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Journal of Chromatography B, 671 (1995) 169–195

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Thin-layer chromatographic procedures for lipid separation

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Abstract

This review focuses on the thin-layer chromatographic (TLC) separation aspects of lipid analysis. Since the space limitations do not permit, the quantitative aspects of the analyses are not discussed at length although some indications of appropriate methodology and detection reagents will be indicated. Many separations carried out by TLC have the prerequisite of proper sample preparation. Using proper sample clean-up prior to TLC enables one to carry out precise separation as well as sensitive quantitation. Thus, the discussions are divided into the two main topics – sample preparation and TLC. Examples of applications are limited to those which illustrate the capabilities of the technique as well as practicability. Since there are a number of reviews in the literature, the discussions herein are focused mainly on reports after 1985.

Contents

0378-4347/95/$29.00
SSDI 0378-4347(95)00232-4

List of abbreviations

BHT	Butylated hydroxytoluene
DG(DAG)	Diacylglyceride
DHEA	Dehydroepiandrosterone
EDTA	Ethylenediamine tetraacetic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GC	Gas chromatography
Gb_4	Globoside b4
HMG	3-Hydroxy-3-methoxyglutaric acid
HPTLC	High-performance thin-layer chromatography
I.S.	Internal standard
LPL	Lipoprotein lipase
MG(MAG)	Monoglyceride
OPTLC	Overpressured thin-layer chromotography
PC	Phosphatidyl choline
SPE	Solid-phase extraction
TG(TAG)	Triglyceride triacylglyceride
TLC	Thin-layer chromatography

1. Introduction

Analysis of lipids by chromatography has progressed along conventional lines. Considerable work has been reported in this area since lipids are involved in so many areas including foodstuffs, myocotoxins, vitamins, and polyaromatic hydrocarbons. This means biochemical and industrial areas must be considered. In this review emphasis is placed on thin-layer chromatographic separations of selected lipids. This of necessity must cover sample preparation since chromatography of poor samples is generally unsatisfactory. The focus here is on the separation, thus the quantitative aspects will be covered only by citation of pertinent reference and selected examples to show how the methods described resulted in viable results. Maximal separation is prerequisite for optimal quantitation. Some reference to the detection methodology is also discussed. Since fluorescence methodology is the method of choice, some discussions of these methods are included.

2. Sample clean-up

Some discussion of sample preparation is appropriate. The success of the TLC, to a large extent, is dependent on the removal of as much of the extraneous material as possible. The discussion herein is limited to more recent methodology involving the so called solid-phase extraction (SPE) devices, and specific examples will be given. A preliminary separation of lipid extracts into acidic and nonacidic species can be done by use of a Sephadex columns as reported by Ledeen and Yu [1]. SPE columns are effective in separating lipid classes differing in polarity. Closely related lipids can then be separated by TLC. Examples follow in the succeeding sections. Polar lipids can first be separated into neutral and acidic fractions as reported by Alvarez and Touchstone [2] using aminopropyl SPE columns. Since these are commercially available they constitute a convenient and highly reproducible means of separating neutral and acidic lipids from biological samples. The example which follows illustrates the procedure which has wide application.

2.1. Solid-phase extraction

2.1.1. Method 1

The SPE method below is a modification of that reported by Luderer et al. [3] for separation of free fatty acids:

(a) Condition octadecyl column with methanol followed by a phosphate buffer (pH = 3).

(b) Acidify the biological fluid to pH 3 with 1 *M* HCl. Apply the plasma samples (1 ml) or buffer (1 ml) directly to the column. Small urine volumes can be applied directly, but when larger volumes (>50 ml) are applied centrifugation of the acidified urine is recommended. Large volumes of urine (400 ml) can be processed using a

6-ml octadecyl column fitted with a reservoir which attaches to the top of the column to accommodate the larger sample size.

(c) Elute the column sequentially with 2 ml of water followed by 2 ml of 25% methanol in water. The fatty acids are retained by the column. Elute either by gravity (1 ml/min), by means of a vacuum manifold, or by gentle application of air pressure to the top of the column for faster rates (20–40 ml/min).

(d) Elute the fatty acid fraction with 2 ml of 100% methanol. This fraction may also contain prostaglandins, leukotrienes, and organic hydroperoxides. The recoveries obtained for the different fatty acids range between 80 and 90%.

(e) Evaporate to dryness under nitrogen and store at −20°C until further processing.

2.1.2. Method 2

The method of Kaluzny et al. [4] (illustrated in Fig. 1) perhaps is an ideal one which will separate a sample into several classes. Several SPE columns and solvents are required. Lipid mixtures are evaporated to dryness under nitrogen and taken up in a minimal volume of chloroform (<0.5 ml). Aminopropyl columns were placed in the Vac Elut apparatus and washed twice under vacuum with hexane. Lipid mixtures in chloroform were applied to the column under vacuum, and the chloroform was pulled through. This left the entire lipid mixture on the column. The scheme is shown in Fig. 1. The eluting solvents are listed in the legend.

2.1.3. Method 3

The following procedure was developed for removal of phospholipids from amniotic fluid and blood plasma as described by Touchstone and Alvarez [5]. It has also been used to separate neutral lipids from the more polar analogues, i.e., cholesteryl esters from phospholipids. Spice C_{18} cartridges from Analtech (Newark, DE, USA) were placed in a vacuum manifold.

(a) The cartridge was conditioned by washing successively under vacuum with 2-ml volumes each of hexane, chloroform and methanol.

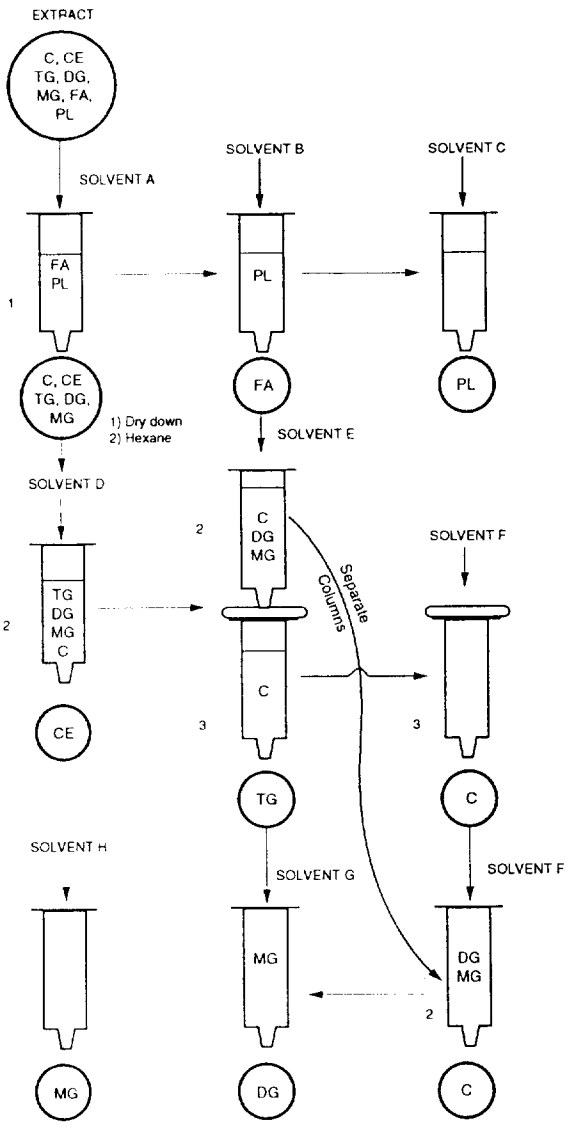

Fig. 1. The Kaluzny method of lipid fractionation from Ref. [4, Fig. 2] with permission. Mobile phases: (A) chloroform–2-propanol (2:1) (B) diethyl ether–acetic acid (98:2); (C) methanol; (D) hexane; (E) hexane–methylene chloride–diethyl ether (89:10:1); (F) hexane–ethyl acetate (95:5), (G) hexane–ethyl acetate (85:15); (H) chloroform–methanol (2:1).

(b) The amniotic fluid is added directly to the cartridge, and the aqueous part allowed to flow through.

(c) The sample is then washed under vacuum with successive 1-ml volumes of hexane and 2-ml volumes of chloroform.

(d) The cartridge is dried by aspirating air through for 4 min.

(e) The analyte is then eluted with 2 ml of chloroform–methanol (1:1).

(f) The eluent is evaporated and reconstituted for analysis (see Fig. 2).

Lipids were separated from human spermatozoa and *Escherichia coli* using the SPE methodology by Alvarez and Touchstone [2]. *Escherichia coli* and human spermatozoa cell suspensions were centrifuged at 800 *g* for 8 min, the supernatants removed, and the resulting pellets resuspended in isotonic phosphate buffer and centrifuged at 800 *g* for 8 min, then were extracted with twenty volumes of a mixture of chloroform–methanol–water (10:10:1). This procedure was repeated twice. The combined extracts were evaporated to dryness, the residue was redissolved in 200 ml of chloroform and loaded onto an aminopropyl column (United Chemical Technologies, Bristol, USA). Nonpolar neutral lipids, fatty acids, neutral phospholipids and glycolipids, and acidic phospholipids and glycolipids, were eluted with isopropanol–chloroform (1:2), 2% acetic acid in diethyl ether or methanol. Fractions were evaporated to dryness and applied to Whatman silica gel layers. The plates were predeveloped in chloroform–methanol (1:1) only to 1 cm from bottom edge, followed by development with chloroform–ethanol–triethylamine–water (30:34:30:8) for 4 cm, thoroughly dried and developed in hexane–diethyl ether (50:5) for separation of polar and non-polar lipids, respectively. The $CuSO_4$-treated chromatogram corresponding to the separation of the different lipid classes from *E. coli* by aminopropyl-bonded silica gel chromatography is shown in Fig. 3.

2.2. Use of preadsorbent or concentrating layers

A relatively underutilized method for sample preparation is that of extraction directly on the layer. Sorbent layers with a preadsorbent zone are available from the manufacturer. This preadsorbent region 2.0 cm wide on 20 × 20 cm plates or 1.0 cm on 10 × 10 cm layers is composed of nonadsorbing material usually Kieselguhr. The sample solution is applied to this area of the layer. After drying, the layer is "predeveloped" with a mobile phase usually much more polar than the mobile phase used for development of the chromatogram. This predevelopment is carried out only to the juncture between the adsorbent and the preadsorbent.

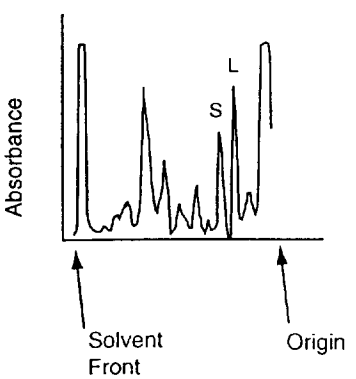

Fig. 2. Phospholipids from amniotic fluid after SPE cleanup according to Touchstone and Alvarez [5] with permission. L = lecithin; S = sphingomyelin.

