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Thin-Layer (Planar) Chromatography

B. Fried, Lafayette College, Easton, PA, USA

Introduction

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

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

Table 1 Lipids frequently separated by TLC

Neutral lipids	*Phospholipids*	*Glycolipids*
Diacylglycerols	Diphosphatidylglycerol	Gangliosides, e.g. monosialoganglosides; disialoganglosides; trisialoganglosides; tetrasialoganglosides
Free fatty acids	Lysophosphatidylcholine	
Free sterols	Lysophosphatidylethanolamine	Plant and bacterial glycolipids, e.g. mono- and digalactosyldiacylglycerols
Monoacylglycerols	Phosphatidic acid	
Triacylglycerols	Phosphatidylcholine	Sphingolipids, e.g. ceramides; sphingomyelin; cerebrosides; globosides; sulfatides
Wax esters	Phosphatidylethanolamine	
	Phosphatidylglycerol	
	Phosphatidylinositol	
	Phosphatidylserine	
	Phosphonolipids	

Herslöf consider that lipids are compounds based on fatty acids or closely related compounds such as the corresponding alcohols or sphingosine bases. Christie noted that a variety of diverse compounds usually soluble in organic solvents are classified as lipids and set up a convenient system of lipid classification that is followed here. His system considers the simple lipids (compounds that upon hydrolysis yield no more than two types of primary products per mole), also referred to as neutral or apolar lipids. According to Christie, the polar or complex lipids (compounds that upon hydrolysis yield three or more primary products per mole) are the glycerophospholipids (or simply phospholipids) and the glycolipids (also termed glyceroglycolipids or glycosphingolipids), including gangliosides. **Table 1** lists the major neutral lipids, phospholipids and glycolipids of interest in studies on the TLC of lipids.

Functions

Lipids are involved in many functions of animals, plants and microorganisms and these functions are often studied using TLC. Lipids are important as storage depots for energy reserve. In mammals the storage depot is usually in the form of adipose tissue and TLC analysis shows that the major storage lipids are triacylglycerols, free fatty acids and mixed glycerides. Less information is available on lipid storage in invertebrates but TLC studies have shown that storage sites exist in invertebrates, including chlorogagen tissue in earthworms, the digestive glands in snails and specialized organs called trophosomes in some nematodes. As shown by TLC, triacylglycerols are major storage components in invertebrates. Lipids (mainly sterols, phosphoglycerides, glycolipids and sphingolipids) are important in the structural integrity of cells and comprise the major components of membranes. Phosphoglycerides in the membranes of nervous tissue are involved in the transmission of electrical signals. Phosphoinosides are involved in cellular communication. Neutral lipids serve as pheromones or carrier of pheromones in both invertebrates and vertebrates. TLC has been used extensively for at least tentative identification of these pheromones.

Christie has documented numerous lipid functions, including their role in abnormal lipid metabolism associated with various disorders; accumulation of lipids associated with coronary blood vessel and cardiac diseases; the importance of lipids in human welfare, including nutrition and disease; the role of lipids as important dietary factors and suppliers of calories for humans and animals; and the importance of lipids to the palatability of foods.

Glycolipids play an important role in cellular metabolism and TLC has helped to elucidate this role. Glycolipids occur at the external surfaces of cell membranes and help regulate cell growth; they also serve as receptors for toxins and hormones and modulate immune responses.

Sample Preparation

Lipid analysis should be done as soon as possible after samples have been obtained from plants and animals. If this is not possible, samples should be maintained at 4°C overnight or at -20°C for longer periods. Tissues that have been fixed in formalin, alcohol or other preservatives should not be used. Glass vessels

