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REVIEW

LIPIDS AND THEIR CONSTITUENTS

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1. INTRODUCTION

Chromatography of lipids is aimed at the resolution of lipid classes and molecular species for the ultimate purpose of complete identification and quantitation of all components. In the course of chromatographic studies, it has become apparent that many tissues, cells and subcellular components possess characteristic lipid compositions that can be recognized without full resolution and quantitation of individual molecular species. Quantitative estimation of a partially resolved lipid profile is frequently sufficient for establishing the origin of the total lipid extract of a tissue or body fluid and for assessing its relationship to normal or abnormal metabolic states. Likewise, the diagnosis and therapeutic management of disorders of lipid metabolism require detailed yet simple analytical procedures for the determination of plasma lipoprotein lipid concentrations. These requirements have led to the development of several specific analytical routines and to the adaptation of other more general chromatographic procedures.

One of the first and most successful methods of rapid profiling of lipids of body fluids and tissues was provided by high temperature gas chromatography (GC), which became possible [1] as a result of the discovery [2] that short packed columns containing non-polar liquid phases can be temperature programmed to provide simultaneous resolution and quantitation of neutral lipid molecules of both low and high molecular weight. The method was subsequently combined with argentation thin-layer chromatography (TLC) to provide resolutions beyond molecular weight or carbon numbers [3]. It was eventually extended to include glycerophospholipids and sphingomyelins following a pretreatment of the sample with phospholipase C, which converts the glycerophospholipids into the corresponding diacylglycerols and the sphingomyelins into the corresponding ceramides [4]. Recently, other methods of lipid profiling of body fluids and tissues have been developed by combining TLC on quartz rods with flame ionization detection (FID) [5] and by reversed-phase high-performance liquid chromatography (HPLC) [6]. In order to improve the identification of the components resolved, mass spectrometry along with gas chromatography (GC—MS) [7,8] and liquid chromatography (LC—MS) [9] have been explored. These later approaches have provided essentially complete assessments of all molecular species in the sample and thus have constituted the ultimate in the profiling of lipids. In the following we have attempted to present an accurate account of the state of the art in the area along with an indication of the various alternatives available for specific practical applications. Detailed analysis of the composition of molecular species of individual lipid classes are beyond the scope of this review.

2. GENERAL EXPERIMENTAL PROCEDURES

Lipid analysis usually involves a chromatographic separation, identification and quantitation of the sample components. As a result, essentially identical routines are employed for the handling of most lipid extracts. These methods have been discussed in great detail in a recent review [10] and only the simplified routines employed in lipid profiling have been included here along with appropriate updating.

2.1. Preparation of standards

Each method of lipid profiling requires the preparation of appropriate analytical standards. The exact standard composition depends on the type of body fluid or tissue to be assayed and to some extent on the method of chromatography used. Neutral lipid and free fatty acid mixtures in proportions comparable to those found in plasma, for example, are prepared by weighing out a minimum of 10 mg of each lipid class and dissolving it in 100 ml methylene chloride in a screw-cap volumetric flask. Appropriate volumes of not less than 1 ml each are combined to give the desired proportions of components [11]. The absolute concentration of the lipid mixture for GC analyses ranging from 0.1—100 μg/μl of each component may be reached by further dilution and/or concentration of the combined mixture. Mixtures containing compounds with free hydroxyl or carboxyl groups may be evaporated to dryness and taken up in an appropriate silylating reagent (150–250 μl). These standards are suitable for the qualitative identification of the peaks in the chromatograms of the unknowns and for the determination of the relative quantitative response (recovery) of the components in the chromatographic detector. Standards for absolute determination of the system are prepared separately and are added to the sample prior to extraction in a known amount. For example, the tridecanoylglycerol internal standard used for the quantitation of plasma total lipid profiles by GC is prepared by weighing out 100 mg of the pure triacylglycerol and diluting it with 1000 ml of chloroform to give a concentration of 100 μg/ml [12]. This standard is added in a volume of 100–200 μl to 0.25—0.5 ml of plasma during the lipid extraction. It is detected in a lipid profile of a normal plasma as a peak making up a minimum of 10% of the total peak area. Similar methods are used to prepare analytical standards for TLC [13] and HPLC [14] determination of the lipid profiles.

2.2. Preparation of sample

Very few methods allow the direct introduction of the original body fluid or tissue sample into the analytical instrument. At least a preliminary solvent extract must be prepared, but both chemical and biochemical transformations may also be necessary before the lipid sample is suitable for effective resolution and quantitation. The actual method of the preliminary work-up necessary depends on the method of analysis [10,15].

2.2.1. Extraction

Manual solvent extracts of 0.5—1.0 ml aliquots of plasma or serum or of

an equivalent amount of lipid from lymph, milk and tissues or cells are prepared in twenty volumes of chloroform—methanol (2:1). The lipid extract is washed with 0.9% potassium chloride as described by Folch et al. [16]. The organic phase is backwashed with distilled water and evaporated to dryness under nitrogen at 40°C. The lipid extracts are diluted to volume in chloroform and stored at −20°C. A similar procedure is used for the extraction of tissues, except that the blotted and weighed tissue is homogenized in the chloroform–methanol extracting solvent. However, losses of water-soluble lipids and the potential for transesterification and chemical alteration inherent in the solvent extraction methods remain.

2.2.2. Dephosphorylation

In order to include the polar glycerophospholipids in the determination of the total lipid profiles by GC, the polar head groups must be removed. This can be accomplished by hydrolysis of the total lipid extract with phospholipase C (either from *Clostridium perfringens* or from *Bacillus cereus*). For this purpose the lipid sample (up to 0.5 mg of lipid) is dissolved in 1.5 ml of peroxide-free diethyl ether in a 9-ml screw-cap vial and 1.5 ml of buffer [17.5 m*M* tris-(hydroxymethyl)-aminomethane adjusted to pH 7.4, containing 1.0 m*M* calcium chloride] is added, along with 10 U of the enzyme [17]. The mixture is agitated using a vortex mixer for 10 s and then shaken mechanically at 37°C for upto 1—2 h, depending on the nature of the phospholipid classes present. The released diacylglycerols and ceramides along with the unchanged triacylglycerols, cholesteryl esters and any other neutral lipids are recovered by extracting twice with 3 ml of diethyl ether each time. The extracts are washed with water and evaporated to dryness [17]. Whole ethylenediaminetetraacetate (EDTA) (0.01%) plasma or serum (0.1—1.0 ml) is digested in a screw-cap centrifuge tube (18 ml capacity) containing 2—4 U phospholipase C in 4 ml of 17.5 m*M* Tris buffer, pH 7.3 along with 1.3 ml of 1% calcium chloride and 1 ml of diethyl ether, and the mixture is incubated with shaking for 2 h at 30°C [11]. The reaction mixture is then treated with five drops of 0.1 *M* hydrochloric acid and extracted once with 10 ml of chloroform—methanol (2:1) containing 100—250 μg tridecanoylglycerol. The solvent phases are separated by centrifuging for 10 min at 200 *g*. The clear chloroform phase is removed from the bottom of the tube and is dried by passing through a Pasteur pipet containing 2 g of anhydrous sodium sulfate. The effluent is evaporated under nitrogen and the residue diluted to volume with chloroform. Individual lipoprotein fractions prepared in EDTA require the addition of extra calcium for complete digestion [18]. The final lipid extracts are suitable for HPLC and TLC in the underivatized form, but for GC analyses it is necessary to provide the free hydroxyl and carboxyl functions with appropriate protective groups. Further general methods for the dephosphorylation of phospholipids by phospholipase C have been given by others [19].

2.2.3. Derivatization

The most suitable derivatives for the analysis of lipid extracts have proved to be the trimethylsilyl (TMS) or *tert.*-butyldimethylsilyl (t-BDMS) ethers. The latter derivatives are also suitable for TLC and HPLC and they offer special

advantages for GC—MS and LC—MS. The derivatization is performed by evaporating an aliquot of the lipid extract to dryness under nitrogen, diluting it with the silylation mixture (150—250 μl) in a screw-cap vial and sealing the vial. The trimethylsilylation is effected with either hexamethylenedisilazane—trimethylchlorosilane—pyridine (12:5:2) or TRISIL—bis(trimethylsilyl)-acetamide (Pierce Chemical Co.) [4]. The t-BDMS ethers are prepared by reacting the lipid extracts with *tert.*-butyldimethylchlorosilane—imidazole reagent at 80°C for 20 min [8]. In addition to the silylation, acetylation of the total lipid extracts before chromatographic profiling has also given satisfactory results [4]. Acetylation, however, does not convert the carboxyl groups into chromatographically inert derivatives. The preparation of benzoates [20], which absorb in the UV, and of α-naphthylisocyanates [21], which fluoresce, is of interest for the HPLC detection of lipid molecules with free hydroxyl functions.

2.3. Determination of lipid profiles

Essentially all chromatographic techniques are suitable for this purpose, but those which combine rapid resolution with effective quantitation are preferable. The various applications of this approach range from a recording of total lipid profiles of plasma and whole tissues to the characterization of the lipid composition of purified lipoproteins of specific cell membranes.

2.3.1. TLC and TLC—GC

The most general technique for obtaining a qualitative profile of a total lipid extract is TLC [10,15]. Usually the polar and non-polar lipids are profiled separately using appropriate solvent systems. However, in most polar solvent systems the neutral lipids run to the top of the plate as a single band and may be quantitated in toto along with the resolved polar lipid classes. Likewise, the total polar lipids may be estimated from the material left at the origin during a neutral lipid separation. The zones in the chromatograms are located by iodine staining and then quantitated by chemical analyses. The determinations can be performed by converting the phospholipids into inorganic phosphorus following recovery of the lipids from the plate [22] or in the presence of the silica gel [23]. Charring of lipids followed by densitometry, is widely practised [24,25], but both steps require standardization [10]. Bitman et al. [26] have developed a routine TLC analysis of lipid classes suitable for in situ quantitation by densitometry. Prior to charring the plate is dipped into a solution of 3% cupric acetate in 8% phosphoric acid for 3 s. Segura and Gotto [27] have described a procedure for the detection and quantitation of organic compounds on TLC plates after inducing their transformation into fluorescent derivatives by thermal treatment of the chromatoplate in the presence of ammonium hydrogen carbonate. More recently, Segura and Navarro [28] have observed that heating the chromatoplates in the presence of silicon chloride induces the fluorescent derivative formation of all classes of compounds much more reproducibly and gives fluorophores that are stable for months. Despite much ingenuity, effective quantitation of all lipid fractions by ordinary TLC has not been entirely satisfactory. This is partly due to a variability in the lipid class

resolution and partly to the variability in response of different lipid classes to charring. Although high-performance thin-layer chromatography (HPTLC) has provided much improved resolution of the lipid classes, the problem of quantitation has not been solved [29].

The most successful method of quantitating the lipid components involves TLC on sintered quartz rods, which can be passed through a hydrogen flame ionization detector [30—33]. This represents a major development in TLC and corrects a basic shortcoming in this analytical system. The method, now commercialized under the name Iatroscan TLC/FID system, combines the technique of TLC with automated quantitative detection, based on the flame-ionization principle employed in GC. Most separations currently performed by conventional TLC can be duplicated on the patented Chromarods, and the FID is applicable to all lipids [31]. A rotary method of sample application has been developed by Read [34] to improve inter-rod and inter-run repeatability. The resolution of neutral and glycerophospholipids on the basis of their degree of unsaturation [35] further expands the total lipid profile. Sample loads ranging from 2 to 20 μg in 1—2 μl of solution are used. These quantities are up to 1000-fold less than those that can be applied and resolved on an ordinary 20×20 cm TLC plate. The Chromarod TLC has become especially attractive for profiling the polar lipid classes, which cannot be readily quantitated by GC or HPLC.

