Chapter 6

An Introduction to Planar Chromatography and Its Application to Natural Products Isolation

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Abstract

Thin-layer chromatography (TLC) is an easy, inexpensive, rapid, and the most widely used method for the analysis and isolation of small organic natural and synthetic products. It also has use in the biological evaluation of organic compounds, particularly in the areas of antimicrobial and antioxidant metabolites and for the evaluation of acetylcholinesterase inhibitors which have utility in the treatment of Alzheimer's disease. The ease and inexpensiveness of use of this technique, coupled with the ability to rapidly develop separation and bioassay protocols will ensure that TLC will be used for some considerable time alongside conventional instrumental methods. This chapter deals with the basic principles of TLC and describes methods for the analysis and isolation of natural products. Examples of methods for isolation of several classes of natural product are detailed and protocols for TLC bioassays are given.

Key words: Thin-layer chromatography, TLC, HPTLC, Bioassays, Natural product isolation

1. Introduction

Planar chromatography utilizes the separation of mixtures of organic compounds on thin-layers of adsorbents which are in most cases coated on glass, plastic, or aluminum sheets. The most widely used form of planar chromatography is thin-layer chromatography (TLC) which is the easiest and cheapest technique 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. This is the essence of separation by TLC as the thin-layer in this case is the blotting paper on which the separation occurs.

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This chapter describes the principles behind this simple technique and gives procedures for analyzing extracts and isolating natural products so that anyone may use TLC to isolate and analyze natural products and synthetic compounds from any source. Examples of TLC isolations of metabolites from plants are given and the methodology behind the isolation of unknown products is detailed. Success in TLC relies on flexibility of approach and experimenting with a variety of methods. There is no perfect separation method for a mixture but logical and sequential experimentation will give rise to a tailored and useful protocol. It is important to try several methods before an ideal method is found, and for those who are beginning their research, some patience will give excellent results!

Natural product extracts are in most cases highly complex and comprise mixtures of neutral, acidic, basic, lipophilic, hydrophilic, and amphiphilic (e.g., amino acids) compounds and consequently there will not be one method that can be used for all eventualities. It is 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 1D NMR experiments are quicker to run than the extensive development of mobile and stationary phases that may be needed. The starting point should always be the simplest method first and examples for the isolation of a number of different classes of natural products are given in Subheading 3 taken from the literature and the author's own research.

The ease of use, the speed and low cost of TLC make it a widely used and versatile technique which can be readily learnt. While HPLC and its ultra-pressure variants (UPLC) are very popular as the methods of choice for a final "clean up" to afford a purified natural product, inspection of papers from journals *Phytochemistry Letters*, *Phytochemical Analysis*, *Phytotherapy Research*, *Planta Medica*, *Phytochemistry*, and *Journal of Natural Products* in the year 2010 still shows that TLC has a central place in natural product isolation and analysis. TLC is also easily interfaced with bioassays and it is likely that many new enzyme inhibition assays that use a colorimetric change on a TLC plate will be developed as a first simple test for biological activity. The author encourages anyone making their first foray into natural product research to experiment with the plethora of sorbents and solvents that TLC has to offer because slight changes in a method can dramatically effect a separation.

1.1. Basic Principles of TLC

Separation by TLC is effected by the application of a mixture or an extract as a spot or thin line on to a sorbent which has been applied to a backing plate. Analytical TLC plates (thickness 0.1–0.2 mm) are commercially available from suppliers, such as Merck (e.g., the commonest analytical silica gel plate is the 20 × 20 cm, plastic or

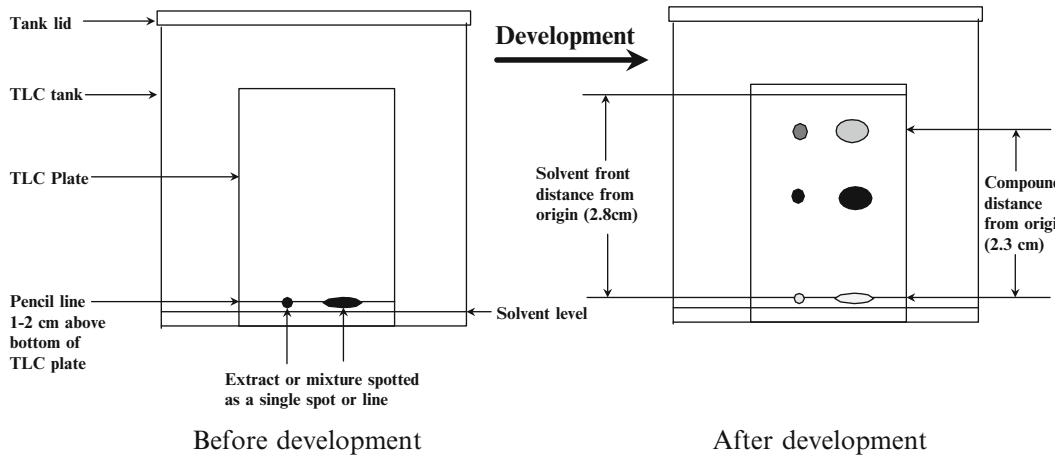


Fig. 1. TLC equipment and development procedure.

aluminum-backed Kieselgel 60 F_{254} plate which has a 0.2 mm thickness of silica sorbent; Merck No. 5554). The plate is then placed into a glass developing tank with sufficient suitable solvent just to wet the lower edge of the plate/sorbent but not sufficient to wet the part of the plate where the spots were applied (the origin). The solvent front then migrates up the plate through the sorbent due to capillary action and this process is known as development (Fig. 1).

A factor in quantifying the movement (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 above example,

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

$$R_f = 0.82$$

R_f values are always ratios, are never greater than one, 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 = $R_f \times 100$ (in our case hR_f = 82). In the case of adsorption chromatography (see below), where the sorbent is silica, polar compounds (e.g., artemisinin; Fig. 2) will have a higher affinity for the sorbent (stationary phase), and will "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. Nonpolar compounds (e.g., psilocybin; Fig. 2) will have less affinity for the

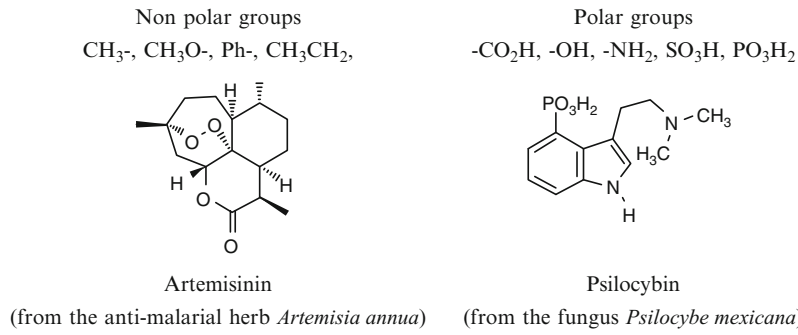


Fig. 2. Polar and non-polar groups in artemisin and psilocybin.

Table 1
Compounds denoted by an asterisk were recorded at 25°C (2)

Solvent	Dielectric constant (20°C)	Solvent	Dielectric constant (20°C)
Pentane	1.8	Ethyl acetate*	6.0
Hexane	1.9	Acetic acid	6.2
Cyclohexane	2.0	Dichloromethane	9.1
Benzene*	2.3	Pyridine	12.3
Toluene	2.4	Acetone*	20.7
Diethyl ether	4.3	Methanol	32.6
Dimethyl sulfoxide	4.7	Acetonitrile	37.5
Chloroform	4.8	Water	78.5

stationary phase, will move comparatively quickly up the plate, and will 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.

