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Isolation by Planar Chromatography

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1. Introduction

Planar liquid chromatography (PLC) involves the separation of mixtures on thin layers of adsorbents that are usually coated on glass, plastic, or aluminium sheets. The most common form of planar liquid chromatography is thin-layer chromatography (TLC); and this particular technique is the easiest, cheapest, and most widely used method for the isolation of natural products. TLC is one of the oldest forms of chromatography, the simplest example being the school experiment of spotting a plant extract near the bottom of thin strips of blotting paper and "developing" in a jar with water or alcohol. As the water moves up the blotting paper, the dark extract is separated into its component colors of light and dark greens and yellows.

The aim of this chapter is to describe the basic principles behind this technique and to give procedures for analyzing extracts and isolating natural products so that the worker unfamiliar with TLC and related techniques may use this simple method to follow and to isolate novel natural products. Examples of TLC isolations, chiefly from plants, will be given and the methodology behind the isolation of unknown products will be discussed. Success in TLC (like all forms of chromatography!) relies on a flexible approach and—by having a variety of methods at your disposal—versatility almost always guarantees success.

Natural product extracts are generally complex and comprise mixtures of neutral, acidic, basic, lipophilic, hydrophilic, and amphiphilic (e.g., amino acids) compounds and, as a consequence, there is rarely one method that will serve for all eventualities. It is sometimes worthwhile to carry out ^{1}H or ^{13}C NMR spectroscopy of the extract or fraction to determine the class of compound(s) to be separated *(1)*—deuterated NMR solvents are cheap ($1.00 for $CDCl_3$) and 1 D NMR experiments are quicker to run than the extensive

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development of mobile and stationary phases that may be needed. The starting point should always be the simplest method; examples for the isolation of a variety of natural products are given in **Subheading 5.**

1.1. Basic Principles of TLC

Separation by TLC is effected by the application of the mixture or extract as a spot or thin line onto a sorbent that has been applied to a backing plate (**Fig. 1**). Analytical TLC plates (thickness 0.1–0.2 mm) are commercially available; e.g., the commonest analytical silica gel plate is the 20 × 20-cm, plastic or aluminium backed Kieselgel 60 F_{254} plate, which has a 0.2 -mm thickness of silica sorbent (Merck no. 5554, Darmstadt, Germany).

The plate is then placed into a tank with sufficient suitable solvent to just wet the lower edge of the plate/sorbent but not enough to wet the part of the plate where the spots were applied (origin). The solvent front then migrates up the plate through the sorbent by capillary action, a process known as development (**Fig. 2**).

An important factor in quantifying migration of a compound on a particular sorbent and solvent system is the R_f value. This is defined as:

$$R_f = \frac{\text{Compound distance from origin (midpoint)}}{\text{Solvent front distance from origin}}$$

In the example shown in **Fig. 2**:

$$R_f = \frac{\text{Compound distance from origin}}{\text{Solvent front distance from origin}} = \frac{2.3 \text{ cm}}{2.8 \text{ cm}}$$

$$R_f = 0\ 82$$

R_f values are always ratios, are never greater than 1, and vary depending on sorbent and/or solvent system. These values are sometimes quoted as hR_f, i.e., relative to solvent front = 100, hR_f = R_f × 100 (in our case hR_f = 82). In the case of adsorption chromatography (*see* **Subheading 1.2.1.**), where the sorbent is silica (i.e., a normal phase), polar compounds (e.g., 2) have a higher affinity for the sorbent (stationary phase), "stick" to the sorbent, and move slowly up the plate as the solvent (mobile phase) migrates. These compounds will have relatively small R_f values in this example. Nonpolar compounds (e.g., Compound 1) have less affinity for the stationary phase, will move comparatively quickly up the plate, and therefore have relatively larger R_f values. As a consequence of development, compounds of a mixture will separate according to their relative polarities. Polarity is related to the type and number of functional groups present on a molecule capable of hydrogen-bonding (cf. 1.2).

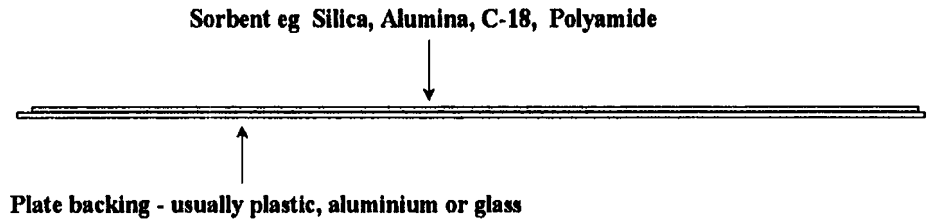


Fig. 1. Basic TLC plate layout.

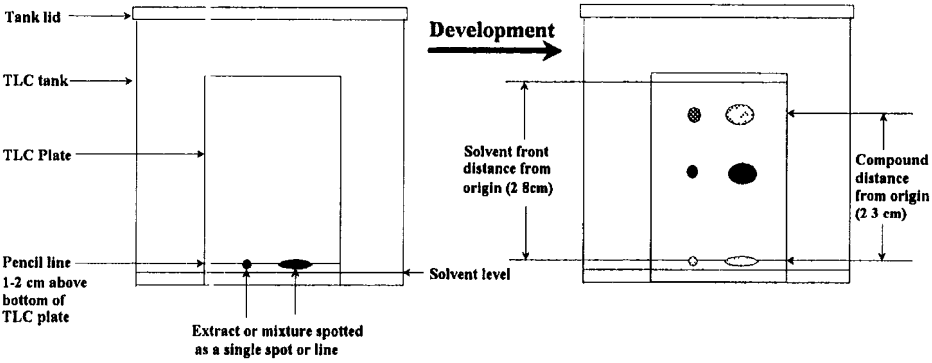


Fig. 2 Simple TLC equipment and procedure.

1. Nonpolar groups: CH_3–, CH_3O–, Ph–, CH_3CH_2, and
2. Polar groups: –CO_2H, –OH, –NH_2, SO_3H, PO_3H_2.

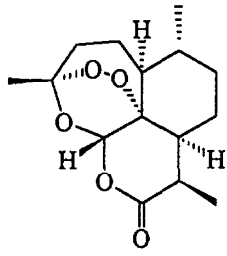
Compound 1 would be considered to be a relatively nonpolar compound compared with **Compound 2**, but it should be noted that this relative polarity will vary according to the type of stationary phase and mobile phase used.

Solvent strengths are also measured in terms of polarity, and dielectric constants are generally used to quantify relative strengths (**Table 1**). A high dielectric constant indicates a polar solvent with a strong power of elution, and a low dielectric constant indicates a nonpolar solvent with a lower ability to elute a component from a sorbent. This elution strength applies to normal phase adsorption chromatography.

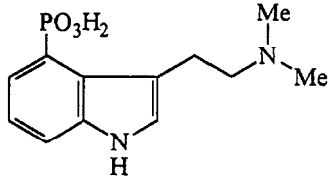
1.2. Mechanisms of Separation

There are four basic mechanisms of chromatography by which separation can occur, and more than one mechanism may be responsible during a given separation:

1. Adsorption chromatography—The most commonly used sorbents utilized in this form of chromatography are silica and alumina. As the components move through



Compound 1. Artemisinin (from the antimalarial herb *Artemisia annua*).



Compound 2. Psilocybin (from the fungus *Psilocybe mexicana*).

Table 1
Dielectric Constants of Solvents

Solvent	Dielectric constant (20°C)
Pentane	1.8
Hexane	1 9
Cyclohexane	2 0
Benzene[a]	2.3
Toluene	2.4
Diethyl ether	4.3
Dimethyl sulfoxide	4.7
Chloroform	4.8
Ethyl acetate[a]	6.0
Acetic acid	6.2
Dichloromethane	9.1
Pyridine	12.3
Acetone[a]	20.7
Methanol	32.6
Acetonitrile	37.5
Water	78.5

[a]Compounds recorded at 25°C *(2)*

the sorbent, their relative rates of migration are affected by their individual affinities for the sorbent. Separation occurs when one compound is more strongly adsorbed by the sorbent than the other components. When the sorbent is silica or alumina, polar natural products move slowly compared to nonpolar natural products. Adsorption takes place because of the interaction between the compound and groups associated with the sorbent; in the case of silica, which has silanol groups (**Fig. 3**), binding occurs between the compound and free hydroxyls on the sorbent. In this particular case, adsorption involves hydrogen bonding between compound functional groups and adsorbent surface hydroxyl groups.

