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Review

General strategies in chromatographic analysis of lipids

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Abstract

Lipid extracts of natural sources contain a large number of lipid classes and molecular species. Completely reproducible samples are obtained only with great care and skill. Analytical methods other than chromatography and/or mass spectrometry are of little use for resolution and identification of lipid molecules even in simple mixtures. The analytical information desired governs the selection of the chromatographic and mass spectrometric method, which determine the sample preparation and derivative needed. Usually a combination of chromatographic methods is necessary to identify specific species of lipids. The recent development of soft ionization techniques, that are readily interfaced with mass spectrometers, have greatly simplified the sample preparation and have largely eliminated the need for derivatization. Because these techniques require expensive equipment and dedicated operators, the methods selected must be consistent with the true analytical needs and the available resources. Although personal preference cannot be eliminated entirely, the general strategies outlined below should help to reduce the number of possibilities facing a lipid analyst to a few practical choices.

Contents

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List of abbreviations

CER	Ceramide
CER-OH	Hydroxyceramide
CH	Cholesterol
CoA	Coenzyme A
CPVPG	N-(*S*)-2(4-Chlorophenyl)isovaleroyl-D-phenylglycine
DNPU	Dinitrophenylurethane
EI	Electron impact
ESI	Electrospray ionization
FAB	Fast atom bombardment
FFA	Free fatty acid
GC	Gas–liquid chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
MS	Mass spectrometry
NEA	(*R*)-1-(1-Naphthyl)ethylamine
NEACV	N-(*R*)-1-(1-Naphthyl)ethylaminocarbonyl-(*S*)-valine
NICI	Negative ion chemical ionization
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
R_F	Relative migration
RP	Reversed phase
SFC	Supercritical fluid chromatography
SPH	Sphingomyelin
TBDMS	*tert.*-Butyldimethylsilyl
TMAP	Tetramethylammonium phosphate
TMS	Trimethylsilyl
UV	Ultraviolet
VLDL	Very low density lipoprotein

1. Introduction[1]

The aim of a chromatographic analysis of lipids is the complete resolution of lipid classes

[1] Definition of terms: in this paper neutral glycerolipids are defined as mono-, di- and triacylglycerols and their neutral derivatives. As neutral lipids are considered also the acylglycerol moieties of glycerophospholipids (GPL) and mono- and digalactosylacylglycerols. Polar lipids are glycerophospholipids, sphingomyelins, galactosylacylglycerols. The different types of chromatography and mass spectrometry are defined as the discussion procedes.

and molecular species for the ultimate purpose of full identification and quantitation of all components. Since organic solvent extracts contain complex mixtures of lipids differing in molecular mass, degree of unsaturation, and in the number and kind of functional groups, no single analytical method is capable of identifying and quantitating all lipid species. Modern lipid analysis therefore involves a multistep extraction, stepwise chromatographic resolution and sequential mass spectrometric identification of lipid mixtures of progressively simpler composition. Complete analysis of the lipid extract is usually performed for the purpose of characterizing a biological system, while abnormalities in known systems can be recognized from partial lipid analyses, e.g. determination of fatty acids, total cholesterol and triacylglycerols. The present chapter reviews the general strategies employed in selecting the analytical procedures to achieve the desired goal of the lipid analysis. Detailed discussion of specific analytical routines, however, is beyond the scope of this chapter.

2. Preparation of samples

The requirement for high sensitivity, selectivity, accuracy, precision and speed in lipid analyses necessitates standardization of the various operations starting with sample isolation. The best analysis cannot salvage the information lost by poor sample selection, careless handling, and inadequate planning of analytical procedures. Effective sample preparation for such analytical techniques as GC, HPLC and mass spectrometry must include chemical and particulate clean-up to provide the component of interest in a solution, free from interfering matrix elements, and in appropriate concentration for detection and quantitative measurement. This usually can be accomplished by sample purification and preliminary segregation by TLC or HPLC.

2.1. Isolation

Lipid samples for analyses are commonly obtained by extraction with organic solvents, which is time consuming and hazardous, and may not result in complete recoveries of the polar components. More recently, solid-phase extraction by means of commercially prepared adsorption cartridges has been introduced to minimize the use of large amounts of organic solvents and to improve the recoveries of the more polar components.

2.1.1. Solvent extraction

Neutral solvent methods of total lipid extraction [1,2] result in low recoveries of acidic phospholipids, lysophospholipids and non-esterified fatty acids [3,4], while acidified solvents generate artifactual lysophospholipids from tissues containing plasmalogens [4–6]. Use of alkaline solvents may result in deacylated products depending upon the temperature and time of exposure [4]. Shaikh [7] has assessed various techniques for the quantitative extraction of lysophospholipids from myocardial tissues and has made recommendations that constitute a good practice for the preparation of total lipid extracts from all tissues. Using lysophosphatidylcholine and lysophosphatidylethanolamine as markers, the acid–butanol technique [8], although effecting a complete recovery, resulted in partial hydrolysis (2–10%) of phospholipids containing 1-alk-1′-enyl-2-acylglycerophospholipids (plasmalogens) and produced lysophospholipids. This problem was avoided using a neutral butanol extraction or the Bligh and Dyer technique, but these methods gave only partial recoveries (60–72% and 75–80%, respectively) of the original lysolipids. Tissue extracted with neutral chloroform–methanol [1] provided virtually complete initial extraction of lysolipids (97–100%), but subsequent losses (up to 15%) occurred during purification of crude extracts with the Folch upper phase. These losses could be avoided by purification of the crude extract on a Sephadex G-25 column [7]. Alternatively, the Folch technique was modified to effect extraction of the tissue with a biphasic chloroform–methanol–saline system. After removal of the lower lipid phase, the upper phase containing residual tissue was reextracted twice more with Folch lower phase and once with lower phase containing HCl. This last extract was neutralized with NH_3 vapor before pooling with the preceeding extracts. This method circum-

vented plasmalogen hydrolysis, avoided use of time-consuming column chromatography, eliminated losses of lipids during purification, and allowed highly reproducible quantitative recoveries of all common lipid fractions including lysophospholipids and nonesterified fatty acids from myocardial tissue [7]. This technique should also recover the polyphosphoinositides, although their recovery was not specifically tested.

2.1.2. Solid-phase extraction

The more complex glycolipids and especially the gangliosides, acyl CoA esters and the more polar prostaglandins are partially or completely lost in the aqueous layer during the solvent extraction [7]. These losses can be avoided by solid-phase extraction, as shown for gangliosides [9], prostaglandins and leukotrienes [10], and acyl CoAs [11]. Christie [12] and Ebeler and Shibamoto [13] have reviewed the methodology and have referenced other applications. In addition to both normal- and reversed-phase cartridges, there are cartridges with other chemically bonded phases for selective adsorption and recovery of specific analytes [12,13]. Despite the apparent success of these routines in specific instances, their general application to the isolation of all lipid classes requires caution [14]. There is ample evidence from TLC and gravity flow column work, that the adsorption can be affected by moisture, temperature and rate of flow, which alter the concentrations and volumes of solvent necessary for optimum recovery of specific lipid classes.

2.2. Purification and preliminary segregation

A lipid extract usually contains water and other non-lipid contaminants, which must be removed from the sample prior to derivatization, storage or chromatographic separation. In many instances a preliminary segregation of lipid classes is also desirable.

2.2.1. Removal of non-lipid components

The small amounts of water in the solvent extract can be removed azeotropically during solvent evaporation, which, however, leaves any non-volatile non-lipids with the lipid residue. Alternatively, small amounts of water along with any water soluble components may be removed by passing the wet organic solvent through a Pasteur pipette packed with powdered anhydrous sodium sulfate. Shaikh [7] purified the crude Folch extracts on Sephadex G-25 column. Kyrklund [15] used a reversed-phase column to purify crude chloroform–methanol extracts following dilution with methanol–water. The pure lipids were recovered by elution with chloroform–methanol. In many instances, the removal of the non-lipids can be combined with a preliminary segregation of the lipid classes.

2.2.2. Segregation of lipid classes

For many purposes it is useful to effect a separation of the neutral and phospholipid fractions by some adsorption chromatographic technique. Shaikh [7] used Fluorosil as the adsorbent, while Ingalls et al. [14] have employed silica gel. The simple polar and non-polar lipid classes may be effectively segregated using commercial adsorption cartridges. Thus, the triacylglycerols, cholesteryl esters and free cholesterol can be readily segregated from the glycerophospholipids and sphingomyelins by sequential elution with mixtures of chloroform and methanol. Christie [12] has reviewed the application of adsorption cartridges for group separations of lipid classes. These separations are similar to those obtained on the Fluorosil column. Attempts to effect a separation beyond that of neutral and polar lipids, however, have been only partially successful, e.g. inclusion of acetone for separate removal of glycolipids. Kyrklund [15] resolved complex acidic lipids and neutral phospholipids and ceramides with methanol–water and chloroform–methanol mixtures, respectively. Ebeler and Shibamoto [13] have discussed the application of solid-phase extraction for both simple and complex separations of lipid classes. The commercial solid-phase extraction cartridges are available with a wide range of chemically bonded stationary phases. To be useful and efficient these cartridges must be used along with a detector or indicator of some kind, which would inform the user of the status of the solute–

column interaction and hence the likely stage of lipid elution.

An effective segregation of the lipid classes can usually be obtained by adsorption TLC. A development with a neutral solvent system retains the glycerophospholipids and sphingomyelins at the origin, while the neutral lipids are carried up the plate according to their R_f values [3]. A parallel development with a polar solvent system carries the neutral lipids together to the solvent front, while the phospholipids are resolved according to their polarity. $AgNO_3$-TLC or $AgNO_3$-HPLC [16], reversed- and chiral-phase HPLC [17] and countercurrent chromatography [18] have also served to purify lipids. Improved conditions for quantitative recovery of glycolipids have been discussed elsewhere [3,19].

2.3. Stability and storage

The extent to which the purification steps can be carried out may depend on the stability of the compounds. Thus, the mono- and diacylglycerols may isomerize, the unsaturated fatty acids and cholesterol may become peroxidized, and the hydroperoxides may decompose or undergo reduction to alcohols. Since partial acylglycerols are known to isomerize even in the solid state, it is recommended that for critical analyses the lipid extract is subjected directly and immediately to derivatization, e.g. acetylation, trimethylsilylation, hydrogenation or sodium borohydride reduction. Any aldehydes in the total lipid extract may need to be converted to the hydrazones and the mono- and diacylglycerols to the dinitrophenylurethanes. The lipid form used for storage depends on the nature of the analyses to be performed. Thus, in case of GC analyses of fatty acids, the lipids may be converted to the methyl esters prior to attempted storage. Appropriate methods for derivatization of various lipid classes have been discussed elsewhere [3,17,20].

The commercial adsorption cartridges can be effectively employed also for purification of derivatives and removal of excess reagents. Thus, methyl ester samples intended for GC analysis can be purified by passing through an adsorption cartridge, which may remove the peroxides and hydroxides [3]. The purified and derivatized (if necessary) lipid extracts are stored under nitrogen at reduced temperature, usually at −20°C but preferentially at −70–80°C [7]. Although addition of antioxidants is frequently advised [21], it should be appreciated that this leads to a contamination of the sample, which must be eventually coped with during the final analyses. When retrieving lipid samples from prolonged storage, they should be inspected for degradation by appropriate chromatographic techniques.

3. Resolution and identification

Having insured the general uniformity of the sample by all possible means, the chromatographic analyses may involve solely the identification of members of a homologous series of molecules. This can be readily done by reference to appropriate reference standards, which provide characteristic peak overlaps. When primary (synthetic) standards are not available, secondary (natural) standards are used. Other retention times are estimated by interpolation from the retention times of the available standards [3,16,17]. In addition, preparation of a chemical derivative, or a reduction product, may be performed as required [3,16,17]. The use of radio- [22] and stable [23] isotope tracers offers unique opportunities for analyte identification and quantitation in chromatographically resolved components. In lipid research the most important combinations of analytical methods are TLC–TLC [24], TLC–GC [25], TLC–HPLC [26], HPLC–GC [27] and GC and HPLC with MS [23,28], or MS–MS [29].

The actual strategy chosen for the analysis of specific lipids depends on the nature of information needed and the need can frequently be met by adopting some established analytical routine. Thus, a need for a fatty acid analysis may require little more than a preparation of fatty acid methyl esters from the total sample and a GC analysis. A more difficult problem may call for the analysis of the molecular species in a glycerolipid, which may require the determination of the composition, molecular association

and positional distribution of the fatty acids in a glycerolipid molecule. This requirement also may be met by following established routines, even when the source of the sample has not been previously analyzed. The following sections provide examples of various approaches that have been taken in the past to deal with both large and small samples of lipids.