Transmethylation of the various fatty esters in presence of gel scrapings, followed by quantitative GC analysis of the fatty acid methyl esters, although time-consuming, is also a satisfactory method for determination of the resolved lipid ester classes [36,37]. This technique, however, has the advantage of providing a quantitative account of the fatty acid composition of the resolved components, which the FID on Chromarods does not give. The identification and quantitation of the fatty acids greatly extends the usefulness of the lipid profile. The glycerolipid classes resolved by TLC may also be quantitated by GC of the diacylglycerol moieties released by phospholipase C [38,39]. This approach, when combined with parallel analyses of the fatty acids, yields extensive structural information about the resolved lipid classes, exceeding the usual needs of quantitative lipid profiling of body fluids and tissues. This method of GC quantitation of lipid classes can be extended to the sphingomyelins, which yield ceramides upon phospholipase C treatment [4,40]. The quantitation of the lipid classes as the diacylglycerols and ceramides has the advantage of avoiding contamination with other lipids, which is a problem during quantitation of minor glycerolipid components by transmethylation.

2.3.2. GC and GC—MS

A procedure that is well suited for a qualitative and quantitative assessment of total lipid extracts is high temperature GC. It so happens that the common natural free fatty acids and their mono-, di-, and tri-esters of glycerol differ greatly in molecular weight from each other, from free cholesterol, and from the fatty acid esters of cholesterol [41]. Because the TMS group has little effect on the elution time of the derivatized components in this GC system, effective resolution of the neutral lipids may be obtained on the basis of molecular weight even after trimethylsilylation of the hydroxyl and carboxyl groups [4,41].

The first successful GC separations of total lipid extracts were achieved on the short, non-polar siloxane columns originally described for work with natural triacylglycerols [2]. Although packed columns of greater length give higher resolution [42], the sharply decreased recoveries of the higher molecular weight components make them impractical for quantitative profiling of total lipid extracts. Capillary columns also have been adopted for this purpose [43, 44]. The best separations and nearly quantitative recoveries have been obtained with capillary columns, 5—10 m in length, coated with methylsiloxane and operated with hydrogen as carrier gas. Under these conditions effective baseline resolution is achieved for molecular species of glycerolipids differing by one methylene unit and between certain saturated and unsaturated homologues. On non-polar 15-m columns, Grob et al. [45] have reported a partial degradation of long-chain triacylglycerols, while on polar 15-m fused-silica columns, Smith [46] has noted hydrogenation of the cholesteryl esters when hydrogen is used as a carrier gas. Geeraert et al. [47] have described a movable on-column injection device in which the injection port is cooled outside the oven during injection. This permits injections at high oven temperatures, simplifying routine capillary GC analysis of high boiling compounds.

Recently, Geeraert and Sandra [48,49] have described capillary columns, which contain a polar liquid phase (a methylphenylsilicone polymer) and possess high temperature stability comparable to that of the methyl silicone polymers. Using such columns it has been possible to obtain triacylglycerol profiles, which, in addition to resolution by carbon number, provide separations based on the degree of unsaturation of the molecular species within a carbon number [50]. The latter columns potentially could provide a complete resolution of all molecular species of glycerolipids, ceramides and cholesteryl esters. The quantitative recoveries of the higher molecular weight components, however, have not been evaluated, and it is possible that extensive calibration of the system is necessary for quantitative lipid profiling. Earlier, Myher and Kuksis [51,52] had described essentially complete resolution of the molecular species of the diacyl-, alkylacyl- and alkenylacyl-glycerol moieties of glycerophospholipids and of triacylglycerols on polar capillary columns prepared with a cyanopropylphenylsiloxane polymer.

All of the above GC systems can be directly interfaced with a mass spectrometer for complete peak identification and quantitation of any components unresolved on the GC column. The original GC—MS analyses involved the use of packed columns with molecular gas separators, which caused difficulties in the recovery of the higher molecular weight components. Capillary columns can be introduced directly into the ion source without the use of molecular separators. The use of hydrogen as a carrier gas in capillary GC makes hydrogen chemical ionization possible for many capillary GC—MS applications [52].

Both capillary GC [53] and GC—MS [54] of fatty acid methyl esters have been recently proposed for the profiling of lipid extracts of tissues and cells. Separation of individual components of fatty acid families (*n*-3, *n*-6 and *n*-9) can be usually achieved. Fatty acid analyses on packed GC columns have been employed extensively in the past, but have given rather limited information.

2.3.3. HPLC and LC—MS

The early work on lipid profiling of body fluids and tissues was performed by column chromatography on silicic acid [55] or similar adsorbents [56]. The separations were based on the polarity of the neutral lipid classes with the phospholipids usually being recovered as a single final fraction. A reversed-phase system for the liquid—liquid chromatographic profiling of total lipid extracts was described by Hirsch [57]. This method allowed the separation of lipid classes and molecular species on the basis of their partition numbers. Although fractions were detected and quantitated by automated refractometry, the elution required much time, the columns were difficult to operate and the results frequently impossible to reproduce. Modern HPLC systems for both adsorption and reversed-phase chromatography of lipid extracts now provide ideal means of profiling most body fluids and tissues. Thus far, however, there have been few efforts in this direction. The difficulty is related to the absence of effective methods for rapid and sensitive quantitation of the resolved components. Lipids in general do not possess strong absorption in the longer ranges of UV light, while the absorptivities in the shorter ranges have been found to be not sufficiently universal and sensitive. Furthermore, few solvent systems are compatible with detection in the short wave UV. Those lipid classes that can be converted into UV-absorbing [20] or fluorescent [21] derivatives, have permitted highly sensitive detection. Therefore, much effort has been expended in the development of other detectors. Privett and Erdahl [58] have proposed a combination of HPLC separation of total lipid extracts with MS identification and quantitation of peaks, followed by computerized evaluation of the data. The interface system for coupling HPLC to the mass spectrometer is based on the endless-chain principle in which a belt of special construction is used as the transport device. After removal of the solvent the sample is converted to hydrocarbons, if it is not volatile, and introduced into the source of the mass spectrometer by a carrier gas. The system was tested with model compounds of common lipid classes and found to be practical. Kuksis et al. [9] have employed HPLC with direct liquid inlet chemical ionization MS, which avoids the difficulties associated with the use of the transport chain. More recently, Phillips et al. [59] have reported HPLC analyses of glycerolipids via hydrogen FID. The peaks were automatically recorded using FID in combination with a transport by the endless chain described previously [58].

The recent introduction of the mass detector [60] for quantitative detection of lipids in HPLC effluents promises to revolutionize lipid profiling by HPLC methods. This method is based on light scattering. The eluate from a chromatographic column is atomized with the aid of air under pressure, and, as the fine mist of lipid droplets progresses down a heated stack, solvent is evaporated. The droplets of particles then pass through a beam of light and the light is scattered and detected as a measure of amount of material present. Robinson et al. [61] have found the detection limits to be less than 1 μg for non-volatile, saturated triacylglycerols, by direct injection, with the reproducibility of responses being about 6% with isocratic elution and 16% with gradient elution. However, the detector response is non-linear for triacylglycerols and probably other lipid classes. The sensitivity is affected by evaporation temperature and

atomiser inlet air pressure within the detector, and by the mobile phase flow-rate and its composition [61]. Although this latter technique is relatively sensitive, it is not adequate for monitoring the narrow-bore HPLC columns, which offer certain advantages for lipid profiling of small samples of body fluids and tissues. These effluents can be detected by MS [62,63].

HPLC on both conventional and narrow-bore columns can be combined with MS, for both detection and identification of the resolved components. LC—MS combinations utilizing direct liquid inlet interfaces [9,64] have proven to be especially efficient. The most extensive resolutions are obtained by reversed-phase HPLC. Direct liquid inlet chemical ionization MS serves both as a sensitive detector of all lipid species and provides structural information for identification purposes. Using the direct liquid inlet interface in combination with conventional HPLC columns about 1% of the column effluent is admitted to the mass spectrometer, which greatly reduces the sensitivity of detection of minor components [9,65]. A higher proportion of the effluent may be admitted to the ion source when using narrower bore columns, but a make-up solvent or make-up gas [66] is needed to help the transfer of the column effluent to the ion source.

3. APPLICATIONS TO BODY FLUIDS

Essentially all the chromatographic techniques have served more or less effectively for profiling the lipids of body fluids and tissues. These applications are reviewed in order of their popularity and according to increasing information, although the chronology has also been considered.

3.1. Plasma lipids

In view of the ready accessibility in man, plasma or serum lipids have been analyzed most often. The lipid profiles complement the estimation of total blood cholesterol and triacylglycerols, which are well established routines in clinical biochemistry. In specific instances it is desirable to record the total lipid profiles of individual plasma lipoprotein classes.

3.1.1. Total plasma

Segura and Gotto [67] and Kupke and Zeugner [68] have utilized a one-dimensional TLC method for the determination of lipids requiring only microliter quantities of plasma. The chromatoplate is developed first with methanol to 3 cm from the origin. After drying, it is developed again in trimethylpentane—diethyl ether—acetic acid (75:25:2). In the first solvent phospholipids move slightly above the origin, while all the other lipids and glucose move to the solvent front. The second solvent resolves the mixture into its major lipid classes. The resolved lipids are detected following transformation into fluorescent derivatives by thermal treatment of the chromatoplate in the presence of ammonium bicarbonate [26]. Another excellent procedure for the determination of the total lipid profiles of plasma or serum by conventional TLC involves developing the plate first with light petroleum—diethyl ether (9:1) and then with light petroleum—diethyl ether—acetic acid (400:100:1)

in the same direction, but to a second front 5 cm below the first. The polar lipids remaining at the origin consist almost entirely of phospholipids [69,70]. The content of the cholesteryl esters, triacylglycerols, free fatty acids, free cholesterol, and phospholipids is determined by charring and densitometry [24]. Gartzke [71] has compared the results of the densitometric determination with those of individual lipid class analyses and has concluded that densitometry yields comparable although somewhat less precise values than chemical or enzymatic measurements. Bitman et al. [26] have developed a routine analysis of lipid classes in blood by conventional TLC giving rapid and reproducible separations suitable for in situ quantitation by densitometry. A two-step development, starting with chloroform—methanol—acetic acid (98:2:1) to 16 cm, is followed by a second development with hexane—diethyl ether—acetic acid (470:30:1) to 20 cm. Prior to charring the plate is dipped into a solution of 3% cupric acetate in 8% phosphoric acid for 3 s.