2. Partition chromatography—This mechanism involves the relative solubility of the compound between the sorbent (stationary phase) and the solvent (mobile phase) Compounds that are more soluble in the mobile phase will migrate up the plate to a greater extent than components that are more soluble in the stationary phase. Reverse phase TLC utilizes sorbents that partition natural products between a hydrophobic, fatty (lipid) stationary phase, and an aqueous mobile phase. The most commonly used reverse phase sorbent is silica that has been reacted with a straight-chain 18 carbon alkyl unit to form an octadecasilyl phase (ODS), and there are a variety of slightly more polar phases commercially available (**Fig. 4**). Nonpolar "fatty" compounds such as the sesquiterpene artemisinin (**Compound 1**) are readily "soluble" in stationary phases such as ODS; and during solvent development a partition is set up between the two phases. Separation is effected by compounds partitioning to different extents between the stationary phase and mobile phase.
3. Size-inclusion/-exclusion chromatography—Compounds may be separated by their relative sizes and by their inclusion (or exclusion) into the sorbent. The most commonly used size-inclusion sorbents are the dextran gels, particularly the lipophilic versions such as Sephadex LH-20, which are of most use for the separation of small hydrophobic natural products from their larger "contaminants," usually chlorophylls, and so on. In organic solvents such as chloroform and methanol these gels swell to form a matrix. As compounds migrate with the solvent through the gel, small molecules become included into the gel matrix and larger molecules are excluded and migrate at a greater rate. It should be noted that separations on gels such as Sephadex LH-20 also involve the mechanisms of adsorption, partition, and possibly ion exchange, and occasionally the trend of larger molecules eluting first and smaller molecules eluting last may be reversed. This form of chromatography has found considerable use in the removal of "interfering" plant pigments such as the chlorophylls, which tend to be larger and more lipophilic than many plant products. This class of chromatography is more usually carried out in open-column form and is less commonly used as a mechanism of separation for TLC, which generally relies on the mechanisms of adsorption and partition.
4. Ion-exchange chromatography—This technique is limited to mixtures containing components that can carry a charge. In this form of chromatography, the sorbent is usually a polymeric resin that contains charged groups and mobile counter-ions, which may exchange with ions of a component as the mobile phase

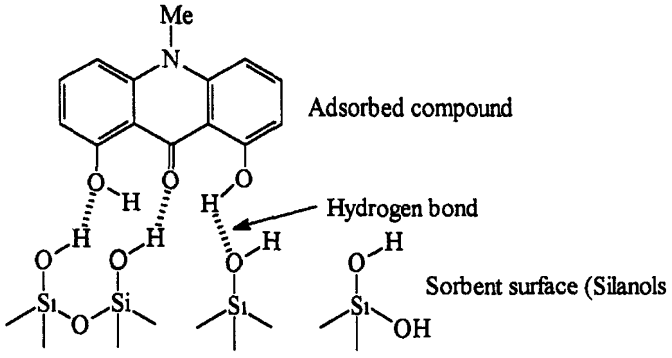


Fig. 3. Adsorption and hydrogen bonding between compound and sorbent face.

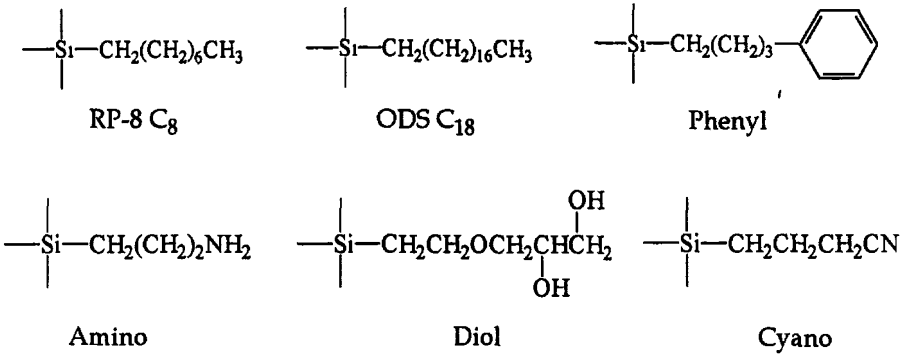


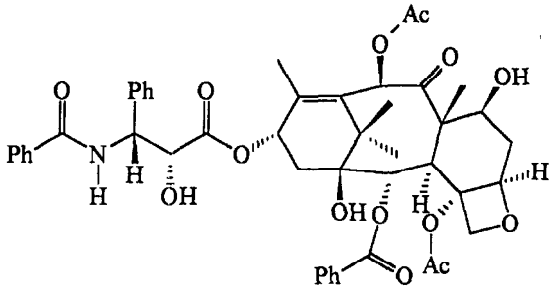
Fig. 4. Common phases for partition chromatography.

migrates through the sorbent. Separation is achieved by differences in affinity between ionic components and the stationary phase.

In cation exchange, acidic groups such as $-CO_2H$ and $-SO_3H$ are incorporated into the resin and are able to exchange their protons with other cations of components to form $-CO_2^-$, H_3O^+, and $-SO_3^-$, H_3O^+, respectively, at particular pH ranges. In anion exchange, basic groups such as quaternary ammonium moieties ($-N^+R_3$), are incorporated into the resin and are able to exchange their anions with anions of components. As with size-exclusion chromatography, this form of separation is generally used in columns, but can be utilized for separations on thin layers.

1.3. Applications of TLC

Traditionally, analytical TLC has found application in the detection and monitoring of compounds through a separation process. In the case of known natural products or other compounds (e.g., pharmaceuticals), qualitative and quantitative information can be gathered concerning the presence or absence



(3)

Solvent System
(1) $CHCl_3$-MeCN, (7 3)
(2) $CHCl_3$-MeOH, (7 1)
(3) CH_2Cl_2-THF, (6 2)
(4) EtOAc-iPrOH (95 5)

Fig. 5. TLC solvent systems for the resolution of taxol (Compound 3).

of a metabolite or breakdown product. An excellent example of this is the production of the antitumor diterpene taxol (**Compound 3**), from the endophytic fungus *Taxomyces andreanae*. Stierle et al. *(3)* isolated fungal taxol that had R_f values identical to that of taxol from the Pacific Yew, *Taxus brevifolia*, on four different solvent systems (**Fig. 5**). Use of the four solvent systems gave a higher degree of confidence that the fungal compound was identical to taxol, although the authors did confirm this by mass spectrometry and immunochemistry. Analytical TLC has been used to chemically classify organisms by their chemical constituents, in particular the filamentous bacteria, the Actinomycetes. The important genus *Streptomyces* generally contains a LL stereoisomer of a cell-wall metabolite known as diaminopimelic acid, whereas the rarer genera possess the *meso* form of this metabolite. By hydrolyzing the organism cell wall and running a TLC of the hydrolysate against the two standards, it is possible to classify loosely the actinomycete *(4)*.

Natural products may be "tracked" by running analytical TLC of fractions from other separation processes, such as column chromatography or HPLC. More than one solvent system should always be used for a TLC separation, as even apparently "pure" spots may consist of several compounds with identical R_f values. The similarity of different extracts from the same species can also be assessed in this way, and the decision to combine nonpolar and polar extracts can be made on the basis of identical or similar TLC chromatograms. Qualitative initial screening of extracts should be routinely performed, and the presence of ubiquitous compounds such as plant sterols and certain phenolics can be ascertained at an early stage by running the appropriate standard alongside an extract. In certain cases, classes of compounds may be determined by spraying developed plates with stains that give a color reaction with a particular compound class (*see* **Subheading 3.**).

Many natural products are still isolated by conventional preparative TLC (PTLC) and examples can be found in the journals *Phytochemistry* and *Journal of Natural Products*. Although preparative HPLC is in "vogue" and is often the method of choice, PTLC is still a very useful isolation method in many cases because of its simplicity, cost, speed, and ability to separate compounds in the 1 mg to 1 g range.

2. System Selection

As much information as possible regarding the extract-producing organism should be gathered—this will aid in the selection of a separation system. After a full literature search, the following points will need to be addressed: Has the species been studied before? If so, what metabolites were isolated? Are there standard TLC methods available? If the chemistry of a species has not been studied, is there any information at the generic level? Chemotaxonomy, or the classification of an organism according to its natural products, may assist in assigning chemically undefined genera ***(5)***—related species *may* produce related secondary metabolites—forewarned is forearmed!

Databases such as NAPRALERT, Berdy, The Dictionary of Natural Products, Chemical Abstracts, and, in the case of plants, certain excellent classical texts such as Hegnauers' "Chemotaxonomie der Pflanzen" ***(6)*** can give a great deal of information regarding the classes of natural products present in certain taxa.

Information about semipurified samples (e.g., column fractions) can also be invaluable. Several workers ***(1,7)*** routinely record ^{1}H NMR spectra of column fractions prior to TLC purification. This may seem a rather expensive detection system, but much information can be gathered about the classes of compounds present, and a separation method can be tailored accordingly.

TLC on silica gel is still the most common method of TLC though it suffers from some drawbacks. These however, may usually be overcome easily:

1. Acidic compounds "tail" on silica because of interactions between acidic groups (e.g., $-CO_2H$, $-OH$) and silanols—This may be reduced by the addition of a small amount of acid (e.g., 1% trifluroacetic acid or acetic acid) to the mobile phase, which will maintain acidic groups in a nonionized form.
2. Basic compounds also may behave poorly (i.e., tail, streak) on silica; the addition of weak bases (e.g., 1% diethylamine or triethylamine) should eradicate poor chromatography.
3. Highly nonpolar compounds such as fatty acids, glycerides, alkanes, and some lower terpenoids require simple nonpolar solvent systems (e.g., cyclohexane, hexane, pentane, diethyl ether.hexane mixtures) and may be difficult to detect by UV (i.e., no chromophore) or by spray detection (use charring reagents, e.g., vanillin-sulfuric acid).
4. Highly polar metabolites such as sugars, glycosides, tannins, polyphenolics, and certain alkaloids require the development of polar mobile phases, and in some

cases such compounds may be irreversibly adsorbed onto silica. Choice of mobile phase should evolve through use of a mono or binary system, i.e., 100% $CHCl_3$ or Hexane:EtOAc (1:1) as a starting point and then on to the addition of acids or bases to improve chromatography, i.e., a ternary system such as toluene:ethyl acetate:acetic acid (60:38:2) and as a last resort to use of quaternary system, e.g., hexane:ethyl acetate:formic acid:water (4:4:1:1).

