and GC-MS is likely to remain the method of choice for analyses of molecular species of most lipid classes for the immediate future.

See Colour Plate 103.

See also: **II/Chromatography: Gas:** Column Technology; Derivatization. **III/Lipids:** Liquid Chromatography; Thin-Layer (Planar) Chromatography.

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Liquid Chromatography

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Introduction

Natural lipids consist of complex mixtures of molecular species, which are found in association with cell membranes, lipoproteins and other subcellular structures. The composition differs among different cell and tissue types, reflecting the function of lipids in these body structures. Much experimental effort and imagination has been expended in determining the exact composition of lipid species during dietary alterations and physiological activity.

The complex nature of natural lipids and their high solubility in organic solvents makes the separation and isolation of individual lipid classes and molecular species by most physical methods difficult or impossible. Only chromatographic methods have proven suitable for this. The earlier thin-layer and gas chromatographic routines have been complemented in recent years by high performance liquid chromatography (HPLC), which has proven to have nearly universal applicability.

Principles of Liquid Chromatography

Normal-phase Columns and Solvents

Normal-phase HPLC provides resolution based on the overall polarity of the lipid molecules. The

stationary phase is typically silica gel. The best column and mobile phase for a specific application are selected by trial and error or based on previous experience using thin-layer chromatography (TLC). More refined solvent selection is based on chemometric methods. The solvents selected for the mobile phase in either isocratic or gradient separations should be excellent lipid solubilizers and should not degrade the stationary phase. Therefore, the addition of buffering salts should be minimized and strong acids should be avoided. Other restrictions on the selection of the mobile phase may be imposed by the choice or availability of the detection system, e.g. ultraviolet (UV) and refractive index (RI) detectors.

Silver ion HPLC provides resolutions based on the number, position and configuration of the double bonds present in the lipid molecule. It complements other methods of separation. For this purpose silica gel columns are impregnated with silver nitrate or silver ions are immobilized on columns containing benzenesulfonic acid groups. Chlorinated solvents as the mobile phase, with acetone or acetonitrile as a polar modifier, afford especially good separations.

Reversed-phase Columns and Solvents

Reversed-phase HPLC is believed to separate lipid molecules based on their partition properties in a biphasic liquid–liquid system, although the exact mechanism is unknown. The most widely used and most important reversed phases for lipid analysis are silicas with relatively long hydrocarbon chains chemically bonded to the surface. Octyl- and octadecyl-bonded chains have been found to provide the best resolution of fatty acids and triacylglycerols.

Chiral-phase Columns and Solvents

HPLC on columns containing a stationary phase with chiral moieties bonded chemically to a silica matrix has proven well suited for the resolution of chiral glycerolipids. The approach has been to prepare 3,5-dinitrophenyl urethane (DNPU) derivatives of mono- and diacyl-*sn*-glycerols and related compounds, where the hydrogen atom on nitrogen in the urethane group is available for hydrogen bonding with the stationary phase. The 3,5-dinitrophenyl moieties of the urethanes contribute to charge–transfer interactions with functional groups having π electrons on the stationary phase; they are also advantageous for detection by UV absorption. A column with a polymer of (*R*)-(+)-1-(1-naphthyl)ethylamine moieties chemically bonded to silica gel (YMC-Pack A-KO3™) has been applied to the resolution of diacyl-*sn*-glycerol derivatives in a similar manner. The best solvents so far have been the hexane/dichloromethane/ethanol mixtures, which, for the purpose of online mass spectrometry, have been replaced by mixtures of hexane/dichloroethane/acetonitrile or isooctane/*tert*-butyl methyl ether/acetonitrile/isopropanol.

There are no foolproof recommendations for the selection of solvents. Previous success appears to be the best guide. However, the polarity of the solvents must be chosen so as not to destroy the hydrogen bonding responsible for the differential affinity between the enantiomers and the stationary phase.

Detectors and Quantification of Solutes

In early work, RI detectors were used in normal-phase HPLC. However, this detector is only applicable to isocratic elution, because RI detection is very sensitive to change in solvent composition. Short wavelength UV (205–210 nm) is also frequently employed for lipid detection but again suffers from sensitivity towards changes in solvent composition. However, UV absorption at longer wavelengths can be effectively used for the specific detection of UV-absorbing solutes. Furthermore, lipids with functional groups can be converted into UV-absorbing or fluorescing derivatives (e.g. free fatty acids, aminophospholipids), which have been widely exploited in reversed-phase HPLC. Many of these derivatives are sufficiently sensitive to permit detection of femtomole levels of the solute (see below).

In HPLC with flame ionization detection (FID), the analyte after solvent removal is burned in a flame and the ions formed are collected by applying an electric field. This nearly universal detector yields a linear relationship between the mass of the solute and peak area over a wide concentration range. More recently, the evaporative light-scattering detector (ELSD) has become the detector of choice in most analytical lipid separations. Like the FID, it is destructive, and must be employed with a stream splitter for peak collection.

It is generally agreed that all detectors require preliminary calibration with reference species to examine the response–structure relationship, and to determine the character of the calibration graph constructed in response–quantity dimensions to be able to work in the linear range. In principle, the quantification is based on measurements of peak area, normalization of the values and calculation of the relative percentage of each component.

Another detector for HPLC of universal application is provided by the mass spectrometer using a variety of ionization techniques to generate total or single ion current response. The use of this detector is

discussed along with the use of online mass spectrometry for peak identification (see below).

Liquid Chromatography–Mass Spectrometry/(LC-MS)

LC-MS

Online mass spectrometry allows one to obtain direct evidence about the nature of chromatographic peak, e.g. purity, molecular weight and characteristic fragment ions. This information, together with the knowledge of the relative retention time, is usually sufficient for peak identification. More complete identification may be obtained by MS-MS, which is based on the specific mass spectrometric fragmentation of primary ions. Recent reviews of LC-MS applications to lipid analyses are available (see Kuksis and Myher, 1995 and Kuksis, 1997 in Further Reading, below).

The online 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 ion source. An early method of interfacing HPLC and a mass spectrometer utilized a direct liquid inlet. Several successful applications to lipid analyses with chemical ionization mode were reported. In the positive ion mode, this method produced mass spectra similar to those recorded in electron impact mass spectrometry. Thus, for a triacylglycerol species, a pseudomolecular ion along with characteristic diacylglycerol-like ions were obtained. These ions are frequently sufficient to identify the molecular weight and degree of unsaturation of the component fatty acids. Furthermore, the regiodistribution of the fatty acids in acylglycerols can also be obtained by this ionization method. More recently, the softer ionization techniques, thermospray (TS), electrospray (ES) and atmospheric pressure chemical ionization (APCI) have been utilized for online monitoring of triacylglycerols resolved by HPLC. The latter techniques also allow direct LC-MS of the molecular species of intact glycerophospholipids, which yield largely or exclusively the pseudomolecular ions $[M + 1]^+$ and $[M - 1]^-$ respectively in the positive and negative ionization modes. This information is sufficient for tentative identification of molecular species when combined with knowledge of HPLC retention times and the overall composition of the fatty acids.

LC-MS-MS

Simple LC-MS is not sufficient to establish the exact composition of all molecular species, which requires the identification of the component fatty acids in each parent acylglycerol molecule. The fast atom bombardment (FAB) and especially the ES ionization techniques are compatible with LC-MS-MS approaches and have been extensively utilized in polar lipid analysis. In many instances, flow ES-MS-MS has also proven adequate for identification of molecular species.

Pseudo MS-MS

LC-ES-MS ionization can be used to produce collision-induced dissociation (CID) spectra of singly charged species with greater sensitivity than can be achieved with flow ES-MS-MS systems. The HPLC effluent is carried into the ES source via a stainless-steel or fixed silica needle at flow rates of 1–40 $\mu L\ min^{-1}$. When analytes are present in the sprayed solution, molecular adduct ions from these analytes, typically protonated ions $[M + H]^+$, are formed. If a low voltage of 50–120 V is applied to the capillary exit, the molecular ion remains intact and the molecular weight of the analyte is obtained. If higher voltages are applied (e.g. 200–300 V) to the capillary exit, extensive and reproducible fragmentation of the molecular adduct ion is realized (pseudo MS-MS).

Isolation of Natural Lipids

In order to determine the composition of the lipid phase associated with a particular function it is necessary to isolate the appropriate subcellular structure and to determine the component lipid classes and molecular species. For the purpose of discussion the analyses are considered separately as those of neutral lipids, glycerophospholipids and sphingolipids, including glycosphingolipids.

Preparation of Lipid Extracts

The neutral lipids can be extracted from natural sources by means of benzene, chloroform and other nonpolar solvents. More complete lipid isolation is obtained by extraction with more polar solvents, such as mixtures of chloroform and methanol. However, use of chloroform/methanol results in low recoveries of acidic phospholipids, lysophospholipids and nonesterified fatty acids, while acidified solvents generate lysophospholipid artefacts from tissues containing plasmalogens.