3.1. TLC and TLC–TLC

TLC is the simplest and still most widely employed technique in lipid analyses. It provides rapid and complete separations of most neutral and phospholipid classes. Numerous reviews [3,16,24,27,30–32] have appeared covering various aspects of the resolution ranging from mere curiosities to practical applications. The availability of precoated plates with acceptable performance has led to the general acceptance of the TLC method by analytical biochemists with few laboratories preparing their own plates.

3.1.1. Normal phase

A large variety of solvents systems and qualitative visualization reagents are available for normal-phase TLC, with the actual selection depending largely on personal preference. TLC of a lipid sample in two or more solvent systems to insure its uniformity and fraction identity constitutes one of the oldest approaches of complementary chromatographic analysis [3,24,27]. Impressive separations and quantitation of both neutral and phospholipids have been reported by Bitman and Wood [33] using a two-step one-dimensional system. The phospholipid fraction from a SEP-PAK (Waters) isolation was placed onto the preadsorbent zone of a 20×20 cm silica gel plate prescored in 2-cm strips. After development, each of the glass strips were snapped off and charred with a copper sulfate visualizing reagent [33]. The efficiency of the TLC separation has been increased further by the introduction of finer gel grades, which, when used in combination with smaller and thinner plates of adsorbent, constitute high-performance TLC [24]. Yao and Rastetter [34] have reported an HPTLC method for separating mixtures of non-polar lipids, glycosphingolipids, and phospholipids in one dimension. More than 20 different lipid subclasses could be separated with three to four consecutive developing solvents. This method was applied to analysis of glycosphingolipids from erythrocytes and other biological membranes, for easy identification of patients with sphingolipidosis.

Additional strategies arise from the development of the TLC plate in two directions at a 90 degree angle with two different solvent systems [7,31]. The solvent systems are chosen to emphasize different properties of the lipid molecules and the plate is dried between the developments. The development in each direction can be carried out with one or more solvent systems and chromatographic conditions, thus giving rise to a large number of variations. The second TLC separation can also be performed following isolation of the components resolved in the first TLC separation. For the purposes of this review emphasis is placed upon those separations complementary to other chromatographic and mass spectrometric techniques, as part of the analytical strategies offered by the hyphenated techniques of analyses of complex lipid mixtures. The introduction of the high-resolution TLC (HPTLC), has lessened the need for the two-dimensional TLC. HPTLC has special advantages for reaction chromatography. Thus, in analyses of the PE, the TLC in the second dimension is preceded by a chemical destruction of the plasmalogens resolved in the first dimension [22]. Such combinations of methods have proven to be well suited to distinguish between alkenylacyl- and diacylglycerolipids.

3.1.2. Modified normal phase

All the above TLC systems are suitable for work with modified adsorbents and have provided an endless variety of strategies for further lipid resolution. TLC may be performed in the presence of special additives, e.g. boric acid, silver nitrate or mercuric acetate.

Boric acid impregnated silica gel is used to prevent or minimize the isomerization of mono- and diacylglycerols during chromatographic separation [35]. The borate is incorporated into

the silica gel at 5–10% and the plates are spread, dried, activated and developed in the usual manner. The boric acid forms complexes with vicinal hydroxyl groups and thus blocks the transfer of fatty acyl chains from one position to another.

Argentation TLC has been extensively utilized for analytical and semipreparative resolution of methyl and glyceryl esters of saturated and unsaturated fatty acids [16,31,36]. The incorporation of 5–20% $AgNO_3$ into the silica gel permits effective complexing of the π double bonds with silver ions, which retard the migration of the polyunsaturated fatty acids more than the oligounsaturated fatty acids and without materially affecting the migration of the saturated fatty acids. The separation is based essentially on the number of double bonds per molecule and separate bands are obtained for compounds with 0–6 double bonds per molecule. With more than 4 double bonds per molecule the resolution becomes progressively more difficult and insignificant with differences among polyenes of more than six double bonds [3,16,37]. The oligoenes (one to a few double bonds per molecule) are also resolved according to the geometric configuration (*cis*- or *trans*-) and the positional location of the double bond. However, not all positional isomers are resolved [3,16,37].

Likewise, the TLC resolution of phospholipid classes can be modified by including additives in the silica gel, e.g. boric acid, silver nitrate. Separations according to number of double bonds, presence of vicinal hydroxyl groups and acidic or neutral functional groups have been obtained [3,16,31]. Carsberg et al. [38] have reported excellent resolution of the dimethyl phosphatidate esters by $AgNO_3$-TLC using ethyl acetate as developing solvent. Silica-bound diethylaminoethyl (DEAE) has been suggested [39] as a support for TLC separation of phospholipids. A complete separation of eight phospholipids was achieved by chloroform–methanol–water–pyridine–5% ammonium hydroxide (130:55:8:4:4, v/v). It should be possible to prepare TLC plates containing silica-bound chiral phases as a support for TLC separation of enantiomeric lipids, but such applications have not been reported. The potential for automation of normal-phase TLC for lipid analysis has been demonstrated [40].

3.1.3. Reversed phase

Improved reversed-phase TLC systems have been developed using RP-18, RP-8 or CN plates [41]. The retention data obtained are roughly comparable to those obtained earlier on TLC plates impregnated with crude paraffin [42], but there have been few applications of this method. Radioactivity provides the best means of locating the separated components.

3.2. HPLC and HPLC–HPLC

The usefulness of HPLC for the separation of neutral lipid classes and molecular species has been well established, as the problems associated with the detection of the non-chromogenic lipids in the column effluent have been largely overcome by the use of the universal light scattering [43,44], hydrogen flame ionization [45,46] and mass spectrometric [3,17,28] detectors. The diradylglycerols and monoradylglycerols, which can be converted into UV absorbing or fluorescent derivatives also have presented no special problems.

3.2.1. Normal phase

Normal-phase HPLC provides neutral lipid class separations of the type originally established for silicic acid adsorption columns and subsequently refined for TLC. Due to long equilibration times and poor resolution of molecular species, normal-phase HPLC is used mainly for lipid class separations. Thus, triacylglycerols, and mono- and diacylglycerols are readily resolved from each other and from cholesteryl esters and glycerophospholipids [44]. Like the triacylglycerols, the long chain diradylglycerols can be readily recovered as a lipid class by normal-phase HPLC, with very limited fractionation. A resolution is obtained for the corresponding X-1,3- and X-1,2-diradylglycerols, with the X-1,3-isomers emerging ahead of the X-1,2-isomers [47], again as established for normal-phase TLC. Normal-phase HPLC also allows the

separation of normal chain and hydroxy fatty acid-containing triacylglycerols [48].

Furthermore, normal-phase HPLC is well suited for the preparative resolution of the diacyl, alkylacyl, and alkenylacylglycerols as the acetates [9], TMS and TBDMS ethers [49,50], benzoyl [51], dinitrobenzoyl [52], naphthyl [53], anthroyl [54], naproxen [55], and pyrenyl [56] derivatives. The elutions are performed with solvents of low polarity and the elution order (when all present) is the same: alkenylacyl > alkylacyl > diacyl. Liu et al. [57] resolved underivatized 1,3- and 1,2-diacylglycerols by normal-phase HPLC but short-chain 1,3-isomers interfered with long-chain 1,2-isomers. The 1(3)-isomers were eluted ahead of the 2-isomers of monoacylglycerols when run in the underivatized form. Normal-phase HPLC has also been used for the isolation of mono- and digalactodiacylglycerols (as reviewed in Refs. [45,46]).

Of great current interest is the normal-phase HPLC resolution of enantiomeric diacylglycerols as the diastereomeric carbamates. These separations are due to differences in the physical properties of diastereomers. Racemic diacylglycerols have been separated as the *S*-(+)- or *R*-(−)-1-(1-naphthyl)ethyl urethanes by HPLC on a column of silica gel with 0.5% 2-propanol in hexane as the mobile phase [58]. The elution order of components derivatized with the (*S*)-form of the reagent was 1,3-, followed by 1,2- and then 2,3-diacyl-*sn*-glycerols. The order was reversed for enantiomers of the (*R*)-form of reagent. Separations of racemic diacylglycerols have been similarly obtained also with *R*-(+)-1-phenylethyl isocyanate derivatives using 0.5–0.7% ethyl alcohol in heptane as the eluting solvent [59]. Toyo'oka et al. [60] have recently described a novel fluorescent chiral reagent for the preparation of diasteromeric alcohol derivatives for normal-phase HPLC.

The separation of phospholipid classes by normal-phase HPLC using a variety of detection systems has found numerous applications as previously reviewed [3,17,44–46]. The systems including ammonia have given some of the best resolutions of the common glycerophospholipids and sphingomyelin. Grieser and Geske [61] used a gradient of chloroform–methanol–ammonium hydroxide and flame ionization detection for the separation of acetyl PE, FFA, PC, PI and lysoPC, while Becart et al. [62] also used a gradient of chloroform–methanol–ammonium hydroxide and light scattering detection for the separation of CER, CER-OH, PE, PI, PS, PC, PA and SPH. Leiter [63] has described a ternary gradient of hexane–isopropanol–water for the separation of standard CH, FFA, PE, PS, PC and SPH. The separations were characterized by near baseline resolution. Caboni et al. [64] obtained similar separations for phospholipids from cooked beef using the chloroform–methanol–ammonia system with light scattering detection. Arnoldsson and Kaufmann [65] have described the development of a method for lipid class analysis by normal-phase HPLC using multivariate optimization strategy to target optimal system conditions. They have given examples of optimized separations, which do not differ much from those obtained by trial and error.

3.2.2. Modified normal phase

Like TLC, normal-phase HPLC separations may be modified by inclusion of various modifiers in the adsorbent phase. HPLC on silver ion loaded silica gel column (5 μm) has given resolution of triacylglycerols based on degree of unsaturation [66]. The separations obtained for vegetable oils are similar to those realized by $AgNO_3$-TLC. Surprisingly, HPLC on $AgNO_3$-treated silica gel gave separations of reverse isomers, as triacylglycerols with the unsaturated fatty acid in the secondary position was eluted ahead of the corresponding isomer with the unsaturated fatty acid in the primary position. Jeffrey [67] has achieved improved resolution of both unsaturates and regioisomers with a 3-μm column. More durable $AgNO_3$-HPLC columns are produced by loading the silver ions on sulfonic acid anion-exchange columns (Nucleosil 5SA) [68]. Although these columns do not permit resolution of positional isomers, they have been employed for effective resolution of fish oil triacylglycerols [69]. The fractions, however, were still extremely complex and required further fractionation by complementary chromato-

graphic techniques, e.g. reversed-phase HPLC (see below). Juaneda et al. [70] have resolved the seven geometric (*cis/trans*) isomers of linolenic (18:3) acid by the silver ion loaded column. For this purpose the temperature was lowered to 10°C and the column was developed with a gradient of dichloromethane and methanol. There have been no reports on the separation of glycerophospholipids by the silver ion HPLC.

El Hamdy and Christie [71] have demonstrated that the cyanopropyl-bonded silica gel used in adsorbent cartridges, can be effectively employed for the separation of neutral lipid classes when used in columns and developed with acetone or other modifiers of hexane–tetrahydrofuran (98:2) as the base solvent. The cyanopropyl-bonded silica gel columns have previously been used for the resolution of phospholipids classes, but the absence of an adequate detector had prevented a proper assessment of the success of such separations. Takagi and Ando [72] have reported the separation of monoacylglycerols as the di-dinitrophenylurethane derivatives by normal-phase HPLC on nitrile-bonded silica (Lichrospher 100 CN, Merck) using *n*-hexane–1,2-dichloroethane–ethanol (40:10:1, v/v/v). The order of increasing elution time was: 20:0, 18:0, 16:0, 18:1, 16:1, 18:2 and 18:3. The 1- and 2-isomers overlapped.

Abidi et al. [73] separated the major soybean phospholipids on β-cyclodextrin-bonded silica using isocratic elution with mobile phases containing hexane, isopropanol, ethanol and water–tetramethylammonium phosphate (TMAP) with UV detection. The presence of TMAP in the mobile phases was critical for good resolution.

3.2.3. Reversed phase

The general usefulness of reversed-phase HPLC in combination with the universal detectors for the resolution of molecular species of neutral glycerolipids has now been well established, numerous practical applications have been described and the subject has been reviewed [43,74]. Aitzetmuller and Gronheim [75] have pointed out the usefulness of short wavelength UV detection (210 nm) for the analysis of triacylglycerols containing highly unsaturated fatty acids, including α- and γ-linolenic acids. This, however, required increased detector sensitivity, which was achieved by employing an interferential refractometer in combination with a thermostat to stabilize the temperature of the entire HPLC system.