Table 2 Frequently used methods for sample preparation of lipids

Lipid extraction technique	*Comments*
Vertebrate and invertebrate organ and tissue samples	
Chloroform–methanol (2 : 1); typically 1 part of tissue or fluid to 20 parts of the solvent	Most widely used method of lipid extraction; useful for TLC of neutral and complex lipids
Chloroform–methanol–H_2O (1 : 2 : 0.8); following extraction, dilute the sample with 1 vol of chloroform and 1 vol of water to get a biphasic system	Particularly useful for extraction of more polar lipids such as gangliosides
Pre-extraction of brain tissue with 0.25% acetic acid followed by chloroform–methanol (2 : 1)	Nonlipid material first removed with the acetic acid; relatively pure lipid fraction then obtained by treatment with chloroform–methanol
Chloroform–methanol (1 : 2); typically 1 part of tissue to 3 parts of solvent mixture	Good for large amounts of tissue where complete recovery of lipid is not needed; does not use as much solvent as the previous extraction techniques
Blood and amniotic fluids	
Chloroform–isopropanol–water (7 : 11 : 2)	Extracts lipids but not pigments; lipids are not contaminated with blood pigments; neutral and complex lipids are quantitatively extracted
Amniotic fluid or blood plasma (about 1 mL) is added directly to a Spice C_{18} solid-phase extraction cartlidge (Analtech, Newark, DE); the analyte is eluted with chloroform–methanol	Good separation of phospholipids achieved, since most extraneous material is removed; technique also useful for separating neutral lipids from steryl esters and phospholipids
Plant tissues	
Tissues first treated with isopropanol and then chloroform–isopropanol (1 : 1) prior to usual chloroform–methanol (2 : 1) extraction procedure	Isopropanol inhibits the action of plant lipases

are recommended for lipid analysis, along with aluminium foil or Teflon-lined lids. Plastic vessels should be avoided because they may dissolve in the organic solvents used during the TLC process. Most samples are extracted in mixtures of chloroform–methanol (**Table 2**) to remove quantitatively the lipids prior to subsequent chromatographic techniques. The first procedure listed in Table 2, usually referred to as the Folch extraction procedure, is the one most frequently used. In brief, this procedure generally uses a 20 : 1 ratio of chloroform–methanol (2 : 1) to sample so that, for example, if 100 mg of tissue is being extracted, 2 mL of chloroform–methanol is suitable for total lipid extraction. The tissue is usually extracted in a glass homogenizer and the extract passed through a glass wool filter; the lipid-containing filtrate is collected and used for TLC following concentration of the sample under nitrogen gas. Many variants of this extraction procedure are available.

Chromatographic Systems

The chromatographic system consists of the sample mixture (the analyte), the stationary phase (the sorbent) and the mobile phase (the development solvent). Along with the sample mixture, lipid standards (usually obtained from a commercial supplier) are run at the same time. Development of the plate in a suitable mobile phase, from the origin to the solvent front, constitutes the essential part of the chromatographic process. Following development, the plate is allowed to dry and the analytes are detected (see detection, next section) and compared to the standards on the plate.

Numerous stationary phases are available for TLC, but the one of choice for lipid work is silica gel. There are many different types of silica gel plates and sheets and most workers use commercially prepared plates. Silica gel plates can be modified for particular purposes. For example, silver nitrate plates can be prepared and used to separate *cis*-enoic compounds based on unsaturation. Other examples exist that show how commercial and home-made silica gel plates can be altered for specialized lipid applications. The 1990s have seen considerable use of high performance thin-layer chromatography (HPTLC) for lipid analysis. HPTLC plates are made of fine silica particles of narrow size distribution, and have excellent resolving power. The quantity of sample applied to such plates can be reduced markedly from that applied to conventional TLC layers. Many samples can be analysed on the same plate with minimal amounts of mobile phase. HPTLC plates are now being used frequently in densitometric studies on lipids.