Purification and Preliminary Separation

Recently, organic solvent extraction has been replaced by solid-phase extraction. It is a simple, rapid technique and can be up to 12 times faster than liquid extraction when executed with commercially

Table 1 Separation scheme for fractionation of lipid classes from wheat flour using combined silica and aminopropyl solid-phase extraction columns

Absorbent	*Solvents (volume ratio)*	*Volume (mL)*	*Lipid class eluted*
Silica	Hexane/Et_2O (200 : 3)	15	SE
Silica	Hexane/Et_2O (96 : 4)	20	TG
Silica	Hexane/HOAc (100 : 0.2)	20	
Silica	Hexane/Et_2O/HOAc (100 : 2 : 0.2)	20	FFA
Silica	Hexane/ethyl acetate (95 : 5)	15	
Silica	Hexane/ethyl acetate (85 : 15)	15	1,2-DG, 1,2-DG
Silica	Et_2O/HOAc (100 : 0.2)	15	α-MG, β-MG
Silica	Et_2O/acetone (50 : 50)	20	MGDG, MGMG
Silica	Acetone	20	DGDG, DGDG
Silica	THF/ACN/isopropanol (40 : 35 : 25)	5	Trace GL
Silica	THF/ACN/isopropanol (30 : 35 : 35)	5	
Silica	THF/ACN/isopropanol (20 : 35 : 45)	5	NAPE
Silica	THF/ACN/MeOH (15 : 45 : 40)	5	NAPE, NAPE
Silica	THF/ACN/MeOH (15 : 35 : 50)	5	
Silica	THF/ACN/MeOH (10 : 35 : 55)	5	PC
Silica	THF/ACN/MeOH (5 : 35 : 60)	5	
Silica	CAN/MeOH (35 : 65)	5	lyso-PC
Aminopropyl	$CHCl_3$/MeOH/ammonium hydroxide (85 : 15 : 0.1)	25	
Aminopropyl	$CHCl_3$/MeOH/ammonium hydroxide (80 : 20 : 0.1)	20	NAPE
Aminopropyl	$CHCl_3$/MeOH/ammonium hydroxide (75 : 25 : 0.1)	20	
Aminopropyl	$CHCl_3$/MeOH/ammonium hydroxide (50 : 50 : 0.1)	20	NALPE
Aminopropyl	$CHCl_3$/MeOH/ammonium hydroxide (0 : 100 : 0.1)	20	

Et_2O, Diethyl ether; HOAc, acetic acid; THF, tetrahydrofuran; ACN, acetonitrile; MeOH, methanol; $CHCl_3$, chloroform. SE, steryl esters; TG, triacylglycerols; FFA, free fatty acids; DG, diacylglycerols; MG, monoacylglycerols; DGDG, diacylglycerol digalactoside; DGDG, diacylglycerol diglucoside; GL, glycolipd; NAPE, *N*-acyl phosphatidylethanolamine; NALPE, *N*-acyllyso phosphatidylethanolamine; lyso-PC, lysophosphatidylcholine. Modified with permission from Prieto JA *et al.*, 1992.

prepared cartridges. These cartridges have proven adequate for rapid removal of nonlipid components from total lipid extracts and for preliminary separation of both neutral and polar lipid classes. For this purpose, the cartridges are eluted by passing through them measured volumes of solvents of appropriate polarity. **Table 1** summarizes a separation scheme successfully applied to the fractionation of lipid classes from wheat flour that is also applicable to the resolution of most lipid classes from animal tissues. A major disadvantage of the solid-phase method is the difficulty of monitoring the separations, so that TLC must be frequently used to assess the results of such isolations.

The currently available techniques concerning extraction and characterization of the different lipids from biological specimens are designed for particular families and do not address consecutive isolation of lipid constituents in their totality. It must be pointed out that conventional TLC remains a convenient, rapid and reliable technique for lipid class isolation which permits efficient extraction of lipid components including gangliosides, without preferential loss of any one group and without the uncertainty of working blindly. The protocol is applicable to biological samples of limited availability.

Derivatization

In order to improve resolution, detection and recovery, natural lipids may be subjected to derivatization prior to HPLC. Since neutral lipids are more readily resolved than polar lipids, the polar functional groups of natural lipids may be removed or masked prior to separation. Thus, the phospholipids may be subjected to dephosphorylation by phospholipase C and the resulting diradylglycerols silylated, acetylated or benzoylated before normal-phase or reversed-phase HPLC. This procedure allows improved resolution of the diradylglycerol classes by normal-phase HPLC and of molecular species by reversed-phase HPLC. The preparation of the benzoates also improves the UV detection of the molecular species by reversed-phase HPLC. The preparation of the benzoates also improves the UV detection of the molecular species, while a preparation of the pentafluorobenzoates improves the sensitivity of mass spectrometric detection of the lipid classes and molecular species. The diacylglycerols generated randomly from natural triacyl-

glycerols by Grignard degradation may be converted into the naphthylethyl urethane derivatives by an enantiomeric reagent prior to normal-phase separation of the resulting diastereomers. Using another approach to stereospecific analysis of triacylglycerols, the racemic diacylglycerols resulting from the Grignard degradation are converted into the dinitrophenyl urethanes of the diacylglycerols prior to separation of the enantiomers. In other instances, free fatty acids, free acylglycerols and aminophospholipids may be converted into UV-absorbing or fluorescent derivatives prior to reversed-phase HPLC. The total lipid extracts or any fraction of them may be hydrogenated, reduced with borohydride, peroxidized or ozonized prior to HPLC to provide reference materials or to improve the chromatographic behaviour of the solutes.

Separation of Neutral Lipids and Free Fatty Acids

Neutral lipids and free fatty acids are made up of monoacylglycerols, diacylglycerols, triacylglycerols, unesterified and esterified sterols, unesterified fatty acids and various other minor components of natural lipids, which migrate with neutral lipids just listed, e.g. tocopherols, alcohols, hydrocarbons, ketones, aldehydes and simple esters. This definition also includes neutral lipids derived from polar lipids by enzymic or chemical transformation (e.g. acylglycerols and ceramides), as well as the peroxidation products of lipids and prostanoids. These lipids possess excellent chromatographic properties.

Normal-phase Separations

Total lipid profiling on adsorption columns was practised in various forms long before the existence of any other comparable method. Reproducibility and quantification were the major problems which have now been resolved by the combination of HPLC and light scattering or mass spectrometric detection. **Figure 1** shows the separation of selected neutral and polar lipid standards and rat liver lipids by automated normal-phase HPLC using a light-scattering detector. Both neutral and phospholipid classes are well separated and this approach is suitable for use with ES-MS. Normal-phase HPLC is also well suited for the separation of natural and peroxidized fatty acid esters. **Figure 2** shows the separation of a mixture of standard oxosterols along with various other mixtures of oxosterols. The peaks were detected by UV at 205 nm.

Normal-phase HPLC can be employed for separation of the diastereomeric diacylglycerol naphthylethyl urethanes for the purpose of stereospecific analysis of the positional distribution of the fatty acids in natural triacylglycerols. **Figure 3** shows the separation of the diastereomeric diacylglycerols as the naphthylethyl urethanes. The *sn*-1,2- and *sn*-2,3-enantiomers are resolved with the elution order depending on the *S*- or *R*-configuration of the reagent. The diastereomeric naphthylethyl urethanes are prepared by reacting the free *sn*-1,2(2,3)-diacylglycerols with either the *S*- or *R*-isocyanate. The partial overlap between molecular species of enantiomeric diacylglycerols from natural sources does not compromise the identification and quantification of the species by ES-LC-MS.

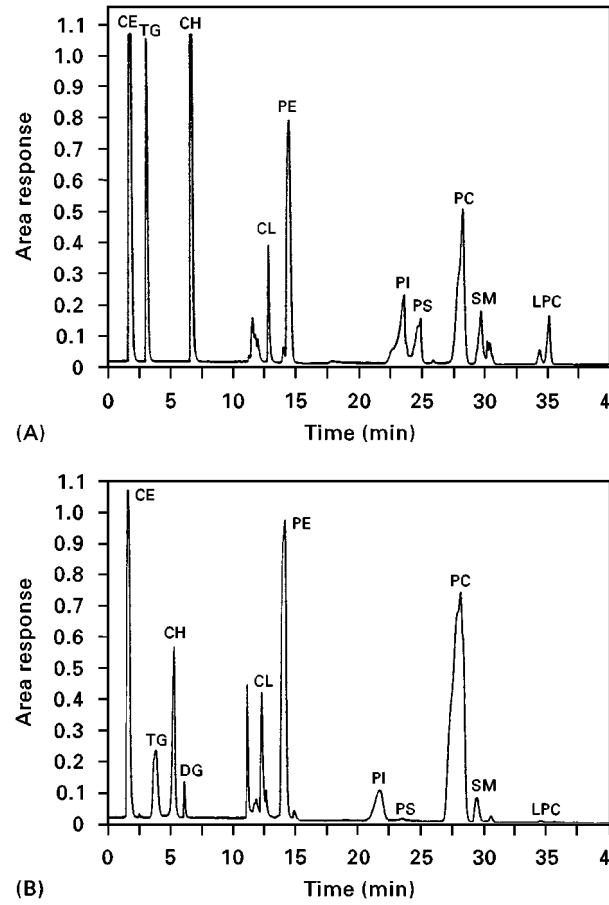

Figure 1 Separation of (A) standard and (B) rat liver lipids by automated normal-phase HPLC with light-scattering detection. Peak identification: CE, cholesteryl esters; TG, triacylglycerols; CH, unesterified cholesterol; DG, diacylglycerols; CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine, PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidyl choline. HPLC conditions: column, 5 μm Ultrasphere Si (25 cm × 4.5 mm); solvent, a binary gradient of three different solvent mixtures made up of hexane/tetrahydrofuran (99 : 1, v/v), isopropanol/chloroform (4 : 1, v/v) and isopropanol/water (1 : 1, v/v). (Reprinted with permission from Redden and Huang, 1991.)

