Thin Layer Chromatography

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ABSTRACT

Thin layer chromatography (TLC) is a quick, sensitive, and inexpensive technique used to determine the number of components in a mixture, verify the identity and purity of a compound, monitor the progress of a reaction, determine the solvent composition for preparative separations, and analyze the fractions obtained from column chromatography. This unit is mainly aimed at novice experimenters, describing in detail the strategies and principal steps for performing a TLC experiment, with illustrations of the relevant instruments, as well as approaches for obtaining and understanding results. Valuable practical tips and troubleshooting solutions are also provided throughout the unit. *Curr. Protoc. Essential Lab. Tech.* 8:6.3.1-6.3.18. © 2014 by John Wiley & Sons, Inc.

Keywords: chromatography • TLC • separation • silica gel • organic solvents • staining

OVERVIEW AND PRINCIPLES

The first widespread application of partition chromatography on a planar surface was paper chromatography, introduced in the 1940s. However, paper chromatography was gradually replaced by thin-layer chromatography (TLC), which has become one of the most routinely used chromatography techniques (Ettre and Kalász, 2001). TLC is also a liquid-solid adsorption technique where the mobile phase ascends the thin layer of stationary phase coated onto a backing support plate (Fig. 6.3.1).

TLC (see *http://www.discoverysciences.com/uploadedFiles/Home/ChromAccessories_TLC_PlatesNAcc_p188_198.pdf* and *http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf*) resembles column chromatography (*UNIT 6.2*), where the solvents (eluents) flow down through the column's adsorbent. However, unlike column chromatography, TLC is a quick, sensitive, and inexpensive technique that only requires a few micrograms of sample for one successful analysis. TLC is commonly used to (1) determine the number of components in a mixture; (2) verify the identity and purity of a compound; (3) monitor the progress of a reaction; (4) determine the solvent composition for preparative separations; and (5) analyze the fractions obtained from column chromatography.

Like all forms of chromatography, TLC involves a dynamic and rapid equilibrium of molecules between the two phases (mobile phase and stationary phase). However, TLC differs from all other chromatographic techniques in the fact that a gas phase is present, which can influence the results of separation significantly. Between the components of the mobile phase and its vapor, an equilibrium will be established gradually (also called chamber saturation). The part of the stationary layer that is already wetted with mobile phase also contributes to the formation of the equilibrium (Fig. 6.3.1).

During development, molecules are continuously moving back and forth between the free and adsorbed states (Fig. 6.3.2A). A balance of intermolecular forces determines the position of equilibrium and thus the ability of the solvent to move the solute up the plate

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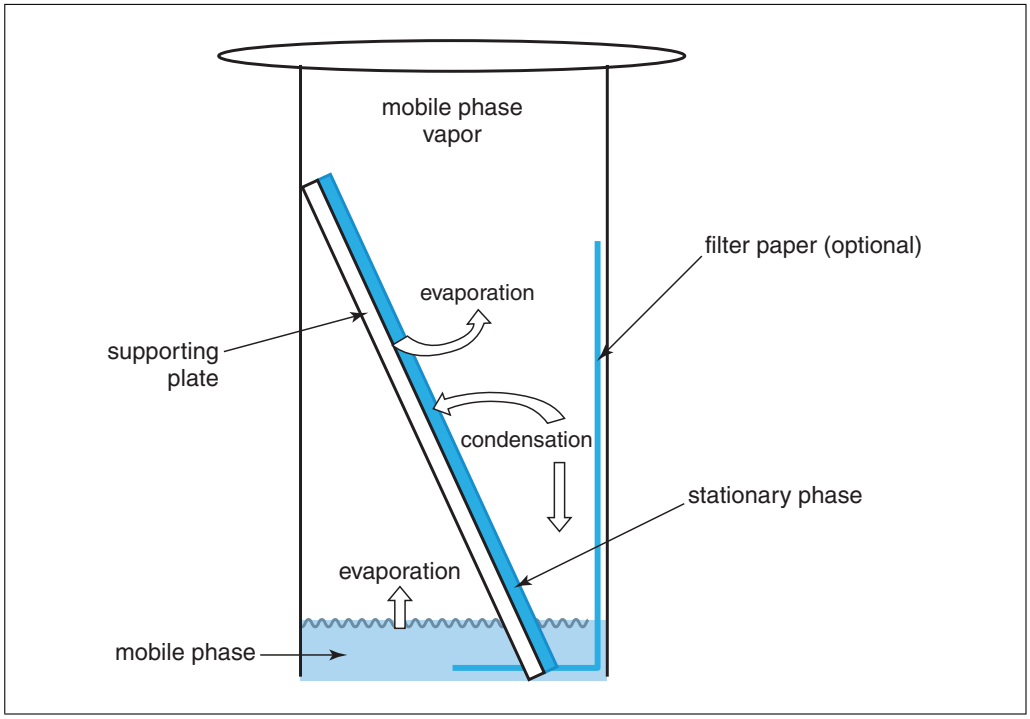