A typical reversed-phase column consists of 5- to 10-μm silica gel. Most separations have been obtained using the C_{18}, but C_5 and C_8 alkyl reversed-phase columns have also been occasionally employed. Programming of solvent temperature has been shown to provide advantages for resolution and peak recovery as well as for expanding the range of solvent types or combinations applicable to the separation of those compounds difficultly soluble in the semi-aqueous solvents [76]. El Hamdy and Perkins [77] and Dobson et al. [78] studied the effects of changes in mobile phase polarity on the critical pair and other isomer resolution using a C_{18} column and a refractive index detector. Critical pairs, which result from the approximate equivalence of one double bond to a chain shortening by two methylene units, were effectively separated with acetone–acetonitrile (63.6:36.4) as an isocratic mobile phase. Other solvent systems are also capable of resolving critical pairs and triplets of triacylglycerols [74–76]. Frede and Thiele [76] adopted the acetone–acetonitrile system to the HPLC resolution of butterfat triacylglycerols. The greatest improvement resulted from simply decreasing the sample load. Marai et al. [79] have reported excellent resolution of fish oil triacylglycerols on reversed-phase HPLC using a gradient of 10–90% isopropanol in acetonitrile. Fig. 1 shows the reversed-phase HPLC profile of menhaden oil triacylglycerols. Effective resolution is seen for molecular species ranging from 66 carbons and 18 double bonds to 54 carbons and no double bonds as shown by LC–MS. Laakso and Christie [69] have reported comparable resolution for herring oil triacylglycerols using a gradient of 1,2-dichloroethane–dichloromethane (1:4) and acetonitrile. Laakso and Christie [69] also used the reversed-phase HPLC system to resolve the fish oil triacylglycerol fractions recovered from a silver ion HPLC column. The

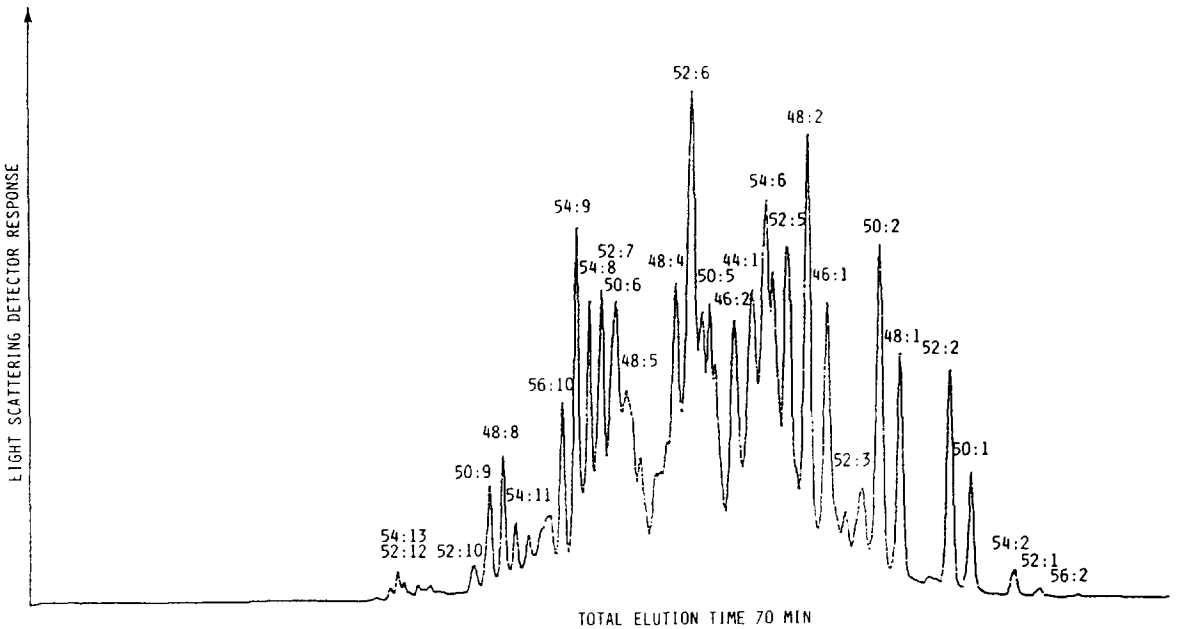

Fig. 1. Reversed-phase HPLC profile of menhaden oil triacylglycerols as obtained with light scattering detector [79]. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph equipped with a Supelcosil LC-18 column (25 cm × 0.46 cm I.D.) coupled to a Varex ELSD II light scattering detector. Solvent: linear gradient of 10–90% isopropanol in acetonitrile at 25°C over a period of 90 min (1 ml/min); recording stopped at 70 min. Peak identification by carbon:double bond number of triacylglycerols.

reversed-phase fractions, however, still contained numerous components per peak.

Shukla and Spener [80] have used reversed-phase HPLC and UV detection at 220 nm to separate triacylglycerols of *Flacourtiaceae* seed oils, which contain cyclopentenyl fatty acids (chaulmoogric acid). A comparison of retention times of triacylglycerols containing cyclopentenyl fatty acids with three straight chain fatty acids showed that CCC is equal to COO, PCC to POO, and PPC to PPO, where C is chaulmoogric acid; O, oleic acid; and P, palmitic acid.

Bergqvist and Kaufman [81] have applied a multivariate optimization to triacylglycerol analysis by reversed-phase HPLC. Molecular species of several natural oils were identified utilizing the Snyder polarity index.

The mobile phase for reversed-phase HPLC of polar lipids consists of an aqueous component and an organic modifier (5–40% for most applications). A third component, an ion pairing reagent is occasionally added. The molecular species of intact glycerophospholipids have been resolved [82] using methanol–water–acetonitrile along with choline chloride or phosphate salts and this method has been effectively reproduced in other laboratories [83]. The reversed-phase HPLC of the aminophospholipids is more readily performed following masking of the polar head groups [84]. Abidi and Mounts [85] have recently reported that the 5-fluoresceinthiocarbinol of phosphatidylethanolamine gives the highest degree of selectivity for component resolution. Kaufmann and Olsson [86] have used a novel multivariate development and optimization strategy to arrive at optimal conditions for resolving molecular species of the choline and ethanolamine phosphatides of bovine milk. Optimum conditions were: from 100% 1-propanol–water–isooctane (52:47:1) to 100% 1-propanol–water–isooctane (58:33:9) in 55 min with 1.56 mmol ammonium acetate at 15°C.

The molecular species are determined more effectively by reversed-phase HPLC of the di-

radylglycerol moieties released from the glycerophospholipids by phospholipase C. Blank and Snyder [51] have provided an updated review of the reversed-phase HPLC methods for the separation of alkenylacyl, alkylacyl and diacylglycerols in the form of various derivatives. Although acetates, trifluoroacetates or silyl ether derivatives are useful, most frequently the separations of the diradylglycerols have been performed following preparation of UV absorbing derivatives, such as the benzoates and dinitrobenzoates. Takamura et al. [52] have accomplished a complete separation of the diacylglycerol moieties of the common glycerophospholipids by HPLC of the derived dinitrobenzoates, using a combination of two solvent systems. The peaks not resolved in acetonitrile–2-propanol (4:1) were separated by methanol–2-propanol (19:1). This method was subsequently applied [87] to the separation of molecular species of alkylacyl and alkenylacyl subclasses of human platelet glycerophospholipids.

Rustow et al. [53] reported the HPLC analyses of the fluorescent naphthylurethane derivatives of diradylglycerols. This routine has been applied to the separation of molecular species of the alkylacyl, alkenylacyl and diacylglycerol subclasses derived from the ethanolamine glycerophospholipid (EGPL) of bovine erythrocytes. Rastegar et al. [55] have employed the fluorescent naproxen derivatives of diacylglycerols for a sensitive HPLC analysis on reversed-phase columns. The fluorescent derivatives can be detected at significantly lower concentrations, but higher temperature (80–85°C) and longer derivatization times (up to 2 h) are required for their preparation, which may adversely affect the polyunsaturates. Ramesha et al. [54] have reported a sensitive HPLC method for the separation and quantitation of GPL subclasses and molecular species as the diradylglycerol-1-anthroyl derivatives. The individual molecular species were separated by reversed-phase HPLC with acetonitrile–2-propanol (70:30) as the mobile phase.

Sempore and Bezard [88] fractionated mixtures of natural diacylglycerols as the 3,5-dinitrophenylurethane derivatives by reversed-phase HPLC using acetonitrile–acetone mixtures. The elution order and resolution was a function of chain length, unsaturation and positional isomerism of the constituent fatty acids. Likewise, Sempore and Bezard [89] used reversed-phase HPLC on a thermostated column to separate mixtures of monoacylglycerols formed by chemical deacylation of natural oil triacylglycerols. Acetonitrile–water mixtures gave excellent resolution of the underivatized monoacylglycerols according to chain length, unsaturation and positional isomerism using differential refractometer.

Kamido et al. [90] have utilized reversed-phase HPLC along with UV and MS detectors for resolution of the molecular species of the diacylglycerol core aldehydes as the dinitrophenylhydrazones. The core aldehydes were derived by phospholipase C from peroxidized choline and ethanolamine phosphatides of plasma lipoproteins.

3.2.4. Chiral phase

Although enantiomeric triacylglycerols can not be resolved, their random deacylation products can be separated by chiral-phase HPLC following conversion into dinitrophenylurethane (DNPU) derivatives [26,91]. The original separations of diradylglycerols were obtained by Itabashi and Takagi [92] on N-(*S*)-2(4-chlorophenyl)isovaleroyl-D-phenylglycine (CPVPG) liquid phase. The *sn*-1,2-enantiomers were eluted well ahead of the *sn*-2,3-enantiomers of the monoacid diacylglycerols. Later N-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine (NEA-CV) liquid phase was found to give improved separation of enantiomeric diacylglycerols [93,94], which could be employed for the resolution of mixtures of homologous diacylglycerols [95]. Fig. 2 shows the separation of saturated synthetic *sn*-1,2- and *sn*-2,3-diacylglycerols as the 3,5-DNPU derivatives on an OA-4100 chiral phase column [95]. These separations were performed with different proportions of hexane–1,2-dichloroethane–ethanol and showed marked increase in resolution with decreasing temperature (−20 to −23°C). Itabashi and co-workers [96,97] showed that the (*R*)-1-(1-naphthyl)ethylamine (NEA) liquid phase could pro-

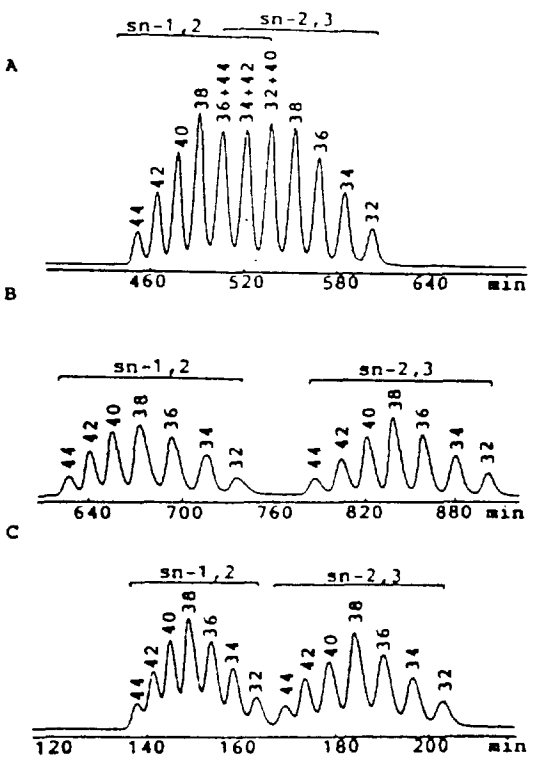

Fig. 2. Separation of saturated diacylglycerol enantiomers as 3,5-DNPU derivatives on an OA-4100 chiral column [95]. Temperature: (A) 19.5°C; B, −23.0°C; C, −20°C. Mobile phase: hexane–1,2-dichloroethane–ethanol, (A) and (B) 170:10:1 (v/v/v) and (C) 150:20:1 (v/v/v). Flow-rate: (A) and (B) 0.25 and (C) 0.5 ml/min. Peaks were monitored at 254 nm after the first recycle. Above each peak is given the acyl carbon number of the diacylglycerol: 32 = $C_{16} + C_{16}$; 34 = $C_{16} + C_{18}$, $C_{18} + C_{16}$; 36 = $C_{18} + C_{18}$, $C_{16} + C_{28}$, $C_{20} + C_{16}$; 38 = $C_{16} + C_{22}$, $C_{22} + C_{16}$, $C_{18} + C_{20}$, $C_{20} + C_{18}$; 40, $C_{18} + C_{22}$, $C_{22} + C_{18}$, $C_{20} + C_{20}$; 42 = $C_{20} + C_{22}$, $C_{22} + C_{20}$; 44 = $C_{22} + C_{22}$. Reproduced with permission from Elsevier.

vide effective resolution of the enantiomers of unsaturated diacylglycerols derived from natural fats and oils by Grignard degradation. The molecular species of the resolved diacylglycerols were identified by on-line mass spectrometry [98]. Yang and Kuksis [99] have employed this method for a stereospecific analysis of chylomicron triacylglycerols obtained from rats absorbing fish oil triacylglycerols.

The amide–urea liquid phases also permit excellent separations of enantiomeric alkylacylglycerols. Thus, 1-hexadecyl-2-hexadecanoylglycerol has been resolved from *sn*-3-hexadecyl-2-hexadecanoylglycerol using the NEA column [100]. The separation of *rac*-1-hexadecyl-3-hexadecanoylglycerols was more difficult and required about 80 min using hexane–1,2-dichloroethane–ethanol (250:20:1) as the mobile phase. The *sn*-1-hexadecyl-3-decanoylglycerol was eluted first.

The amide–urea type chiral phases also provide excellent resolution of the enantiomeric monoacylglycerols [101,102] and monoalkylglycerols [103]. Complete separations of racemic monoacylglycerols were achieved for 1-linoleoyl, 1-linolenyl, 1-arachidonoyl and 1-docosahexaenoylglycerols using a 50 cm × 4 mm I.D. column containing the NEACV phase and hexane–1,2-dichloroethane–ethanol (40:10:1) as mobile phase [102]. The unsaturated monoacylglycerols were resolved in order of increasing number of double bonds, and the *sn*-3-enantiomers were not eluted until after the *sn*-1-arachidonoylglycerol had emerged. However, a separation of *sn*-1-palmitoyl and *sn*-1-oleoylglycerols was not achieved, although *sn*-linolenoyl and *sn*-1-arachidonoylglycerols were partially resolved as were *sn*-eicosapentaenoyl and *sn*-1-docosahexaenoylglycerols.

3.3. TLC–HPLC

Although the lipids can be resolved into individual classes and molecular species by HPLC alone when applied to the total neutral lipid mixture, it is frequently advantageous to effect a preliminary resolution of lipid classes by TLC to avoid potential overlaps among different homologous series. Thus, the individual phospholipid classes resolved by normal-phase TLC during the initial isolation and purification, can be subjected to reversed-phase HPLC either in the intact form [82–85] or following dephosphorylation and conversion into UV absorbing derivatives [51–57]. In other instances TLC and reversed-phase HPLC have been deliberately combined in order to exploit to maximum the advantages of each chromatographic system. For example, normal-phase TLC has been effectively employed for the resolution of the alkenylacyl,

alkylacyl and diacylglycerol subclasses of the ethanolamine phospholipids prior to reversed-phase HPLC resolution of the molecular species of each subclass. For this purpose the diradylglycerol moieties of the glycerophospholipids were converted into the acetyl [9] or benzoyl [51] esters. Other effective combinations of normal-phase TLC with reversed-phase HPLC have been demonstrated in analyses of the more complex triacylglycerols. Thus, either diacylglycerols or triacylglycerols can be resolved on basis of both the number and configuration of the double bonds and the individual fractions recovered and separated further into molecular species by reversed-phase HPLC. Myher et al. [104] have combined argentation TLC with reversed-phase HPLC for increased fractionation of short chain triacylglycerols from butterfat. Bezard and Sempore [105] have recently combined argentation TLC of the DNPU derivatives of 1,2(2,3)-diacylglycerols with chiral phase HPLC for the separation of the molecular species of diacylglycerols derived by Grignard degradation. The *sn*-1,2(2,3)-diacylglycerols were resolved from X-1,3-diacylglycerols by borate TLC.

Fig. 3 shows the application of combined normal-phase TLC and reversed-phase HPLC to the separation of epoxytriacylglycerols (TLC band 9) in peroxidized corn oil [106]. The oil was treated with *tert.*-butylhydroperoxide/Fe^{2+} and the peroxidation products resolved by TLC as shown in the inset. The epoxytriacylglycerols in the single ion plots are listed as monooxygenated species, which possess the same masses as the epoxides.

3.4. GC and GC–GC

GC remains the most valuable and most efficient method of resolution and quantitation of lipids, including the non-polar higher molecular mass components. The development of flexible quartz capillary columns has made capillary GC the method of choice gradually phasing out the packed GC columns along with the operating equipment from the research laboratories. Except for electron capture, flame ionization detector has replaced all others for lipid GC.

The GC of the fatty acid methyl esters, including the importance of the injection technique, is covered by Eder elsewhere in this volume [171], while Shanta and Napolitano [107] and Liu [108] have discussed the preparation of the fatty acid methyl esters along with application in positional analysis. High-temperature GC of neutral lipids has been recently reviewed by Tvrzicka and Mares [109] and Kuksis [110], while the GC of lipid peroxidation products has been discussed by Ebeler and Shibamoto [13].

3.4.1. Non-polar phases

Although essentially complete analyses of the common fatty acids may be obtained with the methyl esters using polar capillary columns, in practice it is more informative first to examine a given fatty acid methyl ester mixture on a non-polar capillary column, which indicates the variety of the chain lengths that are present and that must be accounted for following subsequent analyses on polar capillary columns. The non-polar columns allow also a partial resolution of the saturated and unsaturated compounds, with the unsaturated species emerging slightly earlier than the corresponding saturated species [111]. However, the non-polar liquid phases vary in chemical structure and in their interaction with the fatty acids giving slight differences in relative migration rates, which, along with the film thickness and column length, can be exploited to minimize peak overlap.

A similar strategy is utilized in the analysis of mono-, di- and triacylglycerols on GC columns [112]. An initial run is made on the non-polar column, which provides the molecular mass or carbon number distribution. The recoveries from the polar columns can then be cross-checked with those from the non-polar columns by summing the estimates that belong to the same carbon numbers. The operating temperature constitutes an important practical aspect. Increases in column temperature increase the speed of the analysis, but may decrease the peak resolution. In addition, the useful life-time of the column is reduced. It should also be kept in mind

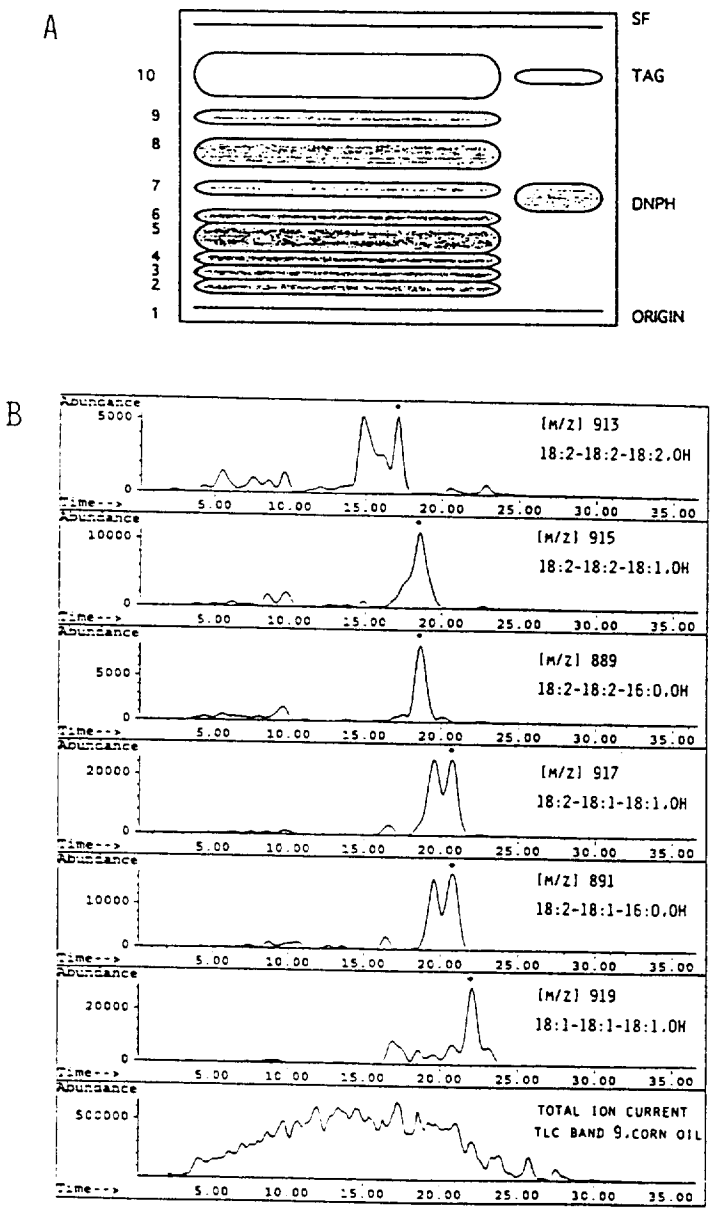

Fig. 3. Combined TLC–reversed-phase HPLC resolution of hydroxytriacylglycerols in peroxidized corn oil with electrospray ionization detection [106]. (A) Normal-phase TLC of *tert.*-butylhydroperoxide/$FeSO_4$-treated corn oil triacylglycerols. Band 9, hydroxytriacylglycerols. Solvent, heptane–diisopropyl ether–acetic acid (60:40:4, v/v/v); (B) single ion (electrospray) profiles of monooxygenated triacylglycerols as obtained by reversed-phase LC–MS. Peak identification as given in figure. HPLC conditions: column, Supelcosil LC-18 (250 mm × 4.6 mm I.D., Supelco, Mississauga, Ontario, Canada); solvent system, 20–80% isopropanol in methanol in 30 min; flow-rate (0.85 ml/min).

that the properties of the liquid phase may change with column temperature. Different liquid phases have different maximum operating temperatures. During the chromatography of triacylglycerols column temperatures exceeding 350°C are necessary and only a few liquid phases are suitable for this purpose.

Other critical aspects of practical GC concern

the method of sample introduction. In conventional GC the sample is evaporated at a temperature that is higher than the column before admission. But practical experience has shown that high temperature resolution of glyceryl and steryl esters is best performed by admitting the sample directly to the column at its starting temperature [113], which may be advantageously combined with programmed temperature vaporization [114]. Likewise, in work with capillary columns, it has proven that the highest recoveries of components are obtained following on-column injection at or below the column temperature. This is performed either by means of flexible quartz needles (Hewlett-Packard, Palo Alto, CA, USA) using wide bore capillaries [110] or by means of special movable ("pop-up") on-column injection apparatus [115], in which the sample is introduced while the front end of the column is outside the oven. Following injection of the sample, the column is returned to the oven held at the desired starting temperature. Capillary columns with split or splitless injection systems, which depend on evaporation of the sample, are not well suited for injection of solutions of compounds of a wide range of molecular masses. Such samples are evaporated and split at different ratios, usually resulting in poor recoveries of the higher molecular mass components. No difficulties were encountered with determination of plasma total lipid profiles, if on-column injection was used [110,116].

GC on non-polar liquid phases possessing low vapor pressure and high thermal stability has been vitally important for lipid chromatography and for GC–MS. High-temperature GC on non-polar columns has been extensively applied in the determination of total lipid profiles, and carbon numbers of diacyl- and triacylglycerols [3]. The original separations obtained on short packed non-polar columns [117] have compared favourably with the separations obtained on capillary columns [118], including columns made with bonded non-polar liquid phases. The use of hydrogen as carrier gas and of capillary columns of minimum length [116] have greatly improved the recovery and shortened the retention times of the higher molecular mass compounds. The non-polar liquid phases still require derivatization of any polar functional groups. TMS [49,119] and TBDMS [120–122] derivatives are especially useful; they increase also the volatility of the molecules and allow their recovery at lower temperatures and slightly shorter retention times than the corresponding acetates.

3.4.2. Polar phases

Essentially complete analyses of the common fatty acids may be obtained with the methyl esters using polar capillary columns and flame ionization detector [107,108]. Major and Wolf [123] have reported a sensitive detection of fatty acid pentafluorobenzyl esters using an electron capture detector. Polar capillary columns consisting of bis-cyanopropylsiloxane phases have recently been optimized by computer simulation [124]. The polar capillary columns yield effective separation of all chain length and double bond number homologues. However, only a few positional double bond isomers, and only certain *cis–trans*-isomers are resolved. Other *cis–trans* isomers and methyl and hydroxyl substitution isomers are not resolved and longer columns or special liquid phases (e.g. 60-100 m, SP-2340 or SP-2560, Supelco) have been sought for their separation [125]. The problem of GC resolution of *cis*- and *trans*-fatty acids has been discussed in great detail by Firestone and Sheppard [126]. The Silar 5CP columns also separate the deuterated from undeuterated fatty acid esters, and a base line resolution is obtained when about 15 deuterium atoms are present per fatty acid molecule [127].