Normal phase plates (silica, alumina) should be stored in a dessicator, as they are prone to pick up water from the atmosphere and become deactivated (lose their ability to resolve compounds). In some cases, plates may be activated by drying in an oven before use.

2.1. Forms of Development

In isocratic development, a solvent of constant composition is used to effect separation; for example, 40% ethyl acetate in hexane. This may be extended to continuous development, in which the TLC plate is left in an isocratic system after the solvent front has reached the top of the TLC plate. This has the advantage that closely eluting bands may be resolved through the use of a nonpolar solvent over several hours. A major disadvantage of this technique is that unstable compounds may degenerate on the adsorbent during this lengthy time period.

In multiple development, the plate is developed, removed from the tank, dried, and then subjected to further development(s). Multiple development may be run isocratically or by use of a step gradient.

In step-gradient TLC, the plate is developed in a nonpolar system (e.g., 10% ethyl acetate in hexane), dried, and then developed in a system of increased polarity (e.g., 20% ethyl acetate in hexane). This method allows great control over a separation in that the polarity may be very gradually increased to achieve separation of closely eluting bands.

2.1.1. Choice of Development

The decision as to whether the system is to be run isocratically or by using a step gradient can be made by running a series of analytical plates with the sample and varying the mobile phase composition. **Table 2** lists some of the more commonly used systems.

3. Detection of Natural Products in TLC

Both at the analytical and preparative stages of TLC, effective visualization or detection is crucial to obtain pure compounds, and poor detection may result in low recovery of product from the sorbent. Detection is either nondestructive, following which the compounds may be recovered from the sorbent (e.g., ultraviolet [UV] detection), or destructive, by which the compounds are contaminated by the detection reagent and are unrecoverable from the sorbent

Table 2
Simple Systems for TLC

Solvent system	Sorbent	Notes
Hexane.Ethyl acetate (EtOAc)	Silica gel	Universal system—can substitute hexane for petroleum spirit or pentane.
Petrol:Diethyl ether (Et_2O)	Silica gel	A universal system for relatively nonpolar metabolites. Excellent for terpenes and fatty acids. Care should be taken with Et_2O as explosive mixtures are formed with air.
Petrol:Chloroform ($CHCl_3$)	Silica gel	Considerably useful for the separation of cinnamic acid derivatives, particularly coumarins
Toluene:Ethyl acetate:Acetic acid (TEA)	Silica gel	Vary the composition, e.g., 80:18.2 or 60 38 2—excellent for acidic metabolites.
$CHCl_3$:Acetone	Silica gel	A general system for medium-polarity products
Benzene:Acetone	Silica gel	Useful for the separation of aromatic products. Care should be taken as benzene is a highly carcinogenic solvent. Substitute toluene for benzene.
Butanol.Acetic acid·Water	Silica gel	A polar system for flavonoid and glycosides.
Butanol:Water:Pyridine: Toluene	Silica gel	Sugar analysis system Try 10:6·6·1. Development may take 4 h on a standard 20 × 20-cm plate.
Methanol·Water	C_{18}	Start with 100% MeOH to determine if metabolite will move from the origin. Increase water concentration to slow migration of metabolites The addition of small amounts of acid or base may improve chromatography
Acetonitrile·Water	C_{18}/C_2	A universal simple reverse phase system
Methanol:Water	Polyamide	Universal
Methanol:Water	Cellulose	Used for the separation of highly polar compounds such as sugars and glycosides

(spray detection). There are some excellent texts available on this subject, such as Wagner, Bladt, and Zgainski's *Plant Drug Analysis* **(8)** or *The Merck Handbook of Dyeing Reagents for Thin Layer and Paper Chromatography* **(9)**; these will cover most eventualities.

3.1. Ultraviolet Detection

UV detection involves the use of UV active compounds (indicators) that are incorporated into the sorbent of TLC plates by the manufacturer. Typical examples of plates with these sorbents include the range of analytical plates produced by Merck: Alumina, 0.2 mm thick, 20 × 20 cm, with a 254-nm UV indicator (Merck no. 5550). Under short-wavelength UV light (254 nm), the indicator, which is usually a manganese-activated zinc silicate, will emit a pale green light. Under long-wave UV light (366 nm), a further indicator will emit a pale purple light. Compounds that absorb light at either 254 or 366 nm will appear as dark spots against a light background when UV light is shone onto the plate. Many compounds, such as the furocoumarins, will also emit a distinctive blue or yellow fluorescence under UV light. Long wavelength (366 nm) light is normally used for compounds that fluoresce, e.g., yellow, orange, blue, or red, as is the case for some chlorophylls. The major disadvantage with UV detection is that compounds that do not absorb UV light at 254 or 366 nm will be invisible and will require spray detection. The primary advantage of UV detection is that it is generally nondestructive and detection of compounds can be observed very readily through a separation process. It should be noted, however, that UV light can promote free radical reactions with certain natural products. UV lamps are widely commercially available from suppliers such as CAMAG (Camag Ref. 022.9230). Care should be taken not to shine light from these lamps into eyes or onto skin, as UV light is mutagenic.

3.2. Spray Detection

This relies on a color reaction between the compound on the TLC plate and a spray reagent (stain) introduced onto the plate as a fine mist from a spray canister. Ten of the most common spray reagents are listed in **Table 3**. Most are universal reagents and will react with many classes of natural products; the most widely used sprays are (1)–(3). Dragendorffs reagent (spray [6]) is especially useful for the detection of many classes of alkaloids and is well worth the effort required to make it. In some cases, heat is required to assist the color reaction and this can be supplied in the form of a hand-held heater (hair dryer!) or a drying oven. All of the compounds required to make the spray reagents are readily available as are the ready-made spray solutions themselves, from suppliers such as Sigma or Aldrich. Each of the spray reagents should be made up and used in a fume cupboard. The subject of partial identification of natural products by spray detection is also discussed in Chapter 10.

When using spray detection in preparative TLC, most of the plate should be covered and only a small proportion of the edge (2 cm) sprayed with reagent. Ideally, a scalpel should be used to score a line 2 cm in from the plate edge so that after spraying, corrosive spray reagent does not migrate into the sorbent and react with compounds to be recovered.

Table 3
Some Simple Spray Reagents for Natural Products TLC Visualization

Detection spray	Recipe	Treatment	Notes
(1) Vanillin/Sulfuric acid	Dissolve vanillin (4 g) in concentrated H_2SO_4 (100 mL).	Heat at 100°C until coloration appears.	A universal spray. Many terpenes give red and blue colors. Natural products with little functionality may give poor coloration—try spray (2) Spray and heat in a fume cupboard.
(2) Phosphomolybdic acid (PMA)	Dissolve PMA in ethanol to make a 5% (w/v) solution.	Heat at 100°C until coloration appears	Useful to detect many terpenes as blue spots on a yellow background. Spray and heat in a fume cupboard.
(3) Ammonium molybdate (VI)	Dissolve ammonium molybdate (VI) (10 g) in concentrated H_2SO_4 (100 mL).	Spray onto plate and heat at 100°C until coloration appears.	A universal spray. Many diterpenes give a blue color. Spray and heat in a fume cupboard.
(4) Antimony (III) chloride	Dissolve antimony (III) chloride in a mixture of glacial acetic acid (20 mL) and chloroform (60 mL).	Spray onto plate and heat at 100°C for 2–5 min or until coloration appears.	Di- and triterpenes give a red-to-blue coloration Care should be taken when handling this spray as antimony compounds are highly poisonous. Spray and heat in a fume cupboard.
(5) Tin (IV) chloride	Add tin (IV) chloride (10 mL) to a mixture of chloroform (80 mL) and glacial acetic acid (80 mL).	Spray onto plate and heat for 5 min at 100°C or until coloration appears.	Useful for the detection of flavanoids and terpenes. Tin (IV) chloride is poisonous and a lachrymator. Spray and heat in a fume cupboard

(6) Dragendorff's Reagent	Add 10 mL of 40% aqueous solution of KI to 10 mL of solution of 0.85 g of basic bismuth subnitrate in acetic acid (10 mL) and distilled water (50 mL) Dilute the resulting solution with acetic acid and water in the ratio 1:2:10.	Generally no heat is required, but if reaction is not spontaneous, heat until coloration appears The procedure may be enhanced by decolorizing the plate with concentrated ammonia vapor.	This is the traditional method for alkaloid detection although care should be taken as some non-alkaloids such as iridoids and some flavonoids give a positive reaction Alkaloids give a dark orange-to-red coloration.
(7) 2,4 Dinitro-phenyl hydrazine	Dissolve 2,4-dinitro-phenylhydrazine (0.2 g) in 2 *N* HCl (50 mL).	Generally no heat is required, but if reaction is not spontaneous heat until coloration appears.	Detects aldehydes and ketones with a yellow-to-red coloration.
(8) Perchloric acid	A 20% (w/v) aqueous perchloric acid solution.	Heat at 100°C until coloration appears.	A universal spray but is especially useful for steroids and triterpenes.
(9) Borntrager Reagent	A 10% (w/v) ethanolic solution of KOH.	Heat until coloration appears.	For the detection of coumarins and anthraquinones
(10) Ninhydrin	Add ninhydrin (0 3 g) to a mixture of butanol (100 mL) and acetic acid (3 mL).	Heat at 100°C until coloration appears.	Especially useful for amino acids, amines, and as a general alkaloid spray. Alkaloids appear as a red coloration.