Table 3 R_F values of common neutral lipids separated in five frequently used solvent systems on silica gel

	$R_F \times 100$				
Compound	S_1	S_2	S_3	S_4	S_5
Cholesteryl esters	97	97	85	94	90
Triacylglycerols	63	79	70	60	82
Free fatty acids	42	21	62	39	50
Cholesterol	28	42	38	19	30
1,3-Diacylglycerols	24	66	46	21	40
1,2-Diacylglycerols	21	53	41	15	25
Monoacylglycerols	8	11	10	2	5

S_1, hexane–diethyl ether–formic acid (80 : 20 : 2). S_2, toluene–diethyl ether–ethyl acetate–acetic acid (80 : 10 : 10 : 0.2). S_3, isopropyl ether–acetic acid (96 : 4) followed by petroleum ether–diethyl ether–acetic acid (90 : 10 : 1) in the same direction. S_4, petroleum ether–diethyl ether–acetic acid (80 : 20 : 1). S_5, heptane–isopropyl ether–acetic acid (60 : 40 : 4).

Numerous mobile phases are available for lipid TLC and most are used with a single development in the ascending mode. However, some systems have been designed for two or more developments in the same direction. A good example of this is the classical Skipski system (**Table 3**) that uses two developments in the same direction. Some mobile phases have been designed with two-dimensional TLC in mind, in which the second development is done after the plate is turned through a 90° angle.

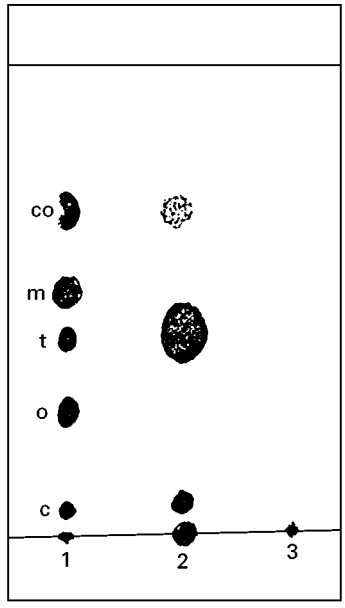

Figure 1 Separation of a hen's egg yolk–saline extract on a silica gel sheet. Lipids were developed 10 cm from the origin in petroleum ether–diethyl ether–acetic acid (80 : 20 : 1) and detected by spraying with 5% phosphomolybdic acid in ethanol. Lane 1 contains a neutral lipid mix consisting of equal parts of cholesterol (c), oleic acid (o), triolein (t), methyl oleate (m) and cholesteryl oleate (co). Lane 2 shows the presence of triacylglycerols and free sterols as the predominant neutral lipids in the yolk–saline extract. Lane 3 contains saline alone that is neutral lipid-negative. (Reproduced with permission from Fried and Sherma (1999).).

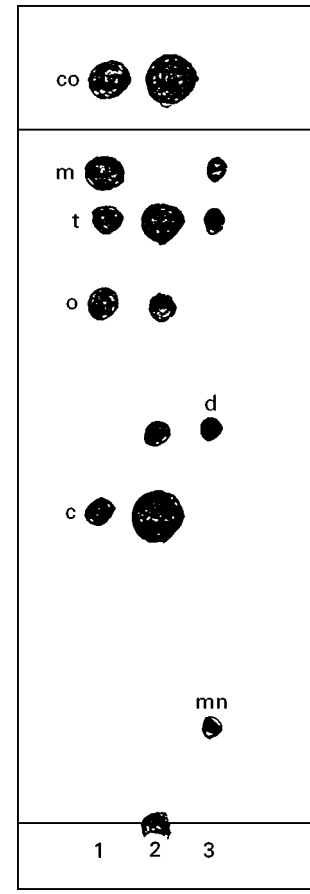

Figure 2 Separation of a snail liver extract on a silica gel sheet using the dual solvent system of Skipski (see text). Lane 1 contains a neutral lipid mix as described in Figure 1. Lane 2 shows the separation of snail neutral lipids; note the abundance of free sterols and cholesteryl esters in this tissue. Lane 3 contains a neutral lipid mix with equal parts of monolein (mn), diolein (d), triolein (t), and methyl oleate (mm). (Reproduced with permission from Fried and Sherma (1999).)