In polar capillary GC of the glyceryl esters of long chain fatty acids, the selection of the column length is critical because the recoveries decrease greatly with increasing column length, although the resolution increases [119]. Usually a satisfactory compromise can be found with the result that the column that yields adequate recoveries also gives acceptable peak resolution. The longer columns, however, require higher operating temperatures (e.g. 280–300°C). At these temperatures of operation it is important that polymerizing catalysts and binders of the polymer to the glass capillary be removed or completely inactivated. The elevated temperatures are necessary for both separation and quantitative recovery of

the components. The more stable polar phases can be subjected to sufficient temperature programming to provide an effective resolution of polyunsaturated species of even very long chain lengths [119]. Myher and Kuksis [128,129] have reported both observed and calculated retention times of long chain diradylglycerols based on primary and secondary standards as obtained on polar capillary columns under isothermal conditions. The relative order of peak elution obtained under isothermal conditions could be used for establishing the order of peak elution under conditions of temperature programming [130].

3.4.3. Polarizable phases

The high-temperature polarizable phenylmethylsilicone liquid phases have permitted high-temperature GC separation of natural triacylglycerols based on degree of unsaturation [131]. The phenylmethylsilicone liquid phase becomes sufficiently polar as the temperature increases above 290°C as indicated by the longer retention of the unsaturated compared to saturated triacylglycerols. As the temperature increases the molecular species of triacylglycerols are eluted in order of increasing number of double bonds per molecule. Below 290°C the liquid phase is non-polar as indicated by the earlier elution of the unsaturated compared to saturated fatty acid silyl esters. Kuksis et al. [132] have demonstrated that high-temperature polarizable capillary columns are suitable for the simultaneous resolution of the diacylglycerol and ceramide moieties of plasma phospholipids, and of the cholesteryl esters and triacylglycerols of plasma. Although significant overlaps occur the method was thought useful for quantitative work. Fig. 4 shows the plasma total lipid profile (lower panel) of a hyperlipidemic male in the fasting state, as obtained by GC on a polarizable column [132]. The method of sample preparation is given in the inset (upper panel). There is an effective resolution of the molecular species of the triacylglycerols, and cholesteryl esters, but the derived diacylglycerols and ceramides overlap. Ruiz-Gutierrez et al. [133] have reported similar resolution of the triacylglycerols from plasma very low density lipoproteins. These studies have permitted the identification of the major molecular species of the intact lipid esters of plasma. The method was especially effective in the resolution of the ceramide moieties of the plasma sphingomyelins, but offered only limited advantages for the resolution of the diacylglycerol moieties of plasma glycerophospholipids, which were better resolved isothermally on the polar liquid phases employed for fatty acid analyses. It also offered only limited advantages for the resolution of the high molecular mass polyunsaturated species of the diacylglycerols derived from fish oil triacylglycerols, due to the high temperature required for their elution. The method was unsuitable for work with fish oil triacylglycerols, which tended to decompose or polymerize at the high temperature necessary for their elution. There were also signs of decomposition of the cholesteryl esters unless the temperature was kept below some 320°C until the cholesteryl esters were recovered [132]. Sassano and Jeffrey [134] have used wide-bore capillary columns containing the polarizable phenylmethylsilicone for high resolution of palm oil triacylglycerol fractions with automated cold on-column injection. Myher et al. [135], Oshima et al. [136] and Kalo and Kemppinen [137] have used polarizable phase capillary GC in combination with mass spectrometry for the determination of the elution profiles and for identification of the eluted species of natural triacylglycerols (see below).

GC–GC combinations have been occasionally employed in the past, where the fractions collected from preparative columns have been examined further on analytical columns [138]. More recently on-line GC–GC has been achieved by admitting the effluent of one GC column to another one connected in series [139]. The non-polar column is operated as the first column. Serial column operation with transient peak trapping has practical potential and appropriate instrumentation has been constructed and is commercially available.

3.5. TLC–GC

One of the oldest approaches to improved identification and quantitation of lipid components is provided by GC examination and quanti-

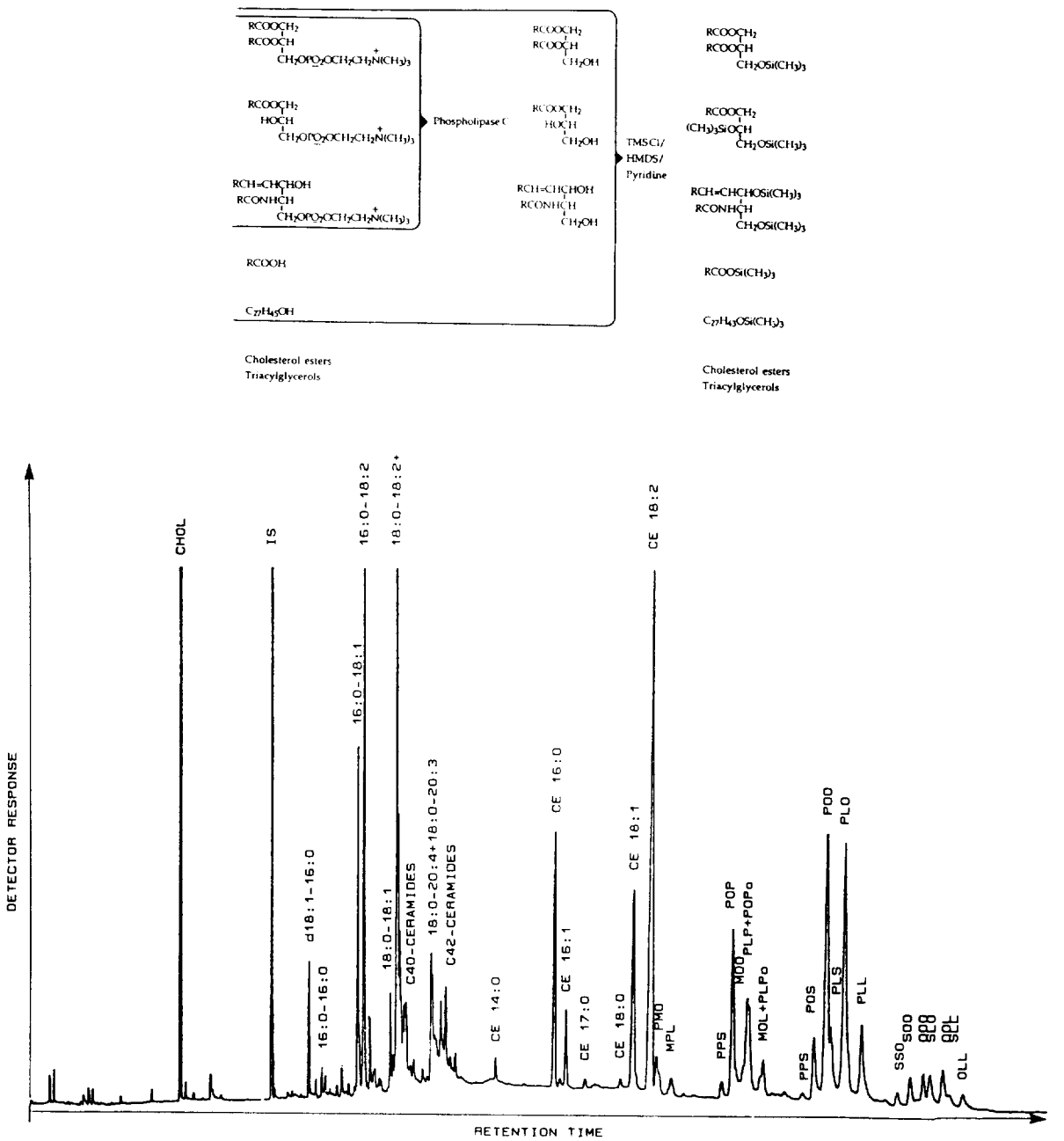

Fig. 4. Plasma total lipid profile as obtained for a hyperlipidemic adult male in the fasting state (lower panel) by automated capillary GC on a polarizable liquid phase [132]. Upper panel, summary of sample preparation: TMSCl, trimethylchlorosilane; HMDS, hexamethyldisilazane. Peak identification: Chol, free cholesterol (as TMS ether); 30:0 tridecanoylglycerol (internal standard); D18:1–16:0, palmitoylsphingosine moiety of plasma sphingomyelin (as di-TMS ether); 16:0–18:1, 16:0–18:2, 18:0–18:1, 18:0–18:2, and 18:0–20:4, major diacylglycerol moieties of plasma phosphatidylcholine (as TMS ethers); CE 16:0, cholesteryl palmitate; CE 16:1, cholesteryl palmitoleate; CE 18:1, cholesteryl oleate; CE 18:2, cholesteryl linoleate; PMO to OOL, triacylglycerols made up of myristic (M), palmitic (P), oleic (O), palmitoleic (P′), linoleic (L) and stearic (S) acids. Column, fused-silica capillary (25 m × 0.25 mm I.D.) coated with methyl 65% phenylsilicone (OV-22); carrier gas, H_2; temperature program as given in figure. *y*-Axis: full scale deflection corresponds to $1 \cdot 10^{-6}$ A. *x*-Axis: retention time. OOO was eluted in 37 min. Reproduced with permission of *Journal of Lipid Research*.

tation of the fractions resolved by TLC [25]. This serves the purpose of identification of both the TLC bands and the GC peaks. The TLC separation may be performed during initial isolation and purification of the sample or during a subsequent TLC fractionation under specific separation conditions. The addition of an internal standard to the fractions recovered from TLC prior to the GC analysis permits the interrelation of the lipid components in the various TLC bands and in the total lipid sample [3,110,139]. Thus, a separation of the phospholipid classes by TLC is frequently followed by the identification of the fatty acids by GC, which in the presence of an internal standard allows the quantitation of the phospholipid classes. In other routines, argentation TLC is used in combination with GC to improve the certainty of identification and quantitation of the lipid components in the TLC fractions and in the total triacylglycerol mixture [3,110,139]. GC on both non-polar [14,140] and polar [123,124] columns provides complementary resolution to argentation TLC of the saturated and unsaturated fatty acids and their esters.

An argentation TLC resolution allows identification of closely running or overlapping GC peaks. This TLC part of the analytical system can be applied sequentially to the resolution and quantitation of the diacylglycerol moieties of glycerophospholipids and triacylglycerols, and subsequently to their fatty acid moieties [3]. Fig. 5 illustrates the application of polarizable capillary GC in combination with silver ion TLC for the resolution of butteroil triacylglycerols [135]. Clearly, argentation TLC of triacylglycerols and diacylglycerols has not become superfluous because the acylglycerol mixtures are so complex that a complete resolution is not achieved by the short polar capillary GC columns, which must be used to obtain adequate recoveries of the higher molecular mass components.

Other TLC–GC combinations result from the need for borate TLC purification of the mono- and diacylglycerols [35], which are then further examined for their fatty acid composition and molecular association by GC on both non-polar and polar capillary columns. Still other combined applications of TLC–GC are represented by reaction TLC–GC, where the plasmalogens are selectively destroyed at an intermediate step in the chromatography, which is followed by GC identification of the fatty acids by transmethylation and GC [3,9,22].

3.6. HPLC–GC

Like TLC–GC, HPLC–GC is also employed for the purpose of identification of the components resolved by normal, silver ion, reversed-phase or chiral-phase HPLC. Normal-phase HPLC may be employed instead of TLC for the isolation of neutral and polar lipid classes for subsequent GC examination on polar or non-polar columns following appropriate derivatization [27]. Thus, the diacyl-, alkylacyl- and alkenylacylglycerol subclasses have been completely resolved by combining normal-phase HPLC with polar capillary GC of the TMS ethers [49]. Both reversed-phase [136,141] and silver ion [142] HPLC can provide small groups of molecular species of triacylglycerols for improved subsequent GC analysis as intact molecules on polarizable capillary columns. In more conventional approaches, silver ion HPLC fractions are analyzed by GC for fatty acid composition [142].