Figure 6.3.1 Schematic representation of ascending development chamber for conventional TLC (side-on view). Adapted from Macherey-Nagel TLC catalog: *ftp://ftp.mn-net.com/english/Flyer_Catalogs/Chromatography/Catalog-en/KATEN200001-4TLC-www.pdf*.

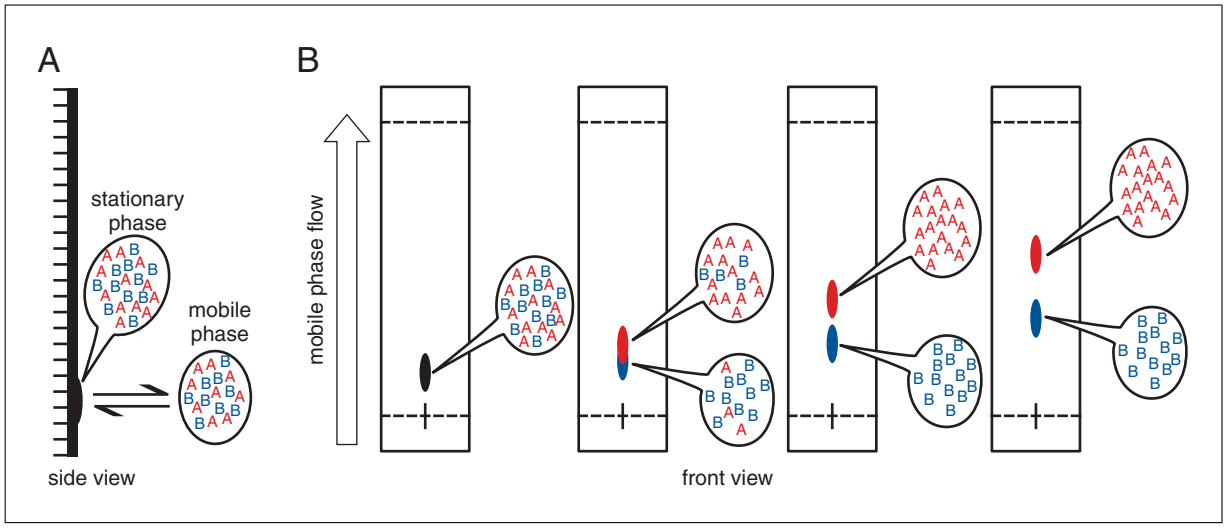


Figure 6.3.2 (**A**) Mixture of A and B adsorbed on the stationary phase and free in mobile phase and (**B**) schematic representations of the principle of separation. Adapted from *http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf*.

(also see Strategic Planning for details). This balance depends on (1) the polarity of the TLC coating material, (2) the polarity of the development solvent, and (3) the polarity of the sample molecule(s). For example, with a sample consisting of two compounds A and B as illustrated in Fig 6.3.2B, if the molecules A spend more time in the mobile phase, they will be carried through the stationary phase more rapidly and move further in a certain time. While molecules B are adsorbed to the stationary phase more than A, B molecules spend less time in the mobile phase and therefore move through the stationary phase more slowly, and do not move as far in the same amount of time. The consequence is that A is gradually separated from B as the mobile phase flows (ascends).

STRATEGIC QUESTIONS

Before performing a TLC experiment and the subsequent analysis, the following questions should be addressed:

1. What type of TLC plate will you use regarding the backing support and coating material?
2. Which solvent system will you choose to achieve the best separation and resolution?
3. How will you handle and develop the TLC plate?
4. How will the compounds in your sample be visualized?
5. How will you solve the problems encountered during TLC experiments?
6. Are there any special techniques and tips for a successful TLC analysis?