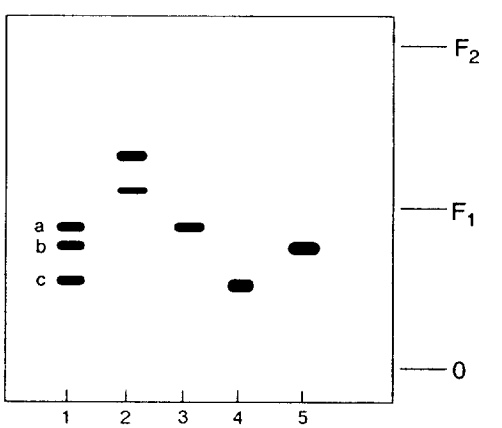

Fig. 3. Lipids from *E. coli* as shown by Alvarez and Touchstone [2] with permission. O = origin, F_1 = solvent front 1, F_2 = front 2. a = Oleic acid; b = phosphatidylglycerol; c = phosphatidylethanolamine.

After drying chromatography is carried out [6] in the usual manner. By judicious use of solvents some provisional fractionation can be performed or the predeveloping solvent can be tailored to remove only the compound class of interest and deposit it on the starting line. This method was used by Touchstone and Alvarez [5] to extract amniotic fluid directly on the layer for analysis of phospholipid.

3. Applications for lipid separations by TLC

3.1. Cholesterol and esters

Cholesterol and its metabolites, particularly the esters with long chain fatty acids, are important in fatty acid transport, storage and maintenance of fat homeostasis in the body. Enzymes for formation of cholesterol esters are found in the cell as well as in plasma. Other esters, such as the sulfate, are present in lipids extracted from keratinizing tissues.

The samples for these analyses are prepared by the classical Folch extraction. The solvent consists of chloroform–methanol (2:1). Usually 1 part of tissue or fluid to 20 parts of this solvent are used. There are numerous modifications of this method. Another useful method is the SPE extraction procedure described by Vanluchene et al. [7]. This is useful for separating neutral lipids from esteryl esters and phospholipids. Cholesterol accumulates in a rare sterol storage disease and can be found in blood and tissues. The diagnosis of the disease can be made after determination of cholesterol in serum. Michalec and Ranny [8] reported the use of TLC for cholesterol determination after extraction of serum with hexane. The extract was subjected to peracetic acid followed by hydrogen sulfide oxidation. The product was separated or silica gel 60 HPTLC using heptane–ethylacetate (60:40). Detection was with the phosphomolybdic acid reagent.

Potential precursors of cholestanol in animals were synthesized by Batta et al. [9]. The compounds were characterized in part by TLC. Cholestanol is formed from cholesterol as the sole precursor. It is present in as much as 10% of cholesterol in various tissues. Patients with the lipid storage disease cerebrotendinous xanthomatosis have increased amounts of cholestanol in their blood and tissues. Cholest-5-enzone and epicholesterol were separated as below. Silica gel plates were used and mobile phases containing different proportions of acetone in methylene chloride, chloroform, or carbon tetrachloride. Spots were visualized by spraying the plates with phosphomolybdic acid (3.5% in isopropanol) followed by sulfuric acid (20%) and heating at 110°C for 2 min. In order to prepare the silver nitrate-impregnated plate, the plate was dipped in an aqueous solution (10%) of silver nitrate in a trough and immediately removed. The plate was air dried in the dark (30 min) and then activated by heating at 110°C for 1 h.

The overpressured and conventional TLC methods were utilized by Pucsok et al. [10] for separation of cholesteryl esters.

For the one-dimensional TLC separation technique used for two successive developments on the same plate, the plates were developed first with solvent to a height of 9 cm above the origin (ca. 15 min). After drying, the plates were developed to 18 cm above the origin using a second mobile phase. Morris et al. [11] used HPTLC to determine cholesterol in egg yolk as well as butter and cream samples. Egg yolks were extracted with chloroform–methanol [1:1] and filtered through glass wool. Samples plus standards were developed by HPTLC on silica gel with mobile phase hexane–acetone (95:5). Cholesterol was detected with cupric acetate–H_3PO_4 reagent. Precision was 2.2%, while recovery was 104%. This is one of the few examples where the differentiation between cupric acetate and copper sulfate was used for making the methodology more specific. Cupric acetate reacts only with the unsaturated species while copper sulfate will react with unsaturated as well as the saturated.

Fei et al. [12] used petroleum ether–ethanol (1:1) to extract lipids from blood. Aliquots were analyzed by TLC on the silica gel plates containing ammonia. A mixture of petroleum ether–

diethyl ether–acetic acid (82:18:1) was used as mobile phase. After development, TLC plates were heated at 180°C and then measured for lipids by scanning densitometry. Recovery of free cholesterol was 99.5% and the relative standard deviation was 1.54%. The concentrations in blood serum of normal human of free cholesterol, cholesterol ester, total cholesterol, and triglycerides were 5.127, 128.3, and 179.7 mg/dl, respectively. Chloroanalogues of cholesterol and its acetate were analyzed by Bhat and Ansari [13]. These compounds have xenobiotic activity. The method was developed for assay in tissues. The lipids were extracted from reaction mixtures for synthesis of the chlorinated derivatives. TLC was performed on silica gel G in a presaturated chromatographic chamber using the mobile phases below. The plates were air dried, sprayed with 50% sulfuric acid and heated at 120°C for 5 min to detect cholesteryl esters. R_F values of cholesteryl acetate were: 0.66, 0.55, 0.37, 0.35 and 0.29 using hexane–ethyl acetate 4:1, 9:1, 19:1, 24:1 and 66:1 as mobile phase. With the mobile phase hexane–ethyl acetate (19:1), cholesteryl dichloroacetate showed a mobility of 1.73 with respect to cholesteryl acetate.

Serizawa et al. [14] separated cholesteryl sulfate from intercellular stratum corneum in studies related to horny layer adhesion. For extraction the surface of the palm was cleaned with 30% ethanol. A cup was pressed tightly to the skin and solvent added to extract the stratum lipids. Various solvents including diethyl ether–acetone (1:1) or chloroform–methanol (2:1) were used to remove the lipids. HPTLC plates were pre-developed in chloroform–methanol (2:1) to the top before use. The procedure for separation of corneum lipids on a HPTLC plate (10×20 cm) follows. First, chloroform–methanol–water (20:95:1) was developed to 6 cm above the origin. Second, this step was repeated. Third, *n*-hexane–diethyl ether–glacial acetic acid (60:30:10) was developed to 10 cm above the origin. Finally, petroleum benzine was developed to the top of the plate. The plates were sprayed with 10% cupric sulfate in 8% phosphoric acid followed by heating at 180°C for 60 min.

Desmosterol in monkey spermatozoa was separated by TLC as reported by Lin et al. [15]. Sterols were extracted by the classical procedures of Folch. Aliquots of the lipid extracts were applied to silica gel G plates after (4-^{14}C) cholesterol and cholesteryl (^{14}C) oleate were added as internal standards. The chromatograms were developed in hexane–chloroform–diethyl ether–acetic acid (80:10:10:1). The free sterol band containing both cholesterol and desmosterol was removed and extracted with diethyl ether. Sterol esters were saponified with alcoholic KOH and the sterols were extracted with hexane. The sterol ester fatty acids were recovered by acidifying the aqueous phase and re-extracting with hexane for fatty acid and analyzed, after transmethylation with boron trifluoride, by GC.

Sterol analyses were carried out by Swaminathan et al. [16] using TLC as follows. TLC was carried out on silica gel G plates or on aluminum-backed silica gel 60 plates. In the case of assays of HMG-CoA reductase activity, Whatman LK5D plates were used. Analytes were visualized after spraying with 5% ammonium molybdate in 10% sulfuric acid followed by heating. Mobile phases were: 35% diethyl ether in hexane; 15% ethyl acetate in hexane; 20% ethyl acetate in hexane. Cholesterol and cholesterol oxide derivatives were separated by TLC using the conditions as below [17]. These products resulted from oxidation of lipoproteins. TLC analysis of the labeled cholesterol and cholesterol oxide derivatives in the lipoproteins was performed using a developing system of hexane–acetone–acetic acid (80:20:1). The R_F values for 7-ketocholesterol, 5,6-epoxycholesterol, unesterified cholesterol, 7-ketocholesteryl ester, 3,5-cholestendien-7-one, and cholesteryl ester were found to be 0.19, 0.22, 0.41, 0.56, 0.63, and 0.96, respectively.

Cholesterol sulfate found in glandular epithelial cells from guinea pigs was separated on TLC by Nicollier et al. [18]. Aliquots of lipid extracts were chromatographed by monodimensional TLC on silica gel developed with chloroform–methanol–acetone–acetic acid–water (10:2:4:2:1). For ^{35}S radioactivity areas with cholesterol sulfate and sulfatide standards were cut out and lipids were visualized by spraying with 50%

H_2SO_4 at 160°C. After locating the radioactive bands by exposure of the plates to a Kodak X-OMAT AR5 film, the silica gel of the bands of sulfolipids were scraped from the TLC plates into a vial and counted by liquid scintillation.

3.2. Fatty acids

The sodium salts of long chain fatty acids are detergents and are not compatible with membranes of cells. Thus, the fatty acids are usually not present in the free form. They are combined with other molecules to form complex substances such as fatty acyl CoA, phospholipids and glycerides. Serum lipid fatty acid compositions have been determined in coronary artery disease, diabetes, as well as renal disease. Preparation methodology is based on the classical Folch et al. [19] extraction technology. The following describes methods developed to determine the free acids as a means to elucidate structures. Two descriptions involve the preparation of fluorescent derivatives. The preparation of the derivative, while greatly increasing sensitivity often increases the ability to enhance resolution.

Many methods for separation of arachidonic acid and its metabolites involve derivatization Rao et al. [20] have described a method for separating monohydroxy acid metabolites of this acid by extraction with ethyl acetate after acidification with 0.5 *M* citric acid. Aliquots were applied to a silica gel G plate. The mobile phase for separation of thromboxane B_2 was diethyl ether–methanol–acetic acid (135:3:3). Hydroxy acids were separated using petroleum ether (60–70°C)–diethyl ether-acetic acid (60:39:1). For characterization of the compounds identified, the extracts from the plate containing the hydroxy acids were concentrated, derivatized to methyl esters using diazomethane. Methyl esters were silylated and analyzed by GC.

Tsunamoto and Todo [21] used two-dimensional TLC for separation of prostaglandins and thromboxane formed from arachidonic acid by neuroblastoma cells. One-dimensional development was used to assess the purity of the standards including arachidonic acid. Sun et al. [22] have reported extensive work in this area with various tissues.

A 20-μg sample of each of the authentic compounds and 104 dpm of each of the radioactive compounds were applied to a silica gel 60 plate, which was pre-washed with acetone. The plates were developed with three different solvent systems: (i) ethyl acetate–isooctane–acetic acid–water (11:5:2:10), (ii) chloroform–methanol–acetic acid (90:8:6), and (iii) diethyl ether–methanol–acetic acid (90:1:2). Detection was by spraying with 10% phosphomolyabdic acid in ethanol [23].

Beneytout et al. [24] separated arachidonic acid and its metabolites on reversed-phase layers. The layers contained phenylmethylvinyl-bonded silica. Cyclooxygenase and lipoxygenase metabolites of arachidonic acid were separated by TLC on silica plates coated with phenylmethylvinylchlorosilane. Hydroxyeicosatetraenoic acids were separated by heptane–methyl formate–diethyl ether–acetic acid (65:25:10:2); prostaglandins and thromboxane B_2 were separated after two developments with heptane–methyl formate–diethyl ether–acetic acid (65:25:10:2), followed with hexane–methyl formate–diethyl ether–acetic acid (50:40:10:1). Resolution was good and took only 15 min.