HPTLC has been applied to plasma lipid profiling by Ando et al. [72]. A sample of the total lipid extract is applied as a 5-mm wide streak to an HPTLC plate (20 × 10 cm, E. Merck, Darmstadt, F.R.G.). The plate is developed up to 4.5 cm from the bottom with chloroform—methanol—acetic acid—formic acid—water (35:15:16:1:2) and dried for 10 min under a hood followed by drying in vacuo for 10 min. The plate was then redeveloped up to the top (10 cm) with *n*-hexane—diisopropyl ether—acetic acid (65:35:2). After drying

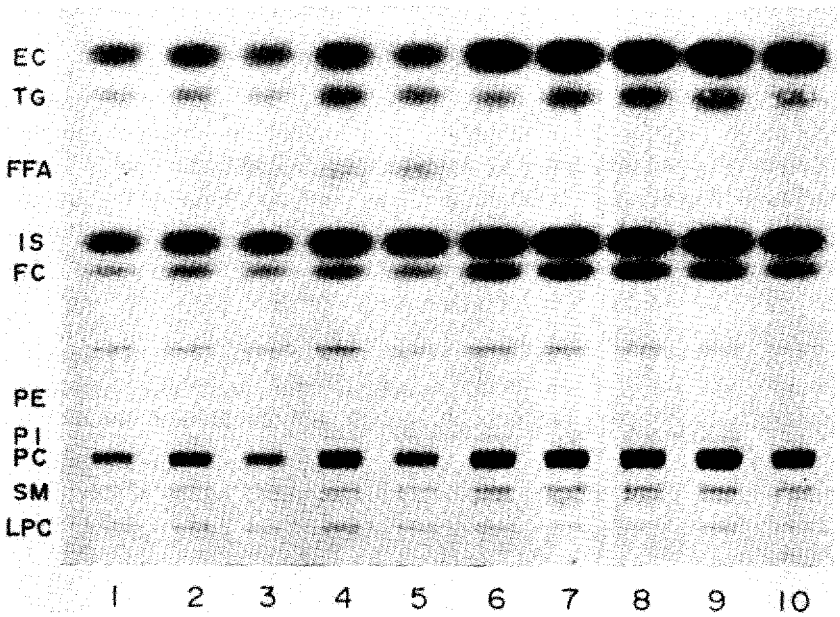

Fig. 1. HPTLC of rat plasma lipids [73]. Lanes 1—5, Sprague—Dawley rats on semisynthetic diet containing 2% safflower oil; lanes 6—10, Nagase analbuminemia rats on the above diet. EC = esterified cholesterol, FC = free cholesterol; TG = triacylglycerols, FFA = free fatty acids, PE = phosphatidylethanolamine, PC = phosphatidylcholine, LPC = lysophosphatidylcholine, SM = sphingomyelin, PI = phosphatidylinositol and IS = internal standard. Lipids were separated on HPTLC plates using the solvent systems given in the text. (Reproduced with permission of Japanese Biochemical Society.)

the plate was sprayed evenly with 0.25% sodium dichromate—15% sulfuric acid solution and heated for 30 min on an aluminum block heater at 125°C. The density of the bands was measured in the transmittance mode at 440 nm. Fig. 1 shows the plasma lipid profiles obtained for control and hyperlipidemic rats [72]. A comparable method has been described by Yao and Rastetter [73].

Excellent separations of plasma total lipids with quantitation by flame ionization detection have been obtained using sintered quartz rods [74]. The separations of the neutral and phospholipid classes are obtained using solvent systems similar to those employed in conventional TLC. Mareš et al. [75] have evaluated it in relation to that of gas–liquid chromatography (GLC). The plasma lipids were spotted on Chromarod S-II and developed in chloroform–methanol–water (40:20:2.5) to a height of 5 cm (two times) and then in *n*-hexane–light petroleum–diethyl ether–formic acid (30:24:6:0.8). Effective separations were obtained for the total phospholipids, free cholesterol, free fatty acids, triacylglycerols and cholesteryl esters, as well as the internal standard, 1-octadecanol.

Due to the advantageous distribution of the chemical classes and the molecular weights of the component species, high temperature GC has proved particularly well suited for obtaining plasma total lipid profiles. For this purpose the phosphatidylcholines and sphingomyelins are first dephosphorylated to the corresponding diacylglycerols and ceramides, which are then estimated together as the choline-containing phospholipids [4,76]. Fig. 2 shows

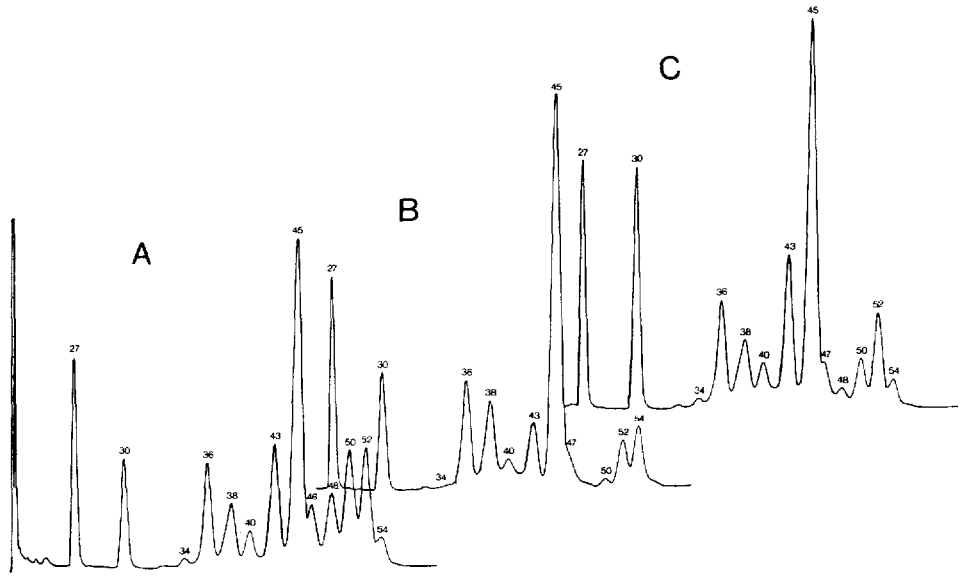

Fig. 2. GC profiles of plasma total lipids of normolipemic young adults on butterfat (A), corn oil (B) and fat-free (C) diets. Fat diets fed at 30% of total calories. Peaks: 16 and 18, TMS esters of free fatty acids with 16 and 18 acyl carbons; 27, TMS ether of cholesterol; 30, tridecanoylglycerol, internal standard; 34, TMS ether of palmitoylsphingosine; 36–42, TMS ethers of diacylglycerols of a total number of 34–40 acyl carbons; 43–47, cholesteryl esters of fatty acids with a total of 16–20 acyl carbons; 48–56, triacylglycerols with a total number of 48–56 acyl carbons. GC conditions as given elsewhere [4]. (Plasma samples courtesy of Dr. J.M.R. Beveridge.)

the plasma total lipid profiles of normolipemic subjects after two weeks on butterfat, corn oil and fat-free diets. Using appropriate correction factors it is also possible to obtain separate estimates for plasma phosphatidylcholine and sphingomyelin [18,77]. The most effective GC profiles of plasma or serum total lipids in the past have been achieved on the short, non-polar siloxane columns originally described for work with natural triacylglycerols [2]. Stainless-steel or glass tubes (30—50 cm × 0.2–0.3 mm I.D.) have generally given the highest recoveries when packed with 1–3% methyl siloxane or equivalent high temperature liquid phases coated on inert supports. These columns are used after preconditioning for 2—3 h at 350°C and a satisfactory recovery test. The column temperature is programmed to rise linearly at the rate of 4—8°C/min in the range 175–350°C during analysis. This method has been utilized for the determination of plasma total lipid profiles of normolipemic subjects [18], subjects with Type III and IV hyperlipemia [78], with ischemic vascular disease [79], on saturated and unsaturated fat diets [80], on cholestyramine [81], on birth control pills [82], with sitosterolemia [83], with Type I hyperlipoproteinemia [84] and in infants on Intralipid [85]. Fig. 3 shows the plasma total lipid profiles from two brothers with lecithin—cholesterol acyltransferase (LCAT) deficiency and from a sister with normal LCAT activity. The cholesteryl ester peaks (peaks 43, 45 and 47) are largely absent from the plasma of the LCAT deficient subjects. In other instances similar GC methods have been used to obtain the neutral lipid profiles of normolipemic [86—90] and hyperlipemic [88, 90] plasma. In these applications, the phospholipids are removed from the total lipid extract either by TLC [87] or adsorbent column chromatography [91]. The replacement of traditional glass columns by ready-made cartridges gives a noticeable improvement both in ease and in speed of separation of neutral lipids and phospholipids without affecting the quality of the final result. GC profiling has also been applied to the plasma lipids of rats [92], gerbils [93] and mice [94].

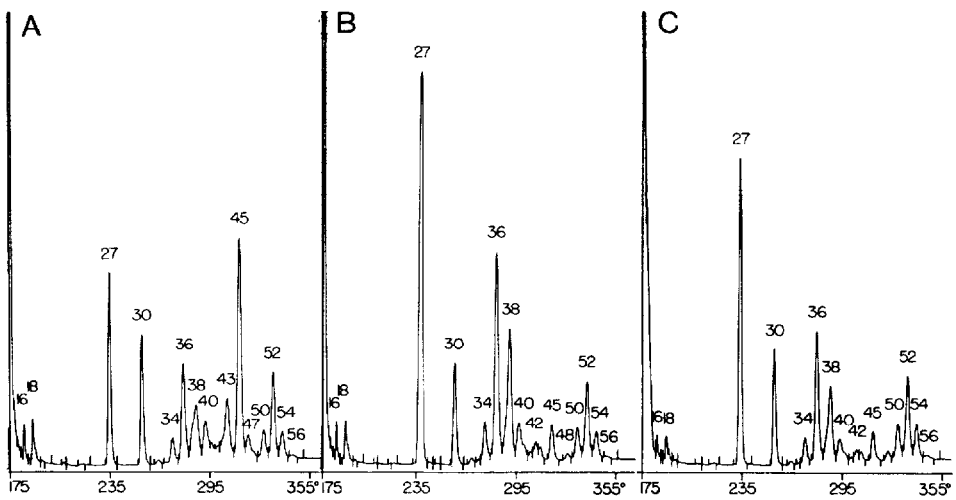

Fig. 3. GC profiles of plasma total lipids from two brothers (B and C) with LCAT deficiency and from a sister (A) with normal level of enzyme activity. Peak identification as given in Fig. 2. GC conditions as given elsewhere [11]. (Plasma samples courtesy of Dr. A. Angel.)

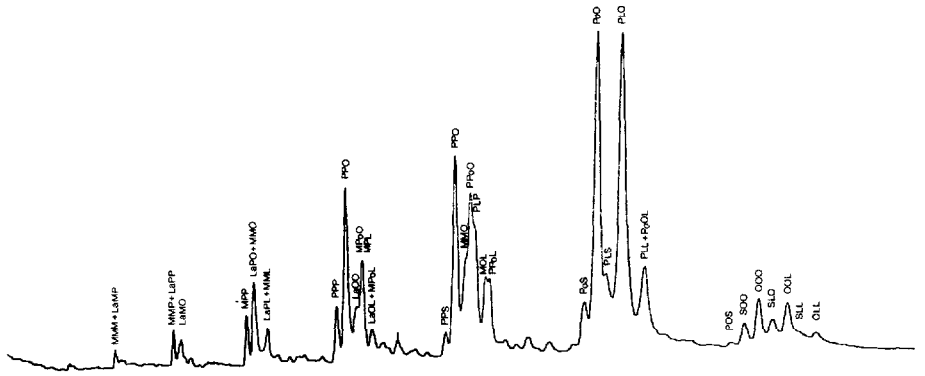

Fig. 4. Polar capillary GC profile of plasma triacylglycerols of a normolipemic subject in the fasting state [95]. Triacylglycerol peaks are identified by component fatty acids as follows: M, 14:0; La, 12:0; P, 16:0; S, 18:0; O, 18:1; L, 18:2; Po, 16:1. Capillary column and operating conditions as given elsewhere [48].