Whether your TLC is successful, as well as the overall time required, depend on each of these decisions. The answers to these questions can be found in Strategic Planning, Protocols, and especially Troubleshooting.

STRATEGIC PLANNING

Precoated TLC Plates

Supports for stationary phases (glass, aluminum, and plastic)

Glass has been found to be a very robust support. It is rigid and transparent, and has high chemical resistance and good heat stability. The glass backing is economical (reusable). However, glass plates are relatively heavy and thick. They cannot be easily cut to desired size (see steps for handling and cutting TLC plates in the Basic Protocol, below). Because glass backing is fragile and highly susceptible to breakage, there is also a potential safety issue.

Aluminum foil is preferable to all other materials for TLC plates. Compared with glass plates, foil plates are thin, lightweight, and easy to handle. They can easily be cut to desired dimensions with scissors and can be stored in a laboratory notebook. Moreover, aluminum plates have strong adsorbent layer adherence and are good for use with eluents containing a high concentration of water. However, they are not as chemically resistant as glass to reagents that contain strong acids, concentrated ammonia, or iodine (i.e., they do not tolerate long treatments in an iodine chamber).

Plastic—polyethylene terephthalate (PET) film—plates are becoming less frequently used. Their advantages (thin, lightweight, easy to handle, can be easily cut, etc.) are similar to aluminum-foil plates, but their flexibility (adsorbent layer may be more susceptible to cracking) and considerably inferior heat stability are very marked disadvantages.

Adsorbent layers and stationary phases

The standard silica coating (silica 60 with a mean pore diameter of 60 Å) is the most commonly used adsorbent in TLC, although for some very sensitive substances less active adsorbents such as aluminum oxide are preferred to prevent sample decomposition. Moreover, in the early days, the use of cellulose, polyamide, and Florisil (magnesium silicate) as adsorbent agents was also described.

For selection of an adsorbent, one considers the properties of the compounds to be separated: first, the solubility of the sample compounds (hydrophilic or hydrophobic); then, whether the compounds can chemically react with the adsorbent or the eluent. Based on these considerations it is recommended that:

1. for lipophilic substances: silica, aluminum oxide, acetylated cellulose, polyamide should be used;

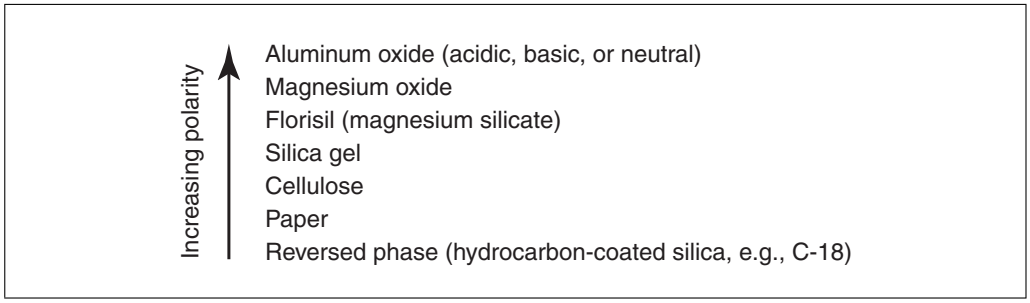


Figure 6.3.3 TLC stationary phase polarities.

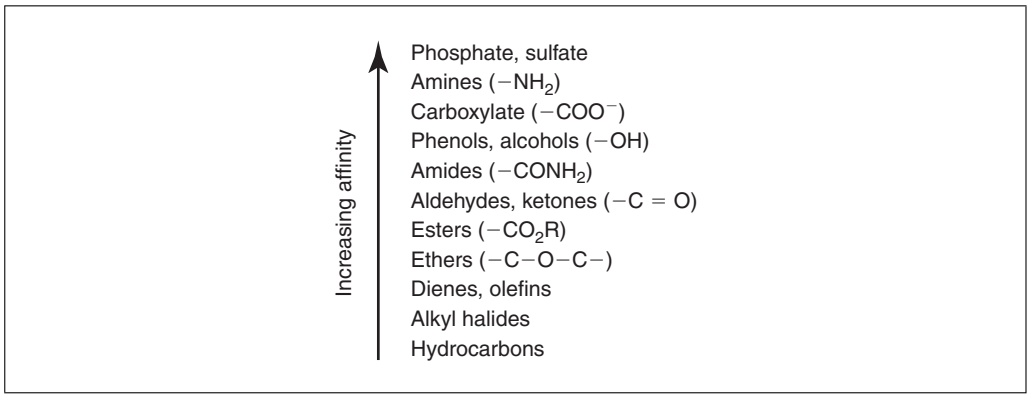


Figure 6.3.4 Affinity of common functional groups for silica gel (approximate).