Alumina argentation TLC was used by Breuer et al. [25] to separate fatty acids or their methyl esters. The alumina sheets were immersed in a 10% $AgNO_3$ solution, giving greater penetration of the adsorbent than did spraying. Development followed as usual. Saturated fatty acids are often contaminated with unsaturated fatty acids or with different chain lengths. To remove the unsaturated species Longmuir and Haynes [26] treated the samples in question with osmium tetroxide. After cleanup by silica gel column chromatography the sample was then subjected to preparative TLC using C_2 layers.

In a typical preparation, 10 μCi (approximately 50 μg) of radiolabeled FFA was treated with 200 μl of osmium tetroxide in carbon tetrachloride (50 mg/ml). After 1 h at room temperature, the solvent was evaporated, and 2 ml of 1 M hydrochloric acid were added. The FFAs were extracted with diethyl ether. The diethyl ether

was evaporated, the residue dissolved in hexane–diethyl ether (90:10) and added to a silica gel column; flushed with 25 ml of hexane–diethyl ether (90:10). The product was eluted with 50 ml of hexane–diethyl ether (75:25), followed by 25 ml of hexane–diethyl ether (50:50). The products were collected into 10-ml fractions and radioactivity counted. RP2 reversed-phase TLC plates were prewashed in dioxane–0.001 *M* hydrochloric acid (7:3). The acids were developed in dioxane–0.001 *M* hydrochloric acid (7:3). Radiolabeled fatty acids were visualized by autoradiography.

Free fatty acids were determined in placenta of rats by Frank and Graf [27]. The method involves derivatization of the fatty acid to monodansyl cadavarides. These derivatives permit a separation of various fatty acids in a very simple way. This technique involves application of RP18 plates and represents an excellent tool for the routine analysis of specific fatty acids in biological tissues. For example, this technique can be used to monitor plasma lipids such as total lipids, triacylglycerols, and cholesterol fractions as well as to determine the fatty acid profiles of all these fractions. It involves solid-phase extraction of samples for the isolation of lipids. No impurities could be detected in the fatty acid fraction. Lipids isolated by solid-phase extraction were derivatized on the plate quickly and completely following application of the monodansyl cadaverine chloride (DCC) solution directly to the layer. Complete separation of saturated, odd and even carbon number, straight chain fatty acids, could be achieved. The R_F values of 20:0 (0.28), 17:0 (0.45), and 15:0 (0.58) and the resolution obtained were in agreement with values published by other authors. Substitution of fluorine for hydrogen in a hydrocarbon gives greater stability to the molecules. This has led to industrial applications of this type of densitive insurfactants corrosion inhibitors and plasticizers. These compounds have heen difficult to analyze from the metabolic viewpoint. Vanden Heuvel et al. [28] have described methods to separate these fluorinated analogues for more complete studies. Plasma was extracted with chloroform–methanol–0.5 *M* H_2SO_4 (2:1:0.1) in a ratio of 6.2 volumes to one of plasma. After centrifugation the lipid-containing lower phase was collected. Lipid content of the final lower phase was determined by a micromethod for the estimation of nonvolatile organic matter. Tripalmitin and phosphatidylcholane yielded equivalent standard curves. The simple lipids, including the perfluorinated fatty acids, were separated from the complex lipids by SPE. The lipid samples to be separated were dried under a stream of nitrogen and dissolved in 0.1 ml chloroform–methanol (2:1). These lipid mixtures were quantitatively transferred to a column using three times 0.1 ml chloroform–methanol (2:1). The mixture was allowed to adsorb to the silica gel for 5 min. Elution of simple lipids, including perfluorinated fatty acids, was carried out with 3 ml diethyl ether–trifluoroacetic acid (100:1) under slight negative pressure. The major simple lipids: cholesterol, cholesteryl esters, triacyglycerols, and the free fatty acids were separated. Silica gel 60 plates were activated in a 110°C oven for at least 12 h. Tissue samples were applied 3 cm from the bottom of the plate and then developed to a height of 16 cm in a solvent system of petroleum ether–diethyl ether–trifluoroacetic acid (70:30:1). The plates were air-dried and developed to a height of 11 cm in the same direction in diethyl ether–petroleum ether–trifluoroacetic acid (70:30:1). Lipids were visualized by spraying the plates with 1′,7′-dichlorofluorescein, followed by examination under UV light.

Ohta et al. [29] have reported that small samples of plasma samples can be applied to the preadsorbent portion of silica gel preadsorbent layers and trans methylation of the lipid fractions without extraction of the lipid from the sorbent. Plasma was transferred to a screw-capped test tube, and 50 μl of I.S. was added. Then 0.1 ml of water, 2.0 ml of methanol and 1.0 ml of chloroform were added with vigorous mixing, to TLC plates.

Plasma (75 μl) or extracts from 75 μl of plasma were applied along with standards in alternate lanes. The TLC plate was developed first in methanol to approximately 1.5 cm above the preadsorbent border. After evaporating the methanol, the plate was again developed to 1.5 cm with chloroform–methanol (1:1), to complete the extraction of lipids from the plasma protein.

The plate was developed to 15 cm above the preadsorbent using hexane–diethyl ether–acetic acid (80:20:1). The lipid bands were visualized by spraying the plate with rhodamine 6G. To each tube containing silica, 20 μl of methanol containing 50 μg BHT was added. All sample tubes were then treated with 1.0 ml of the BF_3 reagent and tightly capped. Methyl docosanoate (22:0) or methyl tricosanoate (23:0) were added as an internal reference. The fatty acid methyl esters were extracted with hexane. This method is an example of using the preadsorbent as a means for extraction as cited in the introduction.

The labeling of fatty acids with the fluorescence probe, 4-bromomethyl-7-methoxycoumarin (Br-Mmc), followed by separation of the Br-Mmc esters by high-performance liquid chromatography has been described. This method of analysis provides both a carboxylic acid-specific mode of labelling, thus excluding aliphatic components that lack the carboxylic acid moiety, i.e. fatty aldehydes, fatty alcohols, and high sensitivity. Alvarez and Touchstone [30] separated 4-bromomethyl-6,7-dimethoxycoumarin (Br-Mdmc)-derivatized fatty acid esters by diphasic-2D-TLC. Diphasic layers with C_{18} in one dimension and silica impregnated with silver nitrate in the other dimension were prepared. The sample is first developed in the reversed-phase C_{18} layer which results in separation based on the number of carbons and/or double bonds. Development of the Br-Mdmc-derivatized fatty acids in the second dimension (silver nitrate in silica gel) served to separate the unsaturated species: faster migration of the saturated fatty acid derivatives (1,2,3,4,5), followed by the mono (6,7,8), di (9,10,11), and polyunsaturated (12,13,14,15) fatty acid derivatives. Due to the hydrophobic effect, the fatty acid derivatives containing longer methylene chains, resulted in the faster migration of the latter (numbers refer to position of double bond).

Aliquots of the reaction mixture were streaked onto the C_{18} part of the layer followed by drying to remove solvent. Predevelop the plates in chloroform–methanol (1:1) to 1 cm from the lower edge of the plate. The first dimension (C_{18}) used chloroform–acetone–methanol–water (60:20:10:10) as the mobile phase. This dimension allows separation based on the number of carbons. Impregnate the silica gel layer with a saturated solution of $AgNO_3$ in methanol to the interface with the reversed-phase C_{18} layer. The plate was turned 90° and predeveloped in chloroform–ethyl acetate–acetonitrile (90:8:2), in the second dimension to the plate interface. Then developed in the same solvent. The layers can be scanned in the fluorescence mode for quantitation or set up of a profile.

Argentation TLC was used by Wilson and Sargent [31] to separate polyunsaturated fatty acids. It has been found that GC or HPLC did not separate these compounds well. Silica gel 60 TLC plates were sprayed uniformly with acetonitrile containing silver nitrate until the plates were saturated. Spraying TLC plates gives a coating of silver nitrate as uniform as dipping. The plates were air-dried in subdued light, heated at 110°C for 30 min to achieve activation and used within 1 h. After isolation by column chromatography, FAME mixtures were applied to the impregnated TLC plates. The plates were developed with toluene–acetonitrile (97:3) to 1 cm from the top. The plates were dried, lightly sprayed with 3% copper acetate–8% orthophosphoric acid in water and charred at 180°C for 20 min to visualize the esters. Using this procedure the dienes could be well separated from trienes and in turn from tetraenes. Recoveries of individual radioactive methyl esters, were 90–92% as determined by elution of individual zones from the plates, radioassaying aliquots of the eluates, and re-chromatographing and radioassay.

Docosahexaenoic acid as well as desmosterol was demonstrated in the spermatozoa of monkeys by Lin et al. [32]. Lipids were extracted by the Folch method. For analysis of free and esterified sterols in sperm, aliquots of the lipid extracts were plated on silica gel G after addition of internal standards. The plates were developed in hexane–chloroform–diethyl ether–acetic acid (80:10:10:1). The free sterol band containing both cholesterol and desmosterol was removed and extracted with diethyl ether. Sterol esters were saponified with alcoholic KOH and the sterols were extracted with hexane. The fatty acids of the lipid classes (phospholipids, free fatty acids, triglycerides, and sterol esters) of

sperm lipids were determined. The four lipid classes were separated by TLC as described above. The individual phospholipids were separated by another TLC system using pre-coated silica gel layers and a mobile phase of chloroform–methanol–petroleum ether–acetic acid–boric acid (40:20:30:10:1.8).

Schwertner and Mosser [33] studying lipids in coronary artery disease extracted lipids with chloroform–methanol with butylated hydroxytoluene as antioxidant. The extract was evaporated and aliquots were applied to silica gel layers. The lipid classes were then separated with 60 ml of *n*-hexane–diethyl ether–acetic acid–BHT (95:5:1:0.1). Plates were sprayed with rhodamine 6G and detected with UV light. The esters were saponified with KOH, then esterified with boron trifluoride and gas chromatography used for quantitation. The concentrations of seven fatty acids were determined by this method.

3.3. Glycerides

Hydrolysis products of reactions with neutral lipids are amenable to separation by TLC. Standards should always be included due to differences in separation between plates. When using radiolabeled substrates, it is often easier to add a mixture of standards containing unlabeled oleic acid and oleoylglycerols to the lipolysis products before application to the TLC layer. This ensures that all of the lipid regions, and especially those products which occur at low concentration, stain with I_2, thus facilitating their identification. The plate may be first developed twice with diethyl ether to 3 cm. This enables the monoacylglycerols to migrate from the origin, and separates them from phospholipids or acyl-CoAs which remain there [34]. The plate may be stained with iodine or a fluorescent dye and the regions corresponding to the various products scraped. If unlabeled lipids are used, the regions corresponding to the various lipids must be scraped, the fatty acids converted to their methyl esters, and an I.S. added before separation by gas chromatography.