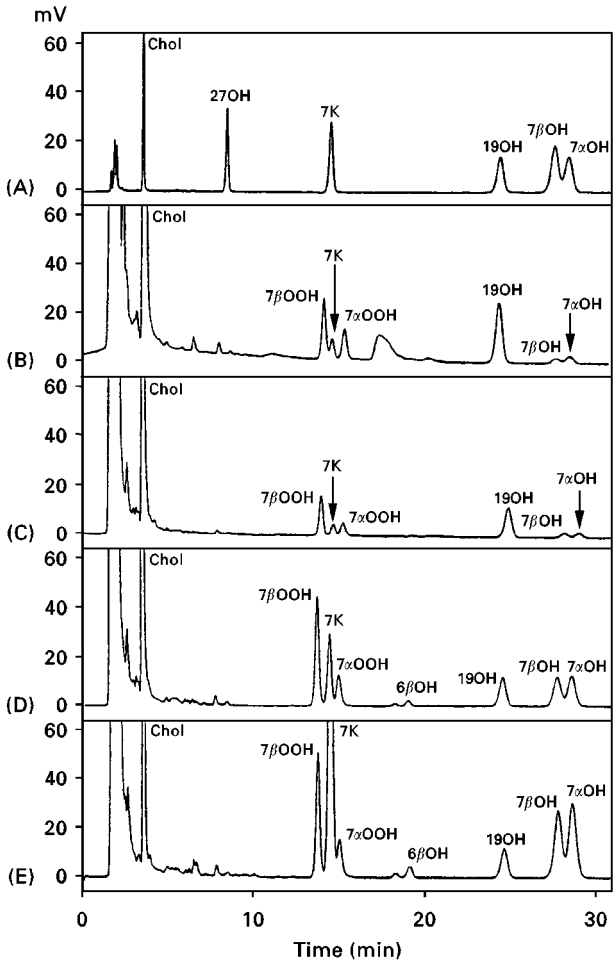

Figure 2 Normal-phase HPLC of (A) standard oxosterols and (B–E) oxidation products of cholesterol. B, Liposomal cholesterol oxidized with azoamidopropane at 37°C for 20 h; C–E, LDL cholesterol oxidized with Cu^{2+} for 4 h, 8 h and 24 h, respectively. Peak identification: Chol, cholesterol; 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 19OH, 19-hydroxycholesterol (internal standard); 7βOH, 7β-hydroxycholesterol; 7αOH, 7α-hydroxycholesterol; 7βOOH, 7β-hydroperoxycholesterol; 7αOOH, 7α-hydroperoxycholesterol; 6βOH, 6β-hydroxycholesterol. HPLC conditions: two columns, 3 μm Ultramex (10 × 0.46 cm) in series with a 3 cm guard column; solvent, hexane/isopropanol/acetonitrile (95.8 : 3.90 : 0.30, by vol) at 1.5 mL min^{-1}. (Reprinted with permission from Brown AJ, Leong S-L, Dean RT and Jessup W (1997) 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *Journal of Lipid Research* 38: 1730–1745.)

Normal-phase HPLC is used for the separation of the leukotriene B_4 (LTB_4) metabolites, with a mobile phase consisting of hexane/isopropanol/acetic acid (96 : 4 : 0.1 by vol; **Figure 4**). The retention times of these compounds are closely related to their polarities, with LTB_4 having the longest and 10,11-dihydro-12-oxo-LTB_4 the shortest time. The 12-oxo-LTB_4 and 10,11-dihydro-LTB_4 have similar retention times but can be distinguished from each other by their UV spectra. Normal-phase HPLC also separates the hydroxyeicosatetraenes (HETEs) and oxo-eicosatetraenes (oxo-ETES) with a mobile phase of hexane/isopropanol/acetic acid (90.05 : 0.45 : 0.5 by vol), as shown in the same figure. As expected from its polarity, 15-oxo-ETE had a retention time considerably shorter than that of 15-HETE. The retention time of 12-oxo-ETE is also shorter than that of 12-HETE, but only marginally so.

Normal-phase HPLC on columns containing immobilized silver ions has been used for the fractionation of simple fatty acid esters and triacylglycerols according to the number and configuration of double bonds. **Figure 5** shows an Ag^+-HPLC separation of a commercial mixture of conjugated linoleic acids (CLA). Using 0.1% acetonitrile in hexane, 12 peaks were obtained, which emerged into three groups of four peaks each. Evidence for the identity of the individual isomers was obtained by comparison with standards and by complementary chromatographic and MS techniques.

Reversed-phase Separations

Figure 6 shows the separation of the molecular species of randomized butterfat by reversed-phase HPLC with light-scattering detection. Butterfat constitutes one of the most difficult mixtures for any chromatographic separation. The analysis of a randomized sample has the advantage that all the

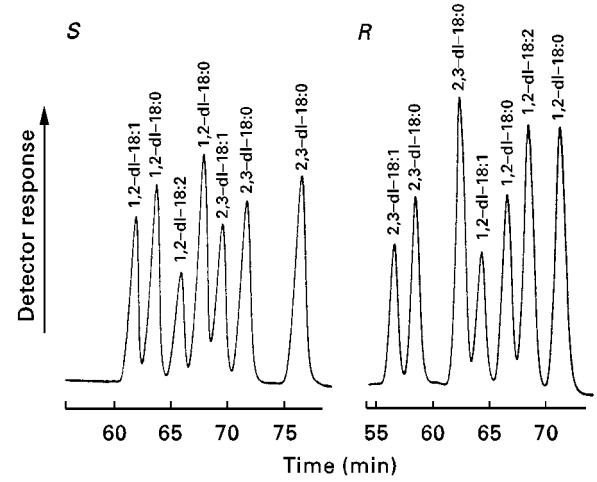

Figure 3 Normal-phase HPLC separation of 1,2- and 2,3-diacyl-*sn*-glycerols in the form of 1-(1-naphthyl)ethyl urethane derivatives. *S*: (*S*)-(+)-1-(1-naphthyl)ethyl urethanes; *R*: (*R*)-(1)-1-(1-naphthyl)ethyl urethanes. HPLC conditions: two columns, 3 μm Hypersil™ (250 × 4.6 mm i.d.) in series; solvents: hexane/isopropanol (99.5 : 0.5, v/v) at 0.8 mL min^{-1} and UV detection at 280 nm. (Reproduced with permission from Laakso and Christie, 1990.)

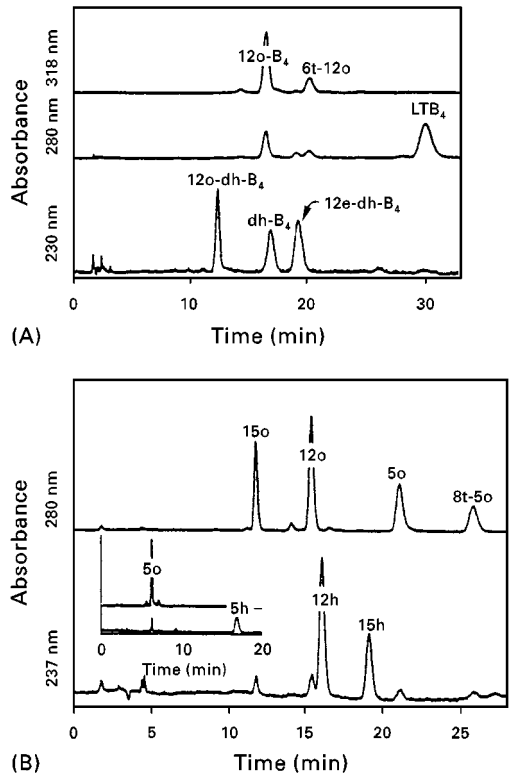

Figure 4 Normal-phase HPLC resolution of (A) LTB_4, 12-oxo-LTB_4 (12o-B_4), 6-*trans*-12-oxo-LTB_4 (6t-12o), 10,11-dihydro-LTB_4 (dh-B_4), 12-oxo-10,11-dihydro-LTB_4 (12o-dh-B_4) and 12-epi-10,11-dihydro-LTB_4 (12e-dh-B_4) with hexane/isopropanol/acetic acid (96 : 4 : 0.1, v/v) and (B) HETEs and oxo-ETEs with hexane/isopropanol/acetic acid (99.05/0.45/0.5, v/v) as the mobile phase. The inset shows the normal-phase HPLC separation of 5-oxo-ETE and 5-HETE using a stronger mobile phase (hexane/isopropanol/acetic acid, 97.2 : 2 : 0.1, v/v). (Reproduced with permission from Powell *et al.* (1997) High-pressure liquid chromatography of oxo-eicosanoids derived from arachidonic acid. *Journal of Biochemistry* 247: 17–24.)

molecular species can be calculated on the basis of random distribution and sorted by number of acyl carbons and double bonds. Although there is nearly complete separation of the various triacylglycerol subclasses, numerous molecular species overlap. It is therefore necessary to employ MS to distinguish among the molecular species within each chromatographic peak. There is no resolution of enantiomers or regio-isomers.

Reversed-phase HPLC provides impressive separation of the complex fish oil triacylglycerols using either light-scattering or MS detection. **Figure 7** shows the separation of a triacylglycerol mixture containing 43% docosahexaenoic acid by LC-ESI-MS. Some 33 major components are detected. An examination of the ions generated from each triacylglycerol peak revealed the presence of both molecular and diacylglycerol-like fragment ions from which the triacylglycerol composition of each peak was determined. Figure 7B and 7C illustrates the identification of peaks 20 and 26 in the chromatographic profile. This extremely powerful method allows identification of species that have the same elution times but different elution times.

The triacylglycerol structures in the HPLC effluent can be determined by atmospheric pressure chemical ionization (APCI) using a corona discharge to ionize vaporized molecules to form both molecular and diacylglycerol-like fragment ions. **Figure 8** compares the elution patterns recorded for a mixture of 35 triacylglycerols by reversed-phase HPLC with FID or APCI detection. Although different gradients of acetonitrile and dichloromethane were used in the two systems, the chromatographic patterns are similar.