Artemisinin is a relatively nonpolar compound when compared with psilocybin (Fig. 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 generally dielectric constants are of use to quantify their 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 mechanisms of chromatography by which separation can occur and more than one mechanism may be responsible during a given separation.

(a) *Adsorption chromatography*. The most commonly used sorbents that are utilized in this form of chromatography are silica and alumina. As the components move through the sorbent, their relative rates of migration are effected 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 when compared to nonpolar natural products. Adsorption takes place due to 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.

(b) *Partition chromatography*. This mechanism involves differences between the relative solubility of the compound between the sorbent (stationary phase) and the solvent (mobile phase). Compounds which are more soluble in the mobile phase will migrate up the plate to a greater extent than components which are more soluble in the stationary phase. Reverse-phase TLC utilizes sorbents which partition natural products between the mobile and stationary phases. These are normally fatty (lipid) stationary phases and aqueous/organic solvent mobile phases. The most commonly used reverse phase sorbent is silica which has been reacted with a straight chain 18 carbon alkyl unit to form an octadecasilyl (ODS) phase and many alternative lipophilic phases are commercially available (Fig. 4). Nonpolar "fatty" compounds, such as the sesquiterpene artemisinin (Fig. 2), 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 having different rates of partition between the stationary phase and mobile phase.

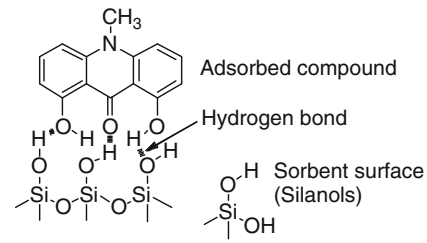


Fig. 3. Adsorption and hydrogen bonding between compound and sorbent face.

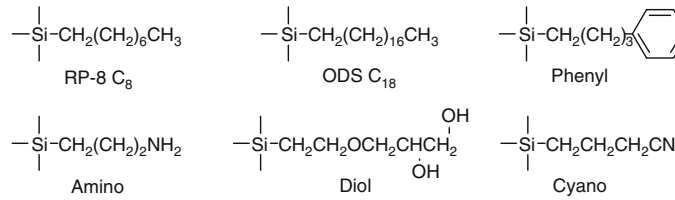


Fig. 4. Common reverse phases for partition chromatography.

(c) *Size inclusion/exclusion chromatography*. Compounds may be separated by their relative sizes and by their inclusion (or exclusion) into a 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, carotenes, fatty acids, and glycerides. 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 (stuck) into the gel matrix and larger molecules are excluded and migrate at a faster rate. It should be noted that separations on gels, such as Sephadex LH-20, also involve, in part, 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 because of these mechanisms. 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 natural products. These large pigments elute quickly through the gel and are easily removed from smaller metabolites.

(d) *Ion exchange chromatography*. This technique is limited to mixtures that contain components which have the ability to carry an electronic charge. In this form of chromatography, the sorbent is usually a polymeric resin which contains polar charged groups and mobile counter ions which may exchange with the ions of a component as the mobile phase migrates through the sorbent. Separation is achieved by differences in affinity between ionic components in the mixture 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 in mixtures 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 present in mixtures (also see Chapter 8).

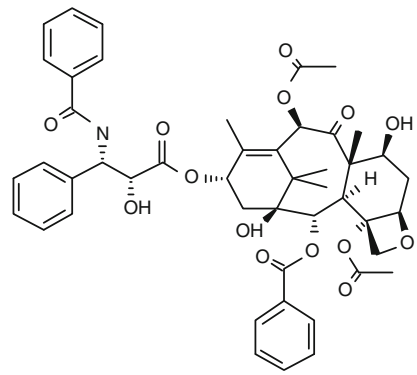
1.3. Applications of TLC

Traditionally, analytical TLC has found application in the detection and monitoring of compounds through a separation process or in the synthesis of a compound. 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 of a metabolite. An example of this is the production of the antitumor diterpene Taxol (Fig. 5) 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 four solvent systems gave a higher degree of confidence of the fungal taxol being authentic, although the authors did confirm this by mass spectrometry. 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 the 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 bacterial cell wall and running a TLC of the hydrolysate against the two standards it is possible to loosely classify the actinomycetes (4).

Natural products may also be "tracked" by running analytical TLC after other separation processes, such as column chromatography or HPLC, and always more than one solvent system should 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 identical or similar TLC chromatograms. Qualitative initial screening of extracts by TLC 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



Solvent systems
(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. Structure of taxol and solvent systems.

by spraying developed plates with stains that give a color reaction with a particular compound class (see Subheading 3.3.2).

Many natural products are still isolated by conventional preparative TLC (PTLC) and many examples can still be found in the journals *Phytochemistry*, *Journal of Natural Products*, *Planta Medica*, *Phytochemical Analysis*, and *Phytochemistry Letters*. Although preparative HPLC and UPLC are currently in "vogue" and are routinely used in many laboratories, 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–1 g range.

2. Materials

Precoated analytical TLC plates are available with various sorbets, including silica, alumina, C_{18}, cellulose, and others, where usually the thickness is 0.2 mm. Precoated PTLC plates with various sorbents also include the range of analytical plates produced by Merck, e.g., Alumina; 0.2 mm thick; 20 × 20 cm; with a 254 nm UV indicator (Merck No. 5550). Commercially available PTLC plates are usually limited to the sorbents silica, alumina, C_{18}, and cellulose and are usually of 0.5, 1.0, and 2.0 mm thicknesses. Glass-backed silica gel 60 plates from Merck have a particle size distribution of 5–40 μm when compared to the corresponding analytical plate of 5–20 μm.

To prepare five preparative Silica plates manually, the following items are needed: five glass backing plates 20 × 20 cm, adjustable gate applicator, two 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), and a 200 mL conical flask with stopper (see Subheading 3.4.4 for procedure).

TLC tank (made of glass) of appropriate size (smaller tank for analytical TLC, and bigger for PTLC) are needed. UV lamps are widely commercially available from suppliers, such as CAMAG (Camag Ref. 022.9230). Spray reagents (Table 2) and developing solvents or mobile phases (Table 3) are required. Hand held heater (hair drier!) to dry TLC plates, and oven to heat up TLC plates after spraying with some reagents, e.g., vanillin–sulfuric acid.

For CPTLC, the plate and motor are housed in an apparatus where a nitrogen atmosphere can be applied. An excellent 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.

Special devices and apparatus that are necessary for specialized forms of planar chromatography, e.g., HPTLC, have been mentioned within individual methods in Subheading 3.