Sodium carbonate (5%, w/w) impregnated silica gel micro-TLC plates have been used to separate the ^{14}C-fatty acids released by the action of lipase from ^{14}C-acylglycerols [35]. The reaction is terminated by the addition of sodium dodecyl sulfate (SDS) and an aliquot (10 to 20 μl) of the lipase reaction mixture is applied directly to the TLC layer. The plate is then dried at 90°C to remove the water. The chromatogram is developed for about 1 min with diethyl ether–methanol (97:3). The fatty acid soaps remain at the origin whereas the acylglycerols migrate at the solvent front. This method was extended to give a complete separation of the fatty acids and all of the various acylglycerols with the sodium carbonate impregnated plates using a solvent consisting of diethyl ether–hexane–methanol (65:35:3).

The enzyme lipoprotein lipase (LPL) hydrolyses circulating lipoprotein triacylglycerol (TAG). Its action appears also to regulate HDL-cholesterol levels, which correlate with LPL activity. The action of LPL thus plays a key role in the modulation of plasma lipid levels and atherosclerotic risk.

LPL action in vitro first produces 1,2(2,3)-diacylglycerol (DAG); further hydrolysis of the 1(3)-ester bond by LPL produces 2-monacylglycerol (MAG), which must then isomerise to 1(3)-MAG before complete hydrolysis [36]. During LPL action in vitro, the partial hydrolysis products may accumulate [37]. It is not known whether they do so in vivo. Partial acylglycerols (AGs) might represent almost 10% of the total acylglycerol in human plasma and as much as 30% in rat plasma [38]. Since partial acylglycerols would be measured as TAG in standard enzymatic assays, current estimations of plasma TAG concentrations and conventional understanding of metabolism might need revision.

The products of lipase-catalyzed hydrolysis of neutral lipid may be separated on silica gel layers. The mobile phase of hexane–diethyl ether–acetic acid (70:30:1) gives a good separation of triacylglycerols, fatty acids, 1,2- and 1,3-diacylglycerols and monoacylglycerols. The R_F values for these compounds are roughly 0.7,

0.45, 0.26, 0.23, and 0.05, respectively. The plate may be first developed twice with diethyl ether to 3 cm. This enables the monoacylglycerols to migrate from the origin, and separates them from phospholipids or acyl-CoAs which remain there [39].

Sodium carbonate (5%, w/w) impregnated silica gel micro-TLC plates will separate the ^{14}C fatty acids released by the action of lipase. Addition of SDS terminates the reaction and an aliquot is applied directly to the TLC layer. The plate is then heated briefly at 90°C. The chromatogram is developed for about 1 min with diethyl ether–methanol (97:3). The fatty acid remains at the origin whereas the acylglycerols migrate at the solvent front. The two regions are well separated. This method was extended to give a complete separation of the fatty acids and all of the various acylglycerols with the sodium carbonate impregnated plates using a solvent consisting of diethyl ether–hexane–methanol (65:35:3). The R_F values of the products were as follows: unesterified fatty acids, 0.0; monoacylglycerols, 0.18; diacylglycerols, 0.79 to 0.85; triacylglycerols, 0.98. This method has been used for the rapid analysis of lipase-catalyzed esterification and interestification reactions of acylglycerols [40,41].

It is important to prevent the migration of acyl groups from the *sn*-2 position of the glycerol to the *sn*-1(3) positions of acylglycerols. This migration occurs at different rates depending on the acyl moiety and can be prevented by using boric acid impregnated silica gel TLC plates. The boric acid interacts weakly with the free hydroxyl groups of the acylglycerols to prevent the acyl migration. The TLC plates are developed with chloroform–acetone (96:4). This resolves both the 1,2- and 1,3-diacylglycerols and the 1(3)- and 2-monoacylglycerols. Fielding et al. [42] used the following method for separation of glycerides in plasma.

The glycerides were extracted from plasma with chloroform–methanol (2:1) based on the method of Folch. After the addition of 1 ml of 1 *M* NaCl, the extraction tubes were centrifuged and the lower solvent layer containing the acylglycerols was separated. The residue was taken up in a small volume of chloroform for TLC. The mobile phase for TLC consisted of toluene–diethyl ether–ethyl acetate–glacial acetic acid (8:1:1:20). After development for 15 min, the layers were dried. The acylglycerol spots were visualized with I_2. The areas were cut out and eluted with chloroform–methanol (2:1). Assay was by the enzymatic hydrolysis of TAG and measurement of the glycerol released.

Human aortic lipids were analyzed using TLC by Elliott et al. [43]. Lipids from the aorta without damaging the elastic lamellae were extracted by three methods. Hydrated and dehydrated 0.5 cm wide bands of human thoracic aorta were used. Extracted with: (A) Me_2CO then butanol, (B) $CHCl_3$–MeOH (2:1), and (C) Me_2CO, BuOH, $CHCl_3$–MeOH, successively. Method A extracted less lipid than B or C, and there was no difference in recovery between dehydrated and hydrated samples treated by the same extraction method. In HPTLC on silica gel with development by hexane–diethyl ether–acetic acid (65:35:1), the aortic and associated plaque lipids were resolved into 5 major bands: cardiolipin, cholesterol, oleate, triolein, and cholesteryl oleate.

Ritchie and Lee [44] determined triglycerides by Iatroscan techniques or by $AgNO_3$ multiphase TLC for isomer separation. Lard and cocoa butter are used as examples. The Iatroscan method allows quicker determinations and good quantitation when linked to a computing integrator, but its accuracy (detection limit 2–3%) is less than TLC coupled with a densitometer (ca. 1%). Multiphase TLC used silica gel plates containing $AgNO_3$ with development in acetone–acetonitrile. This is one of the few reports comparing the Iatroscan procedure with conventional methods.

Bilyk et al. [45] reported the separation of acylglycerol, fatty acids and amides as well as cholesterol. This method may serve when the above classes must all be analyzed in a sample. Two different mobile phases used in sequence enabled the separation by TLC of these compounds from one another (see Fig. 4). TLC layers were washed with methanol for 5 min then dried in air for 30 min. Samples were applied 2

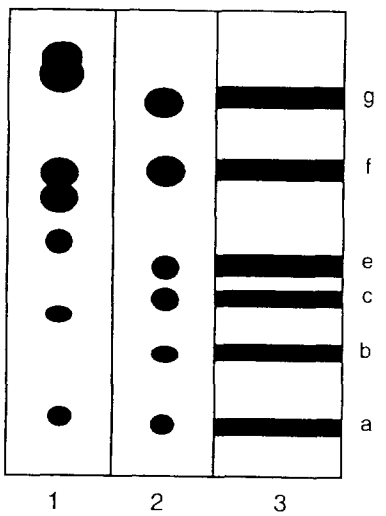

Fig. 4. TLC of acylglycerols from natural lipid mixture as described by Bilyk et al. [45] with permission. Lane 1: standards in toluene–diethyl ether–ethylacetate–acetic acid (75:10:13:12); lane 2: as 1, then hexane–diethyl ether–formic acid (80:20:2); lane 3: preparative separation using method of lane 2.

cm from lower edge of the layer and developed with toluene–diethyl ether–ethyl acetate–acetic acid (75:10:13:1.2) to 8 cm above the level of application. The TLC layers were then developed with hexane–diethyl ether–formic acid (80:20:2) to 14 cm above the level of application. Detection was by spraying the TLC plates with 60% aqueous sulfuric acid charring.

Nikolova-Damyanova et al. [46] separated isomeric triacylglycerols using thin layers impregnated with silver nitrate. They used open cylindrical jars to obtain a continuous development. Plates with 4% to 10% silver nitrate were prepared by incorporation of the salt into the layer. The TG mixture was applied to the plate. Continuous ascending development with a mobile phase of specified composition and volume in open cylindrical jars was done. The plates were dried for 1 h at 110°C and treated consecutively with bromine and sulphuryl chloride vapours and then heated for 15–20 min at 200–220°C. A result of particular importance is the low concentration of the silver nitrate in the impregnating solution. It does not exceed 2% which was found necessary for the separation.

These conditions are interesting in that continuous development in an open chamber was carried out. This also had an effect in change of mobile phase composition as the development progressed due to evaporation in the open tanks.

3.4. Glycolipids

Considerable evidence has accumulated that indicates glycolipid composition undergoes remarkable changes during cellular growth, differentiation and oncogenic transformation. Glycolipids have been reported to be associated with differentiation, development and organogenesis. These glycolipids have an active role in formation of cataracts [47]. The gangliosides are complex glycolipids containing ceramide polyhexosides and one or more sialic acid groupings.

The glycolipid biosynthetic pathway is initiated by the glucosyltransferase-catalyzed synthesis of glucosylceramide. *n*-Butyldeoxynojisimycin is an inhibitor of this synthesis and has been shown to be an inhibitor of HIV replication in vitro [48]. These isolated reports highlight the reason why the research on glycolipids has seen considerable increase in recent years. Sephadex G-25 columns can be used for clean up of samples for TLC. Total lipids were extracted from human cataractous lenses with chloroform–methanol (2:1), and chloroform–methanol–water (30:60:8), successively. The combined extracts were then applied to a DEAE-Sephadex A-25 column, which was further eluted with 15 ml of chloroform–methanol–water (60:40:5). The neutral lipids were eluted in this fraction. Acidic lipids were then eluted with 30 ml of chloroform–methanol–0.9 *M* aqueous sodium acetate (30:60:8). The neutral glycolipid fraction was dissolved in 0.5 ml of 0.3 *M* NaOH in methanol and incubated at 40°C for 2 h in order to remove alkaline-labile phospholipids. The reaction mixture was desalted on a Sephadex LH-20 column and redissolved in a small volume of chloroform–methanol (1:1). An

aliquot of this glycolipid fraction was examined by HPTLC with two different mobile phases: (A) chloroform–methanol–water (60:40:5), and (B) chloroform–methanol–2.5 *M* ammonium hydroxide (60:40:10). The neutral glycolipids were visualized by spraying with the orcinol–sulfuric acid reagent and heating at 120°C.

The acidic lipid fraction was evaporated to dryness, and the sample was desalted by a Sephadex LH-20 column as above. An aliquot of this acidic glycolipid fraction was examined by HPTLC with mobile phase (C) chloroform–methanol–0.2% $CaCl_2$ (55:50:10), and (D) chloroform–methanol–0.4 *M* ammonium hydroxide–0.4% $CaCl_2$ (50:40:5:4) [24]. The gangliosides were visualized by spraying with the resorcinol–hydrochloric acid reagent followed by heating at 95°C or with the orcinol–sulfuric acid reagent.

Minsoon et al. [49] used chloroform–methanol–0.25% KCl (50:40:10) with silica gel layers. For neutral glycolipids, chloroform–methanol–water (65:35:8) might be considered a reasonable starting point. Generalizations can be difficult since some tissues, particularly intestinal mucosa, can yield hexaosyl ceramides containing very long oligosaccharide chains.

Another innovation has been the development of antibodies, and in some cases monoclonal antibodies to various glycosphingolipids [50]. Such antibodies have been used to detect components on TLC plates. Additional ceramides were reviewed by Dyatlovitskaya and Bergelson [51]. Isolation of the arthrotriose and tetraose from the larva of the green-bottle-fly Lucilia caesar has been described [52]. A glucuronic acid derivative of this series has also been reported [53], and could be viewed as being analogous to mammalian acidic glycolipids, gangliosides. The Mullusco series is known with fucose and xylose additions. Antisera produced in rabbits against this mulluscotetraose permitted detection on the TLC layer [54] of very small quantities (0.5 pmol) of this glycolipid and the same glycolipid with a xylose sidechain. The arthro- and mullusco-glycosphingolipids were chromatographed on TLC plates using the mobile phase chloroform–methanol–water (6:4:1).