Figure 9 relates the retention times of various synthetic oxotriacylglycerols to their theoretical carbon numbers used to aid identification of peroxidized

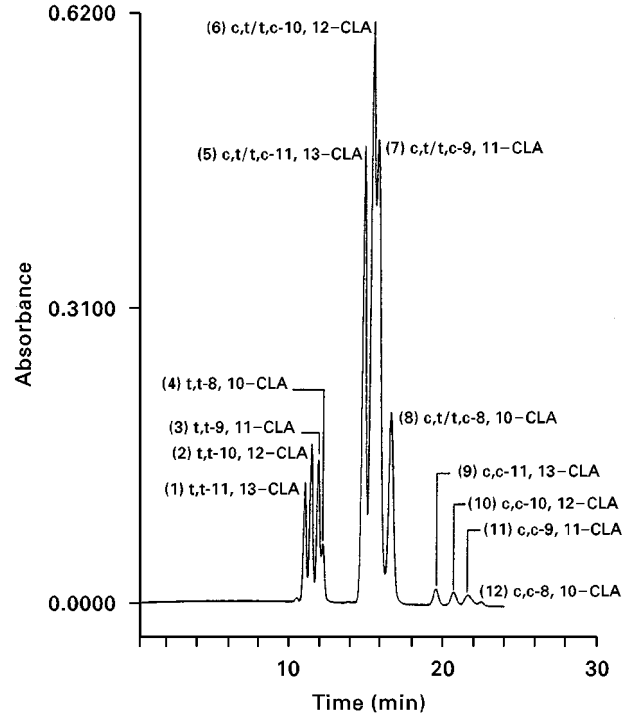

Figure 5 Silver ion HPLC of a commercial conjugated linoleic acid standard. Peak identification is as given in figure: CLA, conjugated linoleic acid. HPLC: column, 5 μm ChromSper, $AgNO_3$ (250 × 4.6 mm i.d.); solvent, isocratic 0.1% acetonitrile in hexane. (Reproduced with permission from Sehat N, Yurawecz MP, Roach JAG *et al.* (1998) Silver ion HPLC separation and identification of conjugated linoleic acid isomers. *Lipids* 33: 217–221.)

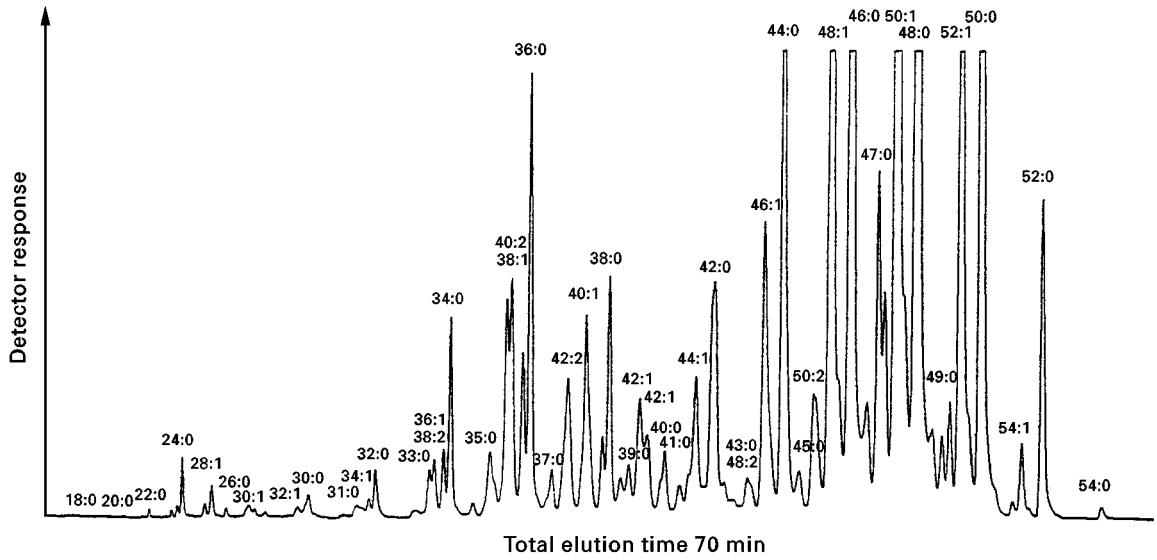

Figure 6 Reversed-phase HPLC elution profile of randomized butterfat triacylglycerols as monitored by light-scattering detection. Peak identification is given in the figure on the basis of total acyl carbon : double bond number. HPLC conditions : column, 5 μm Supelcosil C_{18} (250 × 4.6 mm i.d.) solvent gradient: 10–90% isopropanol in acetonitrile in 90 min. (Reproduced with permission from Marai *et al.*, 1994.)

natural triacylglycerols by reversed-phase HPLC with ES-MS. The theoretical carbon numbers and correction factors for the oxidized and unsaturated triacylglycerols were calculated using the curve for the saturated triacylglycerols as a reference.

Tocopherols exist in nature as a complex mixture of 2-methyl-6-chromanol homologues and aromatic ring position isomers, each having a three-terpene-unit side chain at the C-2-position. These components of closely related structures can be separated by normal-phase HPLC on silica-based columns. However, the most extensive separations have been obtained by reversed-phase HPLC on a column of octadecyl polyvinyl alcohol sorbent. **Figure 10** shows the separation obtained for the α-, β-, γ-, δ- and ε_2-tocopherols on such a column with acetonitrile/water or methanol/water as the mobile phase.

Cholesterol, sitosterol and their metabolic precursors are also separated by reversed-phase HPLC. Conversion to UV-absorbing derivatives greatly facilitates their detection and quantification. Likewise, reversed-phase HPLC is suitable for the separation of cholesteryl esters. **Figure 11** demonstrates the separation of the cholesteryl esters in a lipid extract from cholesterol-loaded J774 macrophages showing esters ranging from eicosapentaenoate (docosahexaenoate) of cholesterol to stearate in order of their partition number. There are several minor peak overlaps. Normally, the cholesteryl esters in a total lipid extract would overlap with the triacylglycerols also present in the mixture. This problem can be solved by a mild alkaline hydrolysis, which destroys the triacylglycerols without affecting the cholesteryl esters.

Similarly, reversed-phase HPLC can be employed for the separation of retinyl esters. **Figure 12** illustrates the separation of 15 synthetic retinyl esters with minimal overlap, except for retinyl linolenate, laurate and arachidonate, which are unlikely to occur together in a natural mixture.

Reversed-phase HPLC has been most extensively employed for the separation of the molecular species of diradylglycerols derived from glycerophospholipids by hydrolysis with phospholipase C and from triacylglycerols by hydrolysis with lipase or Grignard degradation. Both UV-absorbing and fluorescent derivatives are prepared to facilitate detection and quantification (**Table 2**). Reversed-phase HPLC is also excellent for the separation of the molecular species of the diacylglycerol DNPU derivatives recovered from chiral HPLC.

Finally, reversed-phase HPLC is suitable for the separation of fatty acids as UV-absorbing or fluorescent derivatives (**Table 3**) and may rival gas chromatography for specific applications. Thus, excellent separation and sensitive detection of the 9-anthrylmethyl esters and the 1-pyrenyldiazomethane derivatives of free fatty acids has been obtained (**Figure 13**). The method has been applied to the determination of endogenous fatty acids released from a cell culture upon stimulation.