Non-polar capillary GC columns have been utilized in plasma lipid profiling in only a few instances [18,44], but the results have been superior to those obtained on packed columns and more applications are likely to be made in the future, especially using wide- or mega-bore capillary columns. The extra resolution obtained permits improved quantitation of the different lipid classes as well as provides an indication of the relative degree of unsaturation of the glycerolipids. Recently polar capillary columns have been employed in plasma lipid profiling on an experimental basis. Fig. 4 shows the triacylglycerol profile of the plasma of a normolipemic subject in the fasting state [95]. There is an essentially complete resolution of the molecular species based on carbon number and degree of unsaturation, but positional isomers overlap.

Combined GC—MS profiling of plasma total lipids using packed columns has been envisaged on a theoretical basis, but practical difficulties have kept it from being turned into a routine application. Thus, the triacylglycerols [96, 97] have been assayed by these methods on an experimental basis. In isolated instances, direct probe MS has been used to obtain the molecular species profiles of individual phospholipid classes [98,99].

Although HPLC is capable of resolving both polar and non-polar plasma lipids, the published routines report separate runs for the neutral and polar lipids. Duncan et al. [100] have described the HPLC resolution of free cholesterol and various cholesteryl esters on a reversed-phase column with 2-propanol—acetonitrile (1:1) or (5:2) as the mobile phase. Using the same methodology, Smith et al. [101] have obtained characteristic neutral lipid profiles of plasma for normal and diabetic rats. From the total lipid extract, separate estimates were obtained for thirteen chromatographic fractions, including separate estimates for various subfractions of triacylglycerols and cholesteryl esters. The plasma phospholipids under these conditions were apparently eluted so early that they were not separately quantitated. For this analysis, a 25 cm × 4.5 mm O.D. Zorbax ODS column (5 μm particle size, Du Pont) and a UV detector at 215 nm were used. With 2-propanol—aceto-

nitrile (5:2) at a flow-rate of 1 ml/min, the relative standard deviation varied from 4 to 8%, depending on the solute and its peak size. Čoupek and Mareš [102] have compared the resolution of the same sample of neutral lipids by HPLC and GC. For the HPLC separation of the C_{14}—C_{20} esters of cholesterol and glycerol a reversed-phase column (Separon SI VSIL, produced in Czechoslovakia) was used. Reversed-phase HPLC of the molecular species of plasma phosphatidylcholines has given excellent resolution, but peak identification and quantitation of components have required peak collection and GC of the component fatty acids. However, conversion of the diacylglycerol moieties of the phosphatidylcholines to the diacylglycerol benzoate [20] or naphthylurethane [21] derivatives has permitted a highly sensitive detection and quantitation of the individual molecular species.

Reversed-phase HPLC in combination with chemical ionization MS has been used for plasma total lipid profiling by Kuksis et al. [103], who prepared the t-BDMS derivatives of the free sterols and of the diacylglycerols and free ceramides released by phospholipase C treatment of the plasma sample. Effective resolutions of the molecular species of all the neutral lipid classes were obtained, except for the ceramide derivatives, which gave very low response under the chemical ionization conditions. The LC—MS profiles of the neutral lipid classes obtained following a removal of the phospholipids by adsorption were much more informative than those obtained by HPLC alone.

3.1.2. Individual lipoproteins

The methods just outlined for lipid profiling of whole plasma are also suitable for profiling the lipids of individual lipoprotein classes. Thus, the conventional TLC methods have been extensively employed for assessing the neutral and phospholipid [10,15,24] composition of the various lipoprotein classes. Of special interest is the work of Kupke [104], who has developed a procedure for TLC examination of lipoprotein fractions obtained by electrophoresis. Serum lipoproteins from the agarose gel are applied directly to the TLC plate and hydrochloric acid is used to dissolve the agarose structure prior to TLC of the lipids. Lipids were separated and detected as described previously [68]. Typical lipid patterns of serum lipoproteins were demonstrated. Visual examination of the chromatoplates indicated that satisfactory resolution and quantitation [105] was also possible. Detailed profiling of the phospholipids, including the minor classes, of plasma lipoproteins has been reported by Peeters [106], who used conventional TLC. Breckenridge and Palmer [107] used a neomycin column to obtain estimates of the phosphatidylinositol content of plasma lipoproteins.

The most extensive lipid profiling of the individual classes of plasma lipoproteins has been performed by high temperature GC. The lipid composition of the very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions has been characterized in the normolipemic subjects [79], and in subjects with ischemia [81], while the VLDL, intermediate density lipoprotein (IDL), LDL and HDL fractions have been characterized in subjects with hyperlipoproteinemias of Type I [84], Type II [108], and Type III and IV [81]. In other instances [109] character-

istic profiles have been obtained for the HDL2 and HDL3 subfractions of normolipemic and hyperlipemic human plasma. The data have shown highly specific lipid profiles for each lipoprotein class, which presumably reflect their characteristic physico-chemical structure with the free cholesterol and the polar lipids largely restricted to the surface and the neutral lipids to the core of the lipid particles. GC profiles of total lipids of plasma lipoproteins also have been reported by others [110—113]. Tong and Kuksis [114] have recorded the total lipid profiles of the $Sf > 400$ fraction and of total plasma of rats following infusion of chylomicron-like lipid emulsions stabilized with different phospholipid classes.

There have been few applications of HPLC to the lipid profiling of individual plasma lipoprotein classes. Perkins et al. [115] have used reversed-phase HPLC for the resolution of free cholesterol and cholesteryl esters of human plasma lipoproteins. The separations were performed with acetonitrile—chloro-

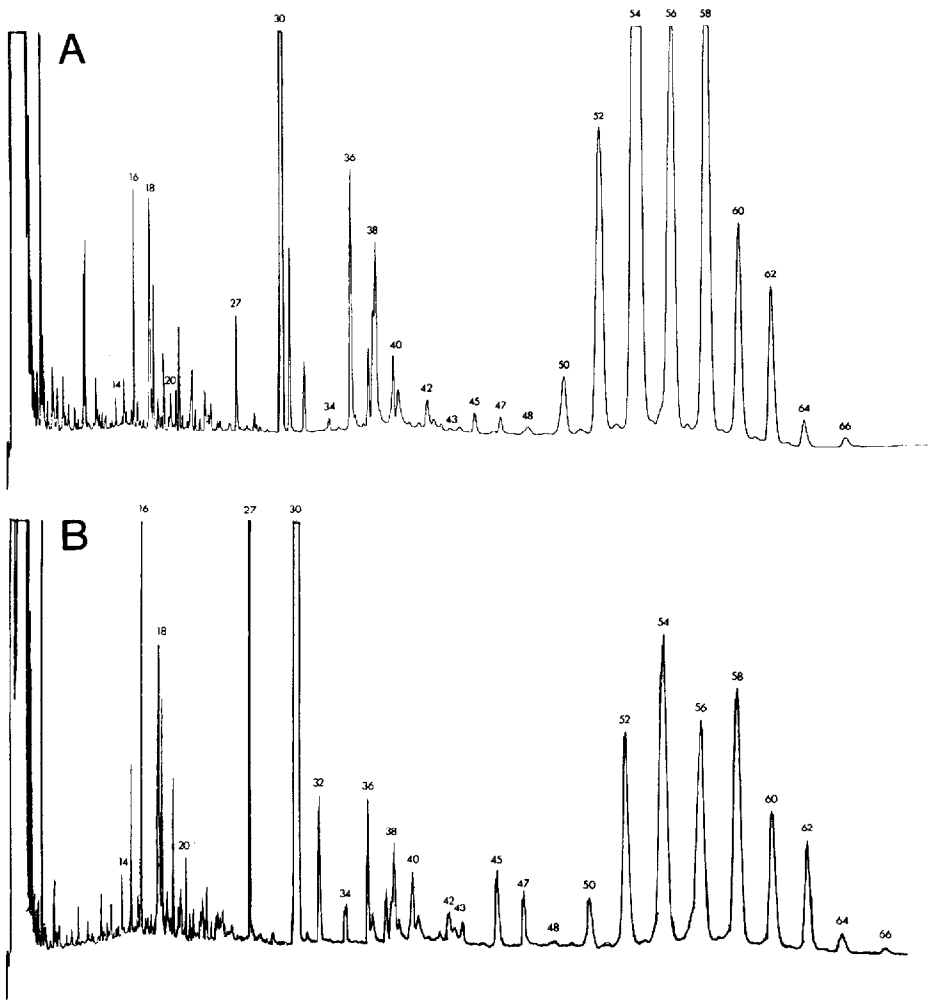

Fig. 5. Capillary GC profile of total lipids of thoracic duct lymph (A) and of plasma (B) of a rat during absorption of mustard seed oil [117]. Peak identification as given in Fig. 2. GC conditions as given elsewhere [18].

form—methanol (1:1:1) as the mobile phase. It was found that within the same equivalent carbon number category, cholesteryl esters with the highest number of double bonds eluted ahead of those with a lower number of double bonds, and with the *cis* isomers eluting ahead of their *trans* partners. The VLDL, LDL and HDL fractions possessed identical patterns of cholesteryl esters, possibly as a result of analyzing a pooled sample.

3.2. Lymph lipids

Lymph is much less readily accessible for analysis and therefore there have been fewer analyses of lymph lipids. Except for specific disease cases where the lymph has been passed either into the urine or has accumulated in the abdominal cavity, lipid profiling has been performed as an aid to research, rather than an assessment of the metabolic state of the subject or patient.

3.2.1. Total lymph

TLC profiles of total lymph lipids of dogs have been obtained by Huang and Kuksis [116] following feeding of corn oil or butterfat. Characteristic differences were observed in the fatty acid composition of the component lipid classes. GC profiles of total lymph lipids have been obtained using the analytical methods described for profiling plasma total lipids. Fig. 5 shows the total lipid profile of rat thoracic lymph collected during the absorption of a meal rich in mustard seed oil [117]. In addition to the large peaks for the

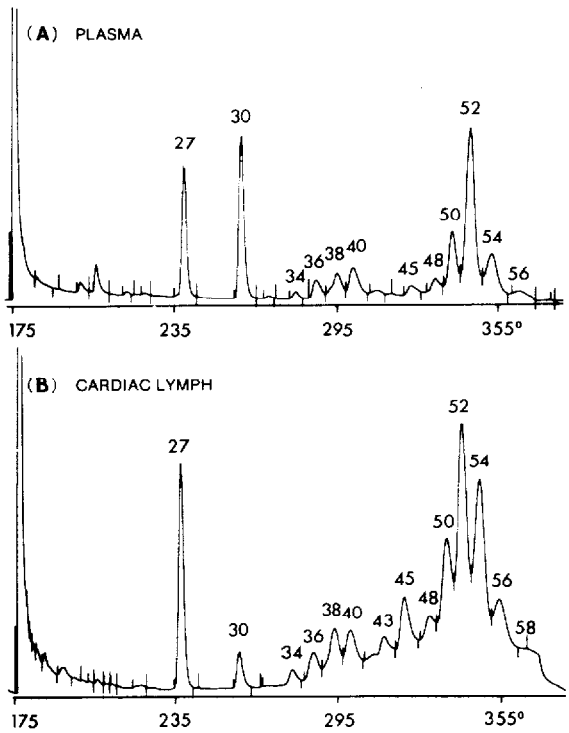

Fig. 6. GC profiles of the total lipids of plasma (A) and of cardiac lymph (B) of a dog. Peak identification as given in Fig. 2. GC conditions as given elsewhere [18]. (Plasma and lymph samples courtesy of Dr. P. Julien.)

triacylglycerols, there are smaller peaks representing the neutral lipid moieties of the phospholipids. Since lymph contains significant amounts of phosphatidylethanolamine (PE) in addition to phosphatidylcholine (PC), the diacylglycerols released by phospholipase C during dephosphorylation represent the sum of the two phospholipid classes. The sphingomyelin (SPH), however, is a relatively minor component in lymph when compared to plasma. Other peaks are seen for free cholesterol and the cholesteryl esters. The total lipid profiles of the peripheral lymph are similar to those of the corresponding plasma lipids. Fig. 6 shows the GC profile of the cardiac lymph of a dog. The major neutral lipid class is due to the triacylglycerols. The overlapping peaks of the diacylglycerols and ceramides have been derived from the mixture of the PC, PE and SPH present in minor amounts in the sample.