Table 2
Ten simple spray reagents for natural products TLC visualization

Detection spray	Recipe	Treatment	Notes
Vanillin/sulfuric acid	Dissolve vanillin (4 g) in concentrated H_2SO_4 (100 mL)	Spray onto plate and 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 PMA spray. Spray and heat in a fume cupboard
Phosphomolybdic acid (PMA)	Dissolve PMA in ethanol to make a 5% w/v solution	Spray onto plate and 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
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
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
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
Dragendorff's reagent	Add 10 mL of a 40% aqueous solution of KI to 10 mL of a 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	This is the traditional method for alkaloid detection although care should be taken as some nonalkaloids, such as iridoids and some flavonoids, give a positive reaction. Alkaloids give a dark orange to red coloration.
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
Perchloric acid	A 20% (w/v) aqueous perchloric acid solution	Heat at 100°C until coloration	A universal spray but is useful for steroids and triterpenes
Borntrager reagent	A 10% (w/v) ethanolic solution of KOH	Heat until color detection	For the detection of coumarins and anthraquinones
Ninhydrin	Add ninhydrin (0.3 g) to a mixture of butanol (100 mL) and acetic acid (3 mL)	Heat at 100°C until coloration	Especially useful for amino acids, amines, and as a general alkaloid spray. Alkaloids appear as a red coloration

Table 3
Simple systems for TLC

Solvent system	Sorbent	Notes
Hexane:ethyl acetate (EtOAc)	Silica gel	Universal system—can substitute hexane for light 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 in air
Petrol:chloroform ($CHCl_3$)	Silica gel	Considerably useful for the separation of cinnamic acid derivatives, in particular the coumarins
Toluene:EtOAc:acetic acid (TEA)	Silica gel	Vary the composition, i.e., 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 flavonoids and glycosides
Butanol:water:pyridine:toluene	Silica gel	Sugar analysis system. Try 10:6:6:1. Development may take 4 h
Methanol (MeOH):water	C_{18}	Start with 100% MeOH to determine if metabolites will move from the origin. Increase the water concentration to "slow" products down. The addition of small amounts of acid or base may improve chromatography
Acetonitrile:water	C_{18}/C_2	A universal simple reverse-phase system
MeOH:water	Polyamide	Universal
MeOH:water	Cellulose	Used for the separation of highly polar compounds, such as sugars and glycosides

3. Methods

3.1. System Selection

As much information as possible regarding the extract producing organism should be gathered as this will aid in the selection of a separation system. After a full literature search on the organism has been performed, the following points need to be addressed.

(a) Has the species been studied before?

(b) If so, what metabolites were isolated?

(c) Are there standard TLC methods available?

(d) If the species has not been study for chemistry, is there any information at the generic level?

Chemotaxonomy or the classification of an organism according to its natural products may assist in dealing with unknown genera (5), and it is quite likely that species which are related to each other may produce related secondary metabolites. Databases, such as Chemical Abstracts, NAPRALERT, Berdy, The Dictionary of Natural Products, and in the case of plants, certain classical texts, such as Hegnauers' Die 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 the ^{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 that are present by this technique and a TLC method can be "tailored" accordingly. TLC on silica gel is still the most common planar chromatography method although it suffers from some drawbacks that may be easily overcome.

(a) *Acidic compounds* can "tail" and "streak" with nonband flow on silica due to 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 and this will maintain any acidic groups in a nonionized form.

(b) *Basic compounds* also may behave poorly on silica and the addition of weak bases (e.g., 1% diethylamine or triethylamine) should also eradicate any tailing and improve chromatography.

(c) *Highly nonpolar compounds*, such as fatty acids, glycerides, alkanes, and some lower (smaller) terpenoids such as mono- and sesquiterpenes require simple nonpolar solvents systems (e.g., cyclohexane, hexane, pentane, diethyl ether:hexane mixtures) and may be difficult to detect by UV light (as they have no chromophore) or by spray detection (use charring reagents, e.g., vanillin–sulfuric acid; Table 2).

(d) *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 the silica. Choice of mobile phase should evolve through the use of a mono or binary system, i.e., 100% $CHCl_3$ or hexane:EtOAc (1:1) as a starting point and if this does not give a good result then the addition of acids or bases to improve chromatography, i.e., toluene:EtOAc:acetic acid (60:38:2) should be tried and as a last resort the use of

tertiary or quaternary systems, e.g., butanol:acetic acid:water (4:1:5) or hexane:EtOAc:formic acid:water (4:4:1:1) should be employed.

3.2. Choice of Development

The decision as to whether the system is run isocratically (using one solvent system of constant composition) or using a step gradient (develop once in a nonpolar solvent and then systematically increasing the solvent polarity after each development) can be made by running a series of analytical plates with the sample at varying mobile phase strengths. Both systems have their merits and can be used with multiple development where the plate is developed, dried, and developed a number of times. This is especially useful in the separation of closely eluting compounds. Table 3 lists some of the more commonly used systems.

3.3. Detection of Natural Products in TLC

At both the analytical and preparative stages of TLC, effective visualization or detection is crucial to obtain pure compounds and poor detection will also result in a low recovery of product from the sorbent. Detection is usually either nondestructive, where the compounds may be recovered from the sorbent [Ultraviolet (UV) detection) or destructive, where the compounds are contaminated by the detection reagent and are unrecoverable from the sorbent (Spray detection). There are some excellent texts available on this subject, such as Wagner and Bladt's Plant Drug Analysis (8) or The Merck Handbook of Dyeing Reagents for Thin Layer and Paper Chromatography (9), and these cover most eventualities.

3.3.1. Ultraviolet Detection

UV detection involves the use of UV active compounds (indicators) which 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 wave 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 florescence under UV light. 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 however, nondestructive and the detection of compounds can be observed readily through a separation process. 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 on skin as UV light is mutagenic.

3.3.2. 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 commonly used spray reagents are listed in Table 2. Most are universal reagents and will react with many classes of natural products and the most widely used sprays are vanillin–sulfuric acid, phosphomolybdic acid and ammonium molybdate (VI) sprays (Table 2). Dragendorff's reagent is especially useful for the detection of many classes of alkaloids and is well worth the effort required to make. 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 drier!) or a drying oven. All of the compounds required to make the spray reagents are readily available from suppliers, such as Sigma–Aldrich. Each of the spray reagents should be made up freshly and used in a fume cupboard.

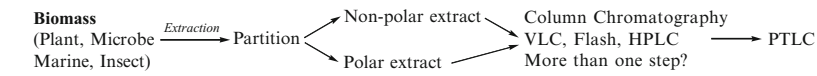
When using spray detection in preparative TLC (see Subheading 3.4), most of the plate should be covered (by tissue paper) and only a small proportion of the edge (2 cm) sprayed with reagent. Ideally, a scalpel should be used to score a line 2 cm in from the plate edge so that after spraying, any corrosive spray reagent does not migrate into the sorbent and damage compounds.

3.4. Preparative Thin-Layer Chromatography

PTLC has long been a popular method of isolation primarily because of its universal accessibility to students and researchers working in natural products chemistry. This popularity has been diminished in recent years due the success of high-pressure liquid chromatography and counter current chromatography. Unlike these two techniques, PTLC does not however, 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 the emphasis on preparing and running plates and some of the advantages and disadvantages encountered with PTLC.

3.4.1. 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 prep plate will ultimately depend on how those compounds behave on a particular system but as a rule the separation of no more than a mixture of three major components should be attempted. The separation of complex mixtures can be carried out on PTLC 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 should, in the first instance, be separated via vacuum liquid

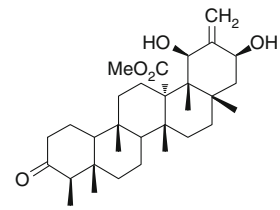


Fig. 6. Structure of friedelane triterpene.

chromatography (VLC), flash chromatography, or conventional column chromatography prior to PTLC. To give an example of how these different separation processes have been used, the friedelane triterpene (Fig. 6) was isolated from the bark of the Camerounian rain forest tree *Phyllobotryon spathulatum* (10) using the following separation process.

1. Extract ground bark of *P. spathulatum* with petrol and $CHCl_3$.
2. Subject the extracts to VLC on Silica gel eluting with hexane–EtOAc mixture.
3. Perform PTLC (mobile phase: toluene:EtOAc:AcOH = 80:18:2) on the fraction eluted with 25% EtOAc in hexane to isolate the friedelane triterpene (Fig. 6).

This triterpene needs only one purification step prior to PTLC and because of its lack of distinctive chromophore, the compound requires visualization by spray detection (vanillin reagent, Table 2).