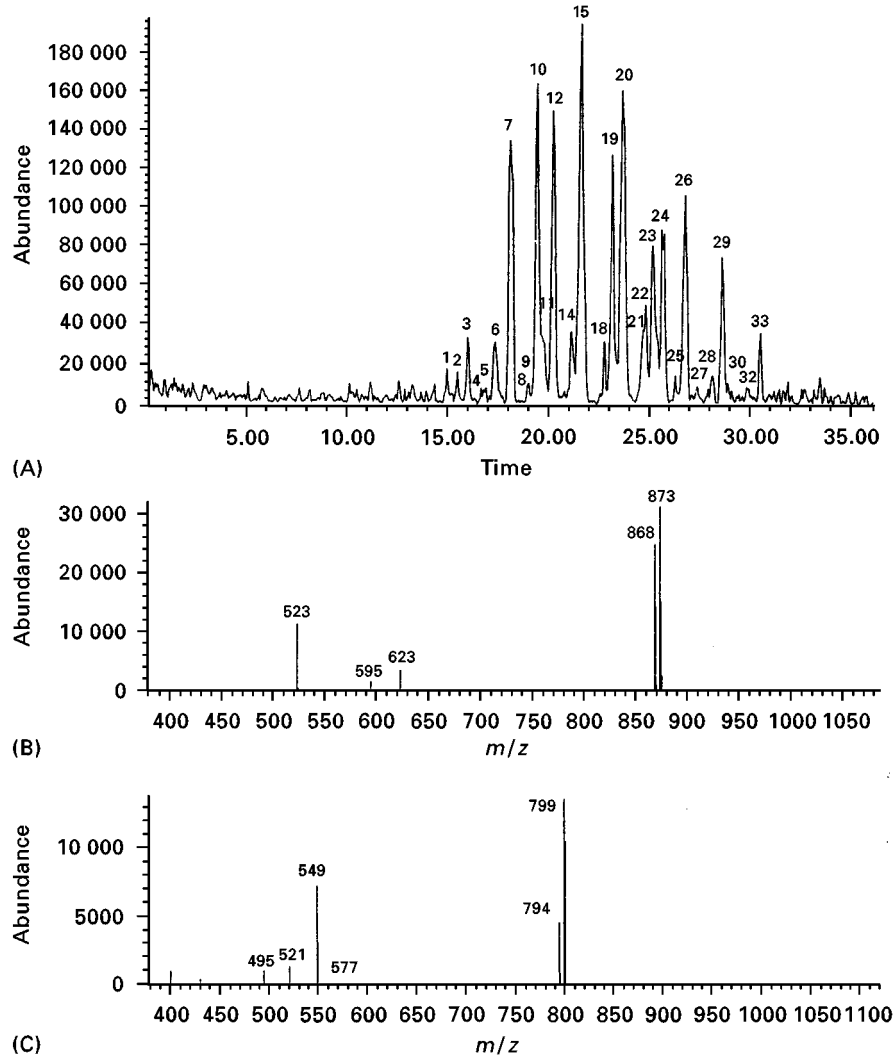

Figure 7 Reversed-phase HPLC of docosahexaenoic acid-rich oil with online ESI/MS. (A) Total positive ion current profile; (B) ESI-CID-MS of peak 20 (14 : 0/16 : 0/22 : 6): *m/z* 868, $[M + 18]^+$; *m/z* 873, $[M + 23]^+$; *m/z* 523, $[M\text{-RCOO}]^+$ (30 : 0 DG); *m/z* 595, $[M\text{-RCOO}]^+$ (36 : 6); *m/z* 623, $[M\text{-RCOO}]^+$ (38 : 6 DG); (C) ESI-CID-MS of peak 26 (14 : 0/14 : 0/18 : 1; 14 : 0/16 : 0/16 : 1): *m/z* 794, $[M + 18]^+$; *m/z* 799, $[M + 23]^+$; *m/z* 495, $[M\text{-RCOO}]^+$ (28 : 0 DG); *m/z* 521, $[M\text{-RCOO}]^+$ (30 : 1 DG); *m/z* 549, $[M\text{-RCOO}]^+$ (32 : 1 DG); *m/z* 577, $[M\text{-RCOO}]^+$ (34 : 1 DG). HPLC conditions: column, 5 μm Supelcosil LC-18 (250 × 4.6 mm i.d.); solvent, linear gradient of 20–80% isopropanol in acetonitrile in 30 min; ESI-CID-MS conditions, capillary exit voltage 215 V. (Reproduced with permission from Myher *et al.*, 1997.)

Chiral-phase HPLC

Chiral HPLC permits the separation of enantiomeric diacylglycerols derived from Grignard degradation or lipase hydrolysis. **Figure 14** shows the separation of the *sn*-1,2(2,3)-diacylglycerols derived from Grignard degradation of a complex triacylglycerol mixture containing 43% docosahexaenoic acid; there is excellent separation of the enantiomers. With this chiral phase, the *sn*-2,3-enantiomers emerge last. There is considerable resolution of molecular species, especially within the longer-retained *sn*-2,3-enantiomers. The *X*-1,3-isomers not removed by borate TLC emerge just ahead of, or overlap with, the *sn*-1,2-enantiomers. A chiral-phase LC-MS analysis of the 3,5-DNPU derivatives of the *sn*-1,2- and *sn*-2,3-diacylglycerols revealed the presence of a high proportion of species containing two long chain fatty acids per acylglycerol molecule, including 20 : 2–20 : 4 and 22 : 6–22 : 6.

Separation of Glycerophospholipids and Sphingomyelins

HPLC analysis of glycerophospholipids and sphingomyelins is usually performed with the total phos-

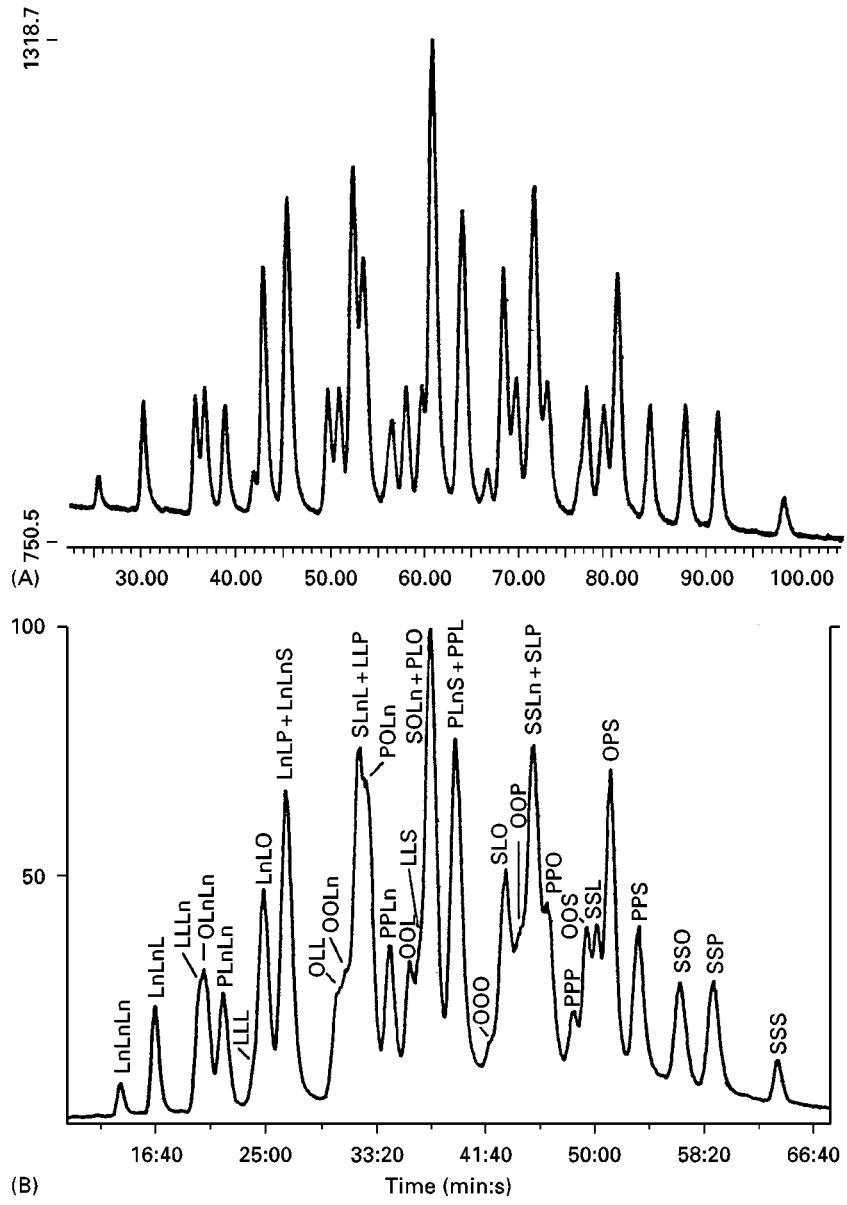

Figure 8 Reversed-phase HPLC separation of synthetic mixture of 36 triacylglycerols containing five randomly distributed fatty acids. Peaks are identified by component fatty acids: Ln, linolenic; L, linoleic; O, oleic, P, palmitic; S, stearic. HPLC conditions: column, 5 μm Adsorbosphere C_{18} (250 × 4.6 mm, i.d.) in series with 10 μm Adsorbosphere UHS C_{18} (250 × 4.6 mm); solvent, linear gradient of acetonitrile/dichloromethane 70 : 30 to 40 : 60, by vol, over 120 min; detector, FID. APCI conditions: initial acetonitrile/dichloromethane 65 : 35, v/v, followed by a 20–25 min linear gradient acetonitrile/dichloromethane 60 : 40, v/v, and held until 85 min. (Reproduced with permission from Byrdwell WC, Emken EA, Neff WE and Adlof RO (1996) Quantitative analysis of triglycerides using atmospheric pressure chemical ionization-mass-spectrometry. *Lipids* 31: 919–935.)

pholipid fraction recovered from the preliminary isolation of the lipid classes, unless it already involved the separation of the individual phospholipid classes. The purified phospholipid classes can be separated further into molecular species by HPLC using the original molecules or their enzymatic or chemical transformation products.

Normal-phase HPLC

Normal-phase HPLC is well suited for the separation of the phospholipids. Various silica gel columns yield excellent separations of the major phospholipid classes which, in many instances, also provide a readily discernible separation of the minor components.

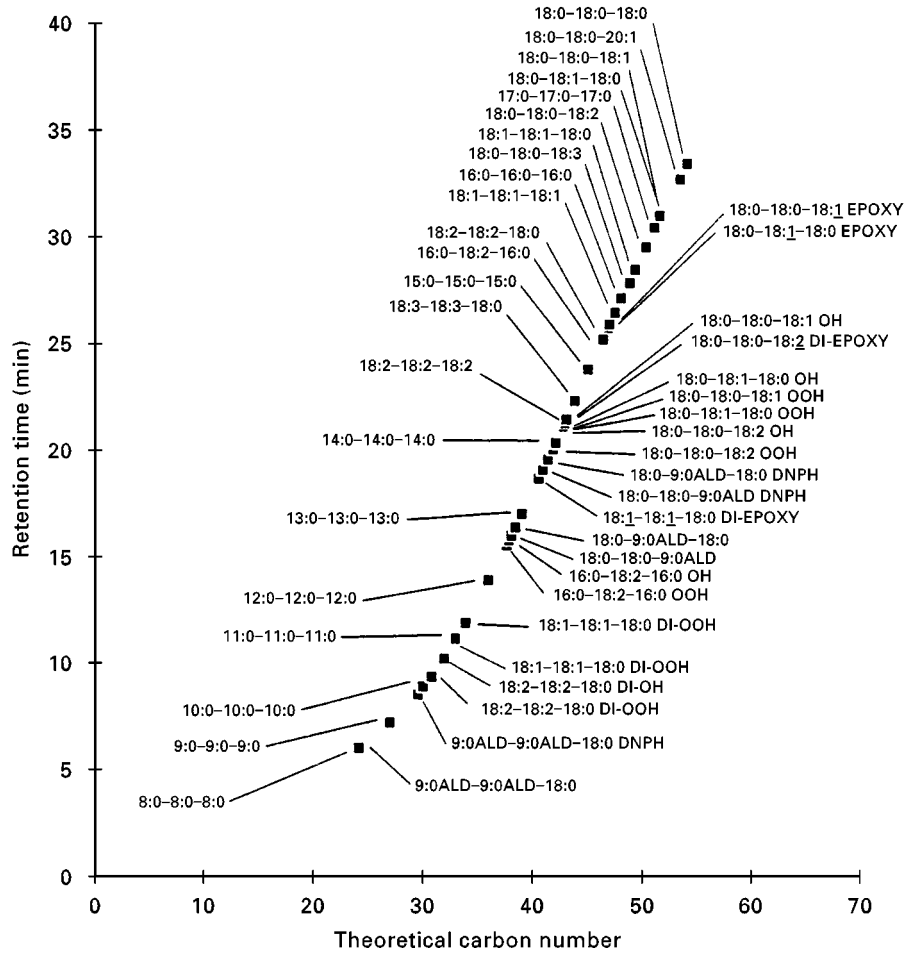

Figure 9 Plot of theoretical carbon numbers (TCN) versus retention times of reference oxo-triacylglycerols along with a series of saturated monoacid triacylglycerols. TCN and correction factors were calculated for oxotriacylglycerols and unsaturated triacylglycerols using the saturated triacylglycerols as a reference curve. (Reproduced with permission from Sjovall O *et al.*, 1997.)