(continued)

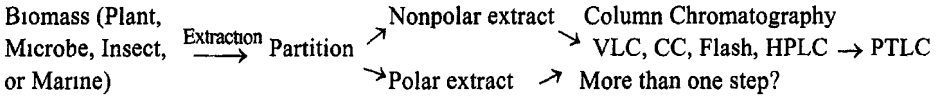
Detection spray	Recipe	Treatment	Notes
(11) Ehrlich Reagent	Spray first with solution of 1 g 4-dimethylamino benzaldehyde in 100 mL 36% HCl/MeOH (3.1), then dimethylamino benzaldehyde in 100 mL ethanol.	Place for 5 min in tank saturated with HCl vapour (or spray with 25% HCl) Gently warm.	Detection of amines, indoles, ergot alkaloids.
(12) Anisaldehyde/sulfuric	Add 1 mL conc. H_2SO_4 to 50 mL acetic acid containing 0.5 mL anisaldehyde	Heat at 100°C until coloration appears	Detection of many compounds, especially terpenes, sugars, phenols, and steroids.
(13) Bial's Reagent (Orcinol-ferric chloride)	Add 10 mL 10% H_2SO_4 containing 1 g ferric chloride to 1 mL 6% orcinol/ethanol	Heat at 100°C for 10–15 min or until coloration appears	Particularly useful for detection of sugars.
(14) Triphenyl-tetrazolium chloride	Add 10 mL 4% triphenyl-tetrazolium chloride/MeOH to 10 mL 1 *N* NaOH	Heat at 100°C until coloration appears	Detection of reducing sugars, corticosteroids.
(15) Fluorescein	0 01% fluorescein/ethanol.	Heat gently, then spray lightly with water or treat with steam	Detection of lipids.
(16) Ferric chloride	5% ferric chloride in 0 5 *N* HCl	Generally no heat required, or heat gently.	For detection of phenolics

4. Preparative TLC (PTLC)

Preparative TLC (PTLC) has long been a popular method of isolation, primarily because of its simplicity and universal accessibility to students and researchers working in natural products chemistry. This popularity has been diminished in recent years because of the success of high-performance liquid chromatography and countercurrent chromatography. Unlike these two techniques, however, PTLC does not require expensive equipment; separations can be effected rapidly and the amount of material isolated generally falls into the 1 mg to 1 g range, which is certainly sufficient for structure elucidation purposes. This section gives a breakdown of the basic steps of PTLC, with emphasis on preparing and running plates, and some of the advantages and disadvantages of PTLC.

4.1. When to Use PTLC

Although separations depend on the level of complexity of an extract, PTLC is nearly always used as a final purification step in an isolation procedure. A broad procedure is given below.



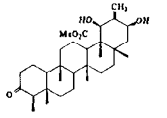
The number of compounds that can be separated on a preparative plate will ultimately depend on how those compounds behave on a particular system, but as a general rule, the separation of no more than a mixture of four major components should be attempted. The separation of complex mixtures can be carried out on preparative TLC as a first stage but larger amounts of material are needed, and as the process of running many plates can be time-consuming, it is more usual to separate partially purified mixtures. Complex extracts can, in the first instance, be separated by vacuum liquid chromatography, flash chromatography, or column chromatography prior to PTLC. These first purification steps are covered in Chapter 4.

The friedelane triterpene (**Compund 4**) was isolated from the bark of the Camerounian rainforest tree *Phyllobotryon spathulatum (10)* using the separation process in **Table 4**.

This triterpene needed only one purification step prior to preparative TLC, and because of its lack of distinctive chromophore, required visualization by spray detection using vanillin reagent (**Table 3**).

4.2. Scale-Up from Analytical to PTLC

The scale-up procedure from analytical (0.1- to 0.2-mm sorbent thickness) to preparative TLC (0.5- to 4-mm sorbent thickness) is of paramount impor-

Species	Extract	Step (1)	Step (2)	Structure
Phyllobotryon spathulatum	Petrol and $CHCl_3$ (Bark)	VLC on Silica gel Fraction eluted with 25% EtOAc in Hexane	Prep TLC Toluene 80 EtOAc 18 AcOH 2	 4

tance, as changing the size of a separation can drastically affect the chromatography of natural products. The chromatography of a compound separated on analytical plates where 1 mg of material is involved can alter significantly when milligrams or tens of milligrams are employed. This is possibly attributable to sorbent particle size. On normal phase silica, when moving from analytical to preparative scale, it may be necessary to reduce the polarity of the solvent markedly in order to achieve the same R_f values. Very often this is a trial and error procedure, but as an example, the separation of a mixture of two components achieved using hexane:EtOAc (60:40) as a mobile phase on an analytical plate would possibly require the less polar system hexane:EtOAc (90:10) on a preparative plate to give comparable R_f values. This is a general rule and will vary according to compound class and the types of stationary phases used—the best method being the sacrifice of a small portion of mixture, and experimentation.

4.3. Commercially Available PTLC plates

These plates are usually limited to the sorbents, silica, alumina, C_{18}, and cellulose and are usually of thicknesses 0.5, 1.0, and 2.0 mm. The glass-backed silica-gel 60 plates from Merck have a particle-size distribution of 5–40 μm, compared to the corresponding analytical plate of 5–20 μm. These silica plates have a high specific surface area, are very homogeneous, and usually give excellent results.

The use of commercially available preparative plates with a concentration zone will enhance separation. This zone is a layer of inert large-pore silica at the bottom of the plate onto which the sample is applied. As the solvent migrates through this zone, the mixture is unretained and focuses at the interface between the zone and "normal" sorbent. Uneven applications of mixtures are focused as discrete lines, and this will greatly improve separation/resolution.

Normal phase plates such as the 2-mm Merck Kieselgel $60F_{254}$ 20 × 20-cm plate (Merck no. 5717) require pre-elution with a nonpolar solvent such as dichloromethane to "clean" and remove contaminants. These impurities will be carried with the solvent to the top of the plate, and then the plates should be

dried prior to use. When a new box is opened, unused plates should be stored in a desiccator as moisture from the air will affect the activity of the sorbent (especially in the case of silica), resulting in poor resolution and poor separation.

4.4. Homemade Preparative Plates

Making your own plates allows greater flexibility of choice of sorbent and, whereas commercial plates are restricted to three or four sorbents, with the correct recipe, homemade plates offer much wider scope for experimentation. These plates also allow the variation of thickness to accommodate the separation of large amounts of material. Binders such as calcium sulphate (gypsum) are require to bind the sorbent to the plate, but some silica sorbents (e.g., Merck 7749) contain sufficient binder for the purpose. Preparing your own plates will also give you the choice of selecting a sorbent with or without a UV indicator and will also enable the incorporation of additives that enhance separation into the sorbent. An example of this (recipe shown below) is the addition of a small quantity of silver nitrate to a silica sorbent that aids the resolution of olefinic compounds.

If cost is an issue, making plates is cheaper than the commercial alternative, and the removal of sorbent from the plate backing during the desorption process is easier than on commercial plates—a point to consider if compounds are poorly resolved. The following example is a method for making silica preparative plates of 0.5 mm thickness with optional silver nitrate additive. For plates of 1 or 2 mm thickness, two or four times the amount of water and sorbent are required, and all of the equipment is readily available from suppliers such as CAMAG or Merck.

Equipment. 5 glass backing plates (20 × 20 cm), adjustable gate applicator, 2 glass spacer plates (5 × 20 cm), Kieselgel 60 (45 g) Merck 7749, plate holder, silver nitrate (1 g) (optional), TLC plate coater, distilled water (90 mL), 200-mL conical flask, and stopper.

4.4.1. Procedure

1. Clean the glass backing plates with 1 *N* aqueous KOH and then acetone and dry prior to spreading.
2. Place the plates into the TLC plate coater with spacer plates at either end. Adjust the applicator to the correct plate thickness required (0.5 mm in this case) and place at one end on the spacer plate.
3. Put silica (45 g) or alternative sorbent and the silver nitrate (if required) into the conical flask and add deionized water (90 mL). Shake the stoppered conical flask vigorously for 30 s and ensure that a homogeneous slurry is produced.
4. Immediately pour the slurry into the applicator, and in one steady movement pull the applicator across the plate faces to rest on the far plate spacer.
5. Air-dry the plates for 1 h and then put into a plate holder and activate in an oven at 115°C for 4 h prior to use

This general method may be applied to other sorbents though additional binder may be necessary. When incorporating silver nitrate into the sorbent, the plates should be stored and developed in the dark to avoid discoloration and degeneration of the sorbent.