3.2.2. Individual lipoproteins

Phospholipid profiles of individual lymph lipoproteins have been determined by conventional TLC as described for the separation of the phospholipids of total plasma and lymph (see Sections 3.1 and 3.2.1). Fernando-Warnakulasuriya et al. [118] have determined the phospholipid profile of the lymph lipoproteins (chylomicrons and VLDL) from suckling rats and have assayed the fatty acid composition of the major phospholipid classes. The common phospholipid classes in the thoracic duct lymph lipoproteins isolated from nonhuman primates has been reported by Klein and Rudel [119]. In the latter case, the sample was spotted on silica gel TLC plates (Silica Gel G-60, Brinkmann, Chicago, IL, U.S.A.). The plates were first developed in hexane—acetone (3:1) and then were developed in chloroform—methanol—glacial acetic acid—water (65:45:12:6). The phospholipids were visualized with iodine vapor. Areas of silica gel containing phospholipid were scraped into tubes and phospholipid was eluted using chloroform—methanol—7 *M* ammonium hydroxide (65:35:5). Appropriate aliquots were then assayed for phosphorus. The major phospholipid present in lymph chylomicrons and VLDL was PC and the phospholipid composition of the particles was not affected by diet. Sloop et al. [120] have determined the common phospholipid classes in the HDL fraction of lymph from the peripheral (prepopliteal) lymphatics of the hind limbs of dogs using chromatography on glass-fiber paper impregnated with silica gel [121]. The chemical composition and physical appearance of the peripheral lymph HDL was markedly different from that of plasma HDL especially in cholesterol-fed animals. The phospholipid content of lymph HDL was higher than that of plasma HDL, while the protein content was lower. Lymph HDL had a higher cholesterol to protein ratio and a markedly increased free cholesterol content.

GC profiles of the thoracic duct lymph chylomicrons and other lipoproteins have been reported by Yang et al. [117]. While those of the chylomicrons and VLDL are characterized by large proportions of triacylglycerols, those due to LDL and HDL contain mainly cholesteryl esters and glycerophospholipids, respectively, and in this respect are not unlike those of the corresponding plasma fractions. The triacylglycerol profile reflects the fatty acid composition of the dietary fat. GC profiles of the thoracic duct lymph lipoproteins of rats receiving other fat meals have also been recorded [117]. These have been shown to differ from each other in the triacylglycerol composition, reflecting the nature of the dietary fat.

Klein and Rudel [119] have used reversed-phase HPLC for obtaining the cholesteryl ester profiles of thoracic duct lymph lipoproteins isolated from non-human primates. The cholesteryl esters of all lymph lipoprotein samples were first isolated by preparative TLC with silica gel H because the amount of triacylglycerols found in the total lipid extracts interfered with subsequent HPLC of cholesteryl esters. The HPLC analyses were performed using the conditions described by Carroll and Rudel [122]. Radioactivity in the cholesterol moiety of the cholesteryl esters was monitored using a radioactivity flow detector (Flo-One R, Radiomatic Instruments and Chemical Co., Addison, IL, U.S.A.). The HPLC column eluate was automatically and continuously mixed with a liquid scintillation cocktail (Budget-Solve R, Research Products International, Elk Grove Village, IL, U.S.A.) to monitor cholesteryl ester radioactivity. Effective separations were obtained for free cholesterol, and cholesteryl linolenate, arachidonate, linoleate, palmitoleate, myristate, oleate, palmitate, heptadecanoate and stearate in the lymph chylomicrons. A peak with a retention time similar to that of free cholesterol was seen with reasonable regularity in cholesteryl ester samples from lymph lipoproteins. This peak did not contain radioactivity and was probably not free cholesterol but may have been either a retinol derivative, or possibly another fat-soluble vitamin. The overlapping cholesteryl ester and triacylglycerol peaks in the total neutral lipid profiles obtained by HPLC can be effectively resolved by LC—MS with selected ion monitoring, as shown above for the total neutral lipid profiles of plasma.

3.3. Milk lipids

Milk represents another body fluid that has been extensively analyzed. Milk triacylglycerols provide a characteristic profile from which the animal family or species can be recognized [123]. However, disease and dietary extremes are known to alter the milk fat profiles [124]. Only the GC and HPLC methods have proved of value in profiling milk fat triacylglycerols. Argentation TLC has given poor resolution because of the complications introduced by the different chain lengths of the saturated fatty acids [125].

3.3.1. Human milk

GC profiles of the neutral lipids of normal human milk have been reported by Breckenridge et al. [126]. These separations are based mainly on carbon number and provide estimates of the free cholesterol, cholesteryl esters and triacylglycerols. The triacylglycerols account for the bulk of the lipid. Since the short chain fatty acids are largely absent, the triacylglycerol profile for subjects on normal diets represents mainly high molecular weight species. Normal subjects on high carbohydrate diets, however, synthesize significantly greater proportions of the shorter chain fatty acids, which leads to the formation of high proportions of short chain triacylglycerols. Likewise, Type I hyperlipoproteinemia, which is characterized by a lipoprotein lipase deficiency, leads to a bizarre pattern for the milk fat triacylglycerols [124]. Fig. 7 shows the GC profile of milk fat triacylglycerols of a Type I hyperlipoproteinemia patient. Since in this condition, plasma free fatty acids or monoacylglycerols were not

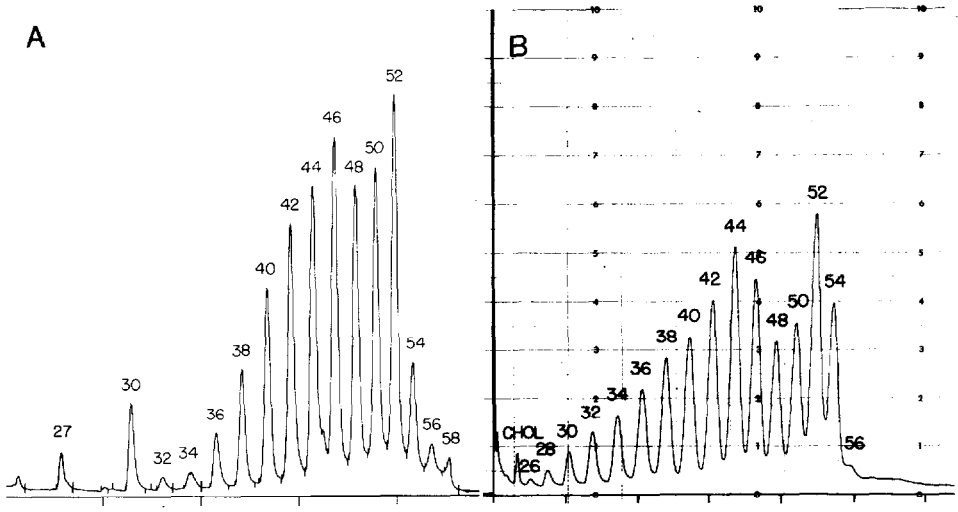

Fig. 7. GC profiles of milk fat triacylglycerols of a patient with Type I hyperlipoproteinemia (A) and of a horse (B). Peaks : 27, TMS ether of free cholesterol; 30, tridecanoylglycerol, internal standard; 23—56, triacylglycerols with a total number of 32—56 acyl carbons. GC conditions as given elsewhere [124].

available, the milk triacylglycerols were formed from the fatty acids generated de novo in the mammary tissue. As a result a high proportion of short chain fatty acids became incorporated in the milk fat triacylglycerols as seen from the high proportion of the low molecular weight triacylglycerols in the elution pattern [127]. This milk fat exhibits a profile similar to that recorded for horse milk triacylglycerols [123].

3.3.2. Ruminant milk

The neutral lipids of the ruminant milk fats have an especially characteristic GC profile. This is due to the high proportion of butyric and other short chain fatty acids in the triacylglycerols [128]. The unusual lipid profile is due to a specific positional distribution of the short chain fatty acids in these triacylglycerols [129]. The resolution can be further enhanced by substituting a capillary for the packed column during the GC separation. Fig. 8 compares the capillary GC profiles of the triacylglycerols from normal human milk and from goat milk [130]. A distinct improvement in the profile of these triacylglycerols, however, results from a separation on a polar capillary column. Fig. 9 shows the resolution of the triacylglycerols of butterfat on such a column [49,131]. Under these conditions, each carbon number subclass of triacylglycerols is resolved into two or more fractions representing the various degrees of polarity of the component fatty acids. Clear resolution is obtained between saturated and unsaturated species, and between species containing short and long chain length fatty acids within the same carbon number subfraction. A much simpler pattern is obtained following hydrogenation of the butterfat [131].

The neutral lipids of cows' milk have also been effectively resolved by reversed-phase HPLC [9], but the small amounts of cholesteryl esters and

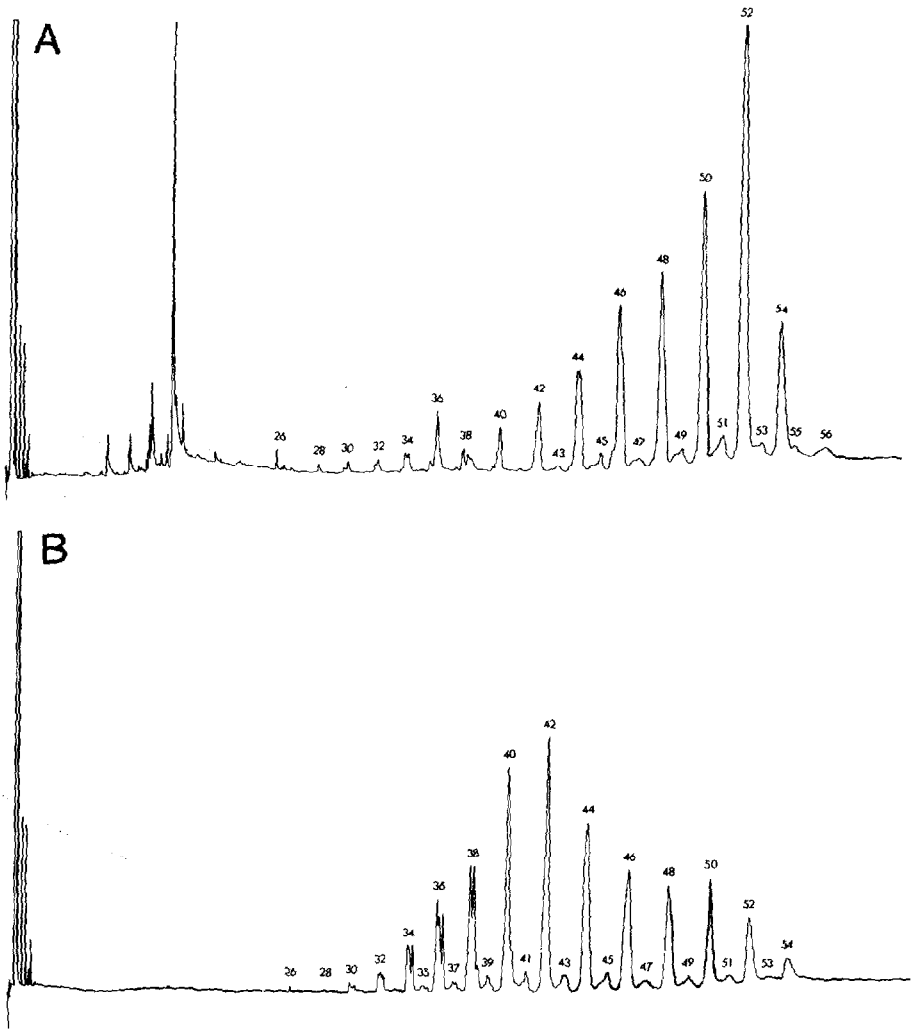

Fig. 8. Capillary GC profiles of the milk fat triacylglycerols of normal milk of man (A) and of a goat (B). Peaks as in Fig. 7. GC conditions as described elsewhere [130].