Figure 15 shows the separation obtained with a silica gel column for the ethanolamine, choline, inositol and glycerol glycerophospholipids and sphingomyelin isolated from a subfraction of human high density lipoprotein preparation with online MS detection. In the positive ion mode only the choline-containing phospholipids are readily detected, although the ethanolamine glycerophospholipids can also be seen at low intensity. The acidic glycerol, inositol and serine phosphatides, along with any ethanolamine phospholipids, are best detected in the negative ion mode. The negative ion mode also registers the choline phospholipids as the chloride adducts. There is a complete baseline separation for all phospholipids without significant resolution of molecular species, except for SM, which is separated into long chain and short chain species.

Normal-phase HPLC with online MS can be used to assess the molecular species present in the individual phospholipid classes. It is possible to obtain single ion chromatograms retrieved from the total positive ion current spectra for the major molecular species of the choline and ethanolamine phosphatides. In this normal-phase system the newly identified glycated diradylglycerophosphoethanolamine migrates with the front of the phosphatidylcholine peak. The single ion chromatograms retrieved by the computer from the total negative ion current permit accurate quantification of the major molecular species of the acidic glycerophospholipids.

Normal-phase HPLC can be used for the separation of the alkylacyl, alkenylacyl and diacyl subclasses of the ethanolamine glycerophospholipids as the trinitrophenyl derivatives. The diradyl subclasses of the choline glycerophospholipids cannot be separated by chromatography of the intact parent molecules. For this purpose, diradylglycerophosphocholines must be dephosphorylated and the re-

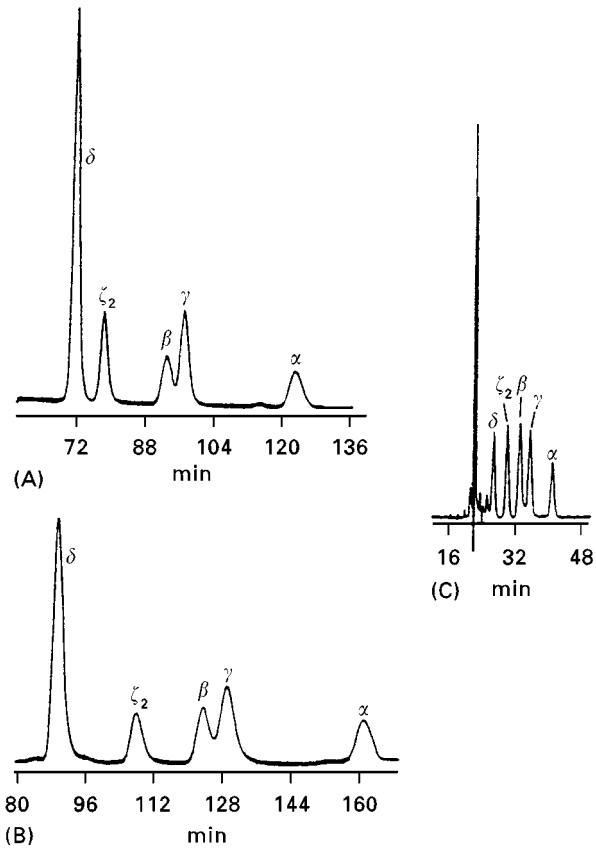

Figure 10 Reversed-phase HPLC separation of tocopherols. HPLC conditions: columns (A and B), 5 μm Asahipak containing octadecyl polyvinyl alcohol phase; column C, 5 μm Phenomenex Curosil-PFP phase (250 × 4.6 mm i.d.); solvents: (A) acetonitrile/water) (85 : 15, v/v); (B and C) methanol/water (87.5 : 12.5, by vol). (Reproduced with permission from Abidi and Mounts, 1997.)

sulting diradylglycerols converted into UV-absorbing or fluorescent derivatives (Table 2) prior to HPLC separation unless an ELSD system is used. The molecular species separation of the diradylglycerols is carried out by reversed-phase HPLC, as described for the diacylglycerols derived from triacylglycerols by Grignard degradation.

Reversed-phase HPLC

The total phospholipid mixture can also be separated on a reversed-phase column. Although this leads to extensive separation of the molecular species, there is little overlap among the different phospholipid classes. Both phospholipid classes and molecular species are readily identified and quantitated by online MS with ES ionization. Using 0.5% ammonium hydroxide in a water/methanol/hexane mixture on a C_{18} column, complex mixtures of phospholipid classes and molecular species were identified mainly as protonated or natriated molecules.

The molecular species of the underivatized phospholipids can be separated by reversed-phase HPLC with a mixture of organic solvents and a counterion. The molecular species of intact aminophospholipids have previously been resolved as the UV-absorbing trinitrophenyl derivatives. The reversed-phase systems are also capable of separating the hydroxylated and hydroperoxidized glycerophospholipids from their unoxidized parent species.

Chiral-phase HPLC

The stereochemical configuration of phosphatidylglycerols has been assessed with chiral phases. Although natural phosphatidylglycerols possess two chiral carbons and are diastereoisomers, they are not readily separable by normal-phase columns. However, the bis-3,5-dinitrophenylurethanes can be separated by chiral-phase HPLC (**Figure 16**). The molecular species of all synthetic phosphatidylglycerol derivatives examined can be separated into diastereomeric peaks in a short time using a mobile phase of hexane/dichloromethane/methanol containing a small amount of trifluorocetic acid.

Separation of Sphingolipids and Gangliosides

The neutral glycosphingolipids or cerebrosides are ceramide monohexosides, lactosides and higher sugar glycosides. The great complexity and number of new glycosphingolipid components being reported challenge the best contemporary methods of characterization. These lipids have been frequently investigated by FAB, chemical ionization and electron ionization MS with prior chromatographic separation. Likewise, the sulfatides and the sialic acid-containing glycosphingolipids (gangliosides) have been separated by HPLC prior to MS.

Normal-phase HPLC

A highly sensitive analytical method that allows the separation of ganglioside mixtures and quantification of individual nonderivatized gangliosides has been reported using Spherisorb-NH_2. Gangliosides in the 2 pmol to 1 nmol range are separated on a 1 mm i.d. column with a gradient of acetonitrile/phosphate buffer. **Figure 17** shows the resolution of standard gangliosides and gangliosides from the serum of a healthy human female and from human oligodendroglioma. Complete separations are obtained for GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b and GQ1b. The

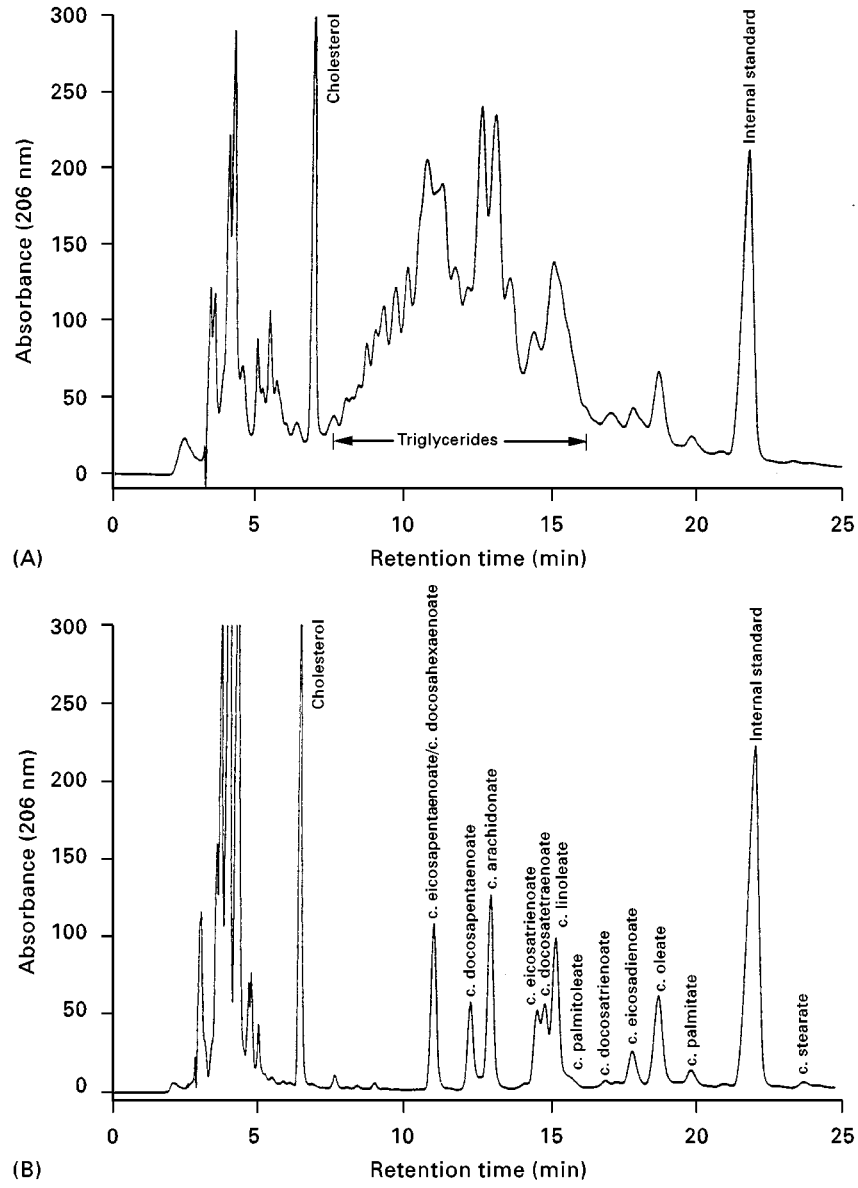

Figure 11 Reversed-phase HPLC of cholesteryl esters isolated from macrophages by lipid extraction and treatment of the extract with a dilute solution of ethanolic potassium hydroxide. Peak identification is given in the figure. HPLC conditions: column, 3 μm Spherisorb ODS2 (250 × 4 mm, i.d.); isocratic solvent, isopropanol/heptane/acetonitrile (35/12/52, by volume) and detected by UV absorption at 206 nm. (Reproduced with permission from Cullen P, Fobker M, Teglkamp K *et al.* (1997). An improved method for quantification of cholesterol and cholesteryl esters in human monocyte-derived macrophages by high performance liquid chromatography with identification of unassigned cholesteryl ester species by means of secondary ion mass spectrometry. *Journal of Lipid Research* 38: 401–409.)

new method of separation bypasses the earlier difficulties regarding baseline stability of the 195 nm absorption by using a high purity phosphate buffer.

Other workers have employed both FAB-MS and ES-MS to characterize monosialogangliosides of human myelogenous leukaemia HL60 cells and normal human leukocytes. The gangliosides were extracted and subjected to extensive segregation and examination of the selectin-binding ability of each fraction. Fractions were resolved on an Iatrobead column pre-equilibrated with isopropyl alcohol/hexane/water (55 : 40 : 5) and subjected to a linear gradient of isopropyl alcohol/hexane/water 55 : 40 : 5 to 55 : 25 : 20 with a flow rate of 1 mL min^{-1}. They were also reanalysed on a semipreparative Iatrobead column with a linear gradient of isopropyl alcohol/hexane/water 55 : 40 : 5 to 55 : 25 : 20 over

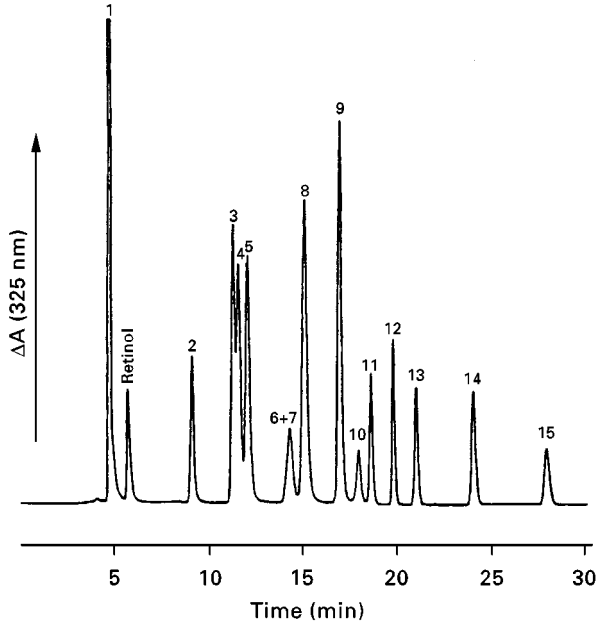

Figure 12 Reversed-phase HPLC of retinyl esters. Peak identification: 1, acetate; 2, caprate; 3, linolenate; 4, laurate; 5, arachidonate; 6/7, palmitoleate/linoleate; 8, myristate; 9, pentadecanoate; 10, oleate; 11, palmitate; 12, heptadecanoate; 13, stearate; 14, arachidate; 15, behenate. HPLC conditions: column, 5 μm Suplex pKb 100 (250 × 4.6 mm) with a 20 mm guard column; solvent, isocratic elution (14 min) with solvent A (acetonitrile/methanol/dichloromethane/hexane, 88 : 4 : 4 : 4, by volume) at 1 mL min^{-1} followed by a linear gradient of 100% B (acetonitrile/methanol/dicholoromethane/hexane, 70 : 10 : 10 : 10, by volume) over a 2 min period; isocratic elution with the final solvent composition continued for 14 min at 1.5 mL min^{-1}. Detection was at 325 nm. (Reproduced with permission from Wingerath T, Kirsch D, Spengler B *et al.* (1997) High performance liquid chromatography and laser desorption/ionization mass spectrometry of retinyl esters. *Analytical Chemistry* 69: 3855–3860.)

200 min with a flow rate of 0.5 mL min^{-1}. The final fractions from the normal-phase HPLC were analysed by TLC and the pure components subjected to negative and positive ion FAB-MS.

Table 2 Selected derivatives of diacyl and monoacylglycerols for UV and fluorescent detection

UV absorption	*Fluorescent detection*
Anthroyl derivatives	
Benzoates	
Dinitrobenzoates	
3,5-Dinitrophenylurethanes	Phenylurethanes
Naphthylethylurethanes	Naphthylurethanes
p-Nitrobenzoates	Anthroylurethanes
Pentafluorobenzoates	

Modified with permission from Bell, 1997.

Table 3 Selected ester derivatives for UV and fluorescent detection of fatty acids

UV detection	*Fluorescent detection*
Anthrylmethyl	9-Anthrylmethyl
Benzyl	9-Aminophenanthrene
p-Bromophenacyl	4-Bromomethyl-7-acetoxycoumarin
p-Chlorophenacyl	9-Anthryldiazomethane
2-Naphthacyl	Dansyl-ethanolamine
p-Nitroanilides	4-Methyl-7-methoxycoumarin
p-Nitrobenzyl	4-Methyl-6,7-dimethoxycoumarin
p-Nitrophenacyl	4-Methyl-7-acetoxycoumarin
Pentafluorobenzyl	2-Naphthacyl
Phenacyl	
p-Phenphenacyl	

Modified with permission from Purdon, 1991.

Reversed-phase HPLC

Of the sphingolipids, the ceramides, cerebrosides and sphingomyelins have been most extensively studied by reversed-phase HPLC. Sphingomyelins obtained from bovine brain, chicken egg yolk and bovine milk fat were separated using a binary solvent system consisting of *n*-butanol/water isopropanol/isooctane on a C_{18} column. The positive ion mass spectra exhibit prominent ions related to the amine base structure and fragments which can be utilized for identification of molecular species.

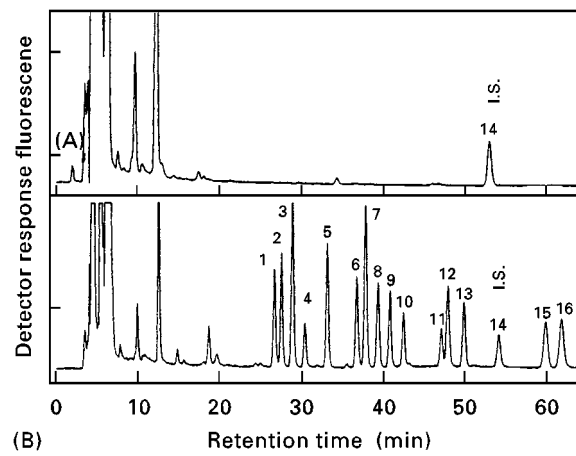

Figure 13 Reversed-phase separation of fatty acids as the 1-fluorescent pyrenyldiazomethane derivatives. Peak identification; 1, 20 : 5*n* − 3; 2, 14 : 1*n* − 9; 3, 18 : 3*n* − 3/18 : 3*n* − 6; 4, 22 : 6*n* − 3; 5, 20 : 4*n* − 6; 6, 14 : 0/16 : 2*n* − 9; 7, 18 : 2*n* − 6; 8, 20 : 3*n* − 6; 9, 22 : 4*n* − 6; 10, 24 : 5*n* − 6; 11, 18 : 1*n* − 9; 12, 16 : 0; 13, 24 : 4*n* − 6; 14, internal standard; 15, 20 : 1*n* − 9; 16, 18 : 0. HPLC conditions: column, 5 μm LC-18 Supelcosil (250 × 4.6 mm i.d.) with a Pelliguard precolumn (4.6 × 20 mm) from Supelco; solvent: a gradient between water (solvent I) and acetonitrile (solvent II) was used as follows: 0–40 min, 90–100% solvent II and 40–70 min, isocratic 100% II at a flow rate of 1 mL min^{-1}. (Reproduced with permission from Brekke *et al.*, 1997.)