4.5. Sample Application

Preparative plates such as those mentioned above should be removed from the oven and allowed to cool to room temperature before use. The sample to be separated should be dissolved in the minimum volume of solvent possible (usually in the concentration range 10–20 mg/mL). This sample is then applied near to the bottom of the plate (~1.5 cm from the bottom) as a thin line (2–4 mm wide) using either a microsyringe, a capillary, or a pasteur pipet that has been drawn out over a Bunsen burner. Thinner capillaries (5–10 µL) give greater control over sample application and result in a finer, more concentrated zone. In order to apply the sample in a straight fashion, it is preferable to lightly draw a pencil line (without scoring the sorbent!) approx 1.5 cm above the plate edge, or alternatively use a piece of A4 paper placed on the plate as a guide, or use a template. Application of the sample as a straight line is necessary as this forms the origin, and assuming that the plate is homogeneous, the sample will separate during development into even bands of individual compounds. If the sample is applied in an irregular fashion (i.e., a wavy line), then during development the sample will separate into compounds as irregular bands that are difficult to remove in a pure form from the plate during the desorption process.

The sample should not be applied right up to the edges of the plate as "edge effects" (the rapid movement of solvent up the plate sides or poor sorbent homogeneity) will lead to uneven movement of solvent up the plate during development, and as a consequence, irregular band shape will result (**Fig. 6**).

4.6. Development and Detection

A suitable solvent system and sorbent phase will have been chosen and some of the simpler cases are given in **Subheading 2.** and in the examples (**Subheading 7.**). Mobile phases for preparative systems should be freshly made up, and usually 100-mL volumes are suitable to run one or two plates in the same tank. A solvent-saturated atmosphere in the tank is favored to improve chromatography, and this can be facilitated by adding some filter paper (e.g., 15 × 15 cm) to the inside walls of the tank.

Silica gel is quite a "reactive" sorbent, and some natural products are unstable on this phase. It should be noted that during development, plates should be kept out of direct sunlight, as degradation of the natural products may occur.

Development is achieved by allowing the solvent front to reach the plate top or within a few millimeters of it and then removing the plate from the tank and

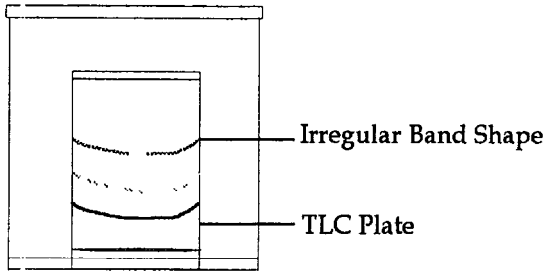


Fig 6 Irregular band shape resulting from "edge effects."

air-drying in a fume cupboard. Handheld dryers should be avoided to remove excess solvent from plates due to the risk of degradation.

Most semipurified samples have some residual color, or the natural products of interest may be colored; in such cases, it is possible to gage how far the compounds have migrated up the plate. In the case of plant extracts, nonpolar pigments such as the chlorophylls or carotenes can give a visual aid to separation and an idea of how far the compounds of interest have migrated relative to the pigments. If the natural products of interest absorb long- or short-wavelength UV light, then the success of the separation can be readily observed—this is especially useful in multiple development—and it is rewarding to see compounds resolved through the isolation process! With poorly UV-absorbing products, use of a spray reagent is required (**Subheading 3.**), and spraying only the plate edge will give an idea of how far the natural products of interest have migrated. If, after spray detection, the compounds have not migrated far enough up the plate to effect separation, then the contaminated sprayed silica (or other sorbent) must be removed to avoid contamination of the solvent system before further redevelopment.

4.7. Desorption and Recovery of Natural Products

Once the decision has been made that the separation of compounds has been satisfactorily achieved, the natural products need to be effectively recovered from the sorbent, dried, and stored for structure elucidation. On the silica example given above, where a UV fluorescent indicator is incorporated into the sorbent, UV-absorbing bands (compounds) may be marked out by a pencil or scalpel and scraped off the backing plate onto tin foil or paper.* With spray detection, bands may be cut from the spray-colored edge (but not incorporating it!) along the plate using a ruler. These bands may be scraped from the plate onto foil.

*The procedure of sorbent removal (especially silica and alumina) from plate backings should always be performed in a fume cupboard, and a dust mask should be worn to avoid breathing dangerous fine particulate material coated in substances of (presumably) unknown composition.

Compounds may be desorbed from the sorbent in three simple ways:

1 The compound-rich sorbent can be transferred to a conical flask and solvent added. The suspension should be left for 30 min to facilitate leaching of compound into the solvent and then filtered. This process should be repeated two or three times to ensure good recovery. The type of solvent used should be slightly more polar than is normally required to dissolve the sample; e.g , if the sample dissolves readily in chloroform, then desorption should be carried out using chloroform:methanol (9:1) or (8:2). This should ensure maximum recovery from the sorbent and minimize the possibility of the product remaining strongly bound to the solid phase.
2. The compound-rich sorbent should be placed in a sintered glass funnel (3 porosity frit) attached to a glass Buchner flask to which a vacuum is applied. The sorbent is then washed with solvent, and the resulting solution can be recovered in the flask and evaporated to yield the product. Repeated washings with solvent will lead to effective recovery of compounds. This is the method of choice for recovery of components from preparative TLC plates.
3. A Pasteur pipet fitted with a cotton wool (defatted) plug or a micro column with a 3-porosity frit can be packed with compound-rich sorbent. This "mini" column can then be eluted with solvent to recover the pure compound. Care should be taken not to overpack these columns as compound elution time may be considerable. However, one benefit of these desorption methods, assuming that the natural products of interest are sufficiently stable, is that they may be set up with sufficient solvent and left to desorb for a long period of time

In all three cases, where silica is the sorbent, methanol can be used as a final wash stage to ensure full compound recovery—many natural products such as the glycosides of flavonoids and triterpenes are highly polar and may require the addition of 1 or 2% acetic acid in this final methanol elution.

Products should be dried quickly after elution, preferably using a high-purity N_2 blow-down apparatus, and stored in a freezer. Rotary evaporators using heat and turbovaps using air should be avoided because of the risk of heat decomposition and oxidation or loss by evaporation.

4.8. Assessing Purity by TLC

After desorption, analytical TLC should be performed on recovered products to ascertain their purity. The smaller particle size of analytical plates compared to PTLC enables better resolution and a greater ability to measure purity. At least two different solvent systems should be used in order to distinguish between compounds that have similar (or identical!) R_f values on a particular system. It should be noted that if a recovered compound appears to be impure, even after what promised to be a successful purification by PTLC, then it is possible that the natural product was unstable on the sorbent or in solution, and an alternative stationary phase or separation process should be sought.

4.9. Advantages and Disadvantages of PTLC

In the last 10 yr, there has been quite a considerable movement away from "wet" techniques (such as PTLC and conventional column chromatography) and toward instrumental techniques such as HPLC and countercurrent chromatography. Many natural products chemists prefer these instrumental methods because of the greater control they afford over a separation process and reproducibility, although the high cost and need for routine servicing of these machines will maintain a significant role for PTLC in isolation procedures. The following lists detail some pros and cons of PTLC.

4.9.1. Advantages

1. PTLC is cost-effective compared to the instrumentation required for HPLC and droplet countercurrent chromatography.
2. PTLC is a simple technique that requires little training or knowledge of chromatography
3. An analytical method may be easily scaled up to a preparative method.
4. Natural products can be quickly isolated in the milligram to gram range
5. Solvent and stationary phase choice are flexible; i.e., the solvent system can be changed quickly during a run.
6. The separation can be optimized readily for one component: It is relatively easy to "zero in" on a particular product.
7. Methods are quickly developed.
8. Almost any separation can be achieved with the correct stationary phase and mobile phase
9. A large number of samples can be analyzed or separated simultaneously.

4.9.2. Disadvantages

1. There is poor detection when compared to diode array HPLC.
2. There is poor control of elution compared to HPLC.
3. Loading of the sample and speed of operation are poor compared to vacuum liquid chromatography.
4. Multiple development methods to isolate grams of material may be time-consuming.
5. PTLC is restricted to simple sorbents such as silica, alumina, cellulose, and RP-2

5. Centrifugal PTLC (CPTLC)

This excellent and underexploited technique can be utilized as a primary "clean-up" process of natural products extracts or as a final purification step. CPTLC makes use of a rotor that is coated with a sorbent to form a circular plate that is then attached to a spindle and rotated using a motor. Solvent is then introduced into the middle of the circular plate by a pump to equilibrate the sorbent. Plates should be saturated with solvent and allowed to equilibrate at a given flow rate for 10 min. The sample mixture can then be introduced to

the plate in the same fashion. As the plate rotates and solvent migrates through the sorbent, the sample is separated into circular compound bands that may be readily collected as they elute from the plate (**Fig. 7**) (cf. TLC in which bands are not generally allowed to reach the edge of the plate).

The plate and motor are housed in an apparatus where a nitrogen atmosphere can be applied. A good example of CPTLC apparatus is the Chromatotron (Harrison Research Model 7924), which has a duct to collect eluting bands and a quartz window that fits over the plate allowing visualization of the plate with UV light.