Chiral-phase HPLC provides effective resolution of the enantiomeric mono- and diacylglycerols, but fails to resolve completely the component molecular species, which must be identified by other methods, including GC. Itabashi et al. [97] have described a combination of chiral phase HPLC with polar capillary GC for the identification of the molecular species of the enantiomeric diacylglycerols derived from triacylglycerols by Grignard degradation. For this purpose the dinitrophenylurethanes needed for the chiral phase HPLC resolution of the enantiomers were subjected to silolysis, which replaced the DNPU groups with TMS groups. The resulting diacylglycerol TMS ethers were effectively resolved by polar capillary GC. Fig. 6 shows the fractionation scheme proposed by Itabashi et al. [97] for the identification of the molecular species of enantiomeric diacylglycerols by a combination of chiral-phase HPLC, silolysis and polar capillary GC. The silolysis is brought about

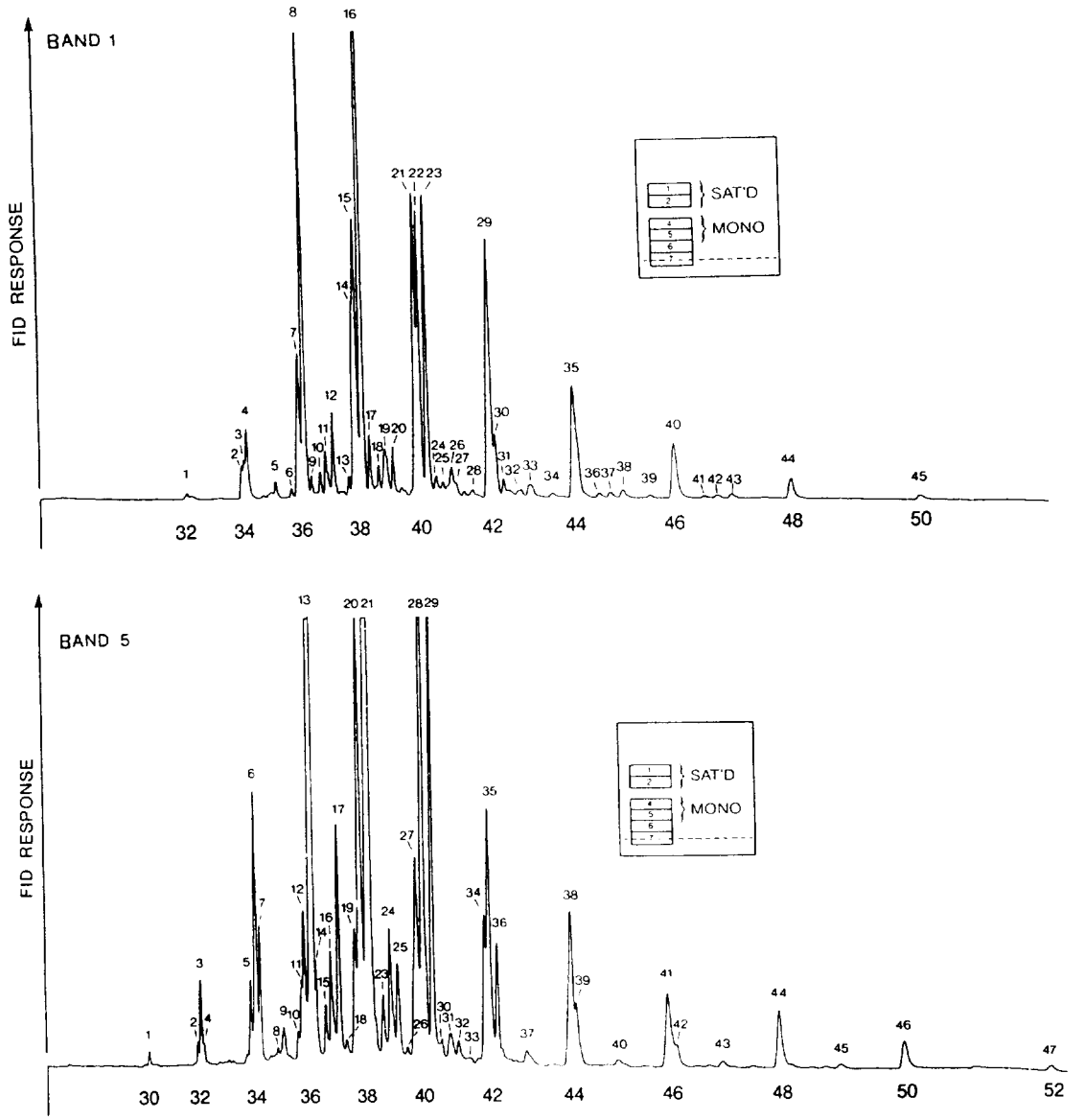

Fig. 5. Polar capillary GC profiles of the saturated long chain-length (band 1) and the short-chain *cis*-monoene (band 5) triacylglycerol fractions isolated from R-4 butterfat distillate by silver ion TLC [135]. Inset; 15% $AgNO_3$-TLC resolution of R-4 distillate in chloroform plus 0.75% ethanol. Peak identification (band 1): 7, 8–14–14 + 8–12–16 + 10–14–12; 8, 6–14–16 + 6–12–18; 14, 10–14–14 + 10–12–16; 15, 8–14–16 + 8–12–18; 16, 6–16–16 + 6–14–18; 21, 10–14–16 + 12–14–14; 22, 8–16–16 + 8–14–18; 23, 6–16–18; 29, 10–16–16 + 10–14–18; 30, 8–16–18; 35, 10–16–18 + 12–16–16 + 14–14–16; 40, 14–16–16; 40, 14–16–16; 44, 16–16–16; 45, 16–16–18. Peak identification (Band 5): 6, 12–18:1–6 + 14–16:1–4; 12, 12–18:1–6 + 14–16:1–6; 13, 14–18:1–4 + 16–16:1–4; 20, 18:1–14–8 + 12–16:1–10; 21, 18:1–16–4 + 16:1–18–4; 27, 18:1–14–8 + 16:1–14–10; 28, 18:1–16–6; 29, 18:1–18–4; 34, 10–18:1–14 + 12–16:1–14; 35, 18:1–16–8; 38, 10–18:1–16; 41, 16–18:1–12 + 14–14–18:1; 44, 14–16–18:1; 46, 16–16–18:1. GC conditions: column, flexible quartz capillary (25 m × 0.25 mm I.D., RSL-300 custom made); carrier gas, H_2; on-column injection temp. 40°C, then ballistically heated to 290°C, then at 10°C/min to 330°C and then at 2°C/min to 350°C. Reproduced with permission from Elsevier.

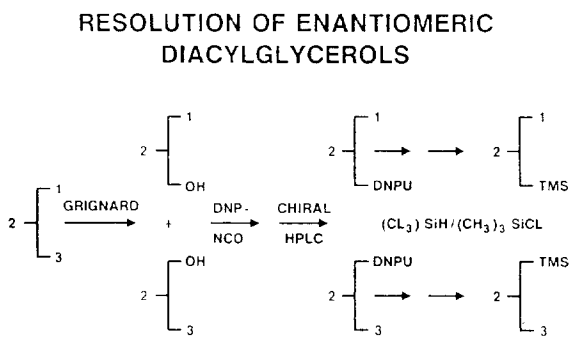

Fig. 6. General scheme of resolution of enantiomeric diacylglycerols generated from triacylglycerols by Grignard degradation [97].

by reaction with trimethylchlorosilane, which replaces the DNPU groups with TMS groups without isomerization. Fig. 7 shows the polar capillary GC profiles of the molecular species of the *sn*-1,2- and *sn*-2,3-diacylglycerols as obtained by the application of the scheme in Fig. 6 [130]. The identification of the molecular species is based on the fatty acid composition and comparison of the retention times of the unknowns with those of knowns [119]. The chiral phase HPLC resolution of the *rac*-1,2-diacylglycerols derived from chylomicron triacylglycerols has been shown elsewhere [99].

3.7. GC–MS and LC–MS

The principle applications of MS in the field of lipids are characterization of newly discovered components and confirmation of the identity of molecular species of known glycerolipids in total sample or in a chromatographic fraction. GC–MS is usually performed under EI or chemical ionization in either positive or negative ion mode, while LC–MS is usually done with the more sensitive negative ion chemical ionization in combination with reversed-phase HPLC, although the positive ion mode may also be used [28]. Reversed-phase LC–MS–MS with FAB or electrospray constitute rapidly expanding technologies in the analyses of lipids [29]. Detailed discussion of the methodology involved in each instance is beyond the scope of this chapter.

3.7.1. GC–MS

The use of GC–MS is normally restricted to low molecular mass lipids, but there have been several important exceptions. For GC–MS analysis the lipid sample has to be transformed by chemical reaction into a derivative, which is thermally stable and volatile. The preparation of the methyl esters of fatty acids and of the methyl ester methoxime-TMS ethers of eicosanoids are well established routine applications of GC–MS [143]. GC–MS of the pyrrolidyl and picolinyl derivatives of fatty acids yield information about the location of double bonds and branched points in the fatty chain [144]. The analysis of fatty acids and eicosanoids as the pentafluorobenzoyl ester derivatives by NCI-MS represents an important advance in methodology [145]. GC–MS of mono- and diacylglycerols is usually performed with TMS and TBDMS derivatives in combination with non-polar capillary GC columns [121]. The latter analyses provide information about the molecular mass of the compound and about the relative proportions of a saturated fatty acid in the primary and secondary positions of the acylglycerol molecule, as well as permits the identification of the mono- and diacylglycerol species in a specific GC peak. The facile loss of the methyl or *tert.*-butyl group from the TMS and TBDMS ethers, respectively, yields the pseudomolecular ion, while the loss of a fatty acyl group results in prominent fragment ions from which the molecular species can be identified. Fig. 8 illustrates the identification of soybean oil triacylglycerols by a combination of capillary GC on polarizable liquid phase and single ion monitoring in the positive mode [136]. The peak identification is accomplished by localizing certain fragment ions having the same retention times in the SIM profile. Three kinds of $[RCO]^+$ corresponding to the fatty acyl residues on the glycerol moiety, and three kinds of $[M-OCOR]^+$ are examined, which is sufficient for peak identification and for establishing their purity. More complete discussion of the characterization of the neutral glycerolipids by capillary GC–MS may be found elsewhere [146].

The GC–MS identification of GC peaks can be improved further by stable isotope labelling. On-

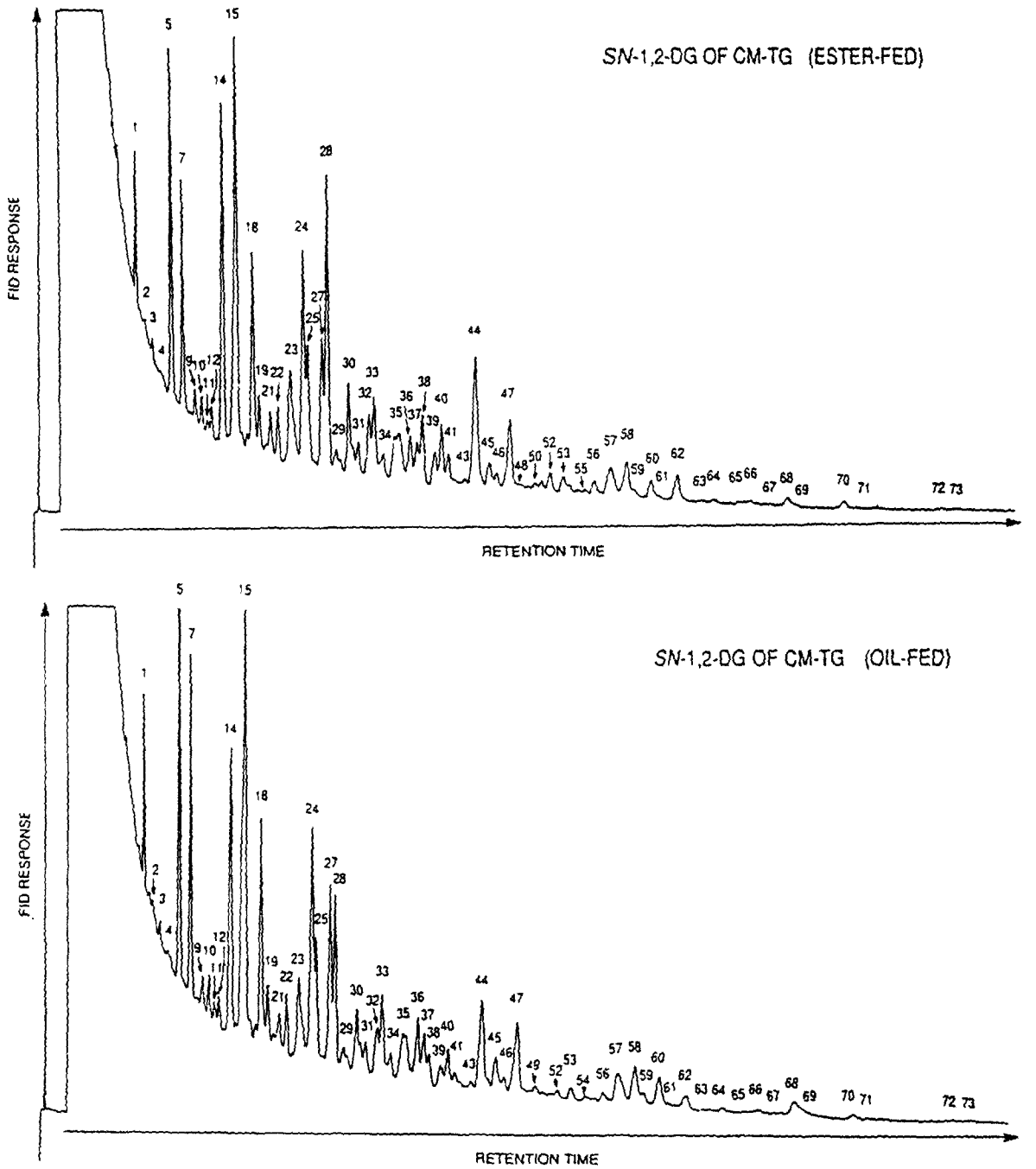

Fig. 7. Polar capillary GLC profiles of *sn*-1,2-(A) and *sn*-2,3-(B) diacylglycerols derived from rat chylomicron triacylglycerols during absorption of fish oil triacylglycerols [130]. GC conditions: instrument, Hewlett-Packard Model 5880 equipped with a polar capillary column (15 m × 0.32 mm I.D.) wall coated with cross-bonded film of RTx-2330 (Restek, Port Matilda, PA, USA). Carrier gas, H_2 at 3 p.s.i (20.7 kPa) head pressure. Temperature program: 240–260°C at 1°C/min, then isothermal at 260°C. Sample: 1 μl of ca. 0.1% solution of TMS-treated lipid mixture in hexane. Reproduced with permission from *Journal of Lipid Research*.

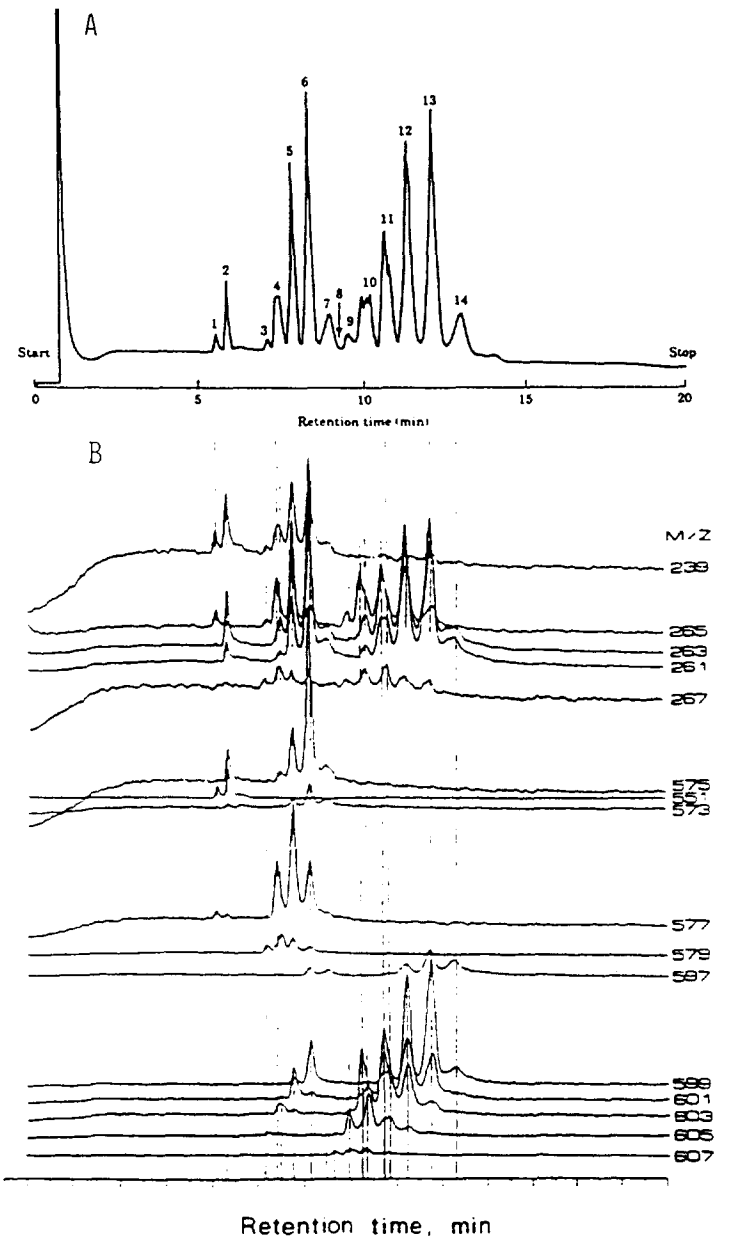

Fig. 8. Capillary GC (A) and GC–MS (B) of soybean oil triacylglycerols [136]. Peak identification: 1, POP; 2, PLP; 3, POS; 4, POO and PLS; 5, PLO and PLNS; 6, PLL and PLnO; 7, PLnL; 8, SOS; 9, SOO and SLS; 10, OOO, SLO and SLnS; 11, OLO and SLnO; 12, OLL, OLnO and SLnL; 13, OLnL, LLL and SLnLn; 14, LLnL. GC conditions: column, MP65HT (25m × 0.25 mm I.D.) at 345°C; injector, a solventless movable injector at 370°C; carrier gas, He at 1.75 kg/cm^2. SIM with Shimadzu QP 1000 quadrupole fitted with an EI source to which the GC column outlet was connected directly. The conditions were 70 eV ion beam, 3 kV accelerating energy with the ion source at 330°C. Reproduced with permnission from *Lipids*.

line determination of the presence of ^{13}C and ^{2}H-labelled metabolites by capillary GC–MS presents no special problems, provided sufficiently enriched preparations are employed [23,147]. The determinations are facilitated by the presence of two or more heavy isotopes per molecule. Consideration should be given to the possibility that internal standards prepared chemically or biologically and stable isotope-labelled metabolites may contain several isologous species differing in the number of heavy isotopes.

3.7.2. LC–MS

As in GC, peak identification in HPLC can be confirmed by mass spectrometry. LC–MS has the advantage that it can be used to study many compounds which are not amenable to GC–MS. Many reviews [28,146–148] of LC–MS combinations for lipid analyses have been published and this book contains a special chapter devoted to the LC–MS–MS application to lipid analyses [29].

The on-line LC–MS analysis of lipids is accomplished using interfaces, which eliminate the HPLC solvent and effect a reliable and efficient transfer of the solute to the mass spectrometer. An early method of interfacing HPLC and a mass spectrometer utilized direct liquid inlet interface and several successful applications to lipid analyses have been reported and the results reviewed [28,146,147]. This method utilized a gradient of 30–90% propionitrile in acetonitrile for the analyses of molecular species of triacylglycerols and of the TMS and TBDMS ethers of the diacylglycerols derived from natural glycerophospholipids [28], as well as for the detection and quantitation of the deuterium-labelled diacylglycerol moieties of rat liver glycerolipids [147]. The sensitivity of the NICI-MS detection of the glycerolipids is increased using the pentafluorobezoates [28,149] or the chloride attachment products of glycerolipids [150,151]. Both techniques yield prominent pseudomolecular ions.

The softer ionization techniques, FAB [146], thermospray [152] and electrospray [153] allow direct LC–MS of the molecular species of intact glycerophospholipids. The FAB and ES ionization techniques are compatable with MS–MS approaches and have been extensively utilized in polar lipid analyses [145–147]. Fig. 9 illustrates the application of chiral phase LC–MS with thermospray to the determination of the molecular species of enantiomeric diacylglycerols as the DNPU derivatives [147]. The overlapping molecular species can be readily identified by combining the HPLC retention time data with the molecular ion plots obtained by LC–MS with single ion monitoring. This method has been used to identify molecular species of *sn*-1,2- and *sn*-2,3-diacylglycerol moieties of hepatic and VLDL triacylglycerols in metabolic experiments [154].

Although the special advantages of on-line LC–MS are obvious, a number of research groups have instituted off-line LC–MS. Off-line LC–MS is frequently the choice for MS–MS work with lipids [29,145,146]. Finally, both on-line and off-line LC–MS techniques can be combined with stable isotope labelling for metabolic studies [23,147]. Both deuterium oxide and deuterated ethanol have been employed extensively for studies of fatty acid and glycerolipid metabolism, while heavy isotope-labelled fatty acids have been utilized in cell culture incubations and organ perfusions.

3.7.3. MS–MS

Electrospray MS–MS has begun a new era in characterization of molecular species of lipids [29,145,146]. In many instances the MS–MS results are comparable to those obtained with LC–MS, where the chromatographic column serves the purpose of the first mass spectrometer. In ordinary LC–MS, it is not always possible to determine with certainty the origin of specific ion fragments when more than one parent ion could yield them. Effective applications of ESI-MS–MS to analyses of molecular species of glycerophospholipids have now been made [155,156]. The rapid, sensitive and quantitative nature of this technique provides an alternative to previously published methods. Fig. 10 illustrates the application of direct ESI-MS–MS analysis to human erythrocyte phospholipids [156]. More than 50 phospholipid constituents

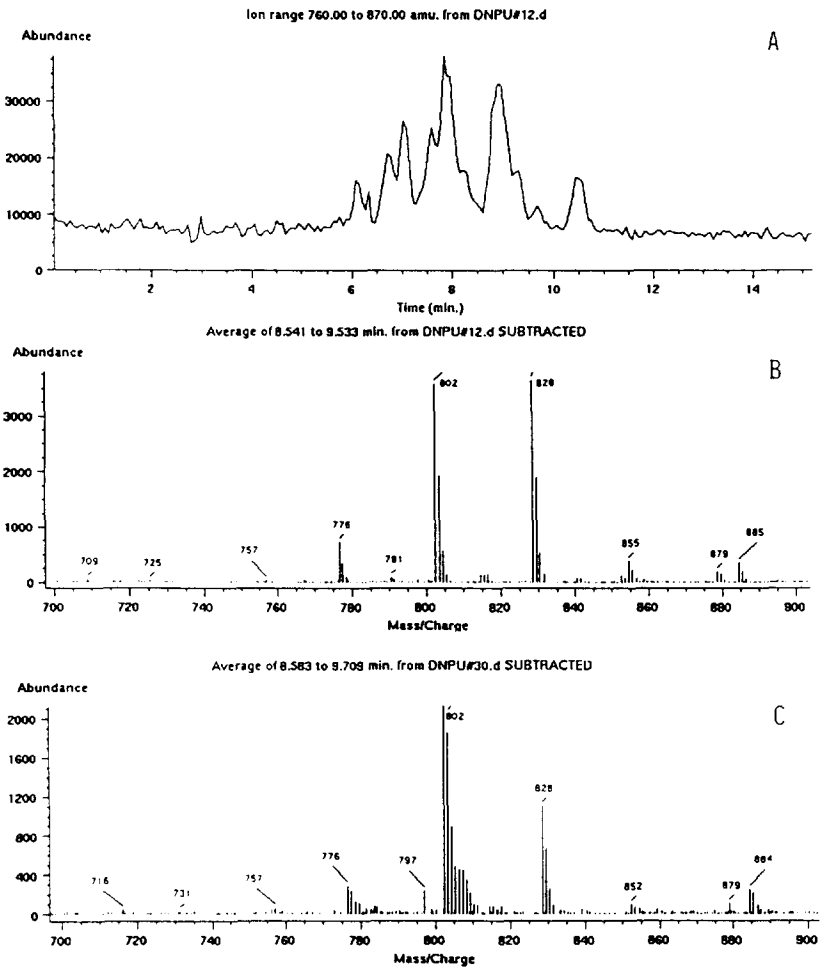

Fig. 9. Total ion profile (upper panel) and abbreviated full spectra (lower panel) of selected *sn*-1,2-diacylglycerol DNPU as obtained by reversed-phase LC–MS with thermospray ionization in the negative ion mode (Hewlett-Packard MS engine Model 5989A, Palo Alto, CA, USA) [147]. The peaks at m/z 776, 802, and 828 represent the $[M-1]^-$ ions of 16:0–16:0, 16:0–18:1, and 18:1–18:1 (18:0–18:2) species, respectively. These diacylglycerols contain deuterium in both palmitic acid and glycerol residues of the molecules, as indicated by the extra masses associated with each species. Sample C: *sn*-1,2-diacylglycerols derived from hepatic triacylglycerols of a rat injected with perdeuterated ethanol for 20 h. Reproduced with permission from John Wiley and Sons, Chichester, UK.

were identified and quantitated directly from chloroform extracts of subpicomole amounts, obviating the need for prior chromatographic separation of phospholipid classes. It is possible that in the future direct probe or flow injection in combination with ESI-MS–MS will substitute for LC–ESI-MS or even LC–ESI-MS–MS because of the speed of analysis. However, ESI-MS–MS did not provide information on the position of fatty acids, and was not capable of differentiating in all instances between alkylacyl and alkenylacyl species without prior separation of these subclasses [155].