Of the many unidimensional solvent systems available to resolve neutral lipids, the Mangold system and its modifications are most frequently used (**Figure 1**). This system consists of different combinations of petroleum ether (or hexanes), diethyl ether and acetic acid with a typical ratio of 80 : 20 : 1 v/v (petroleum ether–diethyl ether–acetic acid; Table 3). Changes in the ratios will affect R_F values of the neutral lipids being separated. Double development in the same direction as in the Skipski system is used to ensure good separation of glycerols from free fatty acids and free sterols (**Figure 2**). Although two-dimensional systems are infrequently used to separate neutral lipids, an example of such use is shown in

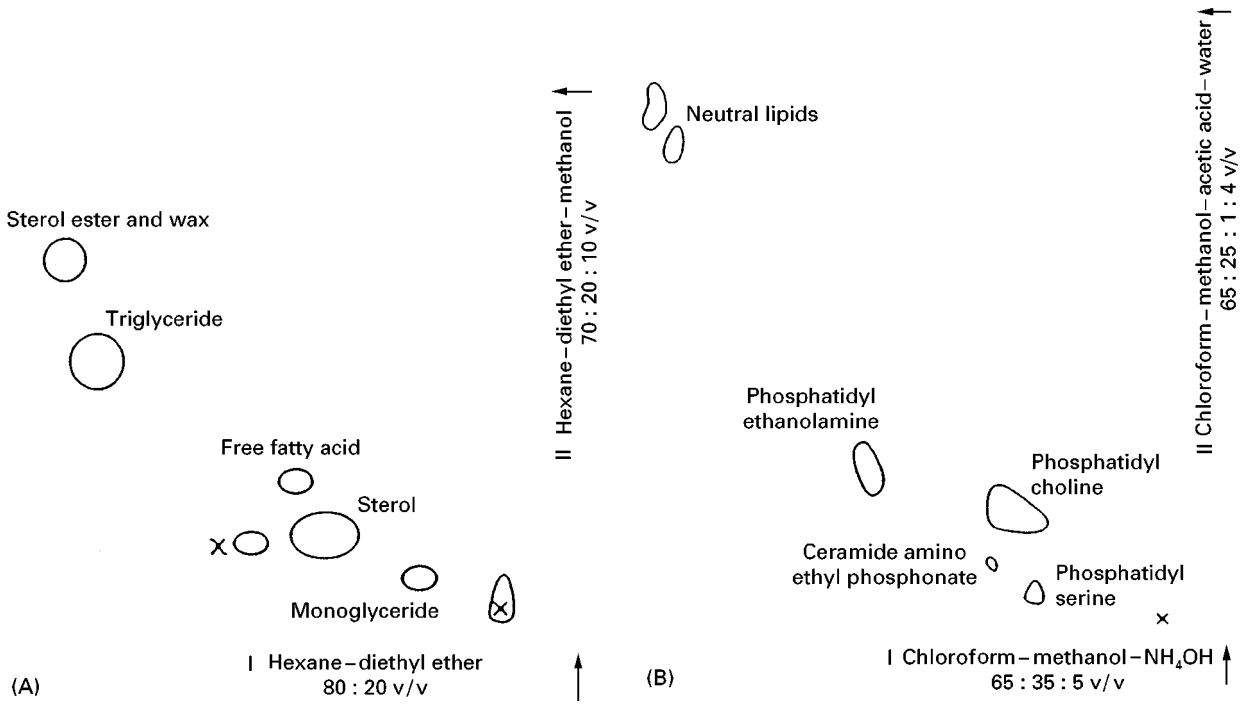

Figure 3(A) Chromatogram of the neutral lipids from an extract of the digestive gland–gonad (DGG) complex of the medically important snail *Biomphalaria glabrata*. The silica gel G plate was developed in the first direction in hexane–diethyl ether (80 : 20) and in the second direction in hexane–diethyl ether–methanol (70 : 20 : 10). Lipids were detected by spraying the plate with H_2SO_4. (Reproduced with permission from Thompson SN (1987) *Comparative Biochemistry and Physiology* 87B: 357–361.) **(B)** Chromatogram of phospholipids from a similar extract as in Figure 3(A). The silica gel plate was developed in the first direction in chloroform–methanol–NH_4OH (65 : 35 : 5) and in the second direction in chloroform–methanol–water–acetic acid (65 : 25 : 4 : 1). Phospholipids were detected using various specific phospholipid detection reagents. (Reproduced with permission from Thompson SN (1987) *Comparative Biochemistry and Physiology* 87B: 357–361.)