free cholesterol overlap with the triacylglycerols. In the LC—MS mode of operation the free sterols and the steryl esters are readily differentiated from the triacylglycerols by means of characteristic ion plots [103]. LC—MS of the triacylglycerols also allows a complete identification of the fatty acid association in the molecular species, although the positional distribution of the fatty acids remains uncertain. Fig. 10 shows a three-dimensional LC—MS profile of goat milk fat triacylglycerols [9]. The LC—MS—computer plot of the composition displays the mass distribution for the ions of the component diacylglycerols and for some of triacylglycerols corresponding to each triacylglycerol peak in the HPLC profile. Although the pattern is too complex for a quantitative evaluation, the qualitative distribution of the peaks provides a good indication of the complexity of the species. Kuksis et al. [132] have analyzed the triacylglycerols of the total neutral lipids of goat milk and have identified the major molecular species. Cerbulis et al. [133] have determined the hydrocarbon profile of goat and human milk fat by GC—MS.

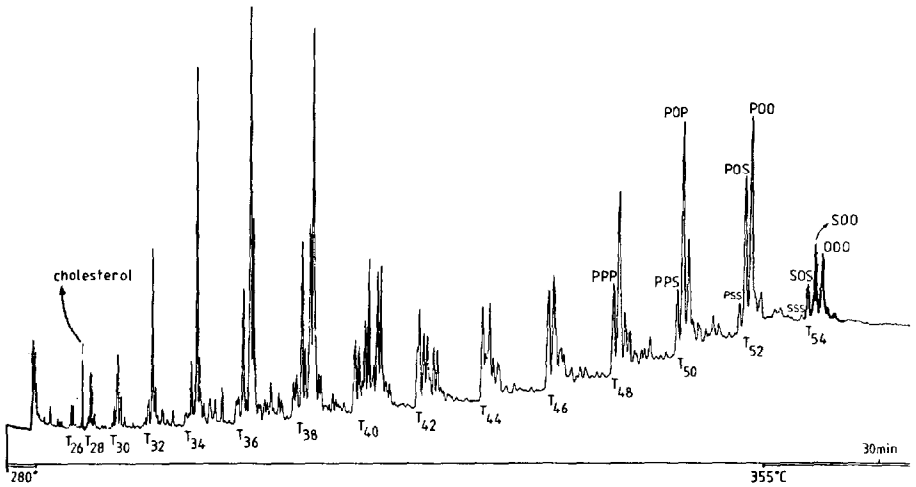

Fig. 9. Polar capillary GC profile of triacylglycerols of butterfat. Peak identification as in Fig. 4. GC conditions as described elsewhere [49,131]. (Reproduced with permission of A. Huethig Verlag, Heidelberg.)

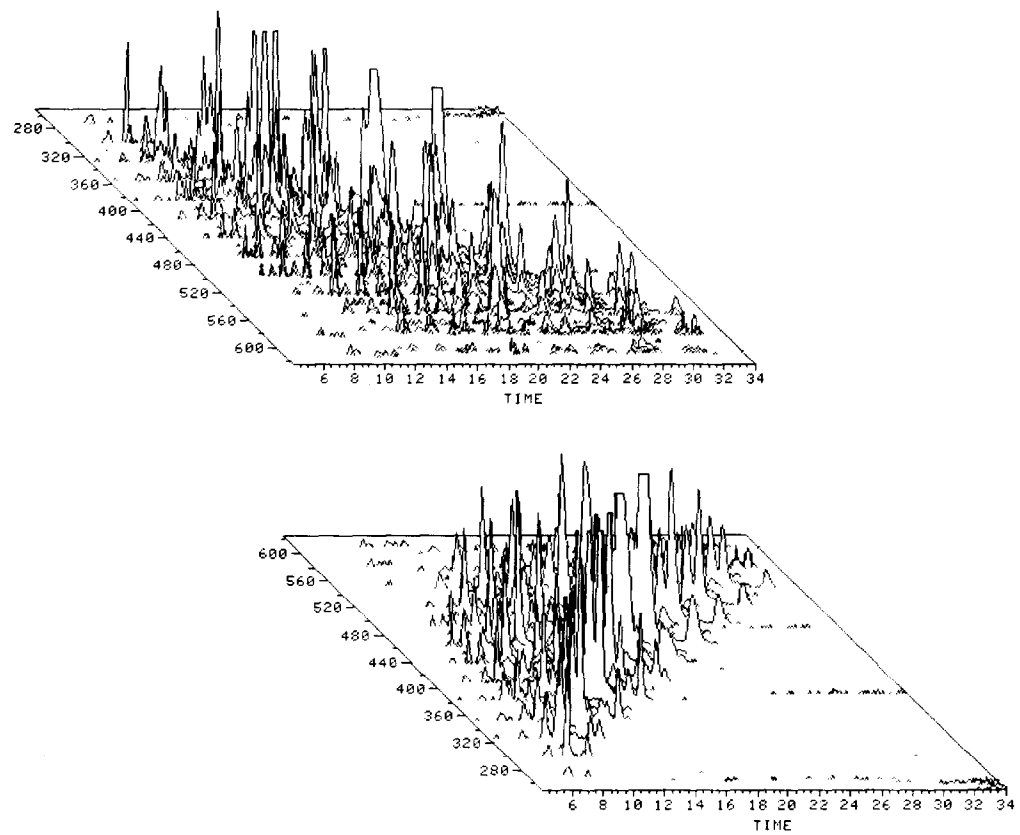

Fig. 10. Three-dimensional LC—MS profile of goat milk fat triacylglycerols. The masses of triacylglycerols and of the diacylglycerol fragmentation products are plotted against the scan number. LC—MS conditions as described elsewhere [9].

3.3.3. Other milks

The triacylglycerols of horse milk contain significant amounts of medium chain length fatty acids, which give rise to a characteristic profile for its total neutral lipids [123,124]. Only small amounts of free cholesterol and cholesteryl esters accompany the triacylglycerols in the milk fat globules. The neutral lipid profiles of rodent milk have been reported by Smith et al. [134], while Parodi [135] has reported the profiles of the milk triacylglycerols of a variety of other animal species.

3.4. Amniotic fluid and lung lavage

Determination of the phosphatidylcholine (lecithin) to sphingomyelin ratio (L/S) provides a means of assessing the development of the fetus in pregnancy and yields reliable information about pulmonary maturity. Kulovich et al. [136] separated the phospholipids in chloroform—methanol—water (65:25:4) and charred the spots with sulfuric acid. Many modifications of the original method have been suggested since [137], including the quantitation of the L/S ratio by HPTLC [25]. Yu et al. [138] and Khan et al. [139] have used the method of Sherma and Touchstone [137] to obtain, respectively, the phospholipid profile of bovine and guinea pig lung lavage. Separate estimates were obtained for PC, which was the major component, and for phosphatidylinositol (PI), phosphatidylserine (PS), SPH, PE, phosphatidylglycerol (PG) and lysophosphatidylcholine (LPC), the minor components. Kuhnz et al. [140] have described an HPLC method for the complete resolution of all pulmonary phospholipids from mouse lung and broncho-alveolar lavage. A LiChrosorb DIOL, 5 μm (Merck, Darmstadt, F.R.G.) column fitted with a 30 × 4.6 mm I.D. LiChrosorb Si 60, 5 μm (Merck) pre-column was used along with a gradient of acetonitrile—0.005 *M* NaH_2PO_4 buffer, pH 5.0 (80:20) in pure acetonitrile. Individual peaks were obtained for PG, PI, PE, PS, PC, SPH and LPC. These results were similar to those obtained by two-dimensional TLC.

3.5. Sebum

Nicolaides [141] has applied conventional TLC to the analyses of human sebum lipids. Marked differences and similarities were found among various samples in lipid class composition and content.

Aitzetmuller and Koch [142] have applied HPLC to the analysis of human sebum lipid classes. The lipid classes were separated by gradient elution from a microparticulate silica column and detected by a moving-wire detector. Sebum lipids were resolved into the following lipid classes: hydrocarbons and squalene, cholesteryl esters and wax esters, fatty acids and their methyl esters, triacylglycerols, 1,3-diacylglycerols, free cholesterol, monoacylglycerols and polar lipids.

4. APPLICATIONS TO TISSUES

The lipids of tissues and cells contain phospholipids as major components. Therefore tissue lipids are most effectively profiled by those techniques, which allow the separation of the polar molecules. In the past TLC methods have

been extensively employed for the characterization of tissue lipid composition [15]. However, many of the separation procedures are tedious and require chromatography in more than one solvent system or in more than one dimension [15,37] and therefore are not suited for rapid lipid profiling. The tissue lipid profiling using the Chromarod technology has been more effective [30]. Presently, HPLC techniques are being developed to give comprehensive polar lipid profiles of both tissues and isolated cells [143,144]. The problem of quantitation of the lipids in the HPLC effluent has been overcome to some extent by the introduction and application of the mass (light scattering) detector [60,61,145].

4.1. Liver

Since the liver contains large amounts of phospholipids, which are actively metabolized in response to various nutritional and physiological stimuli, much of the work on tissue lipid analysis has been done in the liver. A determination of the lipid profile has been of great interest as a method of assessing the overall changes prior to any detailed analyses of the molecular species. The conventional TLC techniques employed for profiling the total phospholipids have been similar to those used for phospholipid analyses in the body fluids and have usually involved one-dimensional TLC followed by a densitometric assessment of the lipid quantities, but two dimensional separations followed by phosphorus analyses have also been used [37]. Fig. 11 shows a separation of rat liver lipids obtained by Macala et al. [29] using HPTLC. Complete resolutions of both neutral and phospholipids are obtained by a double development in a single dimension. Harrington et al. [146] have described a very sensitive procedure for analysis of polyunsaturated phospholipids involving resolution by HPTLC, conversion of the lipids to fluorescent derivatives, and quantitation by densitometric scanning. The formation of the fluorophore is dependent on the presence of at least two double bonds in an acyl chain. As little as 20 pmol of PI can be determined. Therefore, liver samples as little as 10 μg or 1000 dispersed hepatocytes could be analyzed for PI and other phospholipids. Neither dimyristoylphosphatidylcholine nor lysophosphatidylethanolamine, in which the fatty acid moieties are saturated, produced a fluorescent derivative. Oleic acid and its esters gave rise to only slight fluorescence.