Ioneda et al. [55] used TLC for separation of trehatose-6,6′-dimycolate from extracts of bacteria in the generic *Mycobacterium*, *Cormyebacterium* and *Nocardia*. TLC was carried out on either silica gel H or silica gel 60 coated plastic sheets. The eluents used were: chloroform–acetone–methanol–water (50:60:2.5:3), hexane–diethyl ether–acetone–acetic acid (70:30:11:1) and hexane–diethyl ether–acetone–acetic acid (70:20:5:1). Lipids separated by TLC were detected either by: (1) exposure to iodine vapor; (2) by spraying with 0.4% anthrone in benzene followed by spraying 50% sulfuric acid and (3) by spraying with molybdenum-blue reagent for the detection of phospholipids.

The glycolipids in HL-60 cells were extracted from the cells after culture [56]. Briefly, [^{14}C]palmitic acid was added in fetal calf serum and the cells cultured for a further 3 days. The cells were harvested, washed three times with phosphate-buffered saline and extracted in 1 ml of chloroform–methanol (2:1) overnight at 4°C. Extracts were dried under nitrogen and redissolved in chloroform–methanol (2:1). A sample was taken for the determination of radioactivity and an aliquot was applied as a single spot onto a TLC layer. Development in the first dimension used chloroform–methanol–water (65:25:4). Separation in the second dimension was achieved using tetrahydrofuran–dimethoxymethane–methanol–water (10:6:4:1). Plates were air dried and exposed to autoradiography film for location of the separated zones. TLC analysis of the column fractions showed that chloroform, 5% and 10% acetone in chloroform eluted neutral lipids and free fatty acids. The fractions eluted with acetone and methanol contained glycolipids and phospholipids, respectively.

Feize et al. [57] using mixtures of chloroform–methanol–water were able to separate neoglycolipids on TLC as noted. HPTLC plates, of aluminum-backed, 5-μm silica were used. Neoglycolipids are applied as spots or bands. Chromatography is usually performed at ambient temperatures. Solvent is equilibrated in the chromatography tank for at least 1 h. The choice of development solvent depends on the nature of the oligosaccharide portion of the neoglycolipid.

The more polar the neoglycolipid the more polar the solvent should be. Unreacted lipid has the highest mobility, the neoglycolipids have intermediate mobilities, and the free oligosaccharides have the lowest mobilities. The original papers should be consulted for the several reagents for spray reagents used to detect the separated zones.

Merron [58] separated fatty acids from hydrolysis of the glycolipids, as methyl esters. Lipid standards may be detected by exposing the plate to iodine vapor in a closed tank, or by using a variety of spray reagents. The solvent systems listed below were used: (A) chloroform–methanol–water (4:4:1) or (10:10:3); (B) chloroform–methanol–acetic acid–water (25:15:4:2); (C) chloroform–methanol–90% formic acid (50:30:7); petroleum ether–diethyl ether–acetic acid (80:20:1); chloroform–methanol–30% ammonia (65:25:5); (F) acetonitrile–acetic acid (1:1).

To analyze the FAMEs, along with standards, on RP-18 F254S thin-layer plates system F was used. Detect nonradioactivity FAME standards by spraying the plates with the phosphomolybdic acid spray reagent and heating at 120°C until spot formation is attained. Methyl esters of unsaturated fatty acids will give dark blue spots whereas those of saturated fatty acids will show up as relatively unstained spots in the blue background of the plate.

Schnaar and Needham [59] reported separation of glycosphingolipids on silica gel 60 TLC or by HPTLC. HPTLC plates offer the best resolution whereas TLC plates are convenient for loading larger amounts of sample. The same sorbents coated on aluminum plates are preferred for immunoverlay. The plates should be prewashed in the same solvent used for development, especially for quantitative work. The plates are heated in an oven at 125°C to remove water (activate), then allow to cool in a sealed dry box. Use at once, or keep in a dry box prior to use.

Glycolipid TLC frequently requires chloroform–methanol–aqueous mixtures, which form complex vapor–liquid equilibria in the development tank. Therefore tank geometry and conditions during development alter chromatographic migration and resolution.

The most widely used TLC mobile phases for glycosphingolipid are mixtures of chloroform, methanol, and water, because they form a single phase at a range of hydrophobicities well suited for glycosphingolipid resolution on silica gel TLC plates. Chloroform–methanol–aqueous mixtures ranging from 70:30:4 (relative low polarity) to 50:40:10 (relatively high polarity) are chosen depending on the particular glycosphingolipids under study. Smaller, less polar glycosphingolipids will be resolved better in lower polarity solvents, whereas larger, more polar species require more polar solvents.

The use of aqueous salt rather than water is recommended for resolving gangliosides and other anionic glycosphingolipids. Salts alter ganglioside mobility and improve their resolution. Ammonium hydroxide (1–5 *M*) in the aqueous phase results in changes in the relative mobilities of different gangliosides compared to a neutral aqueous phase, and is particularly useful when multiple solvents are used to determine purity.

Alvarez et al. [60] characterized globotriacylceramide (GT) from bovine spermatozoa using HPTLC. Gb_4, GM_1 (globomonoacylceramide), GD_{1a} (globodiacylceramide) GD_{1b}, GT_{1a}; the purified Gb_3 from bovine spermatozoa; LacCer (CDH); and seminolipid (SML), dissolved in chloroform–methanol (1:1) at a concentration of 1 mg/ml, were streaked on Whatman silica gel plates as a thin band 5 mm from the lower edge of the plate. Aliquots of each of the ganglioside mixtures were streaked on Analtech diphasic plates. The plates were then dried, predeveloped in chloroform–methanol (1:1) to 10 mm from the lower edge of the plate, dried, developed in the chloroform–methanol–water (65:30:8) for the Whatman plates and chloroform–methanol–0.6 *M* $CaCl_2$–0.12 *M* sodium acetate (55:45:4:6) for the Analtech plates. Following development the plates were thoroughly dried, sprayed with the orcinol–ferric chloride reagent, and heated in a microwave oven for 5 min. The stained chromatograms were then scanned with a Shimadzu CS-9000

spectrodensitometer at 550 nm in the transmission mode.

3.5. Phospholipids

Phospholipids are primary components of membrane and are essential to cell function. Thus, the heightening interest in analytical methodology for their analysis. With many mixtures relating to the major phospholipids one-dimensional TLC is often sufficient as reported by some of the earlier workers [61]. Silica gel containing 7.5% magnesium acetate was used with a mobile phase of chloroform–methanol–ammonia (65:25:4). The layer could be rotated 90° and the second development carried out with chloroform–ethyl acetate–methanol–acetic acid water (3:4:1:1:0.5). Many mobile phases reported then are still being used with modifications.

Alvarez et al. [62] used TLC to analyze the phospholipids in mouse and rabbit spermatozoa. After aerobic incubation of the cells, aliquots were applied to the preadsorbent zone of scored Whatman LK5 silica gel plates. The layers were predeveloped three times in chloroform–methanol (1:1) to the interface of the preadsorbent zone to extract the phospholipid and deposit it on the starting point of the chromatogram. The mobile phase was chloroform–ethanol–triethylamine–water (30:30:34:8). Development proceeded to 2 cm from the top of the plate. The plates were dried and 2 cm strips from both edges, where the standards had been applied, were cut and dried at 170°C for 2 min to remove residual solvent. The strips were sprayed with 10% $CuSO_4$ in 8% H_3PO_4, or with a 0.2% solution of ninhydrin in acetone to identify the phosphotidal ethanolamine fraction. Then plates sprayed with $CuSO_4$ were dried to 5 min at room temperature, heated in an oven at 110°C for 5 min and finally placed in an oven at 170°C for 2 min. When phospholipid unsaturation was to be measured, a 3% solution of copper acetate in 8% H_3PO_4 which stains only unsaturated phospholipids was used [63]. Plates sprayed with copper acetate were heated at 110°C for 5 min, and then placed in an oven at 180°C for 10 min. Ninhydrin-sprayed plates were placed directly in an oven at 110°C for 5 min.

Korte and Linette [64] separated 6 major phospholipids by one-dimensional TLC on layers provided with a preadsorbent zone. Samples were applied to the preadsorbent area in aliquots. Since all the compounds move with the solvent front as a sharp narrow band to the preadsorbent-silica gel boundary, excellent separation was achieved when up to 650 mg lipid material was applied on each lane. This method is suitable for the separation of relatively large amounts of radiolabeled and nonradiolabeled lipids and free fatty acids from extracts of biological fluids, tissues, or cells maintained in monolayer culture.

Some synthetic phospholipids have shown antineoplastic activity. Rustenbeck and Lenzen [65] used HPTLC to analyze hexadecylphosphocholine to assay the cellular amounts after incubation of cultured cells. After extraction with dichloromethane and cleaned up by SPE, the samples were applied to TLC layers. Development was with chloroform–methanol–triethylamine–water (30:35:34:8). Sorbent saturation was achieved by suspending the plate for 10 min above the elution trough before development. The cooled plate was dipped into a solution of 10% copper sulfate in 8% aqueous phosphoric acid and heated. Densitometry of the charred spots was performed with a Desaga CD60 densitometer at 530 nm.

Coene et al. [66] investigated HPTLC separation of ether phospholipids. HPTLC silica gel layers were washed by predeveloping with methanol, dried at 120°C for 20 min, washed again with cyclohexane and then heated at 120°C for 1 h to activate the silica gel surface. The plates were developed with mobile phases such as chloroform–methanol–water (65:35:5) or chloroform–methanol–water–acetic acid (70:30:3:4) and sprayed with reagents for visualization.

Alvarez and Ludmir [67] used HPTLC for analysis of phopholipids in amniotic fluid. Amniotic fluids were extracted with chloroform–methanol solutions. Aliquots were applied to the

plates as bands, 5 mm from the lower edge of the plate. The plates were predeveloped in chloroform–methanol (1:1) to 1 cm from the lower edge of the plate, thoroughly dried, and developed with chloroform–ethanol–triethylamine–water (30:34:30:8) for the first development. This mobile phase causes separation of polar lipids, including sphingomyelin, phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, and phosphatidylinositol. Cardiolipin and phosphatidylglycerol comigrate as a single band following first development. The plates were then dried at 24°C in a vacuum oven for 5 min and placed in hexane–diethyl ether (50:50) for the second development. This mobile phase separated the cholesteryl ester fraction and resolved phosphatidylglycerol and cardiolipid. The plates were dipped for 5 s in a 10% solution of $CuSO_4$ in 8% H_3PO_3 and heated at 120°C [63] scanning with a spectrodensitometer at 310 nm served for quantitation.