Figure 3A, where a neutral lipid map is obtained. Two-dimensional development is used more frequently to resolve complex lipid mixtures and Figure 3B shows such a chromatogram resolving phospholipids in the medically important snail *Biomphalaria glabrata*. Table 4 shows the most widely used solvent systems for two-dimensional separations of complex lipids in animal and plant tissues.

Numerous one-dimensional systems for phospholipids are available. The commonly used ones for

Table 4 Four recommended solvent systems for two-dimensional separations of phospholipids and glycolipids on silica gel

System	*Direction*	*Solvent composition and ratio*	*Comments*
A	First Second	Chloroform–methanol–water (65 : 25 : 4) *n*-Butanol–acetic acid–water (60 : 20 : 20)	Systems A and B are good for separating polar lipids of animal tissue on silica gel H; 10–15 complex lipids are separated
B	First Second	Chloroform–methanol–28% aq. NH_3 (65 : 35 : 5) Chloroform–acetone–methanol–acetic acid–water (10 : 4 : 2 : 2 : 1)	
C	First Second	Chloroform–methanol–7 mol L^{-1} NH_4OH (65 : 30 : 4) Chloroform–methanol–acetic acid–water (170 : 25 : 25 : 6)	Good for separating bacterial polar lipids on silica gel G; 10–15 polar lipids are separated
D	First Second	Chloroform–methanol–0.2% aq. $CaCl_2$ (60 : 35 : 8) *n*-Propanol–water–28% aq. NH_3 (75 : 25 : 5)	Good for separating a wide variety of ganglioside species on HPTLC plates

separating phospholipids on silica gel are shown in **Table 5**. The most widely used system is that of Wagner (see S_5 in Table 5), consisting of chloroform–methanol–water (65 : 25 : 4 v/v). In this system, the neutral lipids are moved as one or several bands near the solvent front and the common phospholipids of plant and animal tissues are clearly resolved. **Figure 4** shows a chromatogram of such a separation.

Glycolipid separation by unidimensional chromatography can be achieved in mobile phases consisting of various combinations of chloroform–methanol–water or chloroform–acetone–methanol–acetic acid–water. However, unidimensional separation of glycolipids is best done in the absence of phospholipids. Because glycolipids are difficult to separate completely by one-dimensional TLC, various two-dimensional procedures have been designed. A recommended two-dimensional system for glycolipids is chloroform–methanol–7 mol L^{-1} ammonium hydroxide (65 : 30 : 4 v/v) in the first direction and chloroform–methanol–acetic acid–water (170 : 25 : 25 : 6 v/v) in the second direction.

One-dimensional TLC can be used to separate gangliosides with various combinations of chloroform–methanol–water or *n*-propanol–water as solvent systems. As noted for glycolipids, because of the diversity of oligosaccharides associated with gangliosides, it is difficult to achieve complete separation of these lipids using one-dimensional TLC. Thus, various maps have been prepared in which ganglioside species have been separated in two-dimensional systems. These maps follow the nomenclature of Svennerholm and use esoteric designations to indicate the nature of the gangliosides being separated. Details of this work are beyond the scope of this article. Experience, patience, and trial and error are needed to effect good separations of glycolipids and gangliosides with one- and two-dimensional TLC.

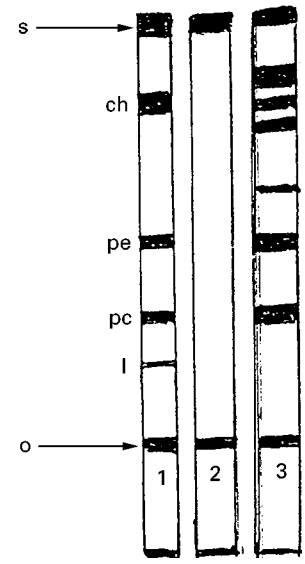

Figure 4 Separation of phospholipids extracted from snail-conditioned water (SCW) on a silica gel plate in the Wagner solvent system (see text). Lane 1 contains a standard with equal amounts of lysophosphatidylcholine (l), phosphatidylcholine (pc), phosphatidylethanolamine (pe) and cholesterol (ch). Lane 2 is a water blank without snails and is lipid-negative. Lane 3 contains SCW and shows that snails release significant amounts of phosphatidylcholine (pc) and phosphatidylethanolamine (pe), along with an unidentified component that migrates ahead of the pe. The bands near the solvent front are neutral lipids. Note the origin (o) and the solvent front (s). (Reproduced with permission from Fried and Sherma (1999).)

Table 5 R_F values of common phospholipids separated in five frequently used solvent systems on silica gel

	$R_F \times 100$				
Compound	S_1	S_2	S_3	S_4	S_5
Diphosphatidylglycerol	91	94	–	–	–
Phosphatidylglycerol	–	–	90	78	–
Phosphatidylethanolamine	65	56	81	59	40
Phosphatidylserine	–	47	51	47	13
Phosphatidylinositol	–	34	34	52	13
Phosphatidylcholine	24	21	90	30	20
Lysophosphatidylcholine	6	6	–	–	6

S_1, chloroform–methanol–water (25 : 10 : 1). S_2, chloroform–methanol–acetic acid–water (25 : 15 : 4 : 2). S_3, chloroform–light petroleum–methanol–acetic acid (50 : 3 : 16 : 1). S_4, chloroform–ethanol–triethylamine–water (30 : 34 : 30 : 8). S_5, chloroform–methanol–water (65 : 25 : 4).

Detection

Following development, lipids are usually detected by a wide variety of detection (visualizing) agents either sprayed or dipped on to the plate. Detection reagents may be nondestructive and reversible such as iodine or destructive and nonreversible such as sulfuric acid. The afore-mentioned agents are considered general because they react with numerous different compound types. **Table 6** provides a list of such general detection reagents frequently used to localize lipids on TLC plates. More or less specific detection reagents are used widely in TLC lipid studies and they generally indicate a particular compound or functional group, e.g. ninhydrin is used to localize amine groups associated with phospholipids such as phosphatidylserine or phosphoethanolamine. A list of spe-

Table 6 Five nonspecific reagents useful for the detection of lipids on TLC plates

Reagents	*Procedure*	*Results*
Iodine	Spray as 1% alcoholic solution or place a few crystals in the bottom of a closed tank	Dark-brown spots on a pale yellow or tan background in a few minutes
2′,7′-Dichlorofluorescein	Spray with a 0.2% solution in 95% ethanol. Observe in UV light	Saturated and unsaturated polar lipids give green spots on purple background
Phosphomolybdic acid	Spray with a 5% solution in ethanol; heat at 100°C for 5–10 min	Blue-black spots on yellow background
Sulfuric acid	Spray with 50% aq. H_2SO_4; heat as above	Black spots on a colourless background
Cupric acetate–phosphoric acid	Dissolve 3 g of cupric acetate in 100 ml of an 8% aq. phosphoric acid solution. Heat at 130–180°C for up to 30 min	Black spots on a colourless background

cific detection reagents frequently used in lipid TLC is shown in **Table 7**.

Quantification

There are many methods available for quantifying lipids using TLC. Some involve scraping and eluting lipids from the plate followed by spectrophotometric, gravimetric or chromatographic determination. The 1990s saw widespread use of direct *in situ* quantification, usually by densitometric methods, and an extensive literature is now available. Densitometry is performed in the reflection or transmittance mode with a specific brand of commercial densitometer. Any lipid that can be detected by ultraviolet or visible light is subject to densitometric analysis. Suitable standards, usually purchased from a commercial supplier, are needed and should match closely the compounds of interest. Quantification involves bracketing the analyte between two standards, one of slightly lower concentration, and the second of slightly higher concentration. Standards can be used to construct a calibration curve.

A list of recent TLC lipid applications by densitometry for the quantification of lipids is shown in **Table 8**.

Concluding Remarks and Future Developments

The most extensive use of TLC is for the analysis of pharmaceuticals, followed by all aspects of lipid analysis. Silica gel TLC is an excellent tool for the separation and identification of neutral and complex lipid classes. Densitometry allows for the quantification of these compounds at least at the class level. Numerous specific detection reagents are helpful for identifying lipids. Moreover, techniques in which

Table 7 Specific chemical detection reagents for various lipids

Compound class	*Reagent*	*Results*
Cholesterol and cholesteryl esters	Ferric chloride	Cholesterol and cholesteryl esters appear as red-violet spots
Free fatty acids	2′,7′-Dichlorofluorescein–aluminium chloride–ferric chloride	Free fatty acids give a rose colour
Lipids containing phosphorus	Molybdic oxide–molybdenum Zinzadze reagent	Phospholipids appear as blue spots on a white background within 10 min of spraying the plate
Choline-containing phospholipids (phosphatidylcholine and lysophosphatidylcholine)	Potassium iodide–bismuth subnitrate Dragendorff reagent	Choline-containing lipids appear in a few minutes as orange-red spots
Free amino groups (phosphatidylethanolamine and phosphatidylserine)	Ninhydrin	Lipids with free amino groups show as red-violet spots
Glycolipids	α-Naphthol–sulfuric acid	Glycolipids (cerebrosides, sulfatides, gangliosides, and others) appear as yellow spots
Gangliosides	Resorcinol	Gangliosides appear as a violet-blue colour; other glycolipids appear as yellow spots

Table 8 Selected applications of densitometric TLC to the quantitative analysis of lipids

Material	*Comments*
Neutral lipids in egg yolk	HPTLC silica gel; Mangold solvent system of petroleum ether–diethyl ether–acetic acid (80 : 20 : 2) for determination of cholesterol, triacylglycerols, and free fatty acids, and *n*-hexane–petroleum ether–diethyl ether–acetic acid (50 : 20 : 5 : 1) for cholesteryl esters. Lipid detection and quantification as described for neutral lipids in *Biomphalaria glabrata* snails in this table
Neutral lipids in *Biomphalaria glabrata* snails	HPTLC silica gel plates; petroleum ether–diethyl ether–acetic acid (80 : 20 : 2) mobile phase; detection by spraying with 5% ethanolic phosphomolybdic acid; lipid zones measured by scanning at 700 nm with a Shimadzu CS 930 TL densitometer operated in the single-beam reflectance mode
Phospholipids in *Biomphalaria glabrata* snails	HPTLC silica gel plates; multiple developments in a chloroform–methanol–isopropanol–0.25% and KCl–ethyl acetate (30 : 9 : 25 : 6 : 18) mobile phase; detection by spraying with 10% cupric sulfate–8% phosphoric acid solution; phospholipids measured by reflectance scanning at 400 nm with a Shimadzu CS-930 densitometer in the single-lane/single-beam mode
Lecithin and sphingomyelin from amniotic fluid	Silica gel plates developed in chloroform–methanol–water (75 : 25 : 4); sprayed with phosphomolybdic acid; scanned in a densitometer at 450 nm in double-beam transmission mode; detection of each lipid at 0.2 μg level
Sphingolipids in the parasitic protozoan, *Blastocystis hominis*	Sphingolipids along with neutral lipids and phospholipids were quantified on HPTLC plates; sphingolipids resolved on plates with chloroform–methanol–water (70 : 22 : 3) and detected with the orcinol reagent; plates scanned with a Shimadzu Flying Spot densitometer operated in the reflectance mode at 580 nm
Brain gangliosides	Complex sample preparation; use of HPTLC plates; chloroform–methanol–0.22% $CaCl_2$ (55 : 45 : 10) solvent system; detection by spraying with resorcinol–hydrochloric acid reagent; chromatogram scanned at 580 nm in transmission mode; separation and quantification of 4–8 brain gangliosides

plates can be impregnated with special agents have allowed for the separation and identification of molecular species within classes, e.g. molecular species of triacylglycerols.