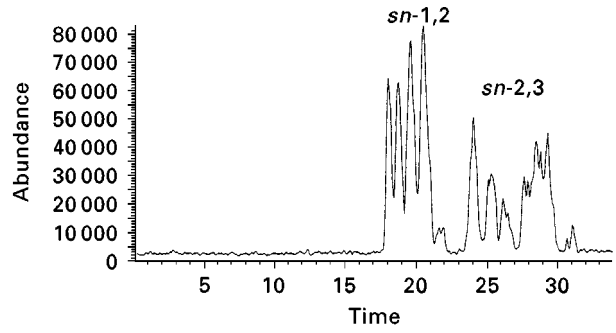

Figure 14 Chiral-phase HPLC of the dinitrophenyl urethane derivatives of the diacylglycerols from an oil rich in docosahexaenoic acid. *Sn*-1-2-, *sn*-1,2-diacylglycerols; *sn*-2,3-, *sn*-2,3-diacylglycerols. HPLC conditions: chiral column, 25 cm × 4.6 mm i.d. tube containing R-(+)-1-(1-naphthyl)-ethylamine polymeric phase chemically bonded to 30 nm wide-pore spherical silica (YMC-pack A-KO_3); solvent: isocratic hexane/dichloromethane/ethanol 40 : 10 : 1 by volume, at 0.5 mL mim^{-1}; UV detector at 254 nm. (Reproduced with permission from Myher JJ, Kuksis A and Park PW (1996) Stereospecific analysis of docosahexaenoic acid-rich triacylglycerols by chiral-phase HPLC with on-line electrospray mass spectrometry. In: McDonald RE and Mossoba MM (eds) *New Techniques and Applications in Lipid Analysis*, pp. 100–120. Champaign, IL: American Oil Chemists' Society.)

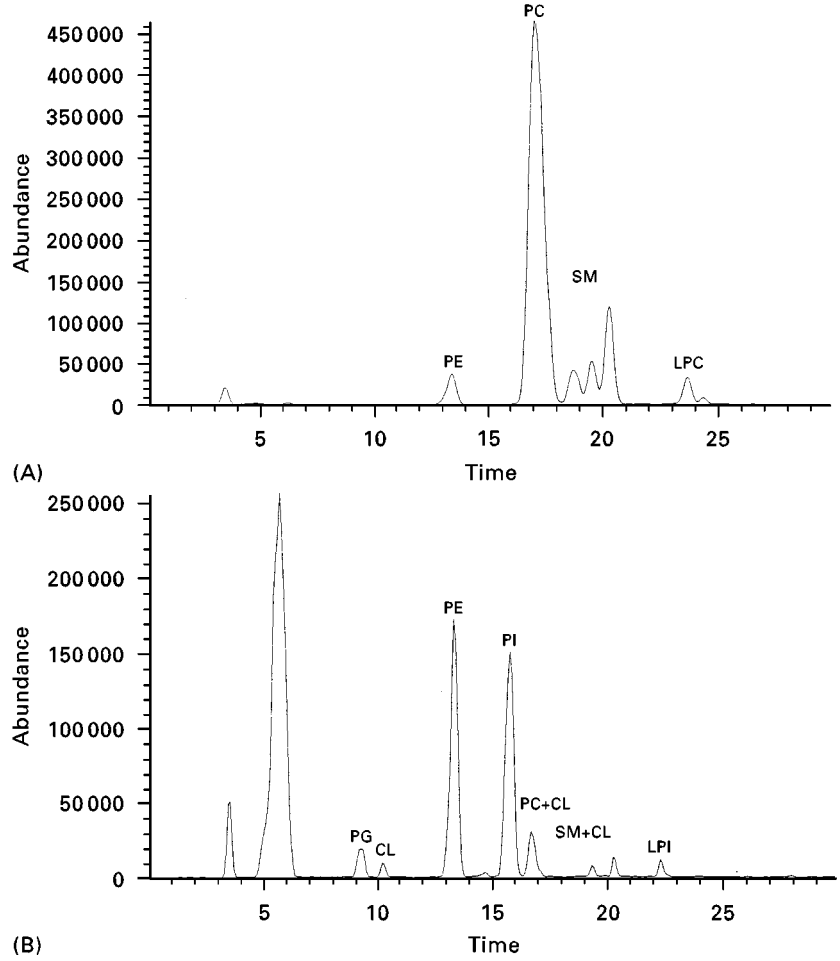

Figure 15 Normal-phase HPLC resolution of high density lipoprotein glycerophospholipids and sphingomyelins in (A) the positive and (B) negative ion mode as recorded by online electrospray mass spectrometry. Peak identification is as given in Figure 1; PAF, platelet-activating factor. HPLC conditions: column, 5 μm Spherisorb (250 × 4.6 mm i.d.); solvent, a linear gradient of 100% A (chloroform/methanol/30% ammonium hydroxide 80 : 19.5 : 0.5, by volume) to 100% B (chloroform/methanol/water/30% ammonium hydroxide 60 : 34 : 5.5 : 0.5, by volume) in 30 min. (Unpublished results of Kuksis A and Ravandi A, 1997.)

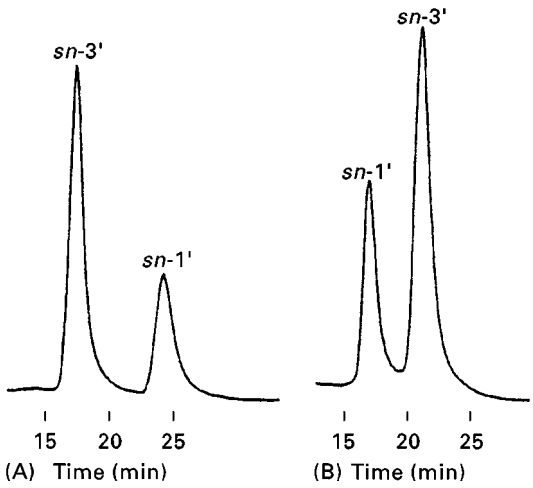

Figure 16 Chiral-phase HPLC resolution of the bis-3,5-dinitrophenylurethane derivatives of the diastereomeric 1,2-dilinoleoyl-*sn*-3-phospho-1′-*sn*-glycerol (*sn*-1′) and 1,2-dilinoleoyl-*sn*-glycero-3-phospho-3′-*sn*-glycerol (*sn*-3) on liquid phases of opposite configuration. (A) (*R*)-(+)-1-(1-naphthyl)-ethylamine column (YMC A-KO3); (B) (*S*)-(−)-1-(1-naphthyl)ethylamine column (YMC A-LO3); solvent, hexane/dichloromethane/methanol/trifluoroacetic acid (60/20/20/0.2, by volume) at 1.0 mL min^{-1}; column temperature 10°C. (Reproduced with permission from Itabashi and Kuksis, 1997.)

See also: **II/Chromatography: Liquid:** Derivatization; Detectors: Mass Spectrometry; Detectors: Ultraviolet and Visible Detection; Mechanisms: Chiral; Mechanisms: Normal Phase. **III/Lipids:** Thin-Layer (Planar) Chromatography. **Silver Ion:** Liquid Chromatography; Thin-Layer Planar Chromatography.

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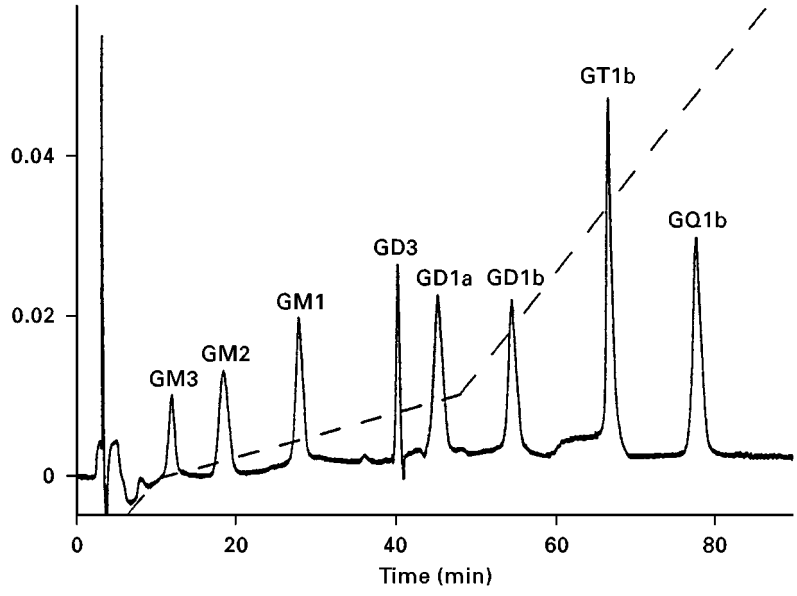

Figure 17 Normal-phase HPLC of standard gangliosides. Peak identification: GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b and GQ1b denote gangliosides according to Svennerholm. HPLC conditions: column, microbore 3 μm, Spherisorb-NH_2 (250 × 1 mm i.d.). A guard column (1 × 20 mm) was filled with the same material; solvent gradient: as indicated by dashed lines; it starts with 100% A, 0% B, and ends with 0% A, 100% B. Solvent A, acetonitrile/5 mmol L^{-1} phosphate buffer, pH 5.6 (83 : 17, v/v); solvent B: acetonitrile/20 mmol L^{-1} phosphate buffer, pH 5.6 (1 : 1, v/v) at 88 μL min^{-1} at 20°C. (Reproduced with permission from Wagener R, Kobbe B and Stoffel W (1996) Quantification of gangliosides by microbore high performance liquid chromatography. *Journal of Lipid Research* 37: 1823–1829.)

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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer chromatography (TLC) is widely used for the separation and identification of lipid classes with silica gel as the most frequently used stationary phase. Numerous mobile phases (solvent systems) are available for the separation of lipids and there are many nonspecific and specific detection reagents (visualization reagents) that are useful for detection.

There is no consensus as to the definition of a lipid. Kates considers lipids as compounds generally insoluble in water but soluble in a variety of organic solvents. He recognized the following classes of lipids: hydrocarbons, alcohols, aldehydes, fatty acids and derivatives such as glycerides, wax esters, phospholipids, glycolipids and sulfolipids. Gunstone and