Rao et al. [147] have employed the Chromarod—FID system for the determination of the total lipid profiles of normal and alcohol-induced fatty livers of rats and have compared these results with those obtained by TLC—GC methods. The triacylglycerol, phospholipid, free cholesterol, and cholesteryl ester contents (65.9, 25.4, 2.9 and 6.8 mg/g, respectively) obtained by the Chromarod—FID system were similar to those observed by other methods (67.9, 27.6, 3.0 and 8.3 mg/g, respectively). The liver lipid content in control rats also was similar to that obtained by other methods. The Chromarod—FID analysis was carried out using Iatroscan TH-10 Analyzer Mark III (Iatron Labs., Tokyo, Japan). The conditions for the storage of Chromarods (Type S-II), spotting the samples and scanning were essentially the same as those used in previous studies [148]. The response of Chromarod—FID to different lipid standards was determined by using the same set of ten Chromarods. Various

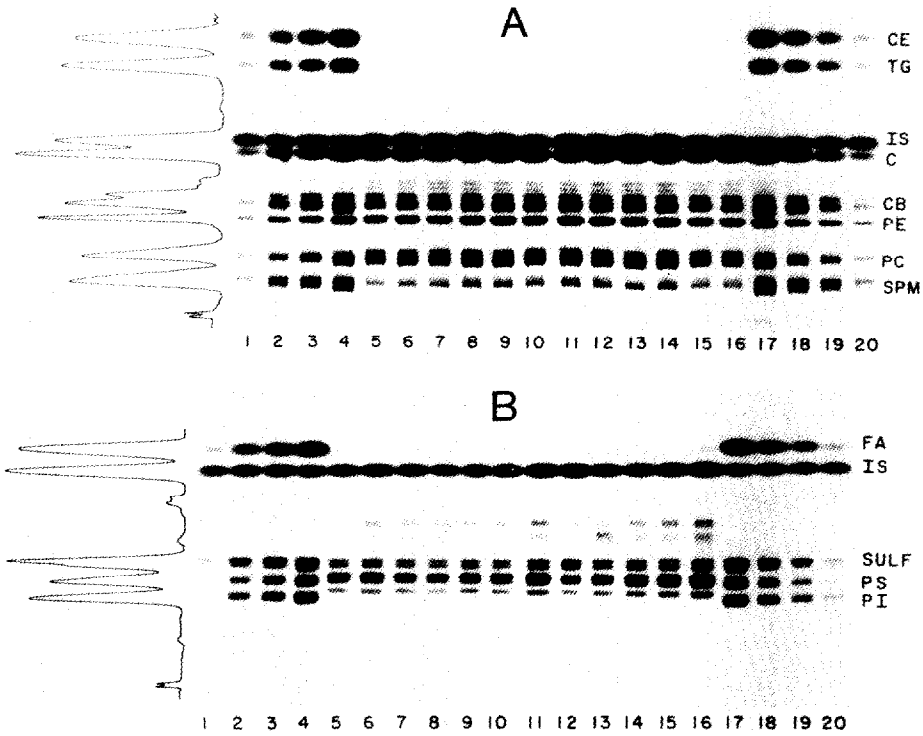

Fig. 11. HPTLC profiles of the acidic (A) and non-acidic (B) lipids of rat liver. CE = cholesteryl ester; C = cholesterol; CB = cerebroside; SPM = sphingomyelin; FA = fatty acids; SULF = sulfatides; PS = phosphatidylserine. Other peak identifications as in Fig. 1. HPTLC conditions as outlined in text and given in detail elsewhere [29]. (Reproduced with permission of J. Lipid Res.)

amounts of standards (tripalmitoylglycerol, PC and free cholesterol) were spotted on Chromarods and scanned either with or without development in a solvent system. The detector response was significantly greater when the spots were not developed. From the results with developed rods, conversion factors (amount/area) were calculated. For the separation of free cholesterol, triacylglycerol and cholesteryl ester fractions from phospholipids, Chromarods were developed in benzene. After scanning to determine the content of neutral lipids, the rods were redeveloped in a solvent system containing chloroform–methanol–water (80:35:3.5) and again scanned to quantitate phospholipids. The TLC–GC analysis of liver lipids was carried out by separating the lipids into different classes by conventional TLC, preparation of the fatty acid methyl esters and quantitating them by GC using methyl pentadecanoate as internal standard [148].

Harvey et al. [149] used the Chromarod–FID system for a rapid measurement of free cholesterol, total bile salts and total phospholipids in a sample of human gallbladder bile. The samples were spotted on S type Chromarods in 5-μl aliquots using a Wiretrol (Drummond Sci. Co., Broomall, PA, U.S.A.) and focused as described. Double development in two solvent systems was required to separate phospholipids, cholesterol and all bile salts as three distinct bands. The first system consisted of chloroform–light petroleum–methanol–acetone (60:20:10:10) and the solvent front run to 8–10 cm. After drying the rods

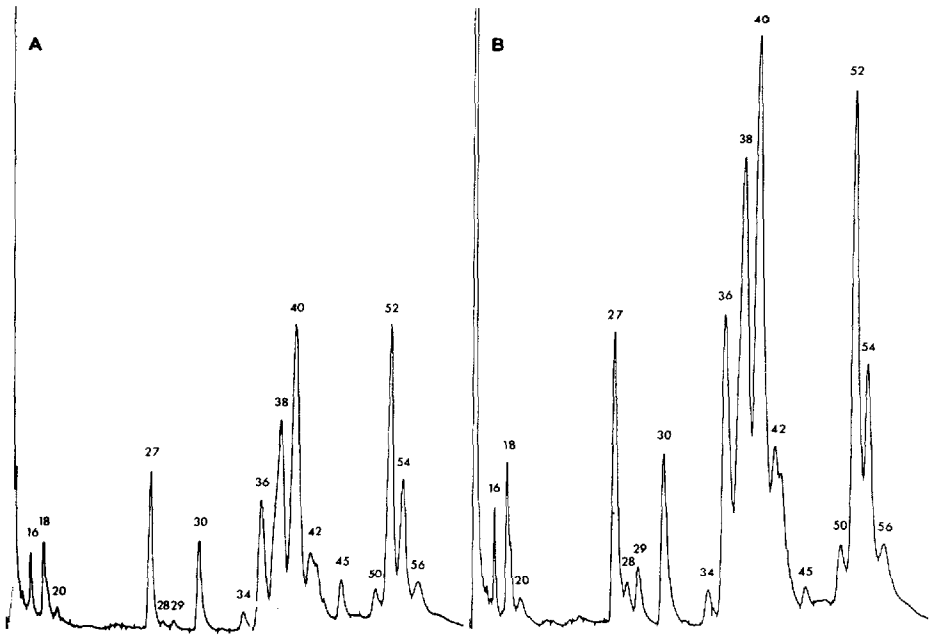

Fig. 12. GC profiles of total lipids of livers of rats following 24-h infusion of Intralipid containing cholesterol (A) or plant sterols (B). Peak identification as in Fig. 2. GC conditions as described elsewhere [150].

(5 min at 100°C), the second system containing acetone—water (50:50) was used and the solvent front run 5 cm. All three components exhibited a linear response over the range of 0.25—8 μg. Samples from ten patients could be processed in less than 1 h.

Kakis and Kuksis [150] have recorded GC profiles of liver lipids, which were obtained by subjecting the total lipid extract to a dephosphorylation by phospholipase C prior to trimethylsilylation and GC analysis. Fig. 12 shows the GC profiles recorded for the total liver lipids of rats following a 24-h infusion of Intralipid containing cholesterol or plant sterols. Separate estimates are obtained for free cholesterol, individual carbon numbers of the cholesteryl esters and the triacylglycerols, while the phospholipids are estimated by carbon number as an unresolved total. A quantitative estimate of the total liver lipid is obtained by comparing the peak areas of the unknowns to that of the internal standard, tridecanoylglycerol, which is added to the sample in a known amount. These lipid profiles also provide the ratios for free to esterified cholesterol and for free cholesterol to total phospholipid. The method requires calibration and appropriate conversion factors for the neutral lipid moieties to the corresponding phospholipids. Complete hydrolysis of all the phospholipids by phospholipase C is critical for effective GC quantitation of the total lipid extracts of liver. Tests with isolated phospholipids have shown that the phosphatidylserines and phosphatidylinositols require longer times for hydrolysis than the phosphatidylcholines and phosphatidylethanolamines. However, in mixtures with neutral phospholipids the acidic phospholipids are hydrolysed more readily and with only a modest increase in the length of digestion time needed [150,151]. The source of enzyme and degree of purification are known to influence the digestion.

Christie [145] has described the use of HPLC with a mass detector for the rapid separation and quantitation of the main lipid classes in rat liver and other tissues, ranging from the least polar cholesteryl esters to the most polar lysophosphatidylcholines. A ternary solvent-proportionating system was used together with a 3-μm silica adsorbent in a short (10 cm) column. The solvent systems were: (A) isooctane—tetrahydrofuran (99:1), (B) isopropanol—chloroform (4:1); and (C) isopropanol—water (1:1). Patton et al. [143] had earlier resolved the total phospholipids of rat liver by HPLC on a silica column with all the major components retaining their fatty acid composition. The only limitation of the method was the incomplete separation of PC from SPH. The molecular species profiles of individual phospholipid classes of the liver lipids have also been resolved by GC [51,52,152], and HPLC [153] of the diacylglycerol moieties and by HPLC of intact phospholipid molecules [143,154], but the discussion of these separations is beyond the scope of this review.

4.2. Heart

Kramer et al. [155] have evaluated the precision and accuracy of the Chromarod—FID method for profiling the total lipids of the heart. A partial scan and repeat development with chloroform—methanol—water (68.5:29:2.5) was introduced to achieve consistently good separations of phospholipid classes. On the whole the Chromarod—FID data were similar to the data from the TLC—phosphorus method, while the TLC—GC method generally gave lower values. For the profiling, the lipid samples were spotted on each of eight silica gel Chromarods (type S). The set of rods was equilibrated before each development for 5 min in a 55% humidity chamber (41% sulfuric acid and 5 min over the developing solvent). The neutral lipids were developed in 1,2-dichloroethane—chloroform—acetic acid (92:8:0.1) [156] to a height of 11 cm, and the rods were partially burned to $R_F = 0.15$. The polar lipids were resolved then by developing the Chromarods twice up to 10 cm in the solvent chloroform—methanol—water (68.5:29:2.5), with an air drying and another equilibration between developments. The double development of the phospholipids improved the resolution of PC and SPH, and provided a larger gap between PE and PC, allowing the enhanced separation of PI and PS. However, PI could not always be resolved from PE, and for this reason a combined estimate for the two phospholipid classes was usually obtained. There was evidence to indicate that the differences in fatty chain length and unsaturation, present particularly in phospholipids, cause peak broadening and poorer resolution on Chromarods [157]. The study showed better precision for the TLC—GC method than for the Chromarod—FID system.

Innis and Clandinin [158] have reported excellent separation and quantitation of the phospholipid classes for rat heart mitochondria using Chromarods (S Type). Lipid extracts applied to each Chromarod were developed for 30 min in light petroleum—diethyl ether (85:15) to separate neutral lipids. After air drying for 20 min, the rods were transferred to the FID scanning frame. Half the rods were scanned in full for quantitation of neutral lipids and total phospholipid. The remaining rods were scanned to the end of the cholesterol band only, thus leaving the phospholipids intact at the origin. Rods were

reactivated by scanning the upper portion a second time. Separation of phospholipids was accomplished in chloroform—methanol—water (80:35:3). Effective separations were obtained in the neutral lipid run for triacylglycerols, free fatty acids, free cholesterol and total phospholipids, while in the polar lipid run effective resolution was obtained for cardiolipin (CL), PE, PI, PS, PC, SPH and LPC. In a previous report [74] chloroform—methanol—water (80:35:5) was found to separate LPC SPH, PC and PE, but not CL, PS and PI when present in the same phospholipid mixture. With the present method reproducible analyses for phospholipids were obtained within 3 h after lipid extraction had been completed. The operating conditions and response factors to standard lipids must be determined in each laboratory.

