For phenacyl esters the resolution tended to be better for 3- to 11-isomers and a maximum was reached at the 7-isomer (Fig. 8). As for silver ion TLC [55], a mixture of all components would not be fully resolved. Indeed substantial but incomplete resolution of a complex mixture of *cis* and *trans* positional isomers was observed for a partially hydrogenated soybean oil [24].

Hydroxyoctadecadienoates have been resolved according to the position of the hydroxyl group as well as the configuration of the double bonds [191]. Columns of the ion-exchange type have been applied to stereochemical analyses of hydroxyeicosatrienoates [192] and hydroxyeicosatetraenoates [193].

Trans-retinoic acid and its photoisomers were separated by using a reversed-phase column and a mobile phase containing silver ions [166]. Retinyl esters with up to two double bonds in the acyl chain were resolved by a similar system [194].

3.2.3. Triacylglycerols

Triacylglycerols with up to three double bonds and low amounts of linoleate were separated on silica gel columns impregnated with silver nitrate [195–198] with, for example, a mobile phase of benzene [198].

Similar separations of cocoa butter, palm oil, sheep adipose tissue [199] and milk fat [200] were performed more easily on columns of the cation-exchange type using gradients of dichloromethane–dichloroethane with acetone. The order of elution of triacylglycerol species was similar to that for silver ion TLC (see Section 2.3). The resolution of SSM species is especially good so that rapid analysis of confectionery fats should be possible by this method.

Samples, including sunflower oil, maize, rat adipose tissue (Fig. 9) and safflower seed oil, with a high proportion of linoleic acid were analysed using a ternary gradient system with the addition of acetonitrile to the gradient described above [199]. By increasing the proportion of acetonitrile, species from linseed oil with up to nine double bonds (trilinolenate as major component) were resolved (Fig. 10). After collecting each triacylglycerol fraction the fatty acid composition was determined by GC following transesterification. This system was used in conjunction with direct GC analysis of triacylglycerols, and with stereospecific analysis to determine the fatty acid compositions at the *sn*-1, *sn*-2 and *sn*-3 positions, to give a comprehensive picture of the triacylglycerol composition of olive oil [201,202]. The silver ion HPLC–GC method agreed well with direct GC analysis of the triacylglycerols although there was overlap of some components (see below for HPLC) by both methods [202].

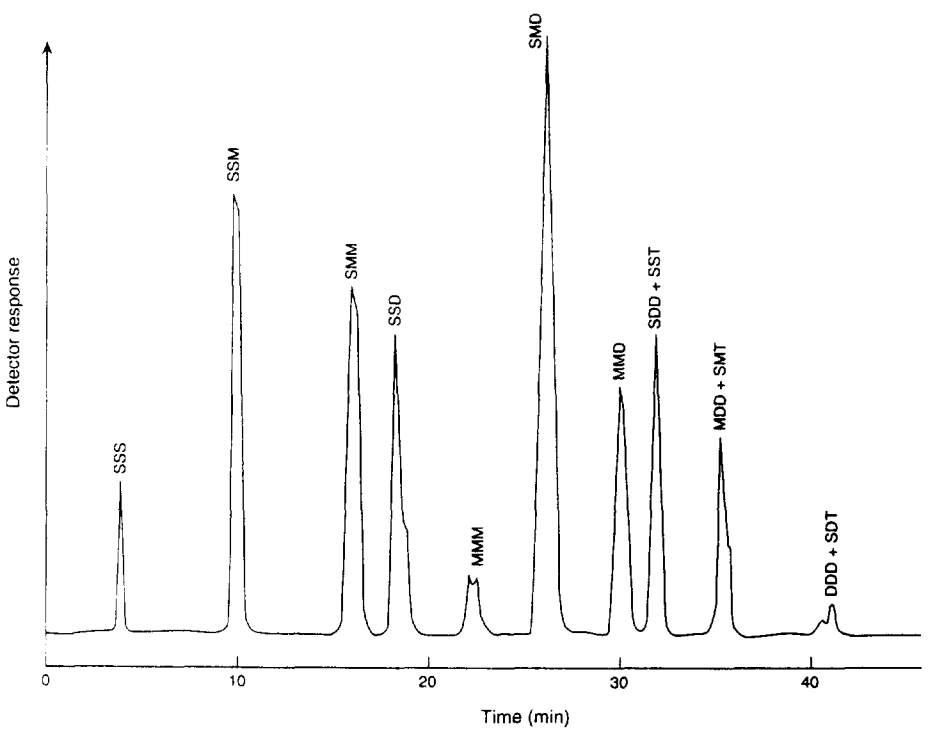

Fig. 9. Separation of triacylglycerols from rat parametrial adipose tissue by HPLC on a Nucleosil 5SA column in the silver ion form with evaporative light-scattering detection [199]. Three solvents [A, 1,2-dichloroethane–dichloromethane (1:1, v/v); B, acetone; C, acetone–acetonitrile (9:1, v/v)] were used at a flow-rate of 0.75 ml/min. There was a linear gradient from 100% A to 50% A–50% B over 15 min, then to 50% B–50% C over a further 25 min and maintained in this state for 5 min. (Reproduced by kind permission of *J. Chromatogr.* and redrawn from the original.)

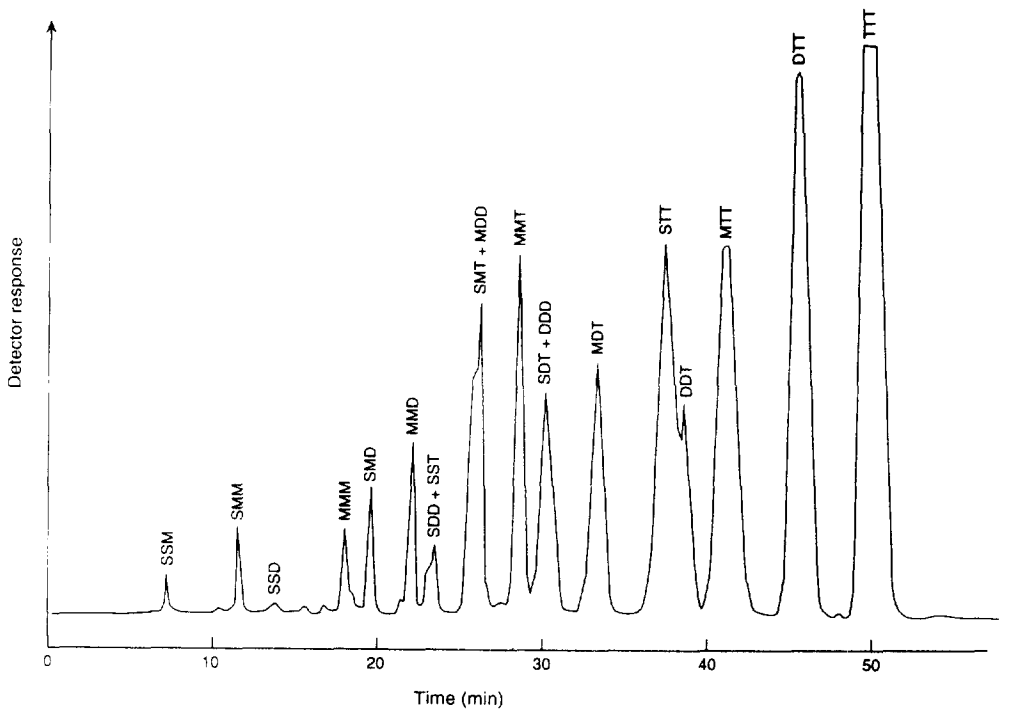

Fig. 10. Silver ion HPLC of triacylglycerols from linseed oil. [194]. Conditions as for Fig. 9, except solvent C was acetone–acetonitrile (4:1, v/v), the flow-rate was 1 ml/min and there was a gradient from 100% A to 50% A–50% B over 10 min, then to 70% B–30% C over a further 20 min and finally to 100% C over another 30 min. (Reproduced by kind permission of *J. Chromatogr.* and redrawn from the original.)

Analogous to silver ion TLC, a linoleate residue is held more strongly than two oleate residues but α-linolenate is retained to the same extent as two linoleates resulting in the overlap of some fractions (SDD and SST, SMT and MDD, SDT and DDD, STT and DDT [200]), although one of these critical pairs (SMT and MDD) was resolved recently [203]. Triacylglycerols with a γ-linolenate residue, in for example evening primrose oil, elute before those with two linoleates [16]. It is worth noting that although most of these separations have been achieved by TLC [94], HPLC methodology is cleaner and more reproducible.

For fish oils, although triacylglycerols up to MMD were well separated, species with up to fourteen double bonds, containing trienoic and residues of greater unsaturation, including eicosapentaenoic and docosahexaenoic acids, overlapped because of the presence of many positional isomers [199,204] (Fig. 11). Some

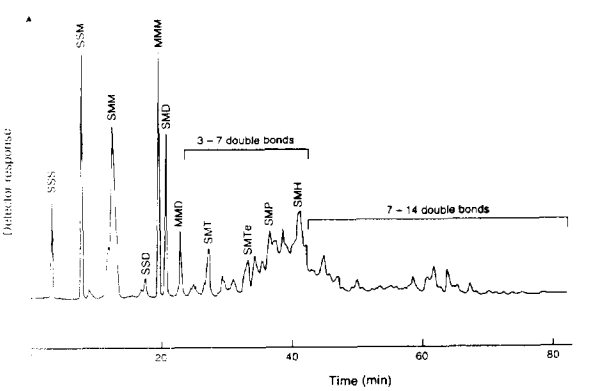

Fig. 11. Silver ion HPLC of triacylglycerols from Baltic herring [204]. Conditions as for Fig. 10, except solvent C was acetone–acetonitrile (2:1, v/v), the flow-rate was 1 ml/min and there was a gradient from 100% A to 50% A–50% B over 10 min, then to 70% B–30% C over a further 30 min, to 50% B–50% C over another 20 min, to 100% C after an additional 20 min and was maintained in this state for 5 min. (Reproduced by kind permission of *Lipids* and redrawn from the original.)

highly unsaturated species, especially those containing either two saturated/monoenoic fatty acids with a polyenoic fatty acid, were separated, however. The triacylglycerols were further resolved by analysing each silver ion fraction by RP-HPLC and this gave many more fractions but each was still a mixture of many species [35]. Similar results for a variety of fish oils were subsequently published by other workers who suggested that the method could be used to rapidly differentiate between triacylglycerols from fish and plant oils, enabling a check for adulteration [36]. The method was reproducible but the authors stressed the importance of standardising the time for re-equilibration of the mobile phase between runs.

The triacylglycerols of *Crepis alpina* oil, which contains a high proportion of *cis*-9-octadecen-12-ynoic (crepenynic) acid together with saturates, oleate and linoleate, were resolved on a commercial silver ion column with a hexane–acetonitrile isocratic mobile phase [165]. Fractions from trisaturated to tricrepenynoyl separated according to the number of double and triple bonds with those containing one triple bond and up to five double bonds (CrL_2) eluting before that with two triple and two double bonds (Cr_2S), indicating the much stronger retention of the triple bond in this system.

Meadowfoam seed oil is unusual in containing mainly *cis*-5-20:1, 5-22:1, 13-22:1 and 5,13-22:2 together with smaller amounts of common C_{18} fatty acids. Silver ion HPLC separated triacylglycerols into three main groups of peaks corresponding to MMM, MMD and MDD [178]. Individual peaks were represented by species differing in the position of the double bond and/or the chain length of only one acyl residue.

The positional isomers SSO and SOS were partially resolved with benzene as the mobile phase on a silver nitrate-impregnated silica gel column [198]. A rapid method, utilising a gradient of toluene–hexane (1:1) to toluene–ethyl acetate (9:1), separated the pairs SSO–SOS, SLS–SSL, SOO–OSO and SLO–OSL from vegetable fats (Fig. 12) and cocoa butter [196]. Separation of SSO–SOS and SOO–OSO pairs has also been achieved with a gradient of *n*-heptane–acetic acid to toluene–acetic acid [205].

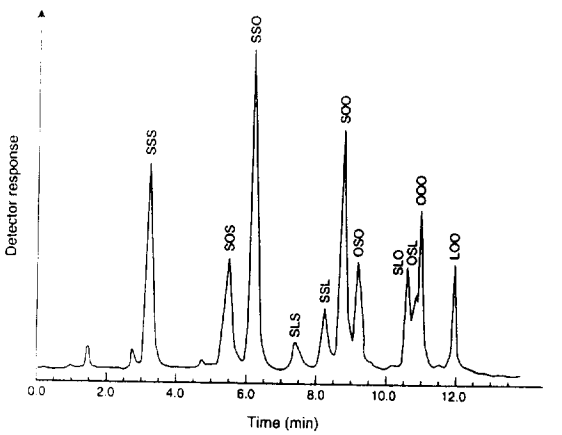

Fig. 12. Separation of positional isomers of triacylglycerols from fully randomized palm oil by HPLC on a silica (Nucleosil 100-3) column impregnated with 10% (w/w) silver nitrate with transport flame ionization detection [196]. Three solvents [A, toluene–hexane (1:1, v/v); B, toluene–ethyl acetate (9:1, v/v); C, toluene–98% formic acid (100:0.008, v/v)] were used at a flow-rate of 1.5 ml/min. There was a linear gradient from 94% A–6% B to 92% A–8% B over 2.5 min, then to 75% A–25% B over a further 4.5 min, to 10% A–90% B over another 3 min and finally to 100% C over 0.1 min and this solvent was maintained for a further 5 min. (Reproduced by kind permission of the author and *J. Am. Oil Chem. Soc.* and redrawn from the original.)

Double bond geometrical isomers of triacylglycerols have seldom been examined. In studies of sheep subcutaneous adipose tissue [199] and bovine milk fat [200], on a column of the silver ion–cation-exchange type, the pairs SSE–SSO, SEO–SOO and EOO–OOO were separated with, as expected, the *trans* isomers eluting first. Comparable separations of the last two pairs appear not to have been reported for silver ion TLC, although it would be interesting to determine whether SEE can be resolved from SSE and SSO by HPLC, as this has been demonstrated by silver ion TLC [66,88,105]. In the same HPLC studies, SOC, where C is the conjugated diene 9-*cis*-11-*trans*-octadecadienoic acid, eluted just before SOO.

In recent studies where the triacylglycerols of human milk were fractionated by silver ion HPLC prior to further fractionation by RP-HPLC and GC into well over a hundred species, separation of SSE–SSM, SEM–SMM, EMD–MMD and SED–SMD pairs was achieved but SED overlapped with MMM [15,205–207] (Fig.

13). Separation of these species would indeed be expected to be difficult to achieve because while elaidate is held less strongly than oleate, linoleate is retained more than two oleates. It is also not surprising that in one of the studies, EMM overlapped with SSD [15] (Fig. 13).

The SSE, SSO, SOE and SOO triacylglycerols in butter fat were fractionated in analyses combining silver ion HPLC with RP-HPLC and GC [208] and RP-HPLC and tandem MS [209,210]. A preliminary separation by silver ion HPLC was necessary not only as a simplification step but also because the MS procedure did not differentiate between geometrical isomers.

A method has recently been developed for separation and quantification of geometrical isomers of partially hydrogenated fats and was applied to soybean and palm oil [33]. The approach was to fractionate samples first by RP-HPLC according to partition number and then to separate the geometrical isomers of each fraction

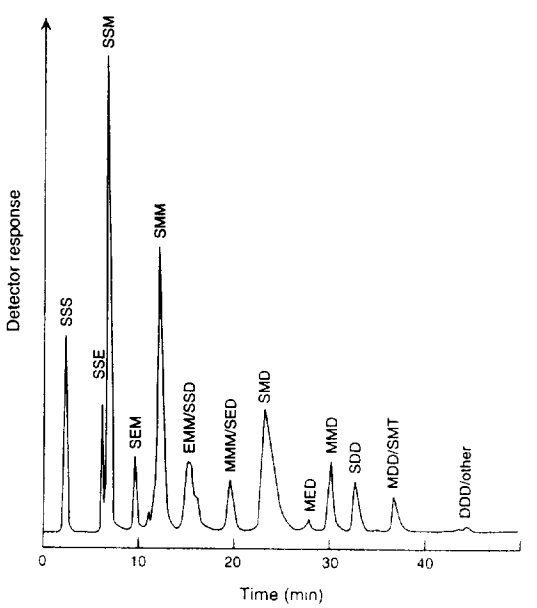

Fig. 13. Separation of human milk triacylglycerols by HPLC on a Nucleosil 5SA column in the silver ion form with evaporative light-scattering detection [15]. Three solvents (A, dichloromethane; B, acetone; C, acetone–acetonitrile [4:1, v/v]) were used. There was a linear gradient from 100% A to 50% A–50% B over 20 min, then to 10% A–70% B–20% C over a further 20 min and, finally, this composition was held for another 10 min. (Reproduced by kind permission of the authors and *J. Chromatogr.* and redrawn from the original.)

by RP-HPLC with silver ions in the mobile phase, a technique which compared to using silver bound to the stationary phase was claimed to reduce separation of positional isomers. Using standards, several series of triacylglycerols were separated that differed in the geometry of one double bond (e.g. OOO, OOE, OEE and EEE, and OOD, OED and EED). OOD_{tt} and OED_{cc} overlapped. The geometrical isomers of the more saturated major fractions of similar partition numbers in the oils were identified. Fractions containing species with a high proportion of linoleate were more complex, because of the greater number of geometrical isomers, and were not completely identified. Further work is obviously needed especially for application to partially hydrogenated oils of greater unsaturation.

3.2.4. Complex lipids

Silver ion HPLC has only been applied to a limited extent to the analysis of molecular species of complex lipids. Intact phosphatidylcholines from rat microsomes were resolved into fractions with up to six double bonds with methanol as the mobile phase on a column of unspecified type and with UV detection at 205 nm [12]. Two highly unsaturated species, 16:0–22:6 and 18:0–22:6, differing only in the chain length of one acyl residue were separated. Brain sphingomyelins were benzoylated and separated into saturated (mainly stearate) and monounsaturated (mainly tetracosenoate) fractions isocratically with methanol–isopropanol mixtures and UV detection at 230 nm [12]. Plant monogalactosyldiacylglycerols were analysed on a C_8 RP column with silver perchlorate in the mobile phase of methanol–water [211,212]. Pairs of species, 18:2–16:3/18:3–16:2 and 18:2–16:4/18:3–16:3, with the same degree of unsaturation were separated.

RP-HPLC has usually been the method of choice for analysing molecular species of complex lipids. It may be worthwhile considering this technique in tandem with silver ion HPLC, as this approach has been successful in analysing triacylglycerols. It would also be necessary to consider whether separations are better with intact lipids or non-polar derivatives since the

latter are usually more suitable for silver ion TLC.

3.2.5. Quantification

Quantification of lipids by silver ion HPLC has been reviewed elsewhere [5,6]. The methods are essentially the same as those employed for HPLC of lipids in general. A common approach is to add an internal standard to collected fractions and analyse by GC in an analogous way to that for silver ion TLC. Quantification based on the response of the HPLC detector may involve either external or internal standards. The merits of the various detectors, namely refractive index, UV, fluorescent, evaporative light-scattering and transport flame ionization, have been considered in other publications [6,213].

4. Supercritical fluid chromatography in the silver ion mode

SFC is at an early stage of development in the analysis of lipids (reviewed elsewhere [214]) but has been used in the silver ion mode by adapting HPLC technology, mainly in Blomberg's laboratory. Fused-silica capillary columns packed with Nucleosil 5SA converted to the silver ion form were used with a mobile phase of carbon dioxide–acetonitrile–isopropanol with UV detection for separation of triacylglycerols from seed oils including corn, olive, palm, soybean and sunflower oils [215,216], and columbine oil which contains the unusual triene, columbinic acid (6*c*,9*c*,13*t*-18:3) [217]. The chromatograms were essentially similar to those published for HPLC, and improved base-line stability as well as quantification of triacylglycerols was attained with the development of a miniature evaporative light-scattering detector [218]. In contrast to conventional silver ion HPLC, but similar to that using RP columns with silver ions in the mobile phase, there is considerable separation according to chain length (often a disadvantage) as well as unsaturation. Also contrastingly, a triacylglycerol with a dienoyl residue is held less strongly than two monoenes and a triene elutes before a species containing both a diene and a monoene [216,32]. In silver ion HPLC, two dienes are held to the same extent as a triene residue resulting in the overlap of DDD and SDT, for example [202]. These species are well separated by SFC.

For columbinic [217] and borage oils [32] the advantages of a two-step separation with RP-HPLC and silver ion SFC, and separation of triacylglycerols differing only in the position of one double bond (also achieved by silver ion HPLC [178]) were demonstrated [32]. Resolution of geometrical isomers of partially hydrogenated triacylglycerols [32] was comparable to that achieved by silver ion HPLC [33]. In a fish oil, many well resolved, although largely unidentified, peaks with up to eleven double bonds were observed [32] but it is likely that, as with silver ion HPLC [35,36,199,204], each peak comprises many triacylglycerol species.

Fatty acid methyl esters with up to six double bonds from a fish oil were separated according to degree of unsaturation and also chain length to some extent, and 20:4($n-3$) and 20:4($n-6$) were resolved [219] (Fig. 14). Although the eight geometrical isomers of linoleate can just be distinguished by silver ion SFC [219], the resolution achieved by silver ion HPLC is superior [68,188].

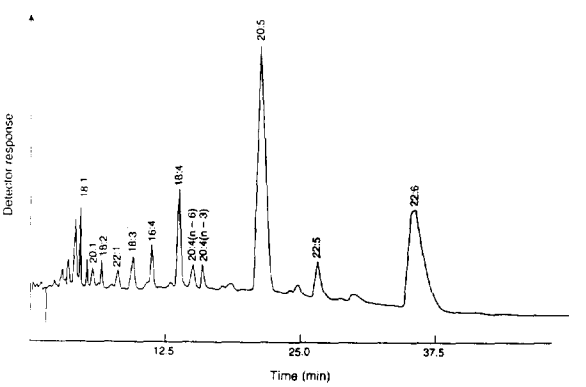

Fig. 14. Supercritical fluid chromatogram of fatty acid methyl esters from CPL fish oil 30 on a capillary column packed with Nucleosil 4SA in the silver ion form with UV detection at 210 nm (219). The mobile phase was carbon dioxide–acetonitrile–isopropanol (97.1:2.6:0.3, mol%). Injection was at 115°C and 280 bar, which was held for 2 min before programming at −1°C/min and 2 bar/min to 75°C and 360 bar. (Reproduced by kind permission of the authors and *J. Chromatogr.* and redrawn from the original.)

SFC with permanganate-impregnated anion-exchange columns have separated fatty acid methyl esters from fish oil [219] and triacylglycerols from seed oils [220,221] by degree of unsaturation suggesting that it may be profitable to investigate ions other than silver.

5. Principles and mechanism of silver ion chromatography

Silver ion chromatography is based on the principle that unsaturated organic molecules react reversibly with transition metals such as silver to form polar charge-transfer complexes. A sigma bond is formed between the occupied 2p π^* electrons of the double bond and the free 5s and 5p orbitals of the silver ion. A π acceptor backbond between the free antibonding 2p π^* electrons of the double bond and the occupied 4d orbitals of the silver ion is also involved [5]. The strength of the complex is determined by accessibility of the electrons in the filled orbitals and steric inhibition of the orbitals.

Much is known about the stability of the complexes from studies of the interaction of silver ions with various types of short-chain olefins. The subject has been reviewed [5,222–225] and will be only briefly summarised here. In the context of silver ion chromatography of fatty acids, it is known that for methylene-interrupted dienes, stability of the complexes increases with increasing number of double bonds, *cis* double bond isomers are held more strongly than *trans* isomers, stability decreases with increasing chain length and tends to increase initially as the distance between the double bonds becomes greater and then diminishes. Conjugated dienes are held less strongly than methylene-interrupted dienes and monoenes are held more strongly than monoynes. Preliminary attempts have been made to study the physics of adsorption of fatty acid methyl esters, namely linoleate and linolenate, to silver ions attached to ion-exchange resins and their desorption by various solvents, particularly with respect to the time taken to equilibrate between the solvent and the resin [226]. The results also apply to triacylglycerols.

The mechanism of the interaction in chromatographic systems, which would have to account for all the observations, is poorly understood. Indeed most of these studies involved silver ion TLC which has drawbacks in that many factors, such as the concentration and topology of silver ions on the silica adsorbent surface, the degree of hydration of the adsorbent, environmental factors such as temperature and humidity and the precise composition of the mobile phase, are difficult to control and result in variable R_F values. Therefore, reproducible numerical values to describe retention characteristics in quantitative terms cannot be obtained. However, attempts at such expressions have been made and they all indicate that as the number of double bonds in an acyl chain increases, the increase in "retention value" is greater than that expected from a simple multiple of the value for a single double bond.

HPLC columns of the type in which silver ions are linked via ionic bonds to phenylsulphonic acid moieties bound to a silica matrix have relatively well-defined properties and chemistry, and many factors, especially the composition and flow-rate of the mobile phase and the column temperature, can be accurately controlled. This system has recently been used to obtain quantitative information on the mechanism of the silver ion–double bond interaction [190,203,227,228]. It must be recognised that silver ion chromatography may involve a mixed retention mechanism even in this system. For example, as well as silver ion complexation with double bonds, free silanol groups may interact with ester groups. Indeed these interactions have been suggested as an explanation for the separation patterns of several series of double bond positional isomers of FAMEs by silver ion TLC [55]. The nature of the mobile phase must also be considered because it greatly affects separations and may interact with both the lipid and the support material.

Quantitative information in terms of capacity factors has been obtained for a series of isomeric *cis*-octadecenoic acids, a partial series of isomeric octadecadienoic acids and several polyunsaturated fatty acids with up to six double bonds

by silver ion HPLC of their methyl and/or phenacyl esters [190]. The results for the monoenes have already been described (see Section 3.2 and Fig. 8). An allenic acid (9,10-18:2) and a conjugated diene (9-*cis*,11-*trans*-18:2) were retained more weakly than oleate, but linoleate was held much more strongly. Isomers with two, three and five methylene groups between the double bonds were held even stronger, those with two the greatest and with five the least. The capacity factors of the polyenes increased with increasing number of double bonds so that the phenacyl ester of docosahexaenoate was retained 30 times as much as oleate.

The explanation proposed for the positional specificity of silver ion HPLC was that there was a dual interaction of a silver ion with the π electrons of the double bond and the free electrons of the carbonyl group of the methyl ester or the benzene ring or oxygenated functions of the phenacyl ester [190]. Indeed, generally monoenes up to 8-18:1 were held more strongly as the electron-rich phenacyl esters than were methyl esters, and capacity factors varied according to the distance between the double bond and the ester group (Fig. 8). Presumably the optimum distance for a dual interaction occurred in the isomer (6-18:1 for methyl and 7-18:1 for phenacyl esters) with the greatest capacity factor. From 9-18:1 onwards the difference between methyl and phenacyl esters diminished, as did the difference between the different positional isomers, presumably because of the diminished likelihood of a simultaneous interaction. An explanation for the shorter elution times of isomers where the double bond is near the ester group may be explained by the inductive effect of the latter resulting in partial withdrawal or delocalization of the π electrons of the double bond as has been suggested for silver ion TLC [76].

The results for the dienes may be explained in a similar way by an interaction between a silver ion and both double bonds simultaneously, with two methylene groups between the double bonds being optimum. Indeed it has been shown by X-ray crystallography that one silver ion can interact with two unsaturated molecules simultaneously [229,230] or with two double bonds in a single molecule [222]. In support of this hypothesis, a triene is held twice as strongly as two dienes in some circumstances and even more strongly under other conditions, suggesting that there may also be interactions with the ester moiety. In addition, the conformation of polyenes may expose a double bond in a more favourable position to interact with a silver ion. The allene and conjugated diene were held weakly, presumably because there was a weak interaction only with the delocalised π electrons of these *bis*-double bond systems.

The retention characteristics of triacylglycerols from seed oils and a series of disaturated triacylglycerols with an unsaturated residue with 1–6 double bonds were also studied on a silver-loaded cation-exchange column [203]. The principal consideration was whether a triacylglycerol molecule participated in complexation as a single entity or whether the general effect was a sum of the different fatty acid moieties. SSS triacylglycerols were not significantly retained. SSM triacylglycerols were considered as unsaturated fatty acid derivatives, and their chromatographic behaviour was compared to that of the methyl esters of the same fatty acids. Triacylglycerols were held about 5–10 times more strongly than the comparable methyl ester. Possible explanations were that the triacylglycerol molecule was more rigid so that the double bond was approached more easily and that there was simultaneous interaction of a silver ion with the free electron pair of one of the carbonyl oxygens. The greatest relative increase in retention occurred when the number of double bonds in the triacylglycerol increased from one to two, suggesting the simultaneous interaction of one silver ion with two double bonds as for simple fatty acid derivatives [190]. From the data on the seed oils [203] it was clear that the retention factor of a monoacid or mixed acid triacylglycerol cannot be derived simply from the sum of the retention factors of the respective SSU triacylglycerols.

In order to better understand the role of the ester group in complexation, a range of esters of octadecenoic acid positional isomers with differ-

ent monounsaturated short-chain alcohols (C_3–C_6) were examined [228]. For any one acyl isomer, the retention increased with increasing distance of the alcohol double bond from the ester group, reflecting the contribution of the alcohol double bond to silver ion complexation (Fig. 15). With the exception of the allyl ester, the patterns of retention were similar for any one series of esters of variable acyl and fixed alcohol moiety (Fig. 15), and the patterns were similar to those previously reported for methyl and phenacyl esters [190]. The results were consistent with a simultaneous interaction between one silver ion and one double bond (on the acyl chain) and the free electron pair of the carbonyl oxygen. This has been confirmed by more recent work with ester moieties containing different electron-donating and electron-withdrawing constituents (Nikolova-Damyanova and Christie, unpublished results).

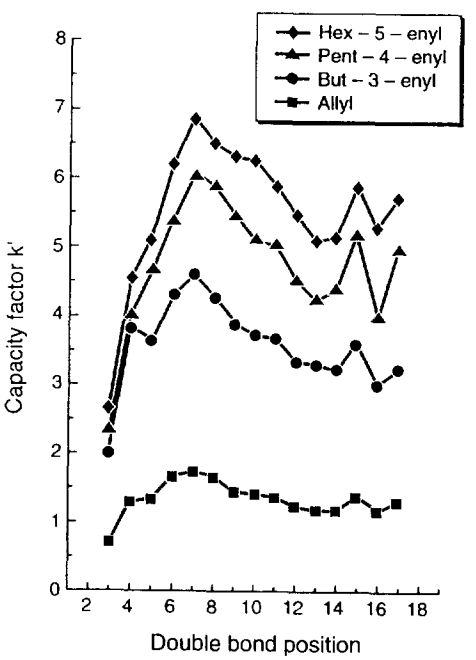

Fig. 15. Capacity factors, k', for HPLC of allyl, butenyl, pentenyl and hexenyl esters of a series of *cis*-octadecenoates at 20°C on a Nucleosil 5SA column in the silver ion form with evaporative light-scattering detection [228]. The mobile phase was 1,2-dichloroethane–dichloromethane–acetonitrile (50:50:0.25, v/v/v) and the flow-rate was 1.5 ml/min. (Reproduced by kind permission of the *J. Chromatogr.*)

Further information on the complexation mechanism was sought by a different system, that of using RP-HPLC with and without silver ions in the mobile phase to examine monoenoic positional isomers and polyunsaturated fatty acids as their phenacyl and phenethyl esters [227]. The difference in the capacity factors under the two conditions gave a measure of the complexation effect. As the degree of unsaturation of the fatty acids increased, the smaller was the effect of silver ions on complexation (Fig. 16). Also, differences in retention of two mono-

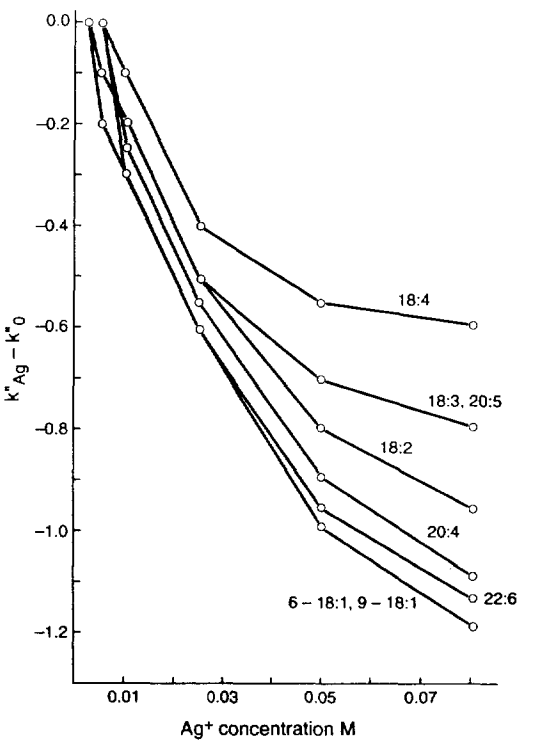

Fig. 16. The k''_{Ag}–k''_{O} values for HPLC of phenacyl derivatives of some polyunsaturated fatty acids at 20°C on a Spherisorb S5C8 column with evaporative light-scattering detection [223]. The mobile phase was methanol–water (87:13, v/v) and the flow-rate was 1 ml/min. The k'' value was defined as the ratio of the capacity factor of the analyte to that of myristic acid phenacyl ester. k''_{Ag} was measured with 0.08 *M* silver nitrate in the mobile phase and k''_{O} was measured with 0.08 *M* ammonium nitrate in the mobile phase. k''_{Ag}–k''_{O} is a measure of the fraction of the capacity factor that corresponds directly with the effect of complexation. (Reproduced by kind permission of the *J. Chromatogr.*)

enoic positional isomers were due to differences in lipophilicity and not in the strength of complexes with silver ions (Fig. 16). Moreover, equivalent chain-length and fractional chain-length values were calculated and indicated that in this system one silver ion complexed with one double bond (i.e. complexation for a diene and triene was twice and thrice as strong as that for a monoene) and the complexation was independent of any possible interaction between the ester group and silver ions. Thus the mechanism of complexation in bulk solvent media is different to that, outlined in preceding paragraphs, for silver ions held on the surface of the support material.

The RP-HPLC–silver ion system allowed the equilibrium constant for the formation of the silver ion–oleate phenacyl ester complex to be determined as having a value of 0.059–0.067 [227]. This is the first time that such an equilibrium constant has been determined for a fatty acid derivative in a chromatographic system.

6. Perspectives

Recent years have seen the emergence and subsequent advancement of silver ion HPLC. The types of separation and resolution that have been achieved generally exceed the possibilities by silver ion TLC. There are only few examples of TLC separations which cannot, as yet, be performed by HPLC. Despite this, the method of choice depends on the required separation and TLC will still have a major role especially for simple, semi-quantitative, analytical applications with a large sample through-put. For simple small-scale preparative separations, especially according to the number of double bonds, a wider application of solid-phase extraction columns can be expected.

Because of the stability and suitability for preparative studies, and moreover of the commercial availability, silver ion HPLC columns based on cation exchangers of benzenesulphonic acid bound to silica can be expected to predominate over other types. SFC in the silver ion mode appeared comparable to silver ion HPLC in selectivity but is limited in practical value, though it may find quality control applications.

Silver ion HPLC has been demonstrated as an essential step in the simplification of an ever-increasing range of samples prior to analysis by other separation techniques and, in conjunction with complementary methods such as RP-HPLC or GC–MS, it is an extremely powerful tool for detailed characterizations. Further advances in separation of positional and geometrical isomers of fatty acids and triacylglycerols are likely. The possible acyl group combinations in, for example, partially hydrogenated fats is enormous. There remains great scope for advancement in the analysis of complex lipids, especially if silver ion HPLC can be successfully combined directly with MS. The foundations for use of silver ion chromatography, especially HPLC, in detailed nutritional and metabolic studies on dietary lipids, for example, have been laid.

Acknowledgements

This paper is published as part of a programme funded by the Scottish Office Agriculture and Fisheries Department and the Bulgarian National Scientific Research Foundation.

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