2. for hydrophilic substances: cellulose, cellulose ion exchangers, polyamide, and reversed-phase silica should be used.

Several different types of TLC stationary phases are listed according to polarity in Figure 6.3.3. Figure 6.3.4 shows affinity of common functional groups for silica gel (approximate). Assuming that a polar adsorbent (silica gel) is used, the more polar compounds will be eluted more slowly and the more nonpolar compounds will be eluted more rapidly. The charts depicted in these figures are very useful to help predict the order of elution; however, the functional groups should always be viewed and considered within the context of a whole molecule. Clear answers come from real experiments!

Solvent System (Mobile Phase)

Finding a suitable solvent system is usually the most difficult part of TLC experiments, and solvent system is the factor with the greatest influence on TLC. Only in a few cases does the solvent consist of only one component, and mixtures of up to five components are commonly used. No matter how many components are present, the prepared solvent system must be a homogenous system with no sign of cloudiness.

Three criteria are usually considered for choosing a solvent system: solubility, affinity, and resolution. The first step in solvent selection is to determine the solubility of the sample. The desired mobile phase will be able to provide the greatest solubility while balancing the sample affinity for the solvent and the stationary phase to achieve separation. Resolution is improved by optimizing the affinity between sample, solvent, and stationary phase. Most TLC solvent systems contain a polar solvent and a chromatographically less polar solvent.

Figure 6.3.5 lists some common mobile phase solvents according to their polarities and elution power with silica 60 as the stationary phase (Halpaap's eluotropic series, Halpaap and Ripphahn, 1976; Hahn-Deinstrop, 2006). With these solvents, there are

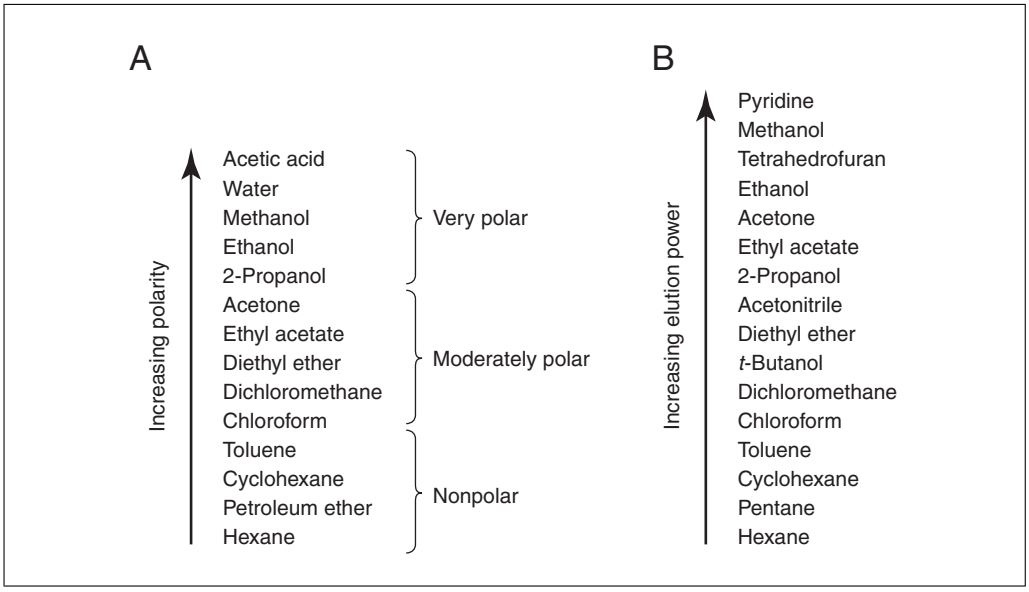


Figure 6.3.5 (**A**) Common mobile phase solvents listed by increasing polarity (adapted from Grace catalog for TLC accessories: *http://www.discoverysciences.com/uploadedFiles/Home/ChromAccessories_TLC_PlatesNAcc_p188_198.pdf*) and (**B**) elution power with silica gel as the stationary phase (Halpaap's eluotropic series, Halpaap and Ripphahn, 1976; Hahn-Deinstrop, 2006).