3.8. Other complementary methods

Other combinations of complementary chromatographic and mass spectrometric methods are represented by supercritical fluid chromatography–mass spectrometry (SFC–MS) [157] and

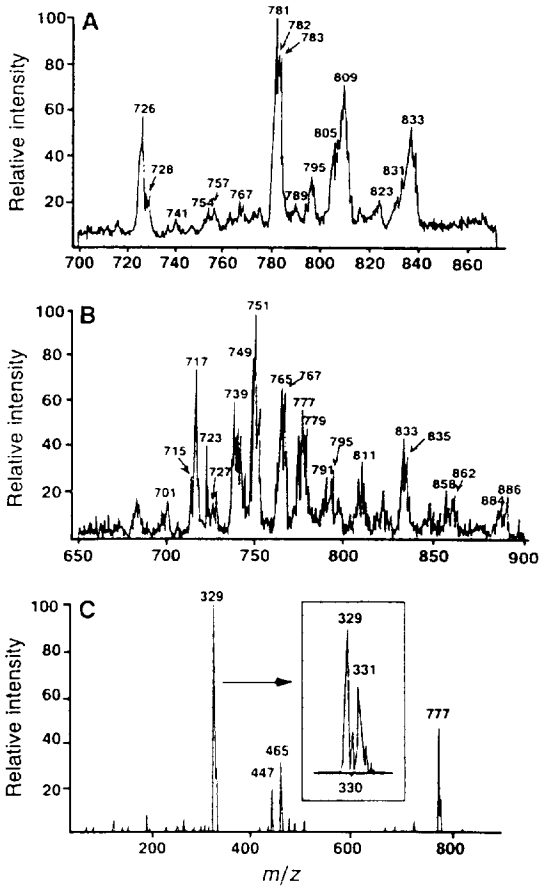

Fig. 10. Direct ESI-MS analysis of human erythrocyte phospholipids [156]. (A) A positive-ion ESI mass spectrum of erythrocyte phospholipid extract (13.5 pmol choline phospholipids from 25 nl of whole blood) shows 14 molecular species of PC and 4 molecular species of SPH; (B) a negative-ion ESI mass spectrum of the same extract shows >25 molecular species of PE and 8 molecular species of PS and PI; (C) ESI-MS-MS analysis of selected isobaric PE (*m/z* 777) after negative-ion ESI demonstrates two carboxylic anions (*m/z* 329 and 331) (inset), corresponding to 22:5 and 22:4 fatty acids, respectively. In addition, several ethanolamine lysoglycerophospholipid-type ions were identified, facilitating the assignment of these species as 18:0–22:5 and 18:1–22:4 plasmenylethanolamines in a 2:1 molar ratio. Reproduced with permission from *Proceedings of National Academy of Sciences USA*

SFC–MS–MS [158,159] as well as by the TLC–MS [41]. An early demonstration of the suitability of SFC–MS was due to Pinkston et al. [157], who used standard methyl arachidate and tristearoylglycerol. The mass chromatograms were obtained using conventional capillary columns with a linear CO_2 pressure gradient and simple ammonia chemical ionization, the spectra of which were dominated by the ammonium adduct ion. Kallio et al. [158] used capillary column SFC–MS–MS for the analysis of butterfat triacylglycerols. The methylphenylsiloxane column provided essentially carbon number resolution. The ratios of triacylglycerols of varying degree of unsaturation in each SFC peak was determined by using the selected ion monitoring of the molecular ions with electron impact mode. The discrimination between the fatty acids at the *sn*-2- and *sn*-1(3)-positions in a triacylglycerol molecule was demonstrated by monitoring the ions $[M - RCO_2CH_2]^+$ from reference compounds. Demirbuker et al. [159] has used micropacked argentation SFC in combination with off-line MS–MS for the identification of the triacylglycerol peaks recognized by their UV absorption at 210 nm. The fractions were collected and reanalyzed by SFC and admitted to the mass spectrometer by direct probe. Staby et al. [160] have recently reported an excellent resolution of fish oil triacylglycerols by SFC on non-polar capillary columns. Molecular mass distributions of eel and salmon oils were given.

The recently discussed [41] in situ analysis of TLC fractions by mass spectrometry must be considered different than a simple off-line TLC–MS combinations and be applicable to lipid analyses.

4. Detection and quantitation in GC and HPLC

The quantitation of the lipid species in the chromatograms is essentially a function of the detector. Quantitation of glycerolipid peaks is simple with detectors that give a reproducible mass or molar response. It is also helpful to have a sensitive detector response, which is linear over the working range for all analytes in the sample. None of the available detectors meet fully all these requirements and a choice must be made in selecting the most appropriate detector for each application.

4.1. Universal detectors

Since natural glycerolipids usually do not contain chromophores, they are best detected by the universal detection systems such as flame ionization, light scattering and total ion current in the mass spectrometer. The flame ionization detector responds essentially to the carbon mass in the solute. It is sensitive and possesses a wide dynamic range. It is not affected by temperature programming in GC or solvent gradients in HPLC. The flame ionization detector response requires minimal calibration except for very high molecular mass components in GC. Moreau [45] and Moreau and Gerard [46] have reviewed the application of flame ionization detectors to HPLC analysis of lipids. They have also discussed the details of the special application of the flame ionization detector to Chromarod or Iatroscan methodology. The Chromarod or Iatroscan approach has the advantage of automation [41].

The development of the light scattering detector [43–45] has revolutionized HPLC of lipids. Detection is achieved by nebulizing the column eluate in a gas stream. Although the response is non-linear and the dynamic range much more limited than that of flame ionization detector, the light or laser light scattering detector responds to all glycerolipids in a reproducible manner [43–45]. Quantitative analysis of triacylglycerol mixtures, carried out without calibration, give results in good agreement with data derived from GC. In earlier work ordinary light scattering had been used with similar results. Mass spectrometry can be effectively employed for a sensitive quantitation of lipids in the effluents from both GC and HPLC. It is especially valuable for the estimation of the relative concentrations of components overlapping in a GC or HPLC peak, where other methods are not applicable. However, the yields are variable for both parent and fragment ions even for members of a homologous series and extensive calibration may be necessary [28].

4.2. Selective detectors

The UV absorbing and fluorescent derivatives yield essentially molar response, which lends readily to quantitation of all molecular species over a wide concentration range [51–56]. Furthermore, wavelength ratios and absorption spectra provide useful, although not definitive, information about the identity or purity of the peak [45]. Bernhard et al. [161] have used a combined UV–fluorescent approach for analysis of phospholipids from different sources.

The refractive index and short-wave UV detection systems employed in early HPLC of glycerolipids are best suited for qualitative work, although important quantitative applications have been reported [76–78,89]. These detectors require extensive calibration and are subject to serious solvent restrictions. Fabien et al. [162] have reported that quantitative analysis are possible with synthetic mixtures of saturated triacylglycerols using HPLC with refractive index detector and an eluent composed of propionitrile and butyronitrile (80:20). A post-column reactor for quantitative analyses of triacylglycerols with high sensitivity has been developed by Kando and Takano [163]. In this reactor triacylglycerols in the HPLC effluent are hydrolyzed with KOH, and the resulting glycerol is oxidized to formaldehyde in the presence of ammonium acetate, and the reaction product is detected calorimetrically at 400 nm. The usefulness of the detector was illustrated by analyses of coconut oil and synthetic triacylglycerols.

On-line measurement of radioactivity in capillary GC is more difficult and no entirely satisfactory systems exist. Kuksis [110] has reviewed the more recent developments in the application of the flow-through detectors to analyses of radiolabelled lipids by HPLC. Another application of a flow-through radioactivity detector (Berthold, Wildbad, Germany) in HPLC analyses of phospholipids has been reported by Truembach et al. [164].

4.3. Validation of quantitation

For quantitation the peak area of each component is reported as a percent of the total area of all peaks. These results reflect the relative composition of a multicomponent sample, provided all the components are eluted, separately detected, and give the same detector response

per weight or mole unit. When the detector response is not equivalent, the area percent of a component is multiplied by a response factor, which must be determined, to reflect the weight or mole percent of the component reaching the detector [3,28,116]. Kuksis [110] has listed the correction factors determined for mixtures of standard triacylglycerols ranging from C_{24} to C_{54}. Various other compilations of correction factors or response curves are available in the literature for GC of triacylglycerols [109,135] and diacylglycerols [3] using hydrogen flame ionization detectors and for HPLC of triacylglycerols using short-wave UV [75] and mass spectrometry [28,165], but these factors should be confirmed in each laboratory. The relative recoveries of triacylglycerols obtained experimentally by nonpolar capillary GC and by calculation from the known positional distribution of fatty acids assuming a 1-random, 2-random, 3-random distribution may be compared, as may be the proportions of the carbon number or partition number distributions obtained by GC or HPLC, respectively, and by calculation [166].

4.3.1. Internal standardization

This is the most satisfactory method for the quantitation of neutral lipids in a sample [116] and has been outlined in detail for fatty acid esters [164]. A small accurately measured aliquot (equivalent to 10–20% of total peak area) of an appropriate standard (e.g. C_{17} or C_{23} for fatty acids and C_{30} triacylglycerol for neutral lipids) is added to the sample before introduction into the chromatograph. Following chromatography the analyte and internal standard peak areas are measured and peak area ratios are determined. The amount of each unknown is calculated in relation to the known concentration of the standard. For the purpose of the calculation it is assumed that the entire sample plus the standard was introduced into the chromatograph. The major advantage of quantitation via an internal standard is that errors arising at each step are selfcompensating, because both standard and unknown are affected proportionally. For this to be true, the internal standard must be closely similar to the unknown, which places extra demands on the separation system. Craske [167] has reported on a collaborative evaluation of the instrumental and chemical errors in analyses of oils by gas chromatography of the fatty acids. He has concluded that the separation of the instrumental and chemical sources of error is a useful concept for validation of the analytical methods.

Mancuso et al. [168] quantitated the tetraethers of acidophilic archaebacteria by Furier transform infrared spectrometry. The availability of a structurally similar standard, 1,2-di-O-hexadecylglycerol enabled quantification of the diether to a 600 pmol detection limit.

4.3.2. External standardization

In this method response curves are established for each unknown by injecting into the chromatograph progressively larger amounts of reference compounds and recording the quantitative response [166,169,170]. Either a linear or non-linear response must be reproducibly obtained for accurate quantitation. The peak area recorded for the unknown is then compared to the corresponding point on the standard curve and the concentration of the unknown read off the graph. For these calibrations the standards should be prepared and run in the same solvent as the unknowns and the standard response should be confirmed before and after the analysis of the unknowns. The requirements for the quantitation of acylglycerols by external standardization are the same as they are for other chromatographic analytes described in greater detail elsewhere [166,169,170]. External standardization is less frequently used in the analysis of neutral glycerolipids.

4.3.3. Definitive methods

The definitive methods of analysis depend on the simultaneous quantitation of the lipid species of interest along with its stable isotope-labelled homologue. Since the two molecular species are identical in all other aspects, they suffer identical fates during extraction, derivatization and chromatography. With all other factors cancelled out, only differences in concentration remain, and these are utilized in the calibration of the detector and hence the concentration of the unknown. Definitive methods are highly sensitive and

specific and permit standardization for various routine methods of analysis. The early applications of definitive methods to analysis of molecular species of lipids have been discussed by Kuksis and Myher [143]. More recent developments in utilization of stable isotope labelled homologues for the characterization and quantitation of lipid molecules, including PAF and prostanoids, have been reviewed by Murphy [146].

5. Conclusions and future prospects

Complete resolution of natural lipids currently requires combined application of two or more chromatographic systems preceded by adequate extraction, purification and sometimes derivatization. Full identification of molecular species requires mass spectrometry preferentially on-line with GC or HPLC. Solid-phase extraction facilitates isolation and purification of various lipids, but cannot replace solvent extraction, or TLC and HPLC. MS–MS systems with soft ionization promise to eliminate the need for separate sample preparation as well as derivatization, but positional analyses of glycerolipids are beyond the reach of present methods, although a distinction between primary and secondary positions can sometimes be made. Therefore, complementary chromatographic techniques, including chiral separations, remain the only effective although time consuming methods of resolution of molecular species for the immediate future. These core methodologies will continue to be coupled with other complementary methods of analysis ranging from traditional GC–MS to LC–SFC–MS. A new trend concerns the miniaturization of columns, systems and the chromatographic process itself, which, following optimization, should yield reduced retention times and increased resolution. Both conventional and miniaturized chromatographic system development is likely to benefit from more wide-spread application of the artificial intelligence available in the computerized expert systems. Expert systems contain knowledge gathered during practical work, which allows improved design of liquid phases and solvent systems in the absence of theoretical knowledge. The above developments should enable the future lipid chromatographer to provide faster, more accurate and better documented analyses that might meet more effectively the future needs of biochemical, clinical and industrial laboratories and the demands of the regulatory agencies.

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