3.4.2. Scale Up from Analytical to PTLC

The scale up procedure from analytical (0.1–0.2 mm sorbent thickness) to prep TLC (0.5–4 mm sorbent thickness) is of paramount importance, as changing the scale (size) of a separation can drastically effect the chromatography of natural products. The chromatography of a compound separated on analytical plates, where microgram to low milligram levels of material are involved can alter significantly when 10s of milligrams are separated. On normal-phase silica, when moving from analytical to prep scale it is required to reduce the polarity of the solvent markedly. 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 prep plate to give comparable R_f values. This is a general rule and will vary according to the compound class being separated and the types of stationary phases used—the best method being to sacrifice a small portion of the mixture and to experiment.

3.4.3. Commercially Available PTLC Plates

These plates are usually limited to the sorbents silica, alumina, C_{18}, and cellulose and are usually of 0.5, 1.0, and 2.0 mm thicknesses. Glass-backed silica gel 60 plates from Merck have a particle size distribution of 5–40 μm when compared to the corresponding analytical

plate of 5–20 μm. These silica plates having a high specific surface area are homogeneous and can give excellent separation results.

The use of commercially available prep plates with a concentration zone also enhances 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 greatly improves separation.

Normal-phase plates, such as the 2 mm Merck Kieselgel 60 F_{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 that should be stored in a desiccator as moisture from the air will effect the "activity" of the sorbent (especially in the case of silica and alumina) resulting in reduced resolution and poorer separation.

3.4.4. Home-Made Preparative Plates

Making your own plates allows for greater flexibility of the choice of sorbent, whereas commercial plates are restricted to 3 or 4 sorbents, with the correct recipe home-made 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 sulfate (gypsum), are required 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 into the sorbent that enhance separation. An example of this (recipe shown below) is the addition of a small quantity of silver nitrate to a silica sorbent that will aid in the resolution of olefinic compounds.

If cost is an issue, then making plates is also 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 prep plates of 0.5 mm thickness with optional silver nitrate additive. For plates of 1 or 2 mm thickness, 2 or 4 times the amount of water and sorbent are required and all of the equipment is readily available from suppliers, such as CAMAG or Merck. The equipment and materials required for preparing PTLC plate are discussed in Subheading 2. The procedure of PTLC (0.5 mm thickness) preparation can be summarized as follows:

1. Clean the glass backing plates with 1 N aqueous KOH and then acetone and finally dry prior to spreading.
2. Place the plates into the TLC plate coater with spacer plates at either end.

3. Adjust the applicator to the correct plate size and place at one end on the spacer plate.
4. Put the silica (45 g) or alternative sorbent and the silver nitrate (if required) into the conical flask (250 mL) and add water (90 mL).
5. Shake the stoppered conical flask vigorously for 30 s to ensure that a homogeneous slurry is produced.
6. 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.
7. Leave the plates to air dry 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 although 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.

3.4.5. Sample Application

Prep 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 a minimum volume of solvent as possible (usually in the concentration range 10–20 mg/mL). This sample is then applied to the bottom of the plate (1.5 cm from the bottom) as a thin line (2–4 mm thick) using either a capillary, or a Pasteur pipette that has been extruded in to a thin applicator by heating over a Bunsen burner. Thinner capillaries (5–10 mL) give greater control over sample application and result in a finer, more concentrated line and therefore better results. In order to apply the sample in a straight and uniform fashion, it is preferable to lightly draw a pencil line (without scoring the sorbent!) approximately 1.5 cm above the plate edge or alternatively using a piece of A4 paper placed on the plate as a guide. Application of the sample as a straight line is necessary as this forms the origin and assuming that the plate is homogeneous, during development, the sample will separate into compounds with even bands. If the sample is applied in an irregular fashion (i.e., a wavy line), then during development the sample will separate into compounds with irregular bands that are difficult to remove in a pure form from the plate during the desorption process.

The sample should also be not applied right up to both edges of the plate as edging effects (the rapid movement of solvent up the plate sides or poor sorbent homogeneity) will result in the uneven movement of solvent up the plate during development and as a consequence irregular band shape.

3.4.6. Development and Detection

A suitable solvent system and sorbent phase should be chosen from experimentation with analytical plates and some of the simpler cases are given in Subheading 3.1 and in the examples

(Subheading 3.10). Mobile phases for prep systems should be made up freshly 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 produced by adding some clean filter paper (of 15 × 15 cm) in the developing tank.

Silica gel is quite a "reactive" sorbent and some natural products are unstable on this phase. Care should also be taken that during development, plates should be kept out of direct sunlight as light-initiated degradation can also occur with some natural products.

This development is achieved by allowing the solvent front to reach the plate top or to be within a few millimeters of it and then removing the plate from the tank and air drying in a fume cupboard. Hand-held dryers should be avoided to remove excess solvent from plates due to the risk of heat-assisted degradation.

Most semipurified samples have some residual color or the natural products of interest may be colored and so it might be possible to gauge how far the compounds have migrated up the plate. In the case of plant extracts, nonpolar pigments, such as the chlorophylls (green) or the carotenes (orange to red) 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 UV light, then the success of the separation can be readily observed under UV light—this is especially useful in multiple developments and rewarding to see compounds resolved through the isolation process! With poor UV absorbing products, use of a spray reagent is required (see Subheading 3.3.2) 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 cut away to avoid contamination before further redevelopment.

3.4.7. 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 scrapped 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 scrapped from the plate onto foil. Compounds may be desorbed from the sorbent in three simple ways.

(a) The compound rich sorbent can be put in a conical flask and solvent then added. The suspension should be left for 30 min

to facilitate the leaching of compound into the solvent and then filtered. This process should be repeated two or three times to ensure good recovery of the natural product. The type of solvent used should be slightly more polar than is normally required to dissolve the sample and as an example if the sample dissolves readily in chloroform then desorption should be carried out using $CHCl_3$:MeOH (9:1) or (8:2). This should ensure maximum recovery from the sorbent and minimize the possibility of a product being strongly bound to the solid phase.

(b) The compound rich sorbent should be put into 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 recover compounds effectively and this is the method of choice for recovery from prep TLC plates.

(c) A Pasteur pipette blocked with a small amount of defatted cotton wool or a microcolumn with a 3 porosity frit can be packed with compound rich sorbent. These "mini" columns can then be eluted with solvent to recover compounds. 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 to ensure full recovery of natural product.

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 due to the risk of heat decomposition and oxidation. Where residual amounts of solvent are trapped as a film in the dried natural product, the use of a drying pistol, vacuum desiccator or a freeze drier will ensure that the product is sufficiently dry for spectroscopic analysis (see Note 1).

3.4.8. Assessing Purity by TLC

After desorption, analytical TLC should be performed on recovered products to ascertain 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 (see Note 2).

3.4.9. Advantages and Disadvantages of PTLC

In the last 20 years, there has been a considerable movement away from "wet" techniques, such as PTLC and conventional column chromatography, toward instrumental techniques such as HPLC, and counter current chromatography. Many natural products chemists prefer these instrumental methods because of the greater control over a separation process and reproducibility they afford although the high cost and need for routine servicing of these machines will ensure that a significant role for PTLC in an isolation procedure will be maintained for some time to come. The advantages of PTLC are outlined below.

(a) PTLC is cost-effective compared to the instrumental methods, for example HPLC or CCC.

(b) A simple technique that requires little training or knowledge of chromatography to use

(c) An analytical method may be easily scaled up to a preparative method

(d) Ability to isolate natural products quickly in the milligram to gram range

(e) Flexibility of solvent and stationary phase choice, i.e., the solvent system can be changed quickly during a run

(f) The separation can be optimized readily for one component, i.e., it is relatively easy to "zero in" on a particular product of interest

(g) Methods are quickly developed

(h) Almost any separation can be achieved with the correct stationary phase and mobile phase

(i) A large number of samples can be analyzed or separated simultaneously

The disadvantages of PTLC are as follows.