some common combinations for organic molecules with silica gel as the stationary phase (see Troubleshooting for combinations of three solvents for very polar compounds):

Hexane (or petroleum ether)/ethyl acetate
Dichloromethane (or chloroform)/methanol
Pentane/ether
Petroleum ether/acetone
Hexane/dichloromethane
Dichloromethane/ethyl acetate
Ethyl acetate/methanol
Toluene/acetonitrile
Water/methanol (for C_{18}-reversed phase silica)
Water/acetonitrile (for C_{18}-reversed phase silica).

The easiest way to find a starting point for development is to look up a reference for chromatography conditions of compounds with similar structure. Meanwhile, consider the affinity for the type of compound (Fig. 6.3.4), as well as the solvent strength (Fig. 6.3.5), to make adjustments.

If the mobile phase has not been previous reported or determined, start with a less polar combination such as hexane/ethyl acetate and observe the separation. If the components do not move very far, try adding a greater volume or a higher ratio/percentage of the polar solvent. Always compare the separation to the previous plate. If the spots stay at the starting line of the plate, add more of the polar solvent or switch to a more polar combination such as dichloromethane/methanol. If they run with the solvent front (or $R_f > 0.8$), then add more nonpolar solvent or switch to an even less polar combination such as pentane/ether. It is common to try three to six solvent systems for the first round of method development. As a general guide, a substitution in the more polar solvent often results in a change in resolution, while a change in the less polar solvent results primarily in a change in R_f of the sample components (see Understanding Results for discussion of R_f).

SAFETY CONSIDERATIONS

1. Take extra caution when breaking scored glass TLC plates. The resulting sharp edges may cause cuts to the hands.
2. Inhaling silica gel (dust form particularly) is highly dangerous and may cause severe lung irritation. Long-term exposure may cause the lung disease silicosis. A safety mask is recommended when handling silica-gel TLC plates.
3. Many organic solvents used for developing TLC are flammable or combustible, and inhalation of their vapors is to be avoided. Some organic solvents are potentially carcinogenic, such as benzene (proven group 1 carcinogenic; should be replaced with toluene), chloroform, and dichloromethane.
4. Many reagents used in TLC staining are toxic and must be handled with care. If heating (with a hot plate or a heat gun) is required for staining, make sure all steps are carried out in a fume hood with care to avoid inhalation of any toxic or irritant smoke or vapor. Personal protections (disposal gloves, safety goggles, and masks) are required.

PROTOCOL

Basic Protocol: Principal Steps of TLC and Required Instrumentation

In this protocol, preparation of the TLC plates, preparation of the spotting capillary, spotting of the sample, development of the TLC plate, and visualization of the components are described. Necessary TLC accessories are illustrated in the associated figures with instructions for their use included in the protocol steps.

Materials

Organic sample solution: the sample for TLC can be dissolved in any compatible solvent because the solvent used to dissolve the sample will be completely dried out after the sample is spotted on the TLC (avoid high-boiling-point solvents which would make it difficult to dry the sample after spotting and cause the TLC look like a smear; see Troubleshooting)
Developing solvents (CH_2Cl_2, hexanes, ethyl acetate, methanol, etc.)
Iodine (I_2; also see Table 6.3.1)
TLC staining reagents (Table 6.3.1)

TLC plates (aluminum, glass, or plastic; e.g., EMD Millipore, SORBTECH, Sigma-Aldrich)
Guillotine paper trimmer
Diamond-tipped glass cutter
Glass Pasteur pipets for capillary spotters
TLC chamber (CAMAG, cat. no. 022.5255; Sigma-Aldrich, cat no. Z126195 or Z243906) or small wide-mouth flat-bottom glass jar/bottle with a lid
Tweezers
Heat gun (optional)
UV lamp
Iodine chamber: a screw-top glass jar with a well-fitting lid can be used as the vaporization chamber (see Table 6.3.1 for preparation details)
Filter paper
TLC spray cabinet equipped with radial fan (CAMAG; *http://www.camag.com*)
Sprayer (see Fig. 6.3.12)
Hot plate or TLC plate heater

Handling and cutting TLC plates

1a. *For an aluminum-foil TLC plate:* Place the aluminum foil TLC plate on a paper trimmer board with the coating side down (Fig. 6.3.6) and use the guillotine to cut

Table 6.3.1 Frequently Used TLC Stains

Name	Application	Preparation
Iodine (I_2)	Temporary stain; insert the TLC plate into the chamber and remove it after it develops a light brown color over the entire plate	To a glass bottle with cap (bottle size depends on how much stain you prepare) add 100 g of silica and 5 to 7 g of iodine crystals (no liquid). Close the cap and shake many times so that iodine is dispersed over the silica.
p-Anisaldehyde	Carbohydrates; heating required to stain the plate; various colors	Dissolve 18 ml of *p*-anisaldehyde in 540 ml 95% ethanol and cool the solution in an ice/water bath. Mix 30 ml of 97% H_2SO_4 and 6 ml of acetic acid. Cautiously add the acid mixture to the prechilled ethanol solution dropwise at 0°C with vigorous stirring, without splashing. Store the resulting colorless solution in a − 20°C freezer before use.
Bromocresol green	Carboxylic acids yield yellow-green spots on blue background; no heating required	Dissolve bromocresol green (0.08 g) in ethanol (200 ml) to get a clear colorless solution. Slowly add 0.1 N NaOH dropwise until blue color just appears in the solution.
CAM	Universal stain; heating required to stain the plate; yields dark blue spots on light background	Slowly add conc. H_2SO_4 (80 ml) to water (720 ml) under stirring followed by ammonium molybdate (40 g) and ceric ammonium sulfate (1.6 g). Stir the resulting mixture to get a clear solution.
Cerium(IV) sulfate [$Ce(SO_4)_2$]	General staining, very effective for alkaloids; should be sprayed on to the plate (not dipped) and then heated for the stain to appear as black spots on yellow-white background	15% aqueous sulfuric acid saturated with cerium (IV) sulfate
Chromic acid	General staining; yields black spots	To a cold (0°C) solution of sulfuric acid (100 ml, 20% v/v aq.), slowly add potassium chromate (2.5 g). Warm the resulting clear bright red/orange solution to room temperature and use directly.
2,4-DNP	Mainly for aldehydes and ketones; yields orange spots, no heating required	Dissolve 2,4-dinitrophenylhydrazine (6 g) in 95% ethanol (100 ml) and add water (40 ml). Stir the resulting mixture to get a clear solution, slowly add conc. H_2SO_4 (60 ml), and stir to get a clear solution.
Dragendorff reagent	Unreactive amines (e.g., carbamate protected amines), alkaloids; yields orange spots, no heating required	Solution A: 1.7 g basic bismuth nitrate in 100 ml water/acetic acid (4:1). Solution B: 40 g potassium iodide in 100 ml water. Mix reagents together as follows: 5 ml A + 5 ml B + 20 ml acetic acid + 70 ml water. Spray plates; orange spots develop. Spots intensify if sprayed later with HCl or 50% water-phosphoric acid.
Ehrlich's reagent	Amines, indole derivatives, antibiotics, steroids; mild heating (lower temperature and shorter heating time; remove the heat source before the background color obscures the spots) required to stain the plate	Dissolve *p*-dimethylaminobenzaldehyde (1.0 g) in 75 ml of methanol and add 50 ml of conc. HCl.

(continued)

Table 6.3.1 Frequently Used TLC Stains, *continued*

Name	Application	Preparation
Ferric chloride spray	Phenols	Dissolve ferric (III) chloride (1 g) in a mixture of methanol (50 ml) and deionized water (50 ml). Stir the above mixture to get a homogenous solution.
Iodoplatinate (PIP)	Alkaloids	Dissolve hexachloroplatinate (0.5 g) and potassium iodide (10 g) in deionized water (295 ml). To the above mixture add conc. HCl (27 ml). Stir the mixture 4 hr at 0°C.
Morin hydrate	General stain; fluorescently active	Dissolve morin hydrate (100 mg) in methanol (100 g) and stir to get a clear solution
Ninhydrin	Excellent stain for amines, amino acids, and aminosugars	Dissolve ninhydrin (1.5 g) in *n*-butanol (100 ml) and then add glacial acetic acid (3 ml). Ethanol can be used in place of butanol.
Potassium permanganate ($KMnO_4$)	Mