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REVIEW

ROUTINE CHROMATOGRAPHY OF SIMPLE LIPIDS AND THEIR CONSTITUENTS

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CONTENTS

1. INTRODUCTION

Application of chromatographic methods to the separation of lipid mixtures has revealed a progressive increase in the number of subfractions with increasing power of resolution of the method. By a combination of complementary chromatographic techniques, it has been possible to obtain pure lipid classes and frequently pure molecular species within a lipid class. Not all of these separation techniques, however, are subject to routine utilization and many require specialized equipment. Since there exists a rapidly rising general interest in the detailed molecular structure and metabolism of various natural lipid classes, a brief summary is presented of those chromatographic methods which in the experience of several lipid research laboratories have reached a routine level of application. It is suggested that this complement of chromatographic methods serves as a guide to the quality of lipid analyses to be expected in general biochemical or clinical studies where lipids are analyzed as part of an overall experimental protocol.

2. PREPARATION OF LIPID EXTRACTS

The selection of the analytical sample and the method of lipid extraction are as important as the methods of lipid separation in the evaluation of the final results. The most efficient and carefully executed analyses cannot retrieve the data lost by poor sample selection and/or non-representative lipid extraction. Furthermore, the more efficient methods of resolution of lipid classes and molecular species require high quality samples that are free of non-lipid material.

A. Initial isolation

There are two basic routines which yield essentially quantitative extraction of the major lipid classes, when applied to homogenates of whole tissue or tissue subfractions. The most popular extraction method is that described by Folch et al. [1] which employs a chloroform—methanol (2:1) mixture in a solvent to tissue ratio of 20:1. This method gives excellent recoveries for neutral lipids and the diacylglycerophospholipids and sphingolipids. Lysophospholipids are

only partly recovered, and the more polar acidic phospholipids may be lost during backwashing with salt solutions and water.

The second most popular extraction method is that proposed by Bligh and Dyer [2], which effects a single phase solubilization of the lipids using a chloroform—methanol (1:1) mixture in a ratio of 4:1. The eventual partitioning of the extracts between chloroform and water results in losses of the more polar acidic phospholipids and lysophospholipids, as already noted for the Folch et al. [1] procedure. The advantages and disadvantages of these methods of lipid extraction have been discussed in great detail by Nelson [3] who has proposed improvements relating to the purification of the initial extracts. Bjerve et al. [4] have shown that complete extractions of the lysophospholipids from aqueous systems may be obtained by means of 1-butanol. Schmid et al. [5] have suggested that for the extraction of free fatty acids and neutral lipids, the chloroform—methanol—water system be replaced with benzene—methanol or toluene—ethanol solvent systems.

B. Purification of extracts

After filtration of the initial lipid extract some 25—75% of the total mass of the extract may represent non-lipid contaminants. These must be removed by purification. The crude lipid extract obtained as the final product of the evaporation of the solvent from a chloroform—methanol extract can be freed of essentially all non-lipid material by column chromatography on dextran gel columns [6, 7]. For this purpose Sephadex G-25 (Pharmacia) is packed in a column as a slurry in methanol—water (1:1) and weighted down with a layer of clean sand to prevent the gel from floating in solvent mixtures containing chloroform. The packing is washed with the entire sequence of solvent mixtures used during the column purification of the lipid extract. The sample is applied in chloroform—methanol (19:1), saturated with water (5 ml/l), which also elutes hydrocarbons and all lipids except gangliosides and conjugated bile salts. For a 10-g Sephadex column about 170 ml of solvent is required to complete the elution. The gangliosides are recovered with chloroform—methanol (9:1) (5 vol.) plus acetic acid (1 vol.). The column can be regenerated by washing it with methanol—water (1:1). This technique of lipid extract purification has been critically reviewed [3]. A batch method of purification of lipid extracts has been described by Williams and Merrilees [8].

C. Sample protection

Since most common lipids contain fatty acids with one or more double bonds, care must be taken to avoid autoxidation of the sample at any time during the manipulation and storage. This can be minimized by working with oxygen-free solvents and by performing all manipulations under a nitrogen atmosphere [9]. In addition, an antioxidant such as 2,6-di-*tert*.-butyl-*p*-cresol (BHT) or a similar compound may be added to the extracting solvents, which effectively prevents oxidative degradation of unsaturated lipids at a concentration of less than 0.005%. Furthermore, this antioxidant may be easily removed at various stages of the experiment by chromatographic means [7].

Before extraction the sample must be protected against the action of degradative enzymes. It should be noted that enzymatic hydrolysis may be significant even at −20° when stored for prolonged periods of time [10].

Finally the sample must be protected from contamination with lipids or other substances in solvents, reagents and on equipment. This can be guarded against by avoiding the use of solvents and reagents that leave lipid residues upon evaporation of appropriate volumes of the solvent and by washing all equipment with pure organic solvents before use.

Purified lipid extracts may be stored in tightly closed vials at low temperatures (−20° or lower) in the presence of inert solvents and inert atmosphere, for short periods of time.

3. SEPARATION OF LIPID CLASSES

There are several well established and reliable routines for the separation of individual lipid classes. A choice between column and thin-layer chromatography is usually made on the basis of sample size. Since the final separations of the individual lipid classes depend upon a careful adjustment of polarity of the eluting solvents, it is best to bring about a general group separation of lipids first and then follow it up with a complete resolution of individual chemical classes.

A. Neutral lipids

The neutral lipids are isolated on the basis of polarity by means of adsorption chromatography and include free fatty acids when present.

a. Initial isolation

A generally applicable strategy is to isolate the non-polar lipids as a mixture by adsorption column chromatography if time and quantity of material permits. This can be effectively accomplished by means of silicic acid columns [11, 12]. The non-polar lipids are recovered as a mixture in chloroform, while the polar lipids are retained on the column. Alternatively, the neutral lipids may be recovered as a group from TEAE-cellulose columns by elution with 5 volumes of chloroform, while the polar phospholipids are retained on the column. Detailed outlines of this method are available [12, 13].

Small amounts of neutral lipids may be isolated as a group by TLC using any of the solvent systems that separate the polar phospholipids and carry the neutral lipids to the solvent front [14, 15]. In all instances, the neutral lipids may be recovered from the scrapings of the silica gel by elution with chloroform.

b. Separation of neutral lipid classes

The most effective resolutions of individual neutral lipid classes are obtained by TLC. There are several excellent solvent systems that yield pure fractions for hydrocarbons, steryl esters, triacylglycerols, free fatty acids, diacylglycerols, free sterols and monoacylglycerols. When the TLC separations are performed in the presence of boric acid, it is possible to recover both *sn*-1,2(2,3)- and *X*-1,3-diacylglycerols as well as *X*-1- and 2-monoacylglycerols as separate neutral

lipid classes free of isomerization [16]. The monoacylglycerols frequently remain at or near the origin and require rechromatography with more polar solvents [17]. Further resolution may be required also for the *X*-1,2-diacylglycerols, which overlap with free cholesterol under these conditions. Any of these separations can be readily performed in quantities that are sufficient for subsequent GLC examination of the individual molecular species and the separation and identification of the component fatty acids.

Effective resolution of the neutral lipid classes may also be obtained by direct GLC on short columns prepared with non-polar liquid phases [18]. For this purpose the free fatty acids are converted into the trimethyl(silyl) (TMS) esters, and the free sterols and mono- and diacylglycerols as well as any free ceramides into the TMS ethers. The various neutral lipid classes including triacylglycerols and steryl esters are then eluted from the column in order of increasing molecular weight by means of temperature programming. There is also a separation within each chemical class of lipids according to the total carbon number. The method is not suitable for a quantitative isolation of the neutral lipid classes on a routine basis.

B. Polar lipids

The polar lipids contain a much more heterogeneous population of functional groups than the neutral lipids and may be subjected to lipid class resolutions that exploit these differences in their structure. By this means it is possible to effect a complete separation of the major lipid classes as well as to isolate many minor components in enriched form and in sufficient amounts for further analysis.

a. Initial isolation

The polar lipids may be recovered in toto or in small groups of related classes by chromatography on anion-exchange cellulose columns. Rouser et al. [12,13] have described the practical aspects of both the DEAE- and TEAE-cellulose column operation for this purpose. Following the initial displacement of the neutral lipids with chloroform, chloroform—methanol (9:1) (8 column volumes) will elute the choline phospholipids (phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin) as well as cerebrosides and glycosyldiacylglycerols; chloroform—methanol (2:1) (9 volumes) will elute the ceramide polyhexosides; chloroform—methanol (2:1) containing 1% glacial acetic acid (8 volumes) will elute phosphatidylethanolamine, dimethylphosphatidylethanolamine, lysophosphatidylethanolamine as well as free fatty acids; while glacial acetic acid (8 volumes) will elute phosphatidylserine. The residual acidic phospholipids (phosphatidic acid, phosphatidylglycerols, cerebroside sulfates, sulfolipids, and phosphatidylinositols) are eluted with chloroform—methanol (4:1) containing 0.1 *M* potassium acetate and 20 ml/l of 28% ammonia (10 volumes). Although the various phospholipids or their subgroups may be further purified by ion-exchange columns, it is usually more efficient to complete the isolation of the fractions along with the lipid class separation by means of TLC.

Alternatively, the polar lipids are recovered from adsorption columns as a residual group of lipids following the elution of the neutral or non-polar lipids [11—13]. Glycosphingolipids can be removed with acetone and/or mixtures of

lipid classes free of isomerization [16]. The monoacylglycerols frequently remain at or near the origin and require rechromatography with more polar solvents [17]. Further resolution may be required also for the *X*-1,2-diacylglycerols, which overlap with free cholesterol under these conditions. Any of these separations can be readily performed in quantities that are sufficient for subsequent GLC examination of the individual molecular species and the separation and identification of the component fatty acids.

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acid—water (5:2:1:1:0.5) for the second dimension. Comparable results may be obtained without the acetone in the second solvent [27].

Another solvent system of comparable resolving power employs chloroform—methanol—aqueous ammonia (65:25:5) in the first dimension and chloroform—acetone—methanol—acetic acid—water (3:4:1:1:0.5) in the second dimension. However, phosphatidylglycerol and phosphatidylethanolamine frequently overlap or cochromatograph in these systems. An improved two-dimensional separation of the glycerophospholipid classes from a total lipid extract of most tissues has been described by Poorthuis et al. [28]. The solvent system for the first dimension is chloroform—methanol—water—concentrated ammonia (70:30:3:2) and for the second dimension chloroform—methanol—water (65:35:5). In comparison with the patterns obtained in the systems of Rouser et al. [12,13] the positions of phosphatidylinositol and phosphatidylserine are reversed and phosphatidylglycerol is more distinctly separated from phosphatidylethanolamine. Both of these differences are due to addition to the silica gel of boric acid, which forms complexes with compounds containing vicinal hydroxyl groups [16] and retards their mobility. The alkyl and 1-alkenyl ether derivatives of the glycerophospholipids cannot readily be resolved from the corresponding acyl analogues [29] and must be left in the mixture until a fractionation stage is reached where it is appropriate to release the acyl glycerols.

c. Separation of glycolipid classes

The diacylglycerol mono- and digalactosides may be resolved by one-dimensional TLC using benzene—acetone—water (30:91:8) [30] or chloroform—methanol—ammonia—water (60:35:5:2) [31] as the developing solvents. A mixture of chloroform—methanol—water (65:35:8) has yielded a separate fraction for glycerophosphoryldiglucosyldiacylglycerol [32]. Excellent separations of galactosyldiacylglycerols from mixtures with glycerophospholipids have been obtained by two-dimensional TLC using chloroform—methanol—7 *N* ammonia (65:30:4) in the first direction and chloroform—methanol—acetic acid—water (170:25:25:6) in the second direction [33,34].

The simple ceramide mono- and oligosaccharides can be separated using the solvent systems developed by Svennerholm and Svennerholm [35], Vance and Sweeley [36], and Skipski et al. [37]. Vance and Sweeley [36] have separated plasma glycosyl ceramides into cerebrosides, dihexosyl ceramides, trihexosyl ceramides and globosides, using chloroform—methanol—water (100:42:6) as the developing solvent. The sphingolipids are separated primarily according to the number of monohexosyl units per molecule. Skipski et al. [37] recommend an initial run with acetone—pyridine—chloroform—water (40:60:5:4) to separate neutral lipids and glycolipids, with the phospholipids remaining at origin of the TLC plate. A second development with a non-polar solvent made up of diethyl ether—pyridine—ethanol—2 *N* ammonia (65:30:8:2) is used to wash away the neutral lipids, while the glycolipids remain more or less stationary. A third run with diethyl ether—acetic acid (100:3) is finally made to wash away the free fatty acids. Under these conditions, the neutral lipids migrate just ahead of the ceramide monohexosides and all phospholipids move more slowly than a ceramide tetrahexoside. The more polar glycolipids occupy the same area of the TLC plate as the phospholipids unless the latter have been removed prior to TLC. An effective way of removing phospholipids from glycosphingolipids has

been described by Saito and Hakomori [19]. The more complex neutral diacylglycerol and ceramide oligosaccharides containing five to eight monosaccharide units may be resolved by TLC after conversion into the fully acetylated form [38].

Glucosyl and galactosyl ceramides which run together on plain silica gel can be resolved on borate impregnated TLC plates [39,40]. The sulfatides, galactosylceramide sulfates, run with similar mobility on TLC as the ceramide di- and trisaccharides in many solvent systems commonly employed for the neutral glycolipids, unless they have been resolved by chromatography on the ion-exchange celluloses [7].

The sialic acid-containing sphingoglycolipids, the gangliosides, are commonly resolved by one-dimensional TLC using propanol—water (7:3) as the developing solvent [41—43]. Complete resolution of the various classes of gangliosides is obtained provided these lipids have been first resolved from other lipids by Sephadex column chromatography [7]. These separations are based on the number of sialic acid residues within each oligosaccharide subclass.