(a) Poor control of detection when compared to HPLC

(b) Poor control of elution compared to HPLC

(c) Loading and speed are poor compared to vacuum-liquid chromatography

(d) Multiple development methods to isolate grams of material may be time consuming

(e) Commercially available plates are restricted to simple sorbents, such as silica, alumina, cellulose, and RP-2

3.5. Centrifugal Preparative Thin-Layer Chromatography

This excellent and under exploited technique can be utilized as a primary "clean up" process of natural products extracts or as a final purification step. Centrifugal preparative thin-layer chromatography (CPTLC) makes use of a glass rotor which is coated with a sorbent to form a circular plate which is then attached to a spindle

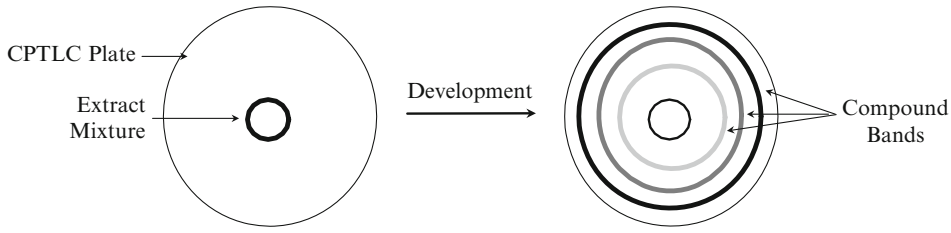


Fig. 7. Circular band separation through CPTLC development.

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 collected readily (Fig. 7).

The plate and motor are housed in an apparatus, where a nitrogen atmosphere can be applied. An excellent 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, firstly, because development is centrifugally accelerated by plate rotation, separation is very rapid. Secondly, 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 on to the plate in one run which is not always possible with PTLC. As with home-made prep plates, CPTLC plates allow the choice of sorbent, additives, and binders which can be tailored for a particular class of compound.

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.

1. Add silica (Kieselgel 60 PF_{254} Merck 7749) (65 or 100 g; for 2 or 4 mm thickness) and binder ($CaSO_4$; 4 or 6 g) to distilled water (100 or 190 mL) and shake thoroughly.
2. Pour the resulting slurry onto the rotor at the edge and the plate tapped gently to remove air bubbles and to ensure a homogeneous layer.
3. Air-dry the plate for 30 min and oven dry at 50°C for 12 h. The resulting plate should then be scrapped 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

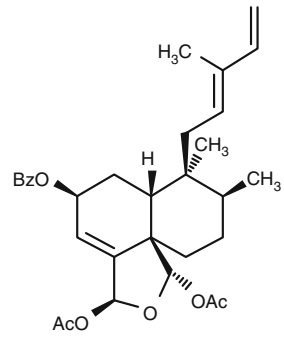
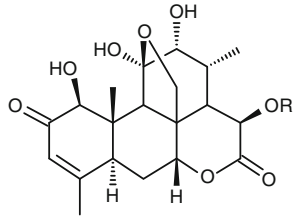
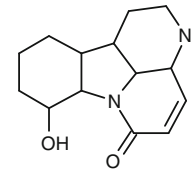


Fig. 8. Clerodane diterpene.



Ailanthinone R = $COCH(Me)CH_2CH_3$
2'-Acetylglaucarubinone R = $COC(OAc)(Me)CH_2CH_3$
(Silica gel; CH_2Cl_2:isopropanol (49:1))



8-Hydroxycanthin-6-one
[Silica gel; toluene:EtOAc:AcOH (5:4:1)]

Fig. 9. CPTLC to isolate the cytotoxic quassinoids ailanthinone, 2′-acetylglaucarubinone and the alkaloid 8-hydroxycanthin-6-one from the rain forest tree *Odyendyea gabonensis* (Simaroubaceae).

determine the R_f values. When using an isocratic system, a solvent system where the R_f of the least polar compound is 0.3 can be used. 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 and 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 and coworkers (11) used a Chromatotron CPTLC apparatus in the separation of some unusual clerodane diterpenes (Fig. 8) in a chemotaxonomic study of the flacourtiaceae species *Zuelania guidonia*. Silica gel sorbent and a mobile phase of petrol-EtOAc (49:1) were used and the compound was visualized under UV light.

Waterman and Ampofo (1984) (12) used CPTLC to isolate the cytotoxic quassinoids ailanthinone, 2′-acetylglaucarubinone and the alkaloid 8-hydroxycanthin-6-one from the rain forest tree *Odyendyea gabonensis* (Simaroubaceae) (Fig. 9).

3.6. Over Pressure Thin-Layer Chromatography

Over Pressure Thin-Layer Chromatography (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-micro chamber (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 for HPLC and consequently the technique can be used to model CC methods. Separations can be carried out 5–20 times faster than conventional TLC and so this method may be applicable to a high numbers of mixtures. 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 annuum* and furocoumarins from *Heracleum sphondylium* have also been separated by Nyirdey et al. (14).

3.7. Automated Multiple Development

This method utilizes a fully automated developing chamber which 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 developments dramatically increases separation power, improves reproducibility and precision and can be run without 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 automated multiple development (AMD) device is the CAMAG AMD system (www.camag.com).

3.8. 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, then this is a 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. 10). 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.

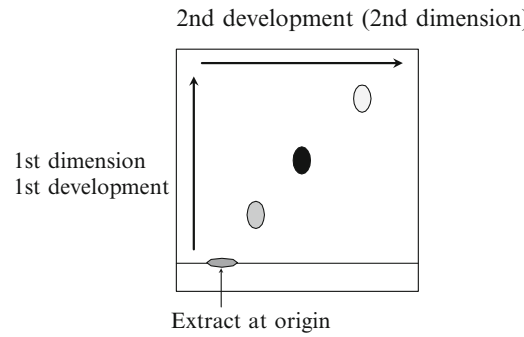


Fig. 10. Two-dimensional TLC plate after two developments.

This technique can be used to detect the presence of a variety of plant metabolites and to help in the taxonomy of plants or to assess their quality by the presence or absence of certain phytochemicals. This method has been used to analyze the root extract of American ginseng (*Panax quinquefolius*) (15), which is rich in triterpene glycosides, known as the ginsenosides. The method employed two solvent systems, the first of which contained $CHCl_3$–MeOH–water (13:7:2) in the first direction followed by the second solvent system which was water–*n*-butanol–EtOAc (5:4:1) in the second direction. This shows the power of 2-dimensional TLC as there are many metabolites present in ginseng and their components are polar and chemically complex and can be resolved using this technique.

An excellent review by Cieśla and Waksmundzka-Hajnos (16) on this subject describes examples from the analysis of coumarins, phenolic acids, flavonoids, alkaloids, triterpenes, and anthraquinones.

3.9. High-Performance TLC

This technique uses plates which have a finer adsorbent particle size and typically analytical plates may have an average size of 5 μm compared to 15 μm for routine plates. This results in a higher resolution of bands, speed and efficiency. High-performance TLC (HPTLC) is often an automated process using instrumentation to facilitate multiple developments and detection using densitometry and imaging to capture an electronic image of the separated bands. This technique has wide applicability in the analysis of natural product mixtures and the improved isolation of compounds.

HPTLC can be used to identify plants which are used as traditional medicines, particularly complex mixtures resulting from the Chinese herbal drugs. This technique can also be used to assess the quality of a herbal drug and to gauge if the sample has been changed or adulterated (addition of a foreign chemical) in any way. HPTLC has also been used to detect poisonous natural products, such as gliotoxin, which is a powerful immune-suppressant natural product

produced by various pathogenic-fungi, such as *Aspergillus* (17). Analysis of biological samples, such as urine to evaluate the presence of controlled substances such as cannabinoids has also been undertaken by HPTLC (18). Both of these examples show the utility and applicability of this technique in clinical evaluation and toxicology. HPTLC is a truly excellent technique and the reader is urged to consult an important review by Reich and Widmer (19) on this valuable part of planar chromatography.

3.10. Analytical and Preparative-TLC, Some Natural Product Examples

The majority of examples cover classes of compounds from plant sources (Tables 4–8). Only the final prep TLC stage is given in the isolation and further details may be obtained from the reference.

3.11. TLC Bioassays

The simplicity of TLC and the ability of this technique to separate mixtures quickly with little expense mean that it can be readily used to detect biological activity of separated components. A number of these assays are described in the literature and the majority relies on the separated compound giving a color reaction with a sprayed reagent either as a final step or as a consequence of enzyme activity, chemical reaction, or organism activity. These assays are easily developed and performed and examples are given below.

3.11.1. Antioxidant TLC Assay

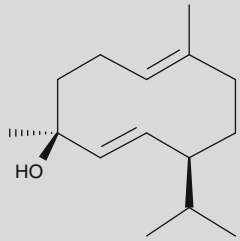
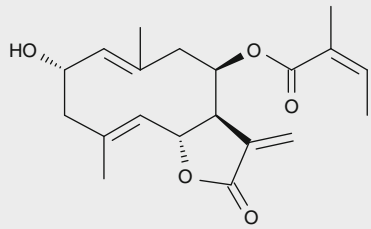
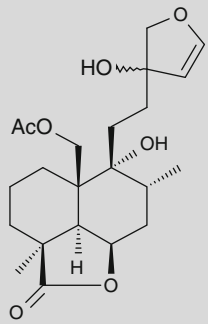
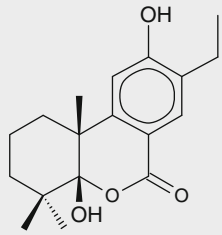
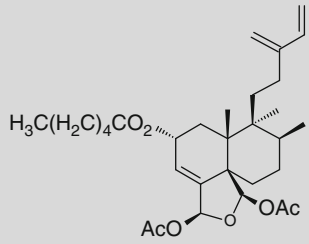
Antioxidant substances are important components of diet and it is widely recognized that they contribute to a healthy state. There is much evidence to suggest that antioxidants are important in retarding cancer cell occurrence and proliferation and consequently a TLC assay which detects this activity is of utility.

Erasto et al. (48) investigated the antioxidant properties of flavonoids using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. This compound is a stable radical and in the presence of radical scavengers (antioxidants) it is converted from a purple to a yellow color. This contrasting difference in color is very distinct and enables easy recognition of antioxidant substances. Figure 11 shows examples of antioxidant compounds isolated using this technique and this is presumably due to their phenolic nature as these groups can form free radicals. Their antioxidant activity was discovered by running a TLC plate with these samples with concentration ranges from 0.1 to 100 μg. The plates were then dried and sprayed with a DPPH solution (2 mg/mL in MeOH) and left for half an hour. Antioxidant compounds appeared as yellow spots against a purple background (48).

3.11.2. Acetylcholine Esterase TLC Assay

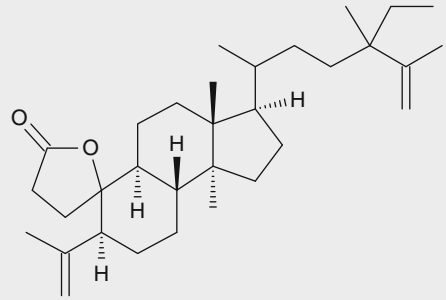
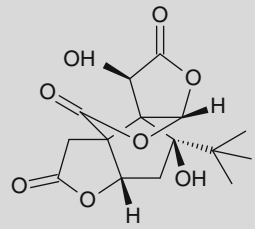
Inhibitors of the enzyme acetylcholine esterase increase the levels of this neurotransmitter at synapses in the cerebral cortex and this has benefit for patients suffering from Alzheimer's disease (49). Galanthamine (Fig. 12), a natural product from the snowdrop

Table 4
Isolation of natural products (terpenoids) by planar chromatography

Compounds and source (references)	Structure	Chromatographic conditions
Sesquiterpene from *Boronia inornata* (20)		Silica gel, toluene:EtOAc (96:4) then toluene:EtOAc (92:8)
Sesquiterpene from *Calea divaricata* (21)		Silica gel, Me_2CO:hexane (4:1)
Diterpene from *Leonitis ocymifolia* (22)		Silica gel, hexane:$CHCl_3$:EtOAc (2:3:2)
Diterpene from *Cupressus goveniana* (23)		Silica gel, CH_2Cl_2:EtOAc (94:6)
Diterpene from *Casearia tremula* (24)		Silica gel, toluene: EtOAc:AcOH (88:10:2) (two developments)

(continued)

Table 4 (continued)

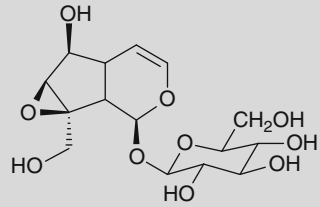
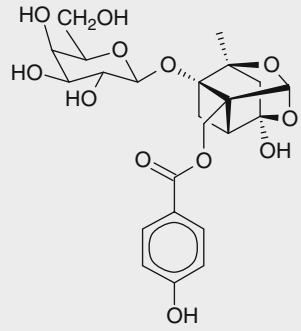
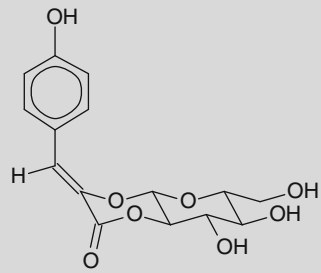
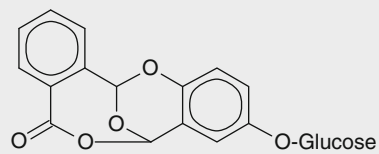
Compounds and source (references)	Structure	Chromatographic conditions
Steroidal triterpene from *Sabal blackburniana* (25)		Silica gel, petroleum ether:EtOAc (4:1), two developments
Bilobalide from *Gingko biloba* (26)		Silica gel, $CHCl_3$:Me_2CO:HCO_2H (75:16.5:8.5)

(*Galanthus nivalis*) is used as an inhibitor of this enzyme in the treatment of this disease and is marketed as a therapy to improve cognition in Alzheimer's patients.

In this assay, the TLC plate is run with prospective inhibitors (e.g., galanthamine, physostigmine, or a plant extract) and after development the plate is dried with a hair-dyer to ensure complete removal of developing solvent. The plate is then sprayed with a stock solution of acetylcholine esterase (1,000 U dissolved in 150 mL of 0.05 M tris–hydrochloric acid buffer at pH 7.8 with bovine serum albumin (150 mg) which was added to stabilize the enzyme during the assay). The TLC plate is then placed in a water humidified chamber for 20 min while ensuring that water does not touch the plate.