Phosphatidylcholine biosynthesis and antagonism of phorbol ester stimulation in HeLa cells was studied by Wieder et al. [68] using TLC for assay. After incubation the cells were washed and extracted by the classical Bligh and Dyer method [69]. Radioactivity was quantified by radioscanning. For lipid quantitation, all lipids were stained using Silica Gel 60 with a $CuSO_4$ solution by the method of Touchstone et al. [63] and quantified by video densitometry. Staining was linear in the range 0.5–6 mg of lipid per band layers.

Phospholipids were isolated by Shamburek and Schwartz [70] using silica-coated plates with $CHCl_3$–MeOH–H_2O–NH_4OH (60:30:3:1) as mobile phase. Plates were sprayed with 0.05% 2,7-dichlorofluorescein in isopropyl alcohol. Phospholipids were identified based on standards applied to adjacent lanes and scraped into scintillation vials for determination of radioactivity. PC was eluted from the silica using $CHCl_3$–MeOH–H_2O–acetic acid (50:39:10:1) and the dichlorofluorescein was removed from the eluate with ammonia. Total phospholipid and PC mass were determined using egg PC as standards.

Phospholipids in cell membranes were analyzed by Mallinger et al. [71] using two-dimensional TLC and laser densitometry. After extraction with chloroform–methanol–HCl solutions aliquots were applied to HPTLC layers of silica gel. The plates had a pre-adsorbent band on one edge for sample application. Then, in order to facilitate resolution of the polyphosphoinositides, the plates were dipped in a solution of 1% potassium oxalate dissolved in methanol–water (3:2 containing 2 m*M* EDTA), and then dried again. Prior to being developed, the plates were activated by heating to 110°C for 15 min. The first development used chloroform–methanol–4.3 *M* NH_4OH (90:65:20) to resolve the polyphophoinositides. Development was carried out to 0.5 cm below the top edge of the plate. After drying, development a second time in the first dimension was with chloroform–methanol–concentrated NH_4OH (130:50:10), in order to separate lysophosphatidyl ethanol from PC. Next, the plates were rotated and developed in the second dimension with chloroform–methanol–acetic acid–water (100:30:35:3) to resolve the remaining phospholipid classes. After being developed in each solvent system, the plates were dried before visualization by charring.

Porn et al. [72] investigated membrane phosphatidyl choline by using TLC to follow the patterns of lipids that were separated. The content of phosphatidylcholine and sphingomyelin in sphingomyelinase-treated cells was determined with an hexane–2-propanol (3:2) extract of cells. Phospholipids were separated on TLC silica gel using chloroform–methanol–acetic acid–water (25:15:4:2). [^{3}H]Sphingomyelin and phosphatidyl[^{3}H]choline areas were scraped into scintillation vials and counted. Phospholipids from unlabeled cells were visualized with 3% cupric acetate in 8% phosphoric acid and heating for 30 min at 150°C. The absorbances of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were then determined with a scanning densitometer.

Goldfine et al. [73] assayed phospholipase activity by TLC of phospholipids from biological membranes. Chloroform extracts were separated on silica gel layers using chloroform–methanol–

acetic acid (65:25:8). Extracts of *C. butyricum* protoplasts were separated by two-dimensional TLC. The plates were exposed to HCl fumes for 20 s between the first and second dimensions to hydrolyze the plasmalogens, permitting separation of the resulting lysophospholipids in the second dimension. Duplicate aliquots of the extracted erythrocyte lipids were chromatographed on silica gel 60 plates with a preconcentrating zone in chloroform–petroleum ether–methanol–acetic acid (5:3:1.6:1). The lipids were stained with iodine vapor and marked. The plates were then sprayed with water, and the lipid-containing areas were scraped from the plates into acid-washed tubes for determination of the amount of phosphate.

This is one of the few methods which utilizes reaction in situ on thin layers for compound identification. Plasmalogens are sensitive to acid hydrolysis without attacking the saturated acyl side chains thus providing a means of identifying this moiety in the molecule.

Ueda et al. [74] showed the presence of Ca^{2+}-independent phospholipase A, in bovine brain. TLC was used for phospholipid analysis as below. Two-dimensional TLC analysis of phospholipids used silica gel 60. Chloroform–methanol–ammonia–water (95:50:5.5:5.5) was used in the first dimension and chloroform–methanol–acetic acid–water (90:40:12:2) in the second dimension. After chromatography, the silica gel plates were thoroughly dried and the phospholipids were visualized with I_2 vapor.

Sajbidor et al. [75] analyzed phospholipids in yeast using chloroform–methanol extracts applied to silica gel layers. For TLC of neutral lipids a mobile phase of *n*-hexane–diethyl ether–acetic acid (70:30:1) was used. Phospholipids were separated by two-step TLC. The plates were developed with acetone to 18 cm above the origin. After drying, the plates were developed to 17 cm above the origin with chloroform–methanol–acetic acid–water (25:15:4:2). They were revealed by exposure to iodine vapor.

Phospholipids in cell cultures were separated by TLC by Mergny et al. [76]. After incubation cells were extracted and aliquots subjected to TLC on silica gel using chloroform–methanol–acetic acid–water (50:30:8:4) as mobile phase.

3.6. Steroids (*not cholesterol*)

TLC is a viable method for steroid analysis. Because of the different chemical structures varying widely even in individual groups, there is no generally applical TLC method for every type of steroid. Considering this the descriptions here in redivided into three groups: now polar, mid-polar and highly polar steroids. However, by nature it is difficult to assign all in to these three classes, thus, these can be considerable overlap. A great deal of TLC is seen in analyses of steroids in biological systems, most frequently seen are urine and plasma. The majority of steroids in these samples are conjugated. Thus, a beginning discussion will be on this aspect with respect to sample preparation. The separation of the conjugation as such has not been met with much success.

Tang and Crone [77] have reported a simple method for methanolysis of steroid conjugates as below. Analyses showed hydrolysis of testosterone glucuronide was complete with no artifact formation. The solvent was evaporated at 60°C under N_2. Residues were reconstituted in MeOH and spotted on HPTLC plates. The plates were developed with butanol–acetone–water (6:5:4) and observed under UV light. The plates were then sprayed with 5% ethanolic H_2SO_4, then heated at 105°C for 2 min. Methanolic HCl was added to steroid samples in Reacti-Vials. The vials were capped and heated at 60°C under nitrogen. NaCl (0.5 g), dilute H_2SO_4 (3.5%), and EtOAc (5 ml) were added to aqueous solutions or urine in Corex tubes. The tubes were capped. Aliquots (1.0 ml) from the organic layer were heated at 50°C for 2 h, then evaporated to dryness at 60°C under nitrogen.

3.6.1. Non-polar steroids

TLC was used successfully to separate metabolites of dehydroepiandrosterone and pregnenolone by Akwa et al. [78]. Extraction of tissue incubates was performed using acetone

and ethyl acetate. The extracts were dried under vacuum, taken up in 0.2 ml of ethyl acetate, and aliquots applied to silica-gel F_{254} thin-layer plates. Standards were applied on separate lanes. The plates were developed once in either chloroform–ethyl acetate (4:1), (system I), ethyl acetate, (system II), or benzene–ethanol (9:1), (system III). Standards were located by spraying with methanol–H_2SO_4 (1:1) and heating at 100°C for 5 min. System II separated 7α-hydroxy-DHEA (R_F 0.25) from its 7β epimer (R_F 0.37), androst-5-ene-3β,7β,17β-triol (R_F 0.38), and 7α-hydroxy-pregnenolone (R_F 0.28) from 3β,7β-dihydroxy-pregn-5-ene-20-one (R_F 0.45). The radioactive areas shown after radiography were located on the chromatograms.

Daeseleire et al. [79] used HPTLC to separate anabolic steroids from tissues of cattle. Extracts were separated on HPTLC plates. Reference standards were applied to the plate. Development was carried out in one direction with chloroform–acetone (90:10) over a distance of 4 cm. After drying, the plate was developed in the other direction in cyclohexane–ethyl acetate–methanol (58.5:39.0:5.5) over a distance of 4 cm. For detection the HPTLC was sprayed with 10% H_2SO_4 in methanol, then heated in an oven and examined in daylight and under UV light at 366 nm.

Medina and Nagdy [80] described the separation of diethylstilbesterol by TLC. Plates were washed in ethanol and activated at 85°C for 30 min. Developing solvent methylene chloride–methanol–2-propanol, (97:1:2) was placed in the tank and allowed to equilibrate for 15 min. Samples were applied on Whatman silica gel channeled plates. Plates were developed for 9 min at room temperature, then dried in an 85°C oven or dried with a hair dryer for 5 min and visualized with iodine-starch or diazonium dyes. TLC plates were exposed to iodine vapors for 3–5 min, then spraying with 0.5% aqueous gelatinized starch solution.

Jansen et al. [81] used two-dimensional TLC for separation of anabolic steroids using a sandwich chamber, samples and standards were applied on the left and on the right side of a HPTLC plate and developed in chloroform–acetone (9:1). Then as the second dimension in cyclohexane–ethyl acetate–ethanol (77.5:20:2.5). After chromatography the plate was sprayed with 10% sulphuric acid in methanol and heated at 95°C for 10 min. Urine extract was purified by high-performance liquid chromatography prior to TLC analysis. 19-Nortestosterone in chloroform was spotted as standard and developed in chloroform–*n*-propanol (95:5). The TLC plate was dried at room temperature. Then the plates were developed a second time perpendicular to the first direction in *n*-hexane–diethylether–dichloromethane (4:3:2). Spraying with a solution of 20 ml of phosphoric acid (75%) and 30 ml of water and heating at 110–120°C for 15 min provided detection.

Steroids derived from testosterone metabolism by skin were analyzed by several TLC methods, both preparative and analytical as reported by Vingler et al. [82]. [4-^{14}C]Androstanedione was chromatographed twice on preparative plates at room temperature in dichloromethane–diethyl ether (90:10). [4-^{14}C]-3β-Androstanediol was purified in the same way on a 0.25-mm TLC plate. [4-^{14}C]Androsterone and [4-^{14}C]epiandrosterone were extracted from silica gel, and separated on a 0.25-mm TLC plate twice in the *y* and *x* directions in toluene–ethyl acetate (50:50). Detection and analyses were carried out by use of a radio-TLC analyser.

3.6.2. Mid-polar steroids

In the mid-polar grouping are included the corticosteroids. These will of necessity show the separation of a wide variety of metabolites of varying polarities, which may tend to push some into the more polar grouping.

A high-performance TLC system for determination of this group of steroids as well as others was described by Golf et al. [83] using silica gel HPTLC and mobile phases. A complete separation of androgens, gestagens, and metabolites was achieved with dichloromethane–cyclohexane–acetone (70:25:5). Corticosteroids, mineralocorticoids, and their derivatives were completely separated with diethyl ether–isooctane–isopropanol (70:25:5). Detection was by fluorescence after derivatization with cinnamic alde-

hyde, 4-dimethylaminobenzaldehyde, and sulfuric acid. The sensitivity of detection was 500 pg–1 ng per spot. The steroid metabolism as catalyzed by rat liver microsomal oxidoreductases was measured by these procedures. According to HPTLC, steroids were reduced by NADPH-5α-reductase in the order progesterone > testosterone > aldosterone > cortisol > corticosterone. The enzyme activities as measured by HPTLC agree well with those obtained by GC.