Horning et al. [44] and Samuelsson and Samuelsson [45] have demonstrated that the ceramide monohexosides can be recovered from conventional GLC columns containing non-polar packings when chromatographed as the TMS ethers or the heptafluorobutyl esters. By means of similar columns Auling et al. [46] and Tulloch et al. [47] were able to resolve the mono- and dihexosyl diacylglycerols as the TMS ethers. The mono- and dihexosides of both diacylglycerols and ceramides have been resolved by Kuksis [48] and Williams et al. [49] in the form of the TMS ethers, acetates and methyl ethers. Using a short GLC column of the type commonly employed in triacylglycerol analyses, it was possible [48] to elute also the TMS ethers of the tri- and tetrahexoside ceramides. The latter separations, however, gave evidence of decomposition of the solutes and could not be recommended for routine applications. The GLC separations of the intact glycolipides also allow a resolution of the molecular species of these lipids as discussed in Section 4F.

The chromatography of phospholipid and glycolipid classes on Whatman SG-81 silica gel loaded paper has been reviewed by Wuthier [50] while Witting [51] has summarized the more recent methods applicable to glycolipids as a class.

4. SEPARATION OF MOLECULAR SPECIES

The most effective and most widely applicable methods of fractionation of molecular species of intact lipid classes utilize differences in degree of unsaturation and in molecular weight of the fatty acid chains of the lipid molecules. The most complete resolutions are obtained by those methods of separation which allow a systematic exploitation of both of these differences.

A. Steryl esters

The steryl esters of various degrees of unsaturation of the component fatty acids may be resolved by argentation TLC. Morris [52] used a double development starting with diethyl ether, which resolved the tetraenes, pentaenes and hexaenes, and ending with diethyl ether—hexane (1:4), which allowed the sepa-

ration of the monoenes, dienes and trienes. The system did not allow the separation of saturated and unsaturated sterols esterified to the same fatty acid. A complete identification of the steryl esters in the various subfractions may be obtained following saponification and a GLC analysis of the component sterols, as described in Section 5D.

Tichy and Dencker [53] and Alling et al. [54] have described routine methods for the separation of serum cholesterol esters by TLC on plain silica gel using multiple developments in *n*-heptane—toluene (80:20) or (65:25), respectively. Separate fractions are obtained for saturated, monoenoic, dienoic, trienoic and tetraenoic, pentaenoic and hexaenoic fatty acid esters. The total steryl ester mixture as well as any of the subfractions derived by argention TLC may be resolved according to molecular weight or carbon number by direct GLC on short columns containing non-polar liquid phases [55, 56].

When more than one type of sterol is present two or more peaks may be obtained for each fatty acid ester [56]. For the resolution of steryl esters with short and long chain saturated fatty acids (C_2—C_{18}) as well as certain unsaturated acids (erucic, oleic, linoleic and linolenic), Kaufmann et al. [57] have utilized hydrophobic layers of paraffin impregnated silica gel, with ethyl methyl ketone—acetonitrile (7:3) as the solvent system. Despite the excellence of the resolution, this method is much inferior to the ease and simplicity of the direct GLC separation of the steryl esters according to molecular weight differences [55, 56, 58].

B. Acylglycerols

The acylglycerols are most effectively resolved by argentation TLC and GLC methods. Complete separations of the molecular species of the triacylglycerols, however, cannot be obtained even by a combination of the two methods, while the molecular species of the monoacylglycerols can be resolved essentially completely by either one of the methods. The completeness of resolution of the diacylglycerols depends on the molecular weight of the species, the lower-molecular-weight homologues being resolved more completely.

a. Triacylglycerols

Argentation TLC of triacylglycerols has given the most useful resolution of the molecular species comprised of mono-, di- and triethylenic fatty acids of uniform chain length of 18 carbons. A fat containing saturated, oleic, linoleic and linolenic acids could contain triacylglycerols with 0 to 9 double bonds. It has proved experimentally feasible [59] to resolve the triacylglycerols into the following classes of unsaturation in order of decreasing rate of migration: 000 > 100 > 200 > 111 > 210 > 211 > 220 > 300 > 221 > 310 > 222 > 311 > 320 > 321 > 322 > 330 > 331 > 332 > 333, where 0 to 3 represent the content of double bonds per molecule of fatty acid. These correlations apply to *cis*-methylene-interrupted polyethylenic acids of the same chain length.

Triacylglycerols containing both short and long chain fatty acids must first be resolved into triacylglycerol subclasses of short, medium and long chain length prior to effective argentation TLC [60, 61]. The position of the acyl group in the triacylglycerol molecule influences the resolution since 1-oleoyl-

2,3-distearoylglycerol is retained longer than 2-oleoyl-1,3-distearoylglycerol [62]. The triacylglycerols containing ethylenic fatty acids with a *trans*-configuration of the double bond migrate faster than those with a *cis*-configuration [59,63].

The triacylglycerols are effectively resolved in hexane—diethyl ether (70:30) when applied to silica gel containing 5—10% silver nitrate [64]. Alternative solvents for the resolution of triacylglycerols of the common fatty acids are mixtures of chloroform—methanol (99:1 to 94:6) [65—67]. A rechromatography of the polyunsaturated triacylglycerol fractions is performed by redeveloping the plate in the more polar solvent system one or more times [67]. The triacylglycerols are recovered from the silver nitrate plates by elution with 10% methanol in diethyl ether.

The GLC resolution of intact triacylglycerols is most easily accomplished with molecules containing short and medium chain length fatty acids, whose molecular weights do not exceed that of tristearoylglycerol [15,68,69]. It is also applicable to triacylglycerols composed of long chain fatty acids exclusively, but these separations require specially optimized columns [15,70] and cannot be utilized routinely at the present time. About 50—60 cm is an optimum length of the column for most triacylglycerol separations for which the losses of the higher molecular weight materials are not too serious [71,72]. A listing of currently available phases for triacylglycerol GLC has been compiled [72].

Triacylglycerols of uniform molecular weight and degree of unsaturation may be isolated by preparative GLC provided a preliminary resolution by argentation TLC has been carried out [73—75]. Owing to the critical nature of the separation conditions, it has been necessary to make repeated collections from essentially analytical columns by means of semi-automatic operation of fraction collectors in conjunction with stream splitters. The collected triacylglycerol peaks may be subjected to partial degradation and positional analysis of fatty acids for complete determination of the molecular structure of the component molecular species, as described in Section 5A.

For the purpose of further resolution of the component triacylglycerols the various subfractions collected from argentation TLC may be submitted to a reversed-phase partition TLC [76]. Although not as simple as direct GLC, this method has yielded some of the most complete resolutions of natural triacylglycerol mixtures. Excellent separations of C_9—C_{56} triacylglycerols according to acyl carbon number have been obtained by Lindqvist et al. [77] using hydroxyalkoxypropyl-Sephadex columns for liquid—liquid chromatography. Complete baseline resolutions were obtained with sample loads as high as 0.5 g/cm^2 of column. The reversed-phase partition TLC as well as the reversed-phase column partition chromatographic systems, however, are not as yet sufficiently reproducible for routine application in a general purpose laboratory.

Trisubstituted acylglycerols containing ether linkages may be separated with relative ease from triacylglycerols by adsorption TLC [78,79]. The relative order of increasing migration in hexane—diethyl ether (95:5 or 90:10) is triacylglycerols, alkyldiacylglycerols, alk-1-enyldiacylglycerols, dialkylacylglycerols and trialkylglycerols. These alkylacylglycerols may be subjected to argentation TLC under the general conditions of triacylglycerol resolution. Likewise they can be effectively resolved by high-temperature GLC under the

conditions employed for the separation of triacylglycerols of comparable molecular weight [72].

b. Diacylglycerols

The fractionation of diacylglycerols by argentation TLC allows separations of molecular species containing 0 to 12 double bonds [80]. A conversion into the acetates helps to prevent isomerization as well as reduces tailing of the components during chromatography. Many positional isomers are also separated. Thus the 1,2-dioleoyl-*sn*-glycerol moves ahead of the 1-palmitoyl-2-linoleoyl-*sn*-glycerol acetate. Likewise, the acetate of 1-oleoyl-2-linoleoyl-*sn*-glycerol moves ahead of the 1-palmitoyl 2-linolenoyl acetate and the acetate of 1-oleoyl-2-arachidonoyl-*sn*-glycerol moves ahead of the acetates containing one saturated and one pentaunsaturated fatty acid. Similar separations are well established among the triacylglycerols [59]. Comparable resolutions of the diacylglycerols have been obtained by argentation TLC of their *tert*.-butyldimethylsilyl ethers [81]. The latter compounds have the advantage of stability to moisture. Dyatlovitskaya et al. [82] have employed the trityl derivatives of diacylglycerols for argentation TLC, while Van Golde et al. [83] have employed free diacylglycerols with equal success.

The argentation TLC usually does not yield pure molecular species and other methods are required to complete the separations. For this purpose GLC methods are best suited [84,85] as they provide effective resolutions based on molecular weight. Complete separations of diacylglycerols as the acetates or the silyl ethers may be obtained according to both molecular weight and degree of unsaturation on certain polar liquid phases [86], but these methods are still at an experimental stage. The identity of the various diacylglycerol fractions obtained by argentation TLC is confirmed by determination of the fatty acid composition as described in Section 5A. Similar separations are obtained with the appropriate derivatives of the 1,3-diacyl-*sn*-glycerols, which exhibit slightly higher R_F values on TLC and slightly longer retention times on GLC than the corresponding *sn*-1,2- or 2,3-diacylglycerols [87].

The diacylglycerols may be readily resolved from the corresponding alkyl acyl and alk-1-enylacylglycerols by adsorption TLC [80]. Furthermore, both alkylacyl- and alk-1-enylacylglycerols can be readily resolved according to degree of unsaturation by argentation TLC [79,80] as well as according to molecular weight and degree of unsaturation by GLC [72] on non-polar and polar columns, respectively.

c. Monoacylglycerols

Mixtures of monoacylglycerols may be resolved according to degree of unsaturation by argentation TLC [62,88]. Preparation of the diacetates prior to resolution avoids isomerization of the monoacylglycerols and facilitates their subsequent analysis by GLC. The monoacylglycerol acetates require a relatively polar solvent system, hexane–diethyl ether (60:40). The monoacylglycerols may be resolved according to the number of double bonds and the position of the fatty acid residue when chromatographed on silica gel impregnated with both silver nitrate and boric acid [89]. The monoacylglycerols are recovered from the silica gel by elution with 20% methanol in diethyl ether [64].

The *tert.*-butyldimethylsilyl ethers of the monoacylglycerols may be resolved by argentation TLC using solvent systems similar to those employed for the separation of the acetates [81].

Mixtures of monoacylglycerols may be resolved by GLC on the basis of molecular weight using non-polar columns and on the basis of molecular weight and degree of unsaturation using polar columns [90]. For this purpose the acetates of monoacylglycerols would appear to be better suited, because of their greater stability on polar liquid phases.

The above TLC and GLC systems also permit the resolution of the acyl-, alkyl- and alk-1-enylglycerols of corresponding carbon number [90].

C. Ceramides

Efficient separations of molecular species of either free ceramides or ceramides released from natural sphingolipids by enzymic [80] or chemical degradation [91] are obtained by ordinary silica gel, or silica gel impregnated with borate or arsenate [92]. The separations are dependent upon the number, position and the stereochemistry of the hydroxyl groups in the ceramide molecules. Borate and arsenate have little effect on the ceramide mobility as determined by the hydroxyl groups of the fatty acid, but borate retards ceramides containing trihydroxysphingosines very strongly, while arsenate has the opposite effect. Borate also retards the ceramides containing an ethylenic double bond at C_4 of the sphingosine base. The effect of chain length on the separation of ceramides on silica gel G is minimal, but compounds with increasing chain length travel faster.

The separations of ceramides on the basis of the number of double bonds are best performed by argentation TLC with the ceramide acetates [92,93].

Further separation based on molecular weight and to a lesser extent on other features may be obtained by GLC.

The GLC separation of ceramides is best accomplished on non-polar columns using the TMS derivatives [18,93]. Prior to GLC the ceramides are resolved as the acetates according to degree of unsaturation, the major chain lengths and the content of hydroxyl groups by argentation TLC. The free ceramides of plasma yield two fractions on argentation TLC, the faster moving one of which corresponds to N-stearoylsphingosine diacetate and the slower one to N-nervonylsphingosine diacetate. After mild methanolysis the ceramides are silylated and examined by GLC on 1% OV-1 columns. Under these conditions the faster moving TLC fraction is resolved into eleven components. Samuelsson and Samuelsson [93] have identified the major peaks by mass spectrometry as the C_{16}—C_{24} fatty acid derivatives of sphingosine. The mass spectra indicated that each GLC peak also contained ceramides with sphinganine and hexadecasphing-4-enine as long chain bases. The ceramides with the latter long chain base contained a fatty acid with two more carbon atoms than the sphingosine ceramides of the same GLC fractions. The slower TLC fraction contained all the same fractions as isolated in the faster TLC band in addition to two major components, one of which was N-docosenoylsphingosine and the other the N-tetracosenoylsphingosine.

The ceramides from the plasma sphingomyelins give four fractions on

argentation TLC of the acetates. On GLC of the TMS ethers two to eleven components may be distinguished in each of the four fractions. The fastest moving TLC fraction was shown by Samuelsson and Samuelsson [93] to contain mainly sphingosine and hexadecasphing-4-enine combined with saturated fatty acids, whereas the second fastest moving TLC fraction consisted primarily of the same long-chain bases combined with monounsaturated fatty acids (mainly N-tetracosenoylsphingosine). The second slowest TLC fraction was a mixture made up of ceramides with sphinga-4,14-dienine as base and saturated fatty acids or sphingosine combined with monounsaturated fatty acids. The slowest moving TLC fraction consisted mainly of two components, N-docosenoylsphinga-4,14-dienine and N-tetracosenoylsphinga-4,14-dienine.