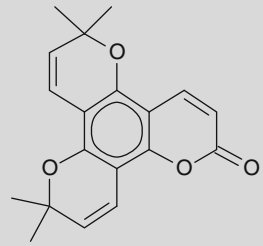
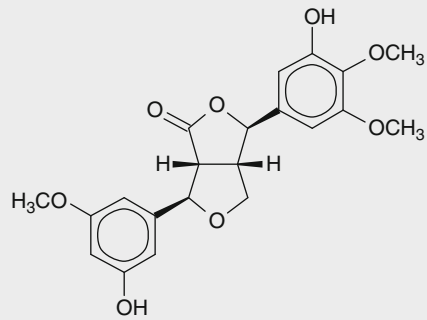
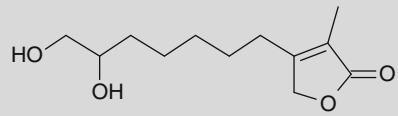
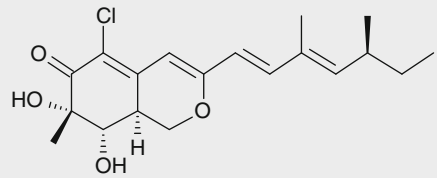
To detect the enzyme, two solutions are prepared: (1) 1-naphthyl acetate (250 mg) in ethanol (100 mL) and (2) Fast Blue B salt (400 mg) in distilled water (160 mL). Following the 20 min incubation of the plate, 10 mL of the naphthyl acetate solution and 40 mL of the Fast Blue B salt solution were mixed together and sprayed onto the TLC to give a purple coloration. Where inhibitors of acetyl choline esterase are present, the white background of the TLC spot is evident.

Table 5
Isolation of natural products (glycosides) by planar chromatography

Compounds and source (references)	Structure	Chromatographic conditions
Glycoside from *Castileja rhexifolia* (27)		Alumina, *n*-BuOH:H_2O:MeOH (7:3:1)
Glycoside from *Paeonia emodi* (28)		Silica gel, $CHCl_3$:MeOH:H_2O (80:19.5:0.5)
Glycoside from *Gunnera perpensa* (29)		Silica gel, MeOH:$CHCl_3$ (3:17)
Glycoside from *Homalium longifolium* (30)		Silica gel, EtOAc:MeOH (1:1)

This assay works by conversion of naphthyl acetate to alpha-naphthol by acetyl choline esterase and the alpha-naphthol then reacts with Fast Blue salt B to give a purple azo dye (49). Where acetylcholine esterase is inhibited, production of alpha-naphthol is stopped and therefore the purple azo dye production is inhibited. Marston et al. (49) have shown that physostigmine inhibited the enzyme at 0.001 μg and galanthamine at 0.01 μg, showing that this assay is very sensitive. The authors state that this assay is rapid,

Table 6
Isolation of natural products (coumarins, lignan, polyketide, and azaphilone) by planar chromatography

Compounds and source (references)	Structure	Chromatographic conditions
Coumarin from *Asterolasia drummondita* (31)		Silica gel, $CHCl_3$:EtOAc (9:1) two developments
Lignan from *Imperata cylindrica* (32)		Silica gel, C_6H_6:EtOAc (1:1)
Polyketide from *Seiridium* sp. (33)		Silica gel, (1) petroleum ether:acetone, (6:4), (2) $CHCl_3$:iso-propanol (9:1)
Azaphilone from *Penicillium sclerotiorum* (34)		Silica gel, CH_2Cl_2:MeOH (19:1)

simple, and easy to apply to several samples at one time. They also postulate that this bioassay could be extended to other enzymes as long as the enzymes are stable under the test conditions. This concept of TLC plus an enzyme for a bioassay has been utilized in the evaluation of the presence of insecticidal organophosphate compounds in fruit juice and eater using HPTLC and an esterase assay (50).

3.11.3. Antimicrobial TLC Bioassays

TLC bioassays against fungi and bacteria have proved exceptionally popular due to their ease of use, cost, rapidity, and because of their ability to be scaled up to assess antimicrobial activity of a high

Table 7
Isolation of natural products (alkaloids) by planar chromatography

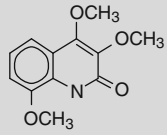
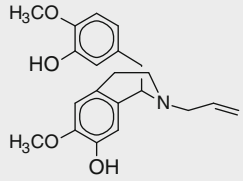
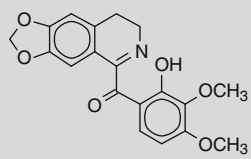
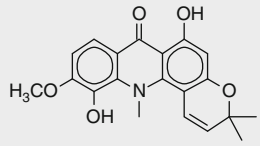
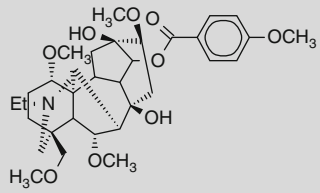
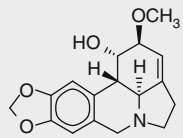
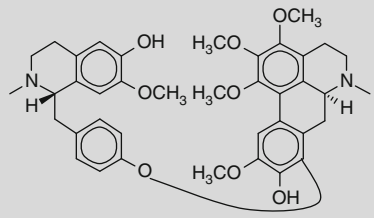
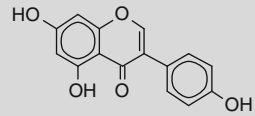
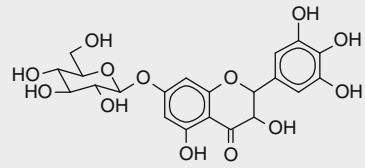
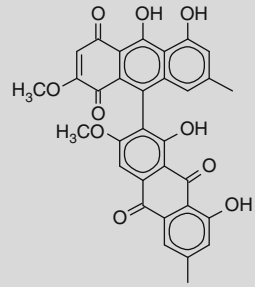
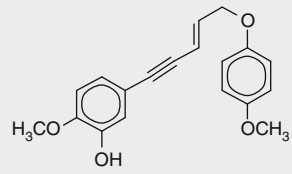
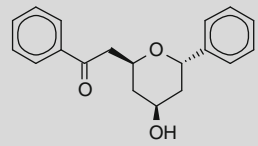
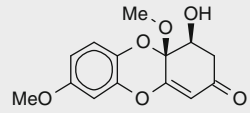
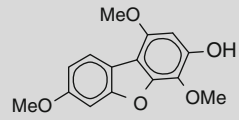
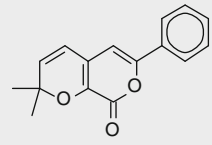
Compounds and source (references)	Structure	Chromatographic conditions
Alkaloid from *Eriostemon gardneri* (26)		Silica gel, (1) Hexane:EtOAc, 8:2. (2) $CHCl_3$:EtOAc, 8:2
Alkaloid from *Papaver somniferum* (35)		Silica gel, $CHCl_3$:MeOH, 8:2
Alkaloid from *Berberis* sp. (36)		Silica gel, (1) $CHCl_3$:MeOH:NH_4OH (90:10:1). (2) C_6H_6:Me_2CO:MeOH:NH_4OH (45:45:10:1)
Alkaloid from *Citrus decumana* (37)		Silica gel, C_6H_6:EtOAc, (19:1)
Alakloid from *Aconitum forrestii* (38)		Alumina, Me_2CO:hexane (45:55)
Akaloid from *Sternbergia lutea* (39)		Silica gel, $CHCl_3$:MeOH (9:1)
Alkaloid from *Thalictrum faberi* (40)		Silica gel, cyclohexane:EtOAc:diethylamine (6:4:1 to 8:4:1)

Table 8
Isolation of natural products (phenolics, e.g., flavonoids) by planar chromatography