A new area of work has used multiphase TLC, in which components are separated in two directions according to different parameters, e.g. conventional silica gel in one direction and reversed-phase in the other. This technique has proved useful in the analysis of triacylglycerols. The use of HPTLC-densitometry has revolutionized our ability to quantify lipids by relatively simple procedures. There are attempts underway to automate various aspects of the TLC process. Certainly with more automated methodology, TLC will be used more widely in the future by both chemists and biologists interested in lipid separations.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Layers; Spray Reagents. **III/Lipids:** Gas Chromatography; Liquid Chromatography.

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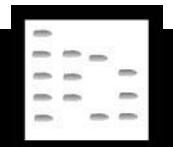
LIQUID CHROMATOGRAPHY-GAS CHROMATOGRAPHY



L. Mondello, P. Dugo and G. Dugo,
University of Messina, Messina, Italy
K. D. Bartle and A. C. Lewis,
University of Leeds, Leeds, UK

Introduction

High resolution gas chromatography (HRGC) is the most suitable technique for the analysis of volatile compounds. If the sample is a complex matrix, such as natural products, food products or environmental pollutants, direct gas chromatographic (GC) analysis is not advisable for several reasons, and a sample pretreatment is necessary. In fact, peaks of different classes of compounds may overlap, rendering the qualitative and quantitative analysis of some compounds difficult. Moreover, if the compounds of interest are present only as trace amount, a preconcentration step is necessary before the GC analysis.

If the mixture is subjected to a preliminary separation by liquid chromatography (LC), the fraction so obtained can be analysed by GC. Offline coupling of LC and GC is laborious, involving numerous steps with the risk of contamination and possible greater loss of part of the sample than if online techniques were used. The online coupling of LC and GC offers a number of advantages compared to offline coupling: the amount of sample required is much lower; no sample work-up, evaporation or dilution is necessary and complex automated sample pretreatment is possible.

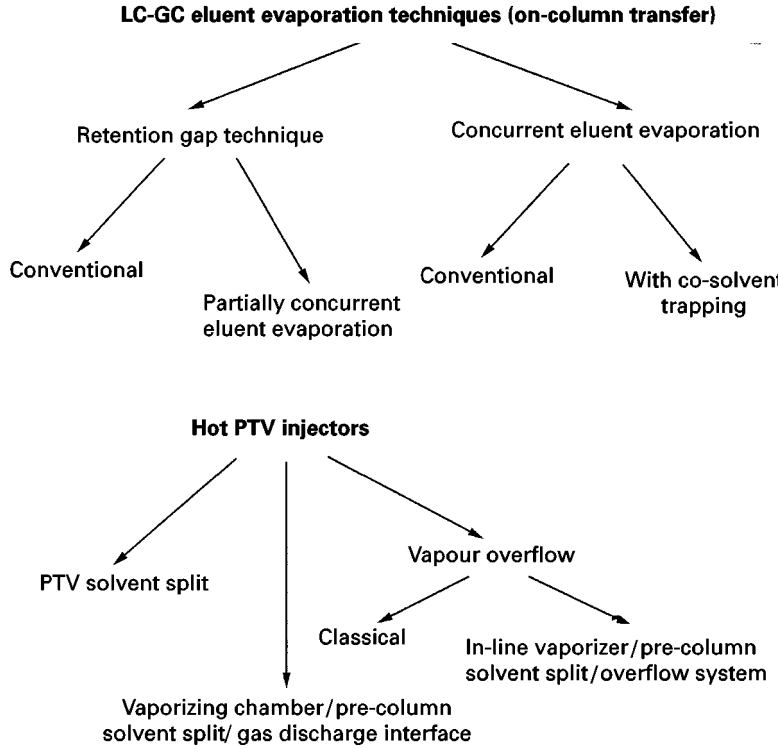


Figure 1 LC-GC transfer techniques. Possible mechanisms for LC-GC eluent evaporation.