Horning et al. [44] have successfully recovered both TMS ethers and heptafluorobutyryl esters of 2-hydroxy fatty acid ceramides from natural sources from relatively long columns containing 1% SE-30 packing. The GLC analysis of ceramides in the form of permethylated derivatives has been described by Huang [94], who has used short columns with non-polar packings and temperature programming.

D. Glycerophospholipids

Intact glycerophospholipids may be resolved into individual molecular species or small groups thereof by both argentation TLC and reversed-phase partition TLC. The most complete separations are obtained by a sequential combination of the two techniques. In several instances a preparation of a suitable derivative greatly enhances the resolution of the molecular species of a given phospholipid class.

a. Phosphatidylcholines

The separation of intact phosphatidylcholines according to degree of unsaturation may be obtained by argentation TLC [95]. An effective solvent is chloroform—methanol—water (60:30:5). This allows the resolution of monoenes, dienes, tetraenes and hexaenes when applied to rat liver phosphatidylcholines. The individual subfractions are recovered from the silica gel by elution with chloroform—methanol—acetic acid—water (50:39:1:10) in a 90—95% yield. Many other laboratories have now employed this procedure with satisfactory results [96—98].

There is minimal resolution of the phosphatidylcholine species according to the fatty acid structures making up the various unsaturation classes, such as that noted for triacyl- and diacylglycerols.

Each of the fractions obtained on the basis of unsaturation by argentation TLC may contain two or more subfractions, which may be resolved according to molecular weight differences by reversed-phase TLC [99]. This separation is effected by rechromatography of the silver nitrate subfractions on calcium sulfate-free silica gel impregnated with undecane and using methanol—water (9:1). By this means the monoenes, dienes, tetraenes and hexaenes of the rat liver phosphatidylcholines may be resolved into their palmitoyl and stearoyl species. However, further separations may be obtained in those instances where more complex mixtures of homologues are present. Many workers have now

reproduced these fractionations with remarkable success [100,101]. The reversed-phase TLC plates may be easily prepared with commercially available hydrophobic silica gel.

b. Phosphatidylethanolamines

The separation of the phosphatidylethanolamines according to the degree of unsaturation of the molecules may be obtained under the general conditions of separation of the phosphatidylcholines using chloroform—methanol—water (55:35:7) as the developing solvent [95]. These separations also have been reproduced in other laboratories with comparable results [101,102].

The reversed-phase TLC of the various unsaturation classes of the phosphatidylethanolamines has been les effective [95].

Masking of the polar groups in phosphatidylethanolamine by O-methylation and N-dinitrophenylation allows a more complete resolution of the molecular species by both argentation TLC and by reversed-phase partition [103]. These derivatives also allow a complete resolution of the alkenylacyl-, alkylacyl- and diacylglycerophosphorylethanolamines on plain silica gel [104] using multiple developments with hexane—chloroform. The order of migration is the same as with diacylglycerol acetates: alkenylacyl > alkylacyl > diacyl. The separations based on the number of double bonds per molecule of these phosphatidylethanolamine subclasses were obtained [105] with the solvent system chloroform—methanol (98:2). Comparable resolution of the molecular species of phosphatidylethanolamine are obtained [106] following N-acetylation and O-methylation when the silver nitrate plates are developed in chloroform—methanol—water (80:15:2) and chloroform—methanol (97:3). These derivatives are more easily prepared and do not leave non-volatile reaction by-products. Work with the N-trifluoroacetyl derivatives of phosphatidylethanolamines [107] has proved that O-methylation is not necessary for effective separation of these derivatives according to the degree of unsaturation of the molecules.

The subfractionation of the dinitrophenylated phosphatidylethanolamines according to molecular weight by counter-current distribution has been extensively utilized by Trewhella and Collins [108] and Collins and Trewhella [109] as well as Shamgar and Collins [110]. Although the latter separations are highly reproducible and subject to routine application, the counter-current distribution equipment may require an experienced operator.

c. Phosphatidylinositols

The phosphatidylinositols may be separated according to degree of unsaturation of the component fatty acids by argentation TLC under the general conditions employed for the analysis of phosphatidylcholines provided the solvent system is properly adjusted. Using chloroform—methanol—water (65:35:5) as the developing solvent, Holub and Kuksis [111,112] obtained four fractions of different number of ethylenic bonds per molecule for the phosphatidylinositols of rat liver.

Luthra and Sheltaway [113] have modified the phosphatidylinositol molecules by periodate oxidation and diazomethylation or acetylation and diazomethylation prior to argentation TLC. Solvent mixtures containing acetone and distilled chloroform are found most suitable for the resolution of the monoenoic, dienoic, trienoic and tetraenoic species.

d. Phosphatidylserines

It has not been possible to separate the phosphatidylserines by argentation TLC of the original compounds. N-trifluoroacetylation, however, has allowed such separations. A development of an ordinary argentation TLC plate with chloroform—methanol—water (65:25:4) has given separate bands for the monoenes, dienes, trienes, tetraenes and hexaenes of phosphatidylserine of rat liver, as well as the appropriate unsaturation classes of the phosphatidylserines of the brain and red blood cells of various animal species [114]. N-Dinitrophenylation and O-methylation [103] has previously allowed a counter-current resolution of the major molecular species of phosphatidylserine.

e. Phosphatidylglycerols

Haverkate and Van Deenen [115] resolved phosphatidylglycerols of spinach leaves into two subfractions on argentation TLC, using chloroform—ethanol—water (65:30:3.5). The slower moving fraction contained three to four double bonds per molecule, while the faster one contained zero to two double bonds per molecule. The 1-linolenoyl-2-Δ^3-*trans*-hexadecenoyl phosphatidylglycerol accounted for about one half of all phosphatidylglycerols of spinach leaves.

The diphosphatidylglycerol (cardiolipin) may be subfractionated [116] by argentation TLC using chloroform—methanol—water (80:20:1) as the developing solvent. Separations are obtained for molecular species containing four linoleic acid residues and species containing two linoleic acid residues along with saturated and monounsaturated fatty acids. The molecular species of cardiolipin may also be assessed [117] by subfractionating the diacylglycerols obtained by acetolysis. These data, however, are more difficult to interpret.

f. Phosphatidic acids

Naturally occurring phosphatidic acids [118] and phosphatidic acids derived from glycerophospholipids by enzymic or chemical degradation [119,120] allow extensive resolution by TLC techniques, when analyzed as the dimethyl esters. TLC on plain silica gel allows the separation [120] of the alkenylacyl, alkylacyl and diacyl derivatives using solvent systems similar to those employed for the resolution of the corresponding disubstituted glycerols. Argentation TLC in chloroform—methanol—water (90:10:1) gives subfractions according to the number and position of double bonds in these molecules [118,120,121]. Individual molecular species of phosphatidic acid dimethyl esters may be resolved [119] by reversed-phase TLC using acetonitrile—acetone—water (8:1:1) and silica gel layers made hydrophobic with tetradecane.