3.6.3. Highly polar steroids

Highly polar steroids could be largely concentrated in the bile acids, the cardiac glycosides and the sulfo and glucuronide conjugates. The sulfur acid glucuronides will not be discussed at length since they are usually assayed after hydrolysis. Zivanovic [84] also separated steroids by TLC and used densitometry for quantitation. Deoxycorticosterone, deoxymethasone, hydrocortisone, and prednisone were determined in pharmaceuticals. Silica gel F254 plates, $CHCl_3$–MeOH (24:1) mobile phase, and a developing reagent containing *p*-acetylaminobenzaldehyde–thiosemicarbazone were used. The method is simple, rapid and precise with satisfactory standard deviations.

Berndtand and Poole [85] reported separation of ethinyl steroids using HPTLC. Simultaneous determination of ethynyl steroids in oral contraceptives by high-performance TLC–scanning densitometry was based on silica gel precoated plates, hexane–chloroform–carbontetrachloride–ethanol (7:18:22:1) mobile phase, and spectrophotometric detection at 220 nm. The detection limits were 3.8–243.0 ng for 7 ethynyl steroids studied. The relative standard deviation was 1.0–1.5%.

Progesterone metabolites in plasma were separated by SPE prior to TLC by Touchstone and Fang [86]. Plasma was extracted with toluene three times. Volumes of 2 ml were used each time for 0.5 ml plasma. The extracts were combined and evaporated in a hot water bath (65°C) using a nitrogen stream and separated by SPE on an aminoprophyl column. Aliquots were taken for reaction with the readily available 7-diethylaminocoumarin-3-carbohydrazide (DACCH) which labeled the ketone group of the tetrahydroprogesterones. Labeling the 20-ketone resulted in derivatives that separated since the isomeric 3-hydroxyl and the hydrogen function at the 5-position were free. High-performance thin-layer chromatography for the separation and quantitation by densitometry was described. 7-Diethylaminocoumarin-3-carbohydrazide of the tetrahydroprogesterone gave a fluorescent derivative that permitted detection of the reduced progesterone at the 25 pg level (see Figs. 5 and 6).

Lafont et al. [87] have done considerable work with evaluating chromatographic procedures for phytoecdysteroids. These authors have reviewed the field well. RP-TLC chromatography on bonded phases (C_2, C_8, C_{12}, C_{18}, aminopropyl and cyanopropyl) as well as on paraffin-impregnated silica gel has also been employed for ecdysteroids. In general, methanol–water (1:1) solvent systems provide good separations but other organic modifiers can be used to achieve

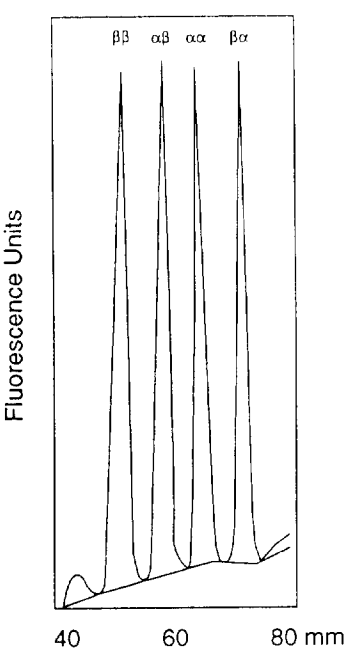

Fig. 5. Separation of the four pregnanediols after labeling with fluorescent probe, modified from Touchstone and Fang [86] with permission.

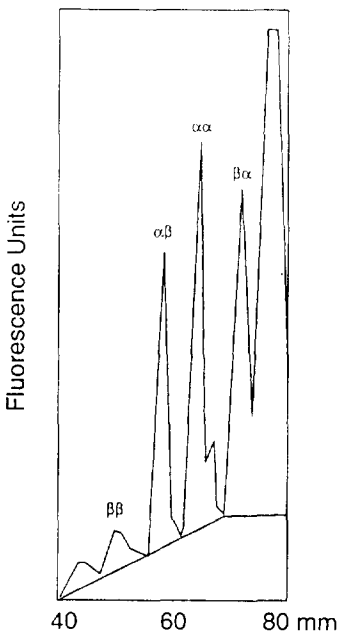

Fig. 6. The pregnanediols in blood plasma of normal women who were administered progesterone. After SPE, the metabolites were labeled with probe. Touchstone and Fang, unpublished.

different selectivities. Reversed-phase systems, like their HPLC equivalents are poor at separating 5β-OH compounds from their 5β-H analogues (e.g. polypodine B from 20-hydroxyecdysone). The effects of various substitution patterns on the RP-TLC of ecdysteroids are discussed in detail elsewhere [88]. Detection methods following RP-TLC are essentially the same as those usually employed.

Separations can also be modified, in both normal-phase and RP-TLC systems by esterifying the 20,22-diol containing ecdysteroids with boronic acids. This has the effect of reducing the polarity with a consequent effect on the chromatographic separation of an ecdysteroid mixture.

Wilson [89] has published an exhaustive review of chromatographic methods from separation of phytoecdysteroids. Twenty five mobile phases for TLC of this class of compounds are listed.

The detection of ecdysteroids on TLC plates can be accomplished in a number of ways. Plates with fluorescence indicator that can provide a general, if rather non-specific method of detection. Spray reagents have been used to provide a higher degree of specificity, and the vanillin-sulphuric acid reagent has been particularly widely employed. This reagent gives a range of colour with different ecdysteroids. The colours produced range from pink (e.g. cyasterone) and red (e.g. 20-hydroxyecdysone) to dark green (e.g. ecdysone), but like many such colour reactions are subject to quite wide inter-laboratory variations [90].

RP-TLC chromatography on bonded phases (C_2, C_8, C_{12}, C_{18}, aminopropyl and cyanopropyl) as well as on paraffin-impregnated silica gel has also been employed for ecdysteroids. In general, methanol–water (9:1) systems provide good separations. Other organic modifiers can be used to achieve different selectivities.

Digoxin and related compounds were separated by HPTLC by Ponder and Stewart [91]. Digoxin preparations were prepared by extraction with chloroform–methanol (1:1) using RPTLC. A 100% wettable HPTLC plate (octadecylsilane) was prewashed with absolute methanol and dried. An aliquot of each standard and tablet sample were applied in duplicate as 5-mm bands at the rate of 10 s/ml. A vertical twin-trough chamber was lined on two sides with saturation pads and equilibrated for no more than 5 min with mobile phase. The plate was developed for 30–35 min, removed and dried. Each lane was scanned for digoxin peak height using 218 nm in the absorbance mode. After scanning for digoxin, the plate was exposed to HCl vapor for 60 min in a conditioning tray. The plate was then heated in an oven for 30 min at 120°C, allowed to cool for 10 min at ambient temperature and each lane was scanned in the fluorescence mode using 365 nm with a 400-nm cut-off filter.

Lithocholic acid has been used as a catalyst of steroid 6-hydroxylation. Chang et al. [92] reported methods to separate the products.

Microsomes isolated from transfected cells were assayed for steroid metabolism. Reaction mixtures were incubated for 20 min at 37°C, then extracted with ethyl acetate and chromatographed on silica-gel and developed sequentially

in multiple solvent systems. For testosterone – first developed in chloroform–acetone (4:1); for the second development chloroform–ethyl acetate–ethanol (40:10:7) was used. Androstenedione was separated with methylene chloride–esthanol (97:3) followed in the same dimension with ethyl acetate–chloroform (1:1). ^{14}C-labeled steroid metabolies formed by CYP 3A10 incubations were initially purified by TLC and subsequently rechromatographed on TLC plates co-spotted with unalbeled, authentic metabolite standards as described previously. Mobilities of the unknown metabolites were then compared with those of the authentic standards.

Monder and Iohan [93] described the separation of steroidal carboxylic acids. It is given as a reminder that these compounds are present in nature. Urines were extracted from urines using ion-exchange columns. The fractions from this column were separated further by TLC on silica gel F layers. Aliquots of column effluents were applied to HPTLC plates (silica gel with fluorescent indicator) along with reference steroids. The plates were placed in the tanks which contained either chloroform–methanol (90:10) or chloroform–methanol–formic acid (90:10:4). For reproducible results, it was found essential to prepare the latter solvent daily, because of the rapid formation of methyl formate. Steroids visible at 254 nm were marked. The plate was sprayed with vanillin–sulfuric acid reagent, air dried, and heated at 120–150°C. Each steroid developed a distinctive color. Separations can also be modified by esterifying 20,22-diol-containing ecdysteroids with boronic acids. This has the effect of reducing the polarity with a consequent effect on the chromatographic separation of an ecdysteroid mixture. This can be used to good effect to resolve ponasterae A and 2-deoxyecdysone, which are otherwise difficult to separate.

Bile acids from extracts of bile were analyzed by TLC by Mili et al. [94] using silica gel 60 F_{254} precoated TLC aluminum sheets. The mobile phases were as follows: *n*-butanol–acetic acid–water (80:10:5); benzene–isopropanol–acetic acid (30:10:1); chloroform–acetone–methanol (70:25:5); isooctane–isopropanol–acetic acid (30:10:1). Aliquots of bile and fecal fractions were applied as bands on the plates along with reference bile salts. After development, bile salts were visualized by spraying the plates with 10% phosphomolybdic acid in ethanol followed by heating at 120°C for 1 min.

Fecal bile acids from hamsters and humans were assayed by TLC as described by Benson et al. [95]. The steroids were extracted with butanol–water (1:1). The extract was cleaned up by SPE. The presence and purity of the chloroform-soluble metabolites was checked using silica HPTLC plates developed in ethyl acetate–cyclohexane–glacial acetic acid (9:9:2). The plates on which the crude chloroform-soluble extract was applied were developed initially in chloroform which did not move the metabolites but made the metabolites mobile when the plates, once dry, were subsequently developed in ethyl acetate–cyclohexane–acetic acid–chloroform (9:9:2:2). Pre-developing the plates in chloroform prior to separating the metabolites in the crude extract probably removed contaminants from the origin which, when present, inhibited the mobility of the metabolites. The plates were sprayed with a manganese chloride reagent until the surface of the plates remained momentarily wet. The plates were then heated uniformly at 100°C for 15 min in an oven to produce colored zones.

3.7. Vitamins

3.7.1. Vitamin A

Some 2500 retinoids have been synthesized and characterized. These compounds have little resemblance to retinol; they are highly unsaturated. The naturally occurring vitamins A in some cases are used as therapeutic agents, but the distinction between the natural and synthetic retinoids means little to their general usage. These compounds are sensitive to oxidation and thus in many cases difficult to analyze. Antioxidants are added to the extraction process to stabilize the compounds.

Vitamin A is necessary for growth and health in higher animals. Retinol is essential for vision, and is capable of preventing xerophythalmia and

blindness. Retinoic acid, the oxidation product of retinol, can maintain all functions of retinol except the function in the eye.

TLC is useful for rapid analysis of reaction products when carrying out synthetic modification of retinoids and carotenoids. Thus, short (7.5–8 cm) commercially available reversed-phase (C_8 and C_{18}) and adsorption (silica and alumina) TLC plates allow more rapid comparisons of chromatographic conditions than do HPLC columns, which must be connected and equilibrated with various mobile phases before analysis. Analysis of radioactivity may be accomplished by autoradiography [96].