Compounds and source (references)	Structure	Chromatographic conditions
Isoflavone from *Derris scandens* (41)		Silica gel, Me_2CO:$CHCl_3$ (2:98)
Flavonoid glycoside from *Pices abies* (42)		Silica gel, *n*-BuOH:AcOH:H_2O (4:1:5)
Anthraquinone dimer from *Senna multiglandulosa* (43)		Silica gel, $CHCl_3$:MeOH (100:2)
Norlignan from *Asparagus gobicus* (44)		Silica gel $CHCl_3$
Diarylheptanoid from *Dioscorea spongiosa* (45)		ODS (reverse phase) MeOH:H_2O (5:2)
Dihydrodibenzodioxinone from *Hypericum* × Hidcote (46)		Silica gel, 35% EtOAc in hexane followed by 30% EtOAc in toluene
Dibenzofuran from *Hypericum revolutum* ssp. *revolutum* (47)		Silica gel, toluene–ethyl acetate–acetic acid (60:38:2)
Pyranone from *Hypericum choisianum* (47)		Silica gel, toluene–ethyl acetate–acetic acid (80:18:2)

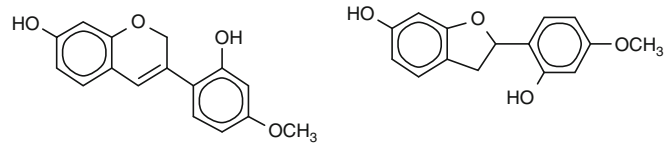


Fig. 11. Antioxidant compounds identified by TLC.

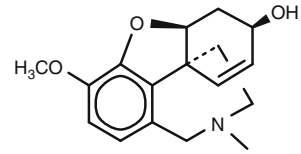


Fig. 12. Structure of galanthamine.

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* (MRSA)) and the need for new antifungal drugs, particularly with activity against resistant *Candida* and *Cryptococcus* species, these simple bioassays continue to prove useful in the assessment of antimicrobial activity of natural product extracts. A review of key antifungal and antibacterial assays has been made by Cole (51) and the reader is referred to a number of authors, namely Spooner and Sykes (52), Holt (53), Rios et al. (54), Homans and Fuchs (55), Betina (56), Ieven et al. (57), and Begue and Kline (58).

(a) *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 (59). 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:

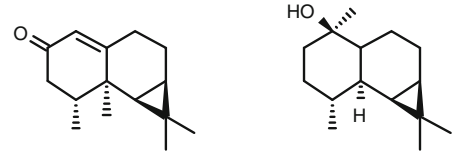
1. Extracts or pure compounds may be spotted on to analytical TLC plates in duplicate (plastic backed, Kieselgel 60 PF_{254}, Merck Art 5735), developed with the appropriate mobile phase and dried.
2. Prepare a slope of *C. cucumerinum* (IMI-299104) from a culture and allow to sporulate for 2 days.
3. A TLC growth medium should be prepared as follows: NaCl (1 g), KH_2PO_4 (7 g), $Na_2HPO_2.2H_2O$ (3 g), KNO_3

(4 g), $MgSO_4$ (1 g), and Tween 80 (20 drops) added to water (100 mL). An aliquot (60 mL) of this solution should be added 10 mL of sterile aqueous glucose (30% w/v).

4. Prepare a fungal suspension by adding the above solution to the fungal slope and shaking.
5. The suspension should be sprayed on to one of the TLC plates and incubated at 25°C for 2 days in an assay tray with moist cotton wool to ensure a moist atmosphere.
6. The inoculated TLC plate should be observed at regular intervals and the presence of antifungal compounds is indicated by inhibition or reduced lack of mycelial growth. This is frequently observed as light spots against a dark green background. This spraying should be performed in a lamina flow cabinet.
7. The remaining TLC plate should be visualized using a spray reagent and/or UV detection and compared with the incubated plate.

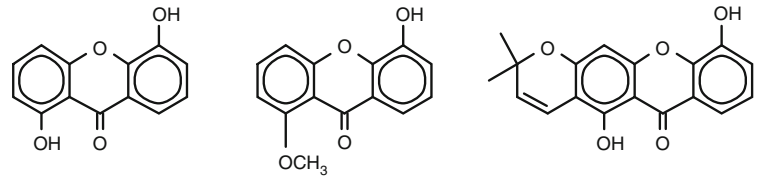
Aspergillus niger a further easily sporulating fungus may be used in the place of *Cladosporium* sp. *but care must be taken with this organism due to risk of aspergillosis and all microbes should be handled aseptically in a lamina flow cabinet.* Controls of antifungal compounds, such as amphotericin B (see Note 3), 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 need to be performed to measure minimum inhibitory concentration (MIC) (51).

Dellar et al. (60) isolated the antifungal sesquiterpenes aristolen-2-one and prostantherol (Fig. 13) from two species of *Prostanthera* (Lamiaceae). Activity was assessed and tracked through the separation procedure by the use of direct bioautography with *C. cucumerinum* as the target fungus. Aristolen-2-one inhibited the growth of *C. cucumerinum* for 70 h at a dose of 1 μg and prostantherol caused inhibition at 10 μg for the same duration.



Aristolen-2-one Prostantherol

Fig. 13. Structures of the antifungal sesquiterpenes aristolen-2-one and prostantherol.



Dose = 0.25 μg Dose = 3 μg Dose = 3 μg

Fig. 14. Antifungal xanthones.

The antifungal activity of many plant phenolic compounds can be readily assessed using this simple procedure. Hostettmann and Marston (61) investigated a series of xanthones (Fig. 14) from *Hypericum brasiliense* (Guttiferae) also for activity against *C. cucumerinum*. One of these compounds (extreme left in Fig. 14) exhibited a low inhibitory dose (0.25 μg) which may warrant further investigation of the antifungal function of these interesting compounds.

(b) *TLC bioautographic overlay assay*: In this 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. (59) have applied this technique for the evaluation of antimicrobial extracts against the yeast *Candida albicans* and the bacterium *Bacillus subtilis*. A simple overlay assay using *Staphylococcus aureus* maybe carried out a follows:

1. A base of nutrient agar (NA) (Oxoid) should be poured into an assay dish and allowed to set.
2. The extract, fraction, or pure compound should be 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 should be accurately measured.
3. An inoculum of *S. aureus* at a titer of 10^9/mL in Mueller Hinton Broth (MHB) should be prepared and nutrient agar added at 7.5 g/L to thicken the medium. This is then diluted out with MHB to give a final titer of 10^5 cfu/mL.
4. The remaining TLC plate should be placed on the NA base and the medium containing the test organism 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 (4 mg/mL in MeOH) (such as *p*-iodonitrotetrazolium chloride (INT) or methylthiazoyltetrazolium chloride (MTT)) which indicate

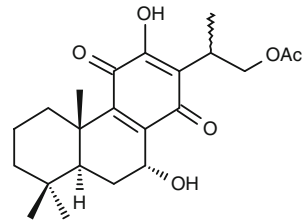


Fig. 15. Antibacterial diterpene from *Plectranthus hereroensis*.

bacterial lactate 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 should be compared with the previously developed TLC plate so that active metabolites may be readily identified and if required, isolated.
7. An appropriate control substance, such as ampicillin or chloramphenicol, should be used.

   Drug-resistant bacteria, such as MRSA will need to be cultured in the presence of methicillin to minimize the risk of loss of resistance.

   Batista et al. (62) used an overlay method in the bioassay-guided fractionation of an acetone extract of the roots of *Plectranthus hereroensis* (Lamiaceae) to isolate the antibacterial diterpene (Fig. 15). *S. 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 (63) used a variant of this assay to investigate the activity of 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.

4. Notes

1. The procedure for removal of sorbents (especially silica and alumina) from TLC plate backings should always be performed in a fume cupboard and a dust mask must be worn to avoid breathing in dangerous fine particulate material.
2. It should be noted that if a recovered compound appears to be impure 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.

3. Amphotericin B is highly toxic and care should be exercised in its use.

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