E. Sphingomyelins

Sphingomyelins frequently yield a double spot on plain silica gel TLC [122, 123]. This is apparently due to a segregation of the molecular species with long-chain fatty acids (C_{22}—C_{26}), which migrate faster, and with short-chain fatty acids (C_{16}—C_{18}), which migrate more slowly. Svennerholm and Svennerholm [35] showed that this separation could also result from the presence of α-hydroxy fatty acids in the ceramide moieties of the sphingolipid. In each instance the slower migrating component contained the hydroxy fatty acids while the faster migrating component did not.

The small differences in the unsaturation of the various ceramide moieties of the sphingomyelins are best exploited by argentation TLC of the ceramides or ceramide acetates derived from them by acetolysis or enzyme degradation [80, 93, 124] (see Section 4B).

F. Glycolipids

Only the simplest glycolipid classes have thus far been separated into molecular species. Nichols and Moorhouse [125] have resolved the monogalactosyldiacylglycerols according to the degree of unsaturation of the fatty acids by argentation TLC using chloroform—methanol—water (60:21:4) as solvent. Five major fractions were isolated corresponding to groups of molecular species containing 1 to 5 double bonds per molecule. A comparable resolution has been obtained for the molecules species of the mono- and digalactosyldiacylglycerols of the broad bean [126]. Since these diacylglycerols were made up largely of 18:3 fatty acids (80—90%), argentation TLC gave strong bands for hexaenes and minor bands for pentaenes, tetraenes, trienes and dienes. Each of the fractions gave a major peak for the component containing two C_{18} and a minor peak for the component containing a C_{16} and C_{18} fatty acid when examined intact on GLC.

The various glycosyl ceramides may be resolved on plain silica gel according to chain length and the number of hydroxyl functions [127] as already noted for free ceramides and sphingomyelins. The simple ceramide glycosides are subject to a limited resolution by argentation TLC because of the absence of large differences in the degree of unsaturation of the different molecular species. Direct GLC allows the separation of the ceramide glycosides according to the carbon number of the nitrogenous base and the component fatty acid [126]. A GLC examination of the intact rat brain cerebrosides in the form of the TMS ethers gave comparable elution patterns for the normal chain and the 2-hydroxy fatty acid derivatives. The major peaks corresponded to cerebrosides with lignoceric and cerebronic acids in the normal and 2-hydroxy acid fractions, respectively. Minor peaks due to acids of 18—23 carbon atoms were also present, which was in agreement with previous results based on fatty acid analyses. A direct GLC of the lactosylceramides from rat bone marrow [126] also gave evidence for a resolution of molecular species. The major peaks represented the 16 and 18 carbon fatty acid derivatives.

The molecules of the more complex glycosphingolipids do not yield readily to the resolution of molecular species within an intact oligosaccharide type. Some progress in this area has been made by high-performance liquid chromatography [128,129], but these techniques are not yet available for routine application.

5. SEPARATION OF LIPID CONSTITUENTS

The ultimate purpose of the separation of the lipid classes and molecular species is their identification and quantitation. The determination of the lipid components of the various lipid classes and molecular species serves to confirm the identification derived by chromatographic methods as well as provides means of their quantitative measurement.

A. Fatty acids

The fatty acid composition of a lipid class or a molecular species is determined after the fraction has been purified, usually by TLC. The fatty acid analyses may be made on the total molecule or on some part of it derived by controlled enzymic or chemical degradation. The lipid sample is methylated with sulfuric acid—methanol, boron trifluoride—methanol, or hydrochloric acid—methanol reagent [3, 130]. The tubes are closed and heated at 90° for a minimum of 2 h. After cooling to room temperature, the fatty acid methyl esters are extracted with several washes of hexane, which may contain 0.01% BHT [7]. Samples containing material other than fatty acid methyl esters may require purification which may be accomplished on small columns of silicic acid using 1% diethyl ether in hexane to elute the methyl esters and the antioxidant and a subsequent wash with methanol to recover any sterols, sphingosine and glyceryl ethers, if present. Alternatively the methyl esters may be purified by TLC using pure benzene as the developing solvent, which also allows the removal of the BHT antioxidant as a separate band [7].

Samples of fatty acid methyl esters are routinely analyzed by GLC using polar columns, which separate the acids according to chain lengths and total number of double bonds, as well as the position of the double bonds. When working with fatty acids from well characterized sources, the separation of the esters on any one of a number of polyester columns may be sufficient to identify all but the minor components on the basis of the retention times or equivalent chain length [130—132]. The conventional polyester columns, however, do not allow the separation of the geometric isomers of the unsaturated fatty acids or the closer positional isomers. Some of these separations can be obtained by means of capillary columns [132]. Despite much progress in the utilization of capillary columns these columns are not yet available for routine application [133—135]. On the other hand, the availability of the polar cyanopropylsiloxane phases, which are capable of effective separation of the geometric isomers of fatty acids using conventional columns [136, 137], may reduce the need for capillary columns to some extent.

In any event, whenever sample size permits, it is helpful to subject the fatty acid methyl esters to argentation TLC and rechromatograph the fractions by GLC [14, 131, 138].

On the basis of the known order of migration of the fatty esters in the TLC and GLC systems it is usually possible to reduce the probable identities of any unknown peaks to a single or a few related components. The combined argentation TLC—GLC approach is much more effective than the comparisons of retention times or equivalent chain length values from two or more different GLC columns where it may be difficult or impossible to decide which peak is which in a changing pattern of the elution profile. Ultimately the unknown fatty acids may be identified by GC—MS and chemical synthesis, which are outside the scope of routine methodology.

B. Carbohydrates

The carbohydrate components of the glycolipids are routinely liberated by

cleavage of the glycosidic bonds with methanolic hydrochloric acid, resulting in the quantitative formation of O-methyl glycosides, although other methods may also be employed [139]. The O-methyl glycosides are resolved by GLC as the TMS ethers [140], N-trifluoroacetyl esters [141] or acetates [142]. Owing to the presence of an anomeric carbon in the sugar molecules, the O-methyl glycosides yield up to four separate peaks for each monosaccharide in the GLC elution pattern obtained with most derivatives on most liquid phases. An exception is provided by the single peaks recorded for each O-methyl glycoside on Apiezon L columns when run as the acetates. Dawson [139] has shown that L-fucose, D-galactose, D-glucose, D-mannitol (internal standard), N-acetylgalactosamine, N-acetylglucosamine, and N-acetylneuraminic acid give single, completely resolved peaks on an 8% Apiezon L column when temperature programmed from 170 to 220° at 2°/min.