For initial "scouting" analysis TLC on silica or alumina plates, mobile phases of hexane–diethyl ether or hexane–acetone (4:1) can be used. By altering the mobile-phase composition, R_F values of desired components can be suitably adjusted.

Kouimtzis and Papadoyannis [97] described the separation and quantitation of vitamin A on silica plates developed with acetone–methanol–benzene. Under these conditions R_F values of 0.96, 0.85, 0.63 were obtained for vitamins A, D_3 and B_2, respectively.

Separation of vitamin A from other vitamins was described by Thielemann [98]. Silica plates were developed with a mixture of benzene–petroleum ether–acetic acid (35:65:1). Vitamin A (R_F 0.71) was separated from other lipophilic vitamins such as D_2 and *E* (R_F values 0.18 and 0.7, respectively). Under these conditions, the water-soluble vitamins present in the multivitamin preparation, remained at the origin.

On silica gel plates impregnated with silver nitrate and developed with petroleum ether–chloroform–acetone (50:10:17), vitamin A palmitate had the highest mobility (R_F 0.82). Retinol and retinal (R_F 0.62) were not separated from each other, while vitamin E eluted before vitamin D_2 (R_F 0.56 and 0.32, respectively) [99]. Changing the mobile phase to hexane–ethyl acetate–isopropyl ether (2:1:1), lowered the R_F values of all these fat-soluble vitamins. With the latter solvent system, retinyl palmitate still moved ahead (R_F 0.77), but vitamin E had higher mobility than the pair retinol/retinal and vitamin D_2 (R_F values 0.54, 0.51 and 0.19, respectively). On HPTLC Kieselgel plates, an aliquot of a petroleum ether extract from serum was developed with petroleum ether–diethyl ether (1:1). Quantitation was done by measuring the absorbance of the sample and a standard spot [100]. A TLC procedure for carotenoids in fruits and vegetables was reported by Premachandra [101], who claimed applicability for quantitative measurement of vitamin A in these matrices. Vitamin A esters could be separated from vitamin A alcohol by developing on alumina layers with 5% diethyl ether in petroleum ether.

With reversed-phase layers, methanol or acetonitrile gives adequate mobility and resolution for retinol and retinyl acetate. Mixtures of less polar solvents (such as dichloroethane) with acetonitrile give good resolution of long-chain retinyl esters, and methanol–water or acetonitrile–water mixtures are suitable for polar retinoids.

Retinol and retinyl esters can be detected on in situ the yellow-green fluorescence under long-wave UV (366 nm). Other retinoids (except anhydroretinol, which fluoresces with a red color), are not fluorescent under such conditions, and must be identified by nonspecific techniques.

Drescher et al. [102] have separated α-carotene using magnesium oxide layers. After extraction of samples of spinach or lettuce leaves by acetone and transfer to petroleum ether aliquots were applied to the TLC layer consisting of Kieselguhr/MgO (1:1) containing $CaSO_4$ binder and a 254 nm fluorescent indicator. Plates were developed 12 cm beyond the origin with a mobile phase of petroleum ether–benzene (3:1) or petroleum ether–acetone (97:3). In situ spectra (370–700 nm) and zone areas were scanned using a Shimadzu CS-930 densitometer.

The literature indicates that some seed plants contain only β-carotene while others also have some of the α isomer [103]. Using the magnesium TLC the α-isomer was not found in any sample tested including spinach and romaine lettuce. The use of the magnesium layer may be one of the first to separate the α and β isomers by TLC.

3.7.2. Vitamin D

Vitamin D analysis now has methods of increased sensitivity and specificity. Picogram

quantities can be readily assayed in physiological fluids. TLC has been used to separate vitamin D and its analogue on silica gel. It is used mainly for prepurification of saponified samples for GC for the separation of vitamin D from cholesterol [104] and in combination with GC for the estimation of 25-OH-D_3 in chick plasma. Care must be taken to avoid oxidation of vitamins on the TLC plate during drying of the applied sample and during removal of the plate from the tank. Such oxidation can be minimized by development at 0°C in an atmosphere of nitrogen, [105]. Vieth et al. [106] used TLC to separate tritiated products in biochemical studies of renal 25-OH-D hydroxylases. Several methods for the measurement of vitamin D_3 and 25-OH-D_3 prior to mass spectrometry utilize TLC as a prepurification step sometimes after derivatization. HPATLC [107] for the separation of vitamin D metabolites using microparticulate silica has been described with a chloroform–ethyl acetate (1:1) mobile phase.

Das [108] has reported the TLC of vitamin D analogues on silica gel layers. The development was first carried out with hexane to 5 cm. The layers were then dried. This was followed by second development with cyclohexane–diethyl ether (1:1) to 7 cm. The chromatograms were developed in the dark and away from any UV light. Absorbance was determined at 268 nm by densitometry for quantitation.

3.7.3. Vitamin E

Recent research has shown a strong link between vitamin E deficiency and atherosclerosis [109]. In epidemiological studies, the mortalities from atherosclerosis-related heart attacks correlated well with low plasma vitamin E levels and even better with plasma vitamin E/cholesterol ratios [110]. The biological manifestations of vitamin E activity appears to be its ability to act as a lipid soluble antioxidant that protects vulnerable polyunsaturated fatty acids in cell membranes and plasma lipoproteins from harmful and potentially fatal lipiperoxidation process [111].

TLC systems are capable of separating tocopherol and tocotrienol homologues. One-dimensional systems are often satisfactory; additional resolution can be achieved by developing a second dimension. Quantitation can be done in situ by densitometry [112]. Few silica-based systems are capable of distinguishing between positional isomers. Of these, the one-dimensional approaches all employ complex mobile phases containing four or five solvent components. More general acceptance was gained by the one-dimensional approach reported by Lovelady [113]. Silica gel G plates were developed with a mixture of cyclohexane–hexane–diisopropyl ether–ammonium hydroxide for the determination of tocopherols in human plasma and red blood cells. Development in the first dimension with chloroform affords a separation of the various homologues. Differentiation of α- and β-isomers was subsequently accomplished in the second dimension by using a mixture of diisopropyl ether–petroleum ether.

α-Tocopherol can be determined in spinach by HPTLC and densitometry following a total lipid extraction and SPE [114]. Alternatively, the unsaponifiable lipid fraction of an extract, without clean-up, can be subjected directly to TLC, as was done for the determination of α-tocopherol in serum [115] or in foods and oils [116]. The total lipid extract can even be applied to the plate without prior saponification [117].

Silica gel lends itself conveniently to the separation of α-tocopherol from its oxidized decomposition products, including α-tocopherylquinone, α-tocopherylhydroquinone, and various partly characterized monomeric and dimeric compounds. These compounds can be prepared by in vitro oxidation, but some of them have also been detected by TLC in biological materials, e.g., spinach [118] and rat liver [119].

The quinone forms of α-, β-, and γ-tocopherol have also been separated on silica gel together with plastoquinones and various plant prenylquinones. A mixed sorbent consisting of alumina–zinc carbonate has been used for the TLC separation of various oxidized decomposition products of tocopherols [120].

3.7.4. Vitamin K

Vitamin K_1 is an essential cofactor in mammals and bids in the posttranslational carboxylation reaction of glutamic acid residues to γ-

carboxyglutamic acid residues in a number of blood-clotting factors and also in some other proteins, such as protein C, protein S, and osteocalcin. These residues provide the proteins with calcium-binding properties essential for the interaction with phospholipids and for their activation.

Silica gel is still undoubtedly the most frequently used sorbent in TLC of vitamin K. One of the major advantages of silica gel is that it has little or no tendency to catalyze the degradation of vitamin K_1. Alumina is more aggressive, and degradation of labile substances often occurs. Selectivity can be enhanced by incorporation of an appropriate modifier and is comparable with high-performance liquid chromatography (HPLC) in that adsorption TLC will not distinguish between different isoprenologs. The method is especially suited for the separation of geometric isomers (*cis* and *trans* isomers) and for the resolution of photochemical degradation products. Silica gel H plates developed with chloroform were used successfully by Baczyk et al. [121] in their study of the decomposition of vitamins D_2 and K_1 due to ultraviolet light. Because of the lability of the vitamin K_1 molecule, degradation on the TLC plate often occurs, even working under subdued light [122] and in an inert atmosphere. TLC was applied to determine vitamin K_1 in infant formulas. An extraction and SPE preceeded TLC analysis. Silica plates were developed consecutively with carbon tetrachloride and benzene [123]. Addition of ethyl acetate or methylethylketone to the mobile phase impaired the separation of K_3 and K_4 but resulted in their separation from decomposition products [124].

On silica plates, vitamin K_1 was separated from α-tocopherol, β-carotene, and vitamins A and D_2 in spinach, fish, and crustaceans. Plates were developed with mixtures of petroleum ether–benzene (6:1), or with hexane–diethyl ether (70:30).

In argentation TLC, R_M values are correlated directly with the degree of unsaturation, whereas in reversed-phase TLC the accent is on the length of the side chain. Therefore, both argentation TLC and RPTLC are complementary techniques for the determination of the length and the degree of unsaturation of the side chains in menaquinones. The same observations were also made by Lichtenthaler et al. [125]. Compounds having the same side chain length that differ only in three double bonds cannot be separated on silica gel TLC. Compounds containing an additional isoprene unit exhibit a similar R_F value on silica gel plates. On silver nitrate-impregnated silica gel plates, however, vitamin K_1 (one double bond) is easily separated from K_4 (four double bonds). Correspondingly, ubiquinones 9 and 10 are also separated. As silver ions are not destructive for vitamin K_1-related compounds, samples can be collected from the plates for further analysis. High-molecular-mass menaquinones are subject to irreversible adsorption on argentation TLC plates [126]. To optimize the separation of epoxy derivatives from the related quinones, Donnahey et al. [127] impregnated Kieselguhr G with paraffin by dipping the plate in a purified 5% solution of liquid paraffin in petroleum ether. They used 90% aqueous acetone (saturated) with paraffin as solvent. Under these (reversed-phase) conditions, epoxy derivatives separated from the quinones, which was impossible on pure silica and on argentation TLC plates.

Madden and Stahr [128] used a Florisil column followed by a Sep Pak cartridge for clean up of liver extracts. Aliquots were applied to a reversed-phase layer containing phosphor and developed with methylene chloride–methanol (70:30). Detection was accomplished by examination of the layer under long and short wave ultraviolet lamps. A vitamin K standard applied before development enabled location of the separated zones.

4. Conclusions

The results seen in this review indicate that TLC remains the technique of choice in separation of the various lipid classes. The trends for the future especially when sensitivity is required point to the use of fluorescence. The several instances described gave methods for either

preparation of the derivative for fluorescence directly on the TLC layer or before application of the sample on the layer. In one instance the preparation of the derivative enabled separation of isomers otherwise not separable [86]. The ready availability of the probes, which also provide specificity makes this methodology the promise for the future. With the continual demand for less usage of isotopes TLC provides means for sensitive assays. Thus, a means is provided for non-isotopic assays in the biomedical field. It must be remembered that fluorescent derivatives can also be prepared by reaction with various reagents directly on the laser. The use of the various sulfuric acids/sprays along with controlled heating can result in highly fluorescent derivatives.

The review presented by no means gives an exhaustive bibliography but rather presents representative descriptions of separations of the lipid classes.

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