CPTLC has a number of advantages over PTLC. First, because development is accelerated centrifugally by plate rotation, separation can be rapid. Sample component zones expand continuously and consequently are drawn into increasingly narrow concentric rings. The mobile phase expands outward so that it is faster at the trailing edge than at the front, and this will compress the component bands. Both of these factors lead to narrow bands and better chromatography than linear development. Solvent changes can be made quickly and one may operate in a gradient or isocratic mode. Larger amounts of material (1–2 g) may be loaded onto the plate at once, than is the case with PTLC. As with homemade preparative plates, CPTLC plates allow the choice of sorbent, additives, and binders.

Recipes for making a variety of plates with different sorbents accompany CPTLC apparatus manuals. However, 2- or 4-mm thickness silica-gel plates may be made in the following way:

Add silica (Kieselgel 60 PF254 Merck Art 7749) (65 or 100 g; for 2 or 4 mm thick) and binder ($CaSO_4$; 4 or 6 g) to distilled water (100 or 190 mL), and shake thoroughly. The resulting slurry should be poured onto the rotor at the edge and the plate tapped gently to remove air bubbles and to ensure a homogeneous layer. Air-dry the plate for 30 min and oven-dry at 50°C for 12 h. The resulting plate should then be scraped to the required thickness and stored in an oven at 50°C prior to use.

The correct choice of solvent system should be ascertained by using a series of analytical plates with increasingly polar solvent to determine R_f values. When using an isocratic system, a solvent system can be used where the R_f of the highest eluting compound is about 0.3. This will result in a steady separation in which fractions (and compound bands) can be collected and analyzed by TLC. A more polar system (e.g., R_f of the least polar component is 0.8) can be used in which concentrated compounds will appear as discrete bands that move quickly through the plate—smaller volume fractions should be collected and analyzed. As with PTLC, use of a sorbent incorporating a UV indicator will aid in the monitoring of UV active compounds.

Khan et al. ***(11)*** used a Chromatotron CPTLC apparatus in the separation of some unusual clerodane diterpenes (e.g., **Compound 5**) in a chemotaxonomic study of the flacourtiaceous species *Zuelania guidonia*. Silica gel sorbent and a

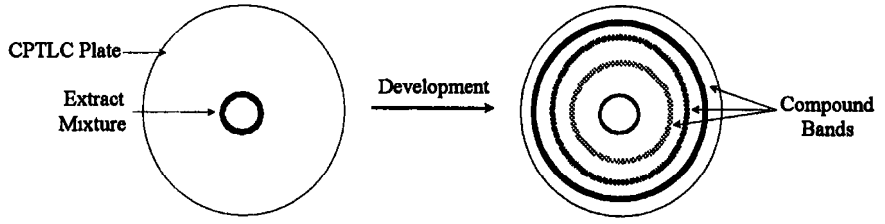
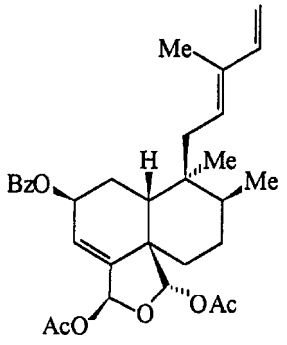


Fig. 7. Circular band separation through CPTLC development.



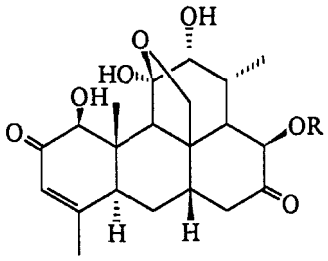
Compound 5.

mobile phase of petrol/ethyl acetate (49:1) were used and the compound was visualized under UV light.

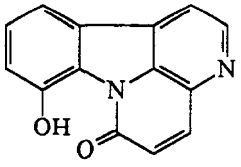
Waterman and Ampofo *(12)* used CPTLC to isolate the cytotoxic quassinoids, ailanthinone (**Compound 6**), 2'-acetylglaucarubinone (**Compound 7**) and the alkaloid 8-hydroxycanthin-6-one (**Compound 8**) from the rainforest tree *Odyendyea gabonensis* (Simaroubaceae).

6. Over-Pressure TLC (OPTLC)

Over-pressure TLC (OPTLC) was introduced by Tyihak et al. in 1979 *(13)* in an attempt to combine the advantages of conventional TLC and HPLC. This technique employs the use of a pressurized circular ultra microchamber (PUM chamber), which houses a TLC plate and inlets for the introduction of sample and solvent onto the sorbent. The thin sorbent layer is covered by a membrane kept under external pressure so that the vapor phase above the sorbent is nearly eliminated. A substantially shorter time is required for separation than in conventional TLC and classical column chromatography (CC) and greater resolution and separation efficiency is achieved. The rate at which solvent migrates is as stable as HPLC and consequently the technique can be used to model CC



Compound 6 and 7. (6) R = $COCH(Me)CH_2CH_3$. (7) R = $COC(OAc)(Me)CH_2CH_3$ (silica gel; CH_2Cl_2·isopropanol [49:1]).



Compound 8. (silica gel; toluene:EtOAc·AcOH [5.4.1]).

methods. Separations can be carried out 5–20 times faster than conventional TLC, and so this method may be applicable to high throughput/repetitions.

Tyihak et al. validated this technique using the separation of the synthetic dyes indophenol, Sudan G, and Butter Yellow. Natural products such as capsaicin from *Capsicum anuum* and furocoumarins from *Heracleum sphondylium* have been separated by Nyirdey et al. ***(14)***.

6.1. Automated Multiple Development (AMD)

This method utilizes a fully automated developing chamber that consists of a sensor to optically detect the solvent front position, a mechanism to lift the plate out of the developing chamber, multiple solvent reservoirs, a solvent pump, and an integrated fan to dry the plate and remove solvent vapor. Modern systems contain microprocessor-controlled programming to vary solvent composition after each run. Multiple development dramatically increases separation power, improves reproducibility and precision, and can be set up to run without continuous supervision. This apparatus can also be used in conjunction with a TLC plate scanner that will detect UV-active bands. This can be interfaced with a PC and linked to a printer for hard copy. An excellent example of an AMD device is the CAMAG AMD system (Merck).

6.2. Two-Dimensional TLC

Two-dimensional TLC is frequently used for the screening of complex mixtures. If the object is to find known compounds, and standards are available,

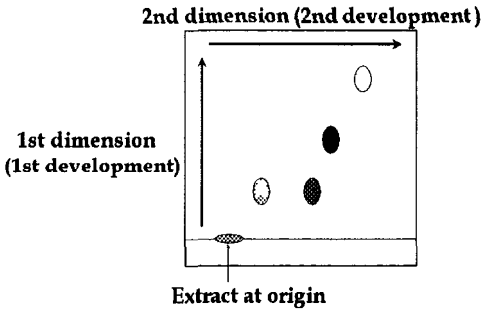


Fig. 8 Two-dimensional TLC plate after two developments.

then this is a very powerful form of TLC. The extract is spotted onto the plate in the normal fashion and the plate is developed, dried, and then turned through 90° and developed a second time (**Fig. 8**). This has the advantage of resolving compounds into the second dimension, which gives further resolution. Also, different solvent systems may be used for the second elution, which further enhances the resolving power of this technique. The resulting chromatogram may then be observed under UV light or stained for detection purposes.

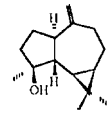
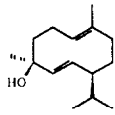
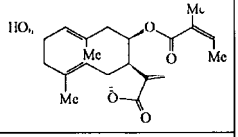
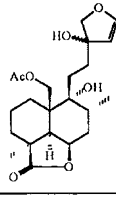
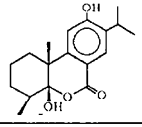
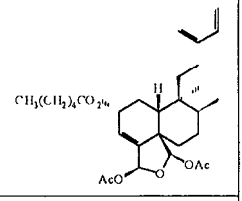
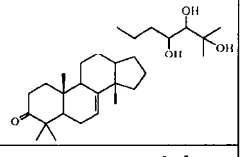
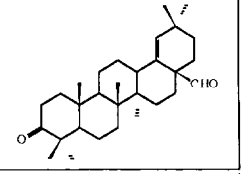
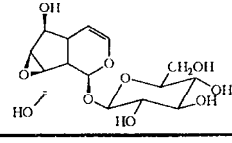
7. Examples of Preparative TLC

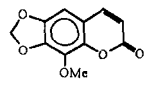
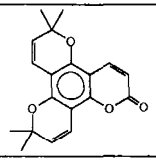
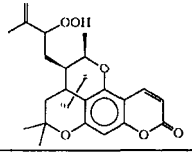
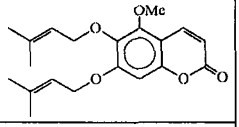
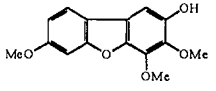
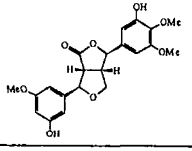
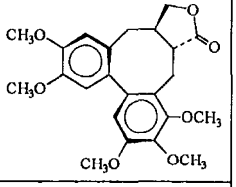
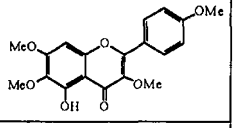
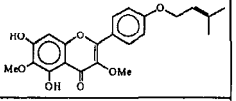
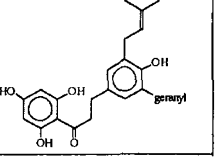
The examples in **Table 5** are taken from the natural products literature, from our own experience, and in particular from the excellent PhD dissertations (University of Strathclyde) of Monira Ahsan *(15)* and Satyajit D. Sarker *(22)*. They cover a range of different classes of plant and microbial natural products. Only the final preparative TLC stage is given in the isolation; further details may be obtained from the reference.

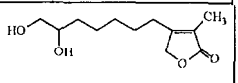
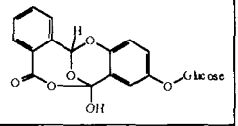
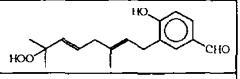
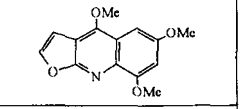
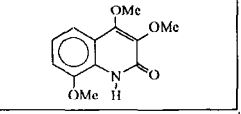
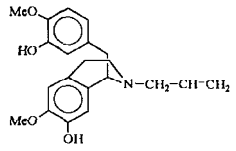
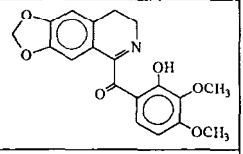
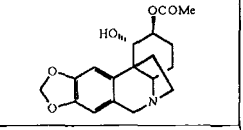
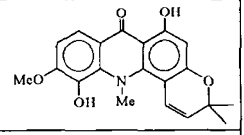
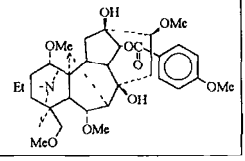
8. TLC Bioassays

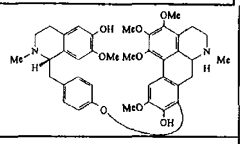
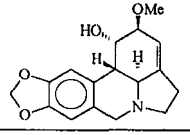
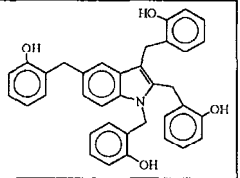
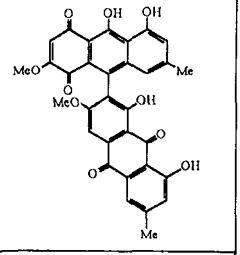
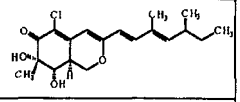
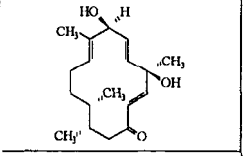
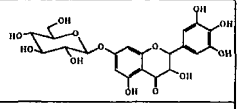
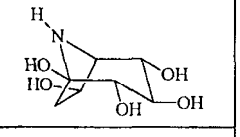
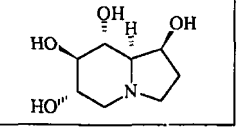
TLC bioassays against fungi and bacteria have proved exceptionally popular because of their ease of use, low cost, speed, and their ability to be scaled up to assess the antimicrobial activity of a large number of samples. Generally, TLC plates are run and then the microorganism is introduced to the plate as a spray (in the case of direct bioautography), or the plate is covered with a growth medium containing the microorganism in a dish or tray (overlay assay). With the occurrence of multiple drug-resistant bacteria (such as methicillin-resistant *Staphylococcus aureus*) and the need for new antimycotic drugs, these simple bioassays will continue to prove useful in the assessment of antimicrobial activity of natural product extracts. A critical review of key antifungal and antibacterial assays has been made by Cole *(46)*, and the reader is referred to a number of authors; namely, Spooner and Sykes *(47)*, Holt *(48)*, Rios et al. *(49)*, Homans and Fuchs *(50)*, Betina *(51)*, Leven et al. *(52)*, and Begue and Kline *(53)*.

Table 5
Examples of Preparative TLC

Species and Compound class	Reference	PTLC Method	Structure
Boronia inornata Sesquiterpene **Spathulenol**	(15) Ahsan (1993)	Silica gel Toluene EtOAc (97 3) R_f = 0 39 Toluene EtOAc AcOH, (89 10 1) Three developments	
Boronia inornata Sesquiterpene	(15) Ahsan, (1993)	Silica gel Toluene EtOAc (96 4) R_f = 0 5 Toluene EtOAc, (92 8)	
Calea divaricata Sesquiterpene **Hydroxyeupatolide-8-*O*-angellate**	(16) Ober *et al*, (1984)	Silica gel Me_2CO Hexane (4 1)	
Leonitis ocymifolia Diterpene ester	(17) Habtemariam *et al*, (1994)	Silica gel Hexane $CHCl_3$ EtOAc (2 3 2)	
Cupressus goveniana Diterpene **Cupresol**	(18) Jolad *et al*, (1984)	Silica gel 60 PF_{254} CH_2Cl_2 EtOAc (94 6)	
Casearia tremula Diterpene ester	(19) Gibbons *et al*, (1996)	Silica gel Toluene EtOAc AcOH (88 10 2) R_f = 0 71 Petrol EtOAc, (1 1) Two developments	
Boronia inornata Triterpene	(15) Ahsan (1993)	1 Toluene EtOAc, AcOH (80 18 12) 2 Petrol EtOAc, 6 4 R_f = 0 20 Toluene EtOAc, AcOH (80 18 12)	
Boronia inornata Triterpene	(15) Ahsan (1993)	Silica gel Toluene EtOAc, (96 4) R_f = 0 49 Toluene EtOAc, (96 4)	
Castilleja rhexifolia Iridoid **Catalpol**	(20) Roby and Stermitz (1984)	Alumina *n*-BuOH H_2O MeOH (7 3 1)	

Species and Compound class	Reference	PTLC Method	Structure
Asterolasia trymalioides Coumarin	(21) Sarker *et al*, (1995)	Silica gel $CHCl_3$ Two developments	
Asterolasia drummondii Coumarin **Dipetalolactone**	(22) Sarker (1995)	Silica gel ($CHCl_3$ EtOAc, 9 1) Two developments	
Eriostemon myoporoides Coumarin	(23) Sarker (1995)	Silica gel Hexane EtOAc, (8 2) R_f = 0 31	
Drummondita hassellii Coumarin	(24) Rashid *et al*, (1992)	Silica gel Toluene EtOAc, (8 1)	
Cotoneaster acutifolius Dibenzofuran **δ-cotonefuran**	(25) Kokubun *et al*, (1995)	Silica gel (1) $CHCl_3$-Me_2CO (19 1) R_f = 0 57 (2) Toluene EtOAc, (2 1) R_f = 0 70	
Imperata cylindrica Lignan **Graminone B**	(26) Matsunaga *et al*, (1994)	Silica gel C_6H_6 EtOAc (1 1)	
Steganotaenia araliacea Bisbenzocyclo-octadiene lignan **Neoisostegane**	(27) Taafrout *et al*, (1984)	Silica gel 60 EtOAc Hexane (1 1) Three developments	
Drummondita hassellii Flavonoid	(24) Rashid *et al*, (1992)	Silica gel Toluene EtOAc (8 3)	
Boronia coerulescens ssp *spicata* Flavonoid	(28) Ahsan *et al*, (1993)	Silica gel Toluene EtOAc (8 2)	
Boronia inconspicua Flavonoid	(15) Ahsan (1993)	Silica gel $CHCl_3$ EtOAc, (95 5) Three developments	

Species and Compound class	Reference	PTLC Method	Structure
Seiridium sp Butenolide **7'-Hydroxyseiridin**	(29) Evidente and Sparapano (1994)	Silica gel (1) Petrol Acetone, (6 4) (2) $CHCl_3$ *iso*-Propanol (9 1)	
Homalium longifolium Glycoside	(30) Shaari *et al*, (1995)	Silica gel EtOAc MeOH (1 1)	
Eriostemon myoporoides Geranylated phenol	(22) Sarker (1995) and (23) Sarker *et al*., (1994)	Silica gel $CHCl_3$ EtOAc, (8 2) R_f = 0 30	
Asterolasia drummondii Quinoline alkaloid **Maculosidine**	(22) Sarker (1995)	Silica gel $CHCl_3$ EtOAc, (8 2)	
Eriostemon gardneri Quinoline alkaloid	(31) Sarker (1995)	Silica gel 1 Hexane EtOAc, (8 2) 2 $CHCl_3$ EtOAc, (8 2)	
Papaver somniferum Alkaloid **N-allylnorreticuline**	(32) Brochmann, Hannssen and Cheng (1984)	Silica gel $CHCl_3$ MeOH, 8 2	
Berberis sp Benzylisoquinoline alkaloid **Dihydrorugosinone**	(33) Valencia *et al*, (1984)	Silica gel 1 $CHCl_3$ MeOH NH_4OH (90 10 1) 2 C_6H_6 Me_2CO MeOH NH_4OH (45 45 10 1)	
Brunsvigia josephinae Alkaloid	(34) Viladomat *et al*, (1994)	Silica gel Pure MeOH	
Citrus decumana Acridone alkaloid	(35) Basa and Tripathy (1984)	Silica gel C_6H_6 EtOAc (19 1)	
Aconitum forrestii Diterpene alkaloid **Forestine**	(36) Pelletier *et al*, (1984)	Alumina, PF_{254} Thick Layer Chromatography (20cm x 20cm x 3mm) Me_2CO Hexane (45 55)	

Species and Compound class	Reference	PTLC Method	Structure
Thalictrum faberi Alkaloid **Thalifaberidine**	(37) Lin, *et al*, (1994)	Silica gel Cyclohexane EtOAc Diethylamine (6 4 1 to 4 8 1)	
Sternbergia lutea Alkaloid **Hippamine**	(38) Evidente, *et al*, (1984)	Silica gel $CHCl_3$ MeOH (9 1)	
Uvaria angolense Indole alkaloid **Uvarindole B**	(39) Muhammad and Waterman (1985)	Silica gel G $CHCl_3$ MeOH (49 1)	
Senna multiglandulosa Anthraquinone dimer	(40) Abegaz *et al*, (1994)	Silica gel $CHCl_3$ MeOH (100 2)	
Penicillium sclerotiorum Azaphilone	(41) Pairet *et al*, (1995)	Silica gel CH_2Cl_2 MeOH (19 1)	
Streptomyces griseoviridis Lactone **Cineromycin B**	(42) Burkhardt *et al*, (1996)	Analytical silica gel TLC $CHCl_3$ MeOH (9 1)	
Picea abies Flavonol glucoside	(43) Slimestad *et al*, (1994)	Silica gel *n*-BuOH AcOH H_2O (4 1 5) Upper layer	
Morus alba *nor*-tropane alkaloid **Calystegine C_1**	(44) Asano *et al*, (1994)	HPTLC Silica gel PrOH AcOH H_2O (4 1 1)	
Castanospermum australe Indolizidine alkaloid **Castanospermine**	(45) Molyneux *et al*, (1988)	CPTLC Silica gel 2mm plate 1 $CHCl_3$ MeOH NH_4OH H_2O (70 26 2 2) 2 EtOH NH_4OH (98 2)	

8.1. TLC Direct Bioautography

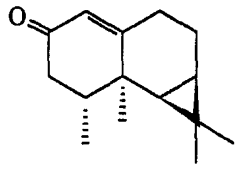
This technique may be utilized with either spore-forming fungi or bacteria and can be used to track activity through a separation process. It is a very sensitive assay and gives accurate localization of active compounds (Rahalison et al. *[54]*). For the assessment of antifungal activity, the plant pathogen *Cladosporium cucumerinum* (IMI-299104) can be used, as it is nonpathogenic to humans, readily forms spores, and can be easily grown on TLC plates with the correct medium. A simple method is outlined as follows:

1. Spot extracts or pure compounds onto analytical TLC plates in duplicate (plastic-backed, Kieselgel 60 PF254, Merck Art 5735), develop with the appropriate mobile phase, and dry
2. Prepare a slope of *Cladosporium cucumerinum* (IMI-299104) from a culture and allow to sporulate for 2 d.
3. Prepare a TLC growth medium as follows. NaCl (1 g), KH_2PO_4 (7 g), $Na_2HPO_4 \cdot 2H_2O$ (3 g), KNO_3 (4 g), $MgSO_4$ (1 g), and Tween-80 (20 drops added to water [100 mL]). To 60 mL of this solution should be added 10 mL of aqueous glucose (30% w/v)
4. Prepare a fungal spore suspension by adding the above solution to the fungal slope and shaking.
5. Spray the suspension onto one of the TLC plates and incubate at 25°C for 2 d in an assay tray with moist cotton wool to ensure a moist atmosphere. This spraying should be performed in a laminar flow cabinet.
6. Observe the inoculated TLC plate at regular intervals—the presence of antifungal compounds are indicated by inhibition or reduced lack of mycelial growth. This is frequently observed as light spots against a dark-green background
7. Visualize the remaining TLC plate using a spray reagent and/or UV detection and compare with the incubated plate

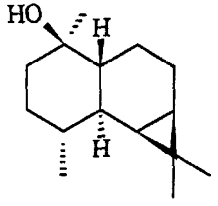
Aspergillus niger, another readily sporulating fungus, may be used in the place of *Cladosporium* sp., but care must be taken with this organism because of risk of aspergillosis—all microbes should be handled aseptically in a laminar flow cabinet. Controls of antifungal compounds such as amphotericin B should be used each time this assay is performed. This assay does not distinguish between fungicidal and fungistatic metabolites, and further assays, such as a liquid broth assay, will be needed to measure Minimum Inhibitory Concentration (MIC) *(47)*.

Dellar et al. *(55)* isolated the antifungal sesquiterpenes aristolen-2-one (**Compound 9**) and prostantherol (**Compound 10**) from two species of *Prostanthera* (Labiatae). Activity was assessed and tracked through the separation procedure by the use of direct bioautography with *Cladosporium cucumerinum* as the target fungus.

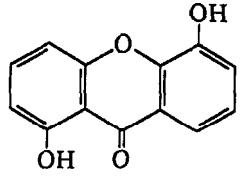
Compound 9 inhibited the growth of *C. cucumerinum* for 70 h at a dose of 1 μg and **Compound 10** caused inhibition at 10 μg for the same duration.



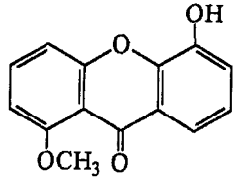
Compound 9.



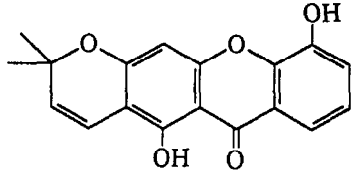
Compound 10.



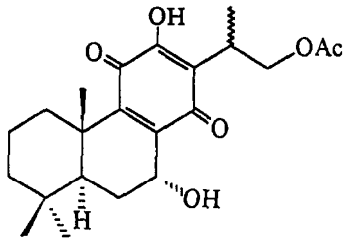
Compound 11. (Dose = 0.25 μg).



Compound 12. (Dose = 3 μg).



Compound 13. (Dose = 3 μg).



Compound 14

The antifungal activity of many natural products, including plant phenolic compounds, can be readily assessed using this simple procedure. Hostettmann and Marston *(56)* have investigated a series of xanthones (**Compounds 11–13**) from *Hypericum brasiliense* (Guttiferae) for activity against *C. cucumerinum*. One of these compounds, **Compound 11**, exhibited a very low inhibitory dose (0.25 μg), which may warrant further investigation of the antimycotic function of these interesting compounds.

8.2. TLC Bioautographic Overlay Assay

In this form of assay, the extract or pure compound is run on a TLC plate, which is then covered by a medium seeded with the appropriate microorganism. As with direct bioautographic assays, both fungi and bacteria may be investigated. Rahalison et al. *(54)* have applied this technique for the evaluation of antimicrobial extracts against the yeast *Candida albicans* and the bacterium *Bacillus subtilis*.

A simple overlay assay against *Staphylococcus aureus* (Manohar *[57]*) may be carried out as follows:

1. A base of nutrient agar is poured into an assay dish and allowed to set.
2. The extract, fraction, or pure compound, is run on a TLC plate (in duplicate) with the appropriate developing solvent. One of these plates should be visualized under UV light and then stained to observe developed compounds. R_f values are then accurately measured.
3. An innoculum of *S aureus* at a titer of 10^9 colony-forming units (CFU)/mL in Mueller Hinton Broth is prepared, and nutrient agar is added at 7.5 g/L to thicken the medium. This is then diluted to give a final titer of 10^5 CFU/mL
4. The remaining TLC plate is placed on the nutrient agar base and then the medium containing the test organism is poured over the plate and incubated at 37°C for 24 h
5. Antibacterial zones appear as clear spots against a background of bacterial colonies Zones may be visualized more clearly by the use of tetrazolium salts (such as *p*-iodonitrotetrazolium chloride [INT] or methylthiazoyltetrazolium chloride [MMT]), which indicate bacterial dehydrogenase activity. These solutions may be sprayed onto the face of the medium. Zones of inhibition (and therefore antimicrobial compounds) appear as clear zones against a purple background.

6. Zones of inhibition can be compared with the previously developed TLC plate, so that active metabolites may be readily isolated.
7. An appropriate control substance such as ampicillin or chloramphenicol should be used.

Drug-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus,* will need to be cultured in the presence of methicillin (1 mg/L) to minimize the risk of loss of resistance.

Batista et al. ***(58)*** used an overlay method in the bioassay-guided fractionation of an acetone extract of the roots of *Plectranthus hereroensis* (Labiatae) to isolate the antibacterial diterpene (**Compound 14**). *Staphylococcus aureus* was used as the test organism.

This compound was then assessed in a broth-dilution assay and found to have an MIC of 31.2 μg/mL.

Hamburger and Cordell ***(59)*** have used a variant of this assay to investigate the activity of some simple plant sterols and phenolic compounds. An overlay of nutrient broth containing the test organism was spread over the TLC plate and then incubated. Interestingly, this assay was insensitive to some cytotoxic compounds, including camptothecin, glaucarubolone, and β-peltatin, when tested at 5 μg. This might be because of poor migration of these compounds through the nutrient medium, and it should be noted that this phenomenon could be a problem with this method.

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