Another method that allows the recording of single peaks for each sugar component in the mixture requires the liberation of the sugar moieties in the free form which are then converted to the alditols. The sugar alcohols are analyzed as the TMS ethers or acetates to yield single peaks for each component when analyzed on either polar or non-polar columns. The most complete resolutions of these derivatives are obtained on polar columns [143]. However, the apparent advantages of obtaining single peaks for each sugar is counterbalanced by the increased complexity in preparing alditol acetates from glycolipids and the apparent inability to estimate sialic acids in the free form.

In the past few years the technique of permethylation of glycolipids followed by GLC and mass spectrometric analyses has greatly increased the understanding of the structure of these compounds. In one of the more successful of these procedures [143] the free hydroxyl groups in the oligosaccharide are methylated in dimethylsulfoxide, methylsulfinyl carbanion and methyl iodide. Following isolation, the glycolipid is subjected to formolysis and hydrolysis, and a reduction with sodium borohydride. The residue is acetylated and the resulting partially methylated partially acetylated sugar alcohols are examined by GLC. Darvill et al. [144] have developed a mixed phase (0.3% OV-275—0.4% XF-1150) column packing which allows the complete separation of the methylated alditol acetates without resorting to multicolumn systems usually employed for complete resolution of these derivatives. Although this technique of structural analysis of oligosaccharides is now extensively used, it cannot be recommended for routine application outside a specialized research laboratory.

C. Nitrogenous bases

The short-chain nitrogenous bases may be released by hydrolysis with 6 *N* hydrochloric acid for 3 h at 100°. Free choline, dimethylethanolamine, monomethylethanolamine and ethanolamine may be separated by TLC using methanol—conc. hydrochloric acid (95:5) or *n*-butanol—methanol—conc. hydrochloric acid—water (50:50:10:10) as the developing solvents. The bases [145, 146] may be recovered from the TLC plates with a mixture of methanol—acetic acid—water (39:1:10). The free nitrogenous bases may be resolved by GLC on non-polar columns containing sodium hydroxide [145], from which they emerge in the order dimethylamine, monomethylamine, ethanolamine,

Choline is not recovered and there is some tailing of all the peaks. Choline may be estimated by GLC following a removal of one of the methyl groups by the Jenden reaction [147,148]. The resulting dimethylethanolamine would overlap with the dimethylethanolamine already present in the sample. Improved separations of the methylated ethanolamines and ethanolamine may be obtained by GLC of their acetates on non-polar columns.

The long-chain bases of ceramides, cerebrosides, sphingomyelins and gangliosides constitute complex mixture of difficultly soluble components which are sensitive to degradation and rearrangement. Heating with conc. hydrochloric acid—methanol—water (3:29:4) for 18 h at 78° has given satisfactory yields of the long-chain bases from most of these compounds [149]. The bases are recovered from an alkaline solution by extraction with chloroform. After drying in vacuo over phosphorus pentoxide the bases are converted to TMS ethers and subjected to GLC. The bases are separated according to chain length, degree of unsaturation and the number of hydroxyl groups present. Carter and Gaver [150] and Karlsson [151] have presented extensive tabulations of the retention times and equivalent chain length values for the TMS ethers of long-chain bases obtained in their laboratories.

D. Sterols

The most convenient methods of resolution of the sterols found in lipids are TLC [152,153] and GLC [152,154]. Argentation TLC allows the separation of saturated and monounsaturated sterols after a development in chloroform [155]. In the same system complete resolution is obtained [156] also for cholesterol—allocholesterol, cholesterol—desmosterol, and other sterol pairs. More recently effective separation of closely related sterols has been achieved by argentation TLC using chloroform—acetone (9:1) [156] or chloroform—dimethylketone (95:5) at 4° [157] as developing solvents. The TLC separation of many other sterol mixtures has been reviewed by Lisboa [153]. The simple sterols are routinely resolved according to molecular weight and the overall shape of the molecule by GLC on both polar and non-polar packings using conventional columns [152, 154]. A combination of argentation TLC and GLC methods insures the greatest success in routine analyses [152, 156—158].

E. Partial hydrolysis products

Chromatography of the partial hydrolysis products of a phospholipid or a glycolipid yields information about the structure of the molecular species or lipid class [159]. The lipid-soluble components derived from enzymic or chemical degradation of the lipid molecules are analyzed using the methods described for the various neutral and simple polar lipid classes, such as the mono- and diacylglycerols, phosphatidic acids, ceramides and lysophospholipids. Completely or partially water-soluble products are obtained by mild alkaline hydrolysis of the lipids, which selectively removes the O-fatty acyl groups leaving the N-fatty acyl, alkyl and alk-1-enyl groups intact. The water-soluble products such as the glycerolphosphate esters of the nitrogenous bases can be separated

by paper chromatography, paper chromatography and ionophoresis, or ion-exchange chromatography [9,159]. TLC has proved to be well suited for the separation of the partial hydrolysis products of plasmalogens [159].

Ohashi and Yamakawa [160] have described a GLC method for the analysis of the oligosaccharides released from glycosphingolipids by ozonolysis and alkaline cleavage. With short columns containing non-polar packings and temperature programming (110–350°) excellent separations were obtained for the TMS glycitols of mono-, di-, tri-, tetra- and pentasaccharides.

6. SPECIFIC APPLICATIONS IN BIOMEDICAL RESEARCH AND CLINICAL CHEMISTRY

Chromatographic methods of lipid analyses are extensively utilized in biomedical research but only a few of them have become established routines in clinical chemistry. In the following section reference has been made to selected applications of lipid chromatographic methods in analyses of clinical material in both routine and research laboratories.

A. Total lipid profiles

TLC has been extensively utilized in the determination of plasma lipid profiles in various clinical conditions [161]. TLC using densitometry and other techniques of charred spot measurement offer rapid micromethods for quantitative analysis of lipid classes [162]. A review of literature on this subject reveals that different procedures are used in virtually every laboratory engaged in this work. Recent improvements in the method relate to charring of the lipids following removal of the gel from the plate [163] and incorporating the charring reagent in the silica gel [164]. Under these conditions the results for serum free cholesterol, cholesterol esters and triglycerides compare favourably to the results obtained with the conventional Sperry-Web and Van Handel-Zilversmit methods for cholesterol and triglyceride determination, respectively. Extensive use of TLC techniques in the separation and quantitation of the phospholipids of red blood cells has been made by Nelson [7] who has also reviewed the subject [3]. Applications of TLC in analyses of complex lipids in health and disease have been discussed by Witting [51].

Along with determination of plasma lipid patterns by TLC may be mentioned the profiling of plasma lipids achieved by GLC. Kuksis et al. [165] and Horning et al. [44] have demonstrated that glycerophospholipids can be analyzed directly by pyrolysis GLC. For routine application, however, these techniques are inadequate. A modified procedure involving dephosphorylation of phospholipids by phospholipase C prior to GLC, however, is satisfactory [18]. The latter technique has been employed for a preliminary examination of plasma lipid profiles of a limited number of normolipemic and hyperlipemic subjects, as well as for assessing the effect of diet and hypolipemic drugs on plasma lipid levels and profiles [166]. The method has been recently automated and the calculations facilitated by computer programming [167]. By this means several thousand plasma samples from normo-

lipemic and hyperlipemic subjects have been examined in a study of the prevalence of hyperlipemia in a free living urban population [168]. In addition to providing quantitative estimates for free and esterified cholesterol, triacylglycerols and the glycerophospholipids and sphingomyelins, the method also gives quantitative values for plasma free fatty acids [169] and any plant sterols [170] that may be present.

High-temperature GLC has been evaluated [171] as a technique to monitor changes in composition of intact triacylglycerols of human serum lipoproteins after ingestion of oils containing fatty acids of widely differing chain lengths. The technique proved useful in this type of study since it measured the appearance of triacylglycerols of specific molecular weight. Random or non-random distributions of fatty acids in serum lipoproteins could be detected after ingestion of the oils. With simple fatty acid analysis, only changes in the proportions of fatty acids in triacylglycerols can be measured.

B. Lipid class ratios

When properly recorded the total lipid profiles provide both absolute quantities and characteristic ratios of the different lipid classes which are of clinical interest. In many instances, however, it is convenient to determine the ratios of specific lipid classes without obtaining a total lipid profile. Thus the ratio of free to esterified or total cholesterol can be readily obtained by quantitating the corresponding fractions from neutral lipid TLC [162, 163]. This ratio is of interest in familial lecithin:cholesterol acyltransferase deficiency [172, 173]. TLC can also provide a ratio for free cholesterol and total phosphatidylcholine, which is of interest in assessing the stability of certain abnormal plasma lipoproteins, such as the LPX component appearing in cholestasis [174] and during Intralipid infusion [175]. A lysolecithin index defined as the ratio of log lysolecithin phosphorus to total phosphorus has been computed to characterize uremia patients [176].

Determination of the ratio of phosphatidylcholine (lecithin) and sphingomyelin (L/S) provides means for assessing the development of the fetus in pregnancy and yield reliable information about the pulmonary maturity. Glueck et al. [177] have observed that the concentration of phosphatidylcholine in amniotic fluid increases to approximately four times that of sphingomyelin at 35 weeks and in subsequent weeks continues to rise, while sphingomyelin declines. Glueck et al. [177] separated the phospholipids in chloroform—methanol—water (65:25:4) and charred the spots with sulfuric acid. Many modifications of the original methods have been suggested since, including the development of the plate and the quantitation of the spots [178, 179]. Others have proposed the quantitation of the L/S ratio from GLC analysis of the component palmitic acid moieties [180]. The need for standardization of the Glueck test has also been pointed out [181].

C. Separation and quantitation of lipid constituents

The separation and quantitation of lipid constituents by TLC is also a well established routine in many clinical laboratories. For this purpose, use may be

made of most of the systems employed for determination of neutral lipids. These analyses are performed either for a more detailed assessment of the structure of complex lipids or for quantitation of the lipid classes separated on TLC plates, as an alternative to charring.

There have been several successful applications of GLC in the determination of cholesterol in clinical materials [182, 183]. These methods have employed relatively large samples of plasma or tissue and generally have not provided the sensitivity obtained by spectrometric methods. However, MacGee et al. [184] have reported a practical micromethod using 50 μl of plasma for the determination of total cholesterol by GLC. The authors have revised the original method to utilize 5 to 20 μl of plasma [185] with the same accuracy and precision as reported for the 50 μl samples. At this sample level the GLC methods become fully competitive with spectrometric methods in sensitivity and surpass them in specificity. This latter method has been recently adopted for the analysis of cholesterol in the high density plasma lipoprotein fraction (corresponding to 50 μl of plasma) to complement the GLC analyses for total cholesterol [186].

Comparative studies of plasma cholesterol concentrations by GLC, colorimetric and enzymatic methods have given essentially identical results [187].

Bjorkhem et al. [188] have described a highly sensitive and accurate reference method for estimation of total cholesterol in serum. A fixed amount of [2,2,3,4-D_4] cholesterol is added to a fixed amount of serum (usually corresponding to 10 μl). After saponification and extraction with hexane, the amount of unlabeled cholesterol is determined from the ratio between recordings at *m/e* 384 and *m/e* 389 obtained after analyses with a mass spectrometer equipped with a multiple ion detector. The method can be used for determination of cholesterol down to a level of 10 pmoles (4 ng).

The GLC analysis of fatty acids is an important and well established routine in most clinical laboratories. It permits to establish the existence of normal fatty acid profiles in specific clinical samples, as well as allows the recognition of any unusual fatty acid components. Of specific interest is the identification in serum of methylbranched fatty acids in Refsum's disease [189], and of fecal hydroxy fatty acids in sprue [190]. Other important applications relate to the assessment of the adequacy of essential fatty acid levels in infants, and in adults with massive dissection of the small bowel [191]. For this purpose the triene/tetraene ratio, 20:3ω9/20:4ω6, provides an adequate analytical measurement [192].

The utilization of conventional GLC columns in the analysis of fatty acids in biomedical research and clinical chemistry, however, presents a number of difficulties, which arise from the temperature limitations imposed by high-bleed liquid phases and the relatively low resolution. As a result most conventional analyses of fatty acids are lacking in estimates of long-chain components as well as of geometrical isomers of unsaturates. Lin et al. [133] have developed a new gas chromatographic method for a simultaneous analysis of long-chain fatty acids, α-tocopherol and cholesterol. This method has been applied to the analysis of plasma free fatty acids [193] and of the acids of plasma phosphatidylcholines and cholesteryl esters [134] in stroke patients and in normal young adults. The analysis of fatty acids of plasma lipids with

special emphasis on the geometric isomers of the unsaturated fatty acids has been reported by Jaeger et al. [135].

There has been a gradual increase in the utilization of GC—MS systems for separation and identification of lipid compounds in clinical chemistry, and many clinical laboratories now have access to such facilities. Lawson [194] has reviewed the scope of mass spectrometry in clinical chemistry and instrumentation, as well as of structure identification of physiological compounds including lipids. A recent review of the biomedical applications of mass spectrometry and GC—MS in the analysis of lipids has been prepared by Burlingame et al. [195].

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8. SUMMARY

This review summarizes the basic chromatographic routines commonly employed in lipid research laboratories in the analysis of the lipid mixtures normally isolated from natural sources. Emphasis is placed upon a systematic application of complementary chromatographic techniques as a means of ensuring maximum resolution and complete identification of lipid classes and molecular species. Many lipid samples, however, are simple enough to be analyzed completely by means of one or a few of the analytical sequences discussed. Regardless of the chromatographic routine selected, the analysis should be preceded by an effective isolation of the lipid sample free of contamination and in the absence of decomposition. Both aspects of sample handling are considered in the early part of the discussion.

The bibliography has been selected to call attention to the most recent comprehensive coverage of each subject from which the original references, if other, can be located. Hopefully this survey will show that for many purposes adequate analyses of known lipids can be obtained with conventional equipment of thin-layer and gas chromatography.

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