Christie [145] has utilized an HPLC method with a mass detector for the rapid separation and quantitation of the main lipid classes in the heart tissue, ranging from cholesteryl esters to SPH. A ternary solvent-proportionating system was used, together with a 3-μm silica adsorbent column.

4.3. Brain

The great heterogeneity of the lipid classes in the brain yields an extremely complex lipid profile and intelligible comparisons can be made only by separating and quantitating specific lipid classes or molecular species. Vitiello and Zaneta [159] separated the major phospholipid classes on HPTLC, along with cerebrosides and sulfatides, but did not resolve the neutral lipid classes. Ando et al. [160] developed an HPTLC method which separates the major neutral and phospholipid classes, with the exception of the serine and inositol phosphatides. Furthermore, sulfatides were found to co-migrate with PE. Macala et al. [29] have modified the procedure of Ando et al. [160] to resolve LPC, SPM, PC, PE, PI, PS, cholesterol (C), triacylglycerol (TG), esterified cholesterol (CE), fatty acids (FA), cerebroside (CB) and sulfatide (SULF). The total lipid extract was separated first into neutral and acidic lipid fractions by DEAE-Sephadex column chromatography. The lipid fractions were then spotted on separate HPTLC plates and chromatographed in one dimension using two solvent systems. The non-acidic lipids of rat brain were developed first with chloroform—methanol—acetic acid—formic acid—water (35:15:6:2:1) to about 4.5 cm which separated SPM, PC, PE and CB. A second development with hexane—diisopropyl ether—acetic acid (65:35:2) to the top of the plate separated C, internal standard, TG and CE. The acidic lipids of rat brain were first developed with chloroform—methanol—acetic acid—formic acid—water (35:15:6:2:1) to about 6 cm to separate PI, PS and SULF. A second development with hexane—diisopropyl ether—acetic acid (65:35:2) to the top of the plate separated the internal standard from FA. Approximately 1.0 μg of each lipid class could be quantitated and a triplicate analysis could be done with less than 400 μg of total lipid from 5 mg of tissue. The charring agent was 3% cupric acetate in 8% phosphoric acid.

Månsson et al. [161] have used a new type of strong anion-exchange resin in the separation of brain gangliosides. These were separated into mono-, di-, tri- and tetrasialoganglioside fractions by a discontinuous gradient of potassium acetate in methanol. The procedure was applied to the separation of

gangliosides from normal human and sialyl hexosaminyl dihexosyl ceramide (GM2)-gangliosidosis brain. A comparable separation using gradient elution has been subsequently reported [162]. A simple method for the separation of free fatty acids from neutral lipids with simultaneous resolution of major phospholipids by HPLC in brain extracts has been described by Chen and Chan [163]. The separation is achieved on a silica column using hexane, 2-propanol and water as the mobile phase. Lipid fractions were monitored by UV absorption at 206 nm. With this HPLC method, the authors were able to successfully monitor the changes in the free fatty acids and phospholipids in cold injured, free radical treated and ischemic brains.

4.4. Arteries

Bowyer and King [37] have described methods for the rapid separation of the major individual phospholipids and neutral lipids of arteries and other tissues by TLC on small plates. This is followed by microchemical estimation of the separated lipids and the determination of fatty acid composition and radioactivity. The overall method, involving tissue extraction, TLC separation and assay was evaluated using pure standards and biological samples and gave good reproducibility with almost complete recovery of lipids.

4.5. Intestine

Since the intestinal mucosa is specifically involved in the uptake and re-synthesis of dietary lipids, much effort has been expended to determine the effect of dietary fat on the lipid profile of the mucosal cells [164—167]. In these studies extensive use was made of the total lipid and neutral lipid profiling by GC on non-polar and polar columns and by combined TLC—GC methods. In other studies detailed analyses have been made of the molecular species of the choline and ethanolamine phosphatides [151]. Fig. 13 shows capillary GC profiles of the molecular species of the diacyl, alkylacyl and alkenylacylglycerol moieties of the phosphatidylethanolamines of mucosal scrapings of rat small intestine [168]. It emphasizes the great complexity of the composition of a single phospholipid class in this tissue.

4.6. Other tissues and cells

Korte and Casey [169] have recently described a simple and rapid method for the separation of six major phospholipids and four major neutral lipids from cell extracts by one-dimensional preadsorbent TLC. The compounds are applied to the preadsorbent (Celite) area in 10-μl aliquots (total volume 0.15 ml). The phospholipids from human endometrial stromal cells were resolved using chloroform—ethanol—water—triethylamine (30:34:8:35) as the solvent system and the neutral lipids using *n*-heptane—diethyl ether—acetic acid (75:25:4) as the solvent system. The lipid profiles were determined by radiometric scanning following the incubation of the cells with [^{14}C]arachidonic acid.

Christie [145] has obtained the combined neutral lipid and phospholipid profile and red blood cells by means of HPLC on an adsorption column using a

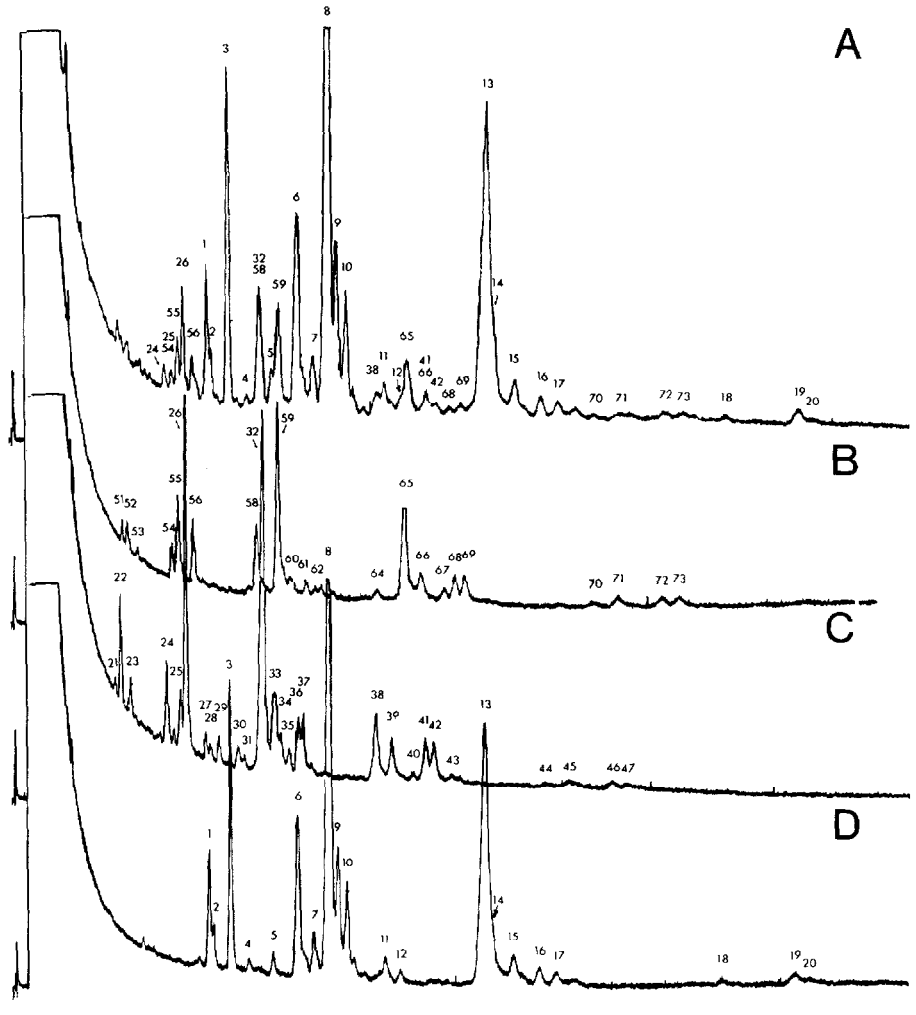

Fig. 13. Polar capillary GC profiles of the diradylglycerol moieties of the phosphatidylethanolamines of mucosal scrapings of rat small intestine. (A) Total; (B) alkylacyl; (C) alkenylacyl; (D) diacyl. Peak identification and chromatographic conditions as given elsewhere [168].

ternary solvent-proportionating system. The solutes were detected and quantitated by a mass detector. The components ranged from free cholesterol and traces of free diacylglycerols to LPC.

5. BIOMEDICAL APPLICABILITY AND PERSPECTIVES

Chromatographic analyses of lipids of body fluids and tissues are extensively utilized in biomedical research but only a few of them have become established routines in clinical chemistry. The limited clinical use of these methods is due to the relatively higher expense of operating complicated analytical instruments in comparison to chemical or enzymatic analyses, and to the lack of relevance of the chromatographic data to present day clinical practice. Thus, determina-

tions of plasma total cholesterol and triacylglycerols are made much more cheaply by chemical methods than by GC. As well the results are equivocal in establishing the presence or absence of hyperlipidemia. With further modification, the chemical methods can provide also estimates for free and esterified cholesterol. The additional information supplied by chromatographic analyses about the nature of the cholesteryl and glyceryl esters is of marginal clinical interest. When felt to be absolutely necessary, a determination by GC can be made of the fatty acid composition and the relative proportions of the saturated and unsaturated, or the essential and non-essential fatty acids. Usually this information on the dietary fat and the body lipids is implied from the analyses of the fatty acid composition of the dietary lipids.

The additional information provided by a chromatographic profile of plasma total neutral lipids, e.g. free and esterified cholesterol and plant sterol levels, relative proportions of different carbon numbers of steryl and glyceryl esters, is relevant only to such rare clinical conditions as sitosterolemia and LCAT deficiency. The information gained beyond the determination of total lipid phosphorus by profiling the total polar lipids is of greater current clinical interest. Thus, a determination of the ratio of phosphatidylcholine (lecithin) and sphingomyelin (L/S) in the amniotic fluid provides means for assessing the development of the fetus in pregnancy and yields valuable information about the pulmonary maturity. Furthermore, analyses of polar lipids of tissues have been helpful in a post-mortem confirmation of sphingolipidoses and ischemia. A full utilization of the data provided by profiling either the neutral or polar lipids of body fluids and tissues in clinical diagnosis must await a better understanding of disease processes and of their preclinical manifestations in lipid abnormalities. Eventually one could visualize that determinations of the molecular species of all lipids and of their ratios might become clinically relevant as an indication of membrane and lipoprotein structure and function. At the present time such analyses are only at an experimental level and the potential of the clinical relevance remains unrecognized.

6. SUMMARY

Many tissues, cells and body fluids possess characteristic lipid composition that can be readily recognized without full resolution and quantitation of individual molecular species. Various chromatographic methods have been adopted for this purpose and are extensively employed in biomedical research. Although lipid profiles are known to change with disease and lipid profiling holds considerable potential for clinical diagnosis, few routines have been established for this purpose. This is partly due to the laborious nature of the simpler methods and the high cost of automated systems. A combination of thin-layer or liquid chromatography with universal detection systems promises to provide more attractive analytical routines for clinical application in the future. At present thin-layer chromatography is the simplest and most rapid qualitative assay for both neutral and polar lipids. Low-temperature gas chromatography is still the method of choice for fatty acid analyses, while high-temperature gas chromatography is eminently suited for quantitative analysis of intact neutral lipids. The availability of the flame ionization and mass detec-

tors now makes high-performance liquid chromatography more useful for profiling both neutral and polar lipids. Combinations of gas or liquid chromatography with mass spectrometry must remain of specialized interest only because of the prohibitive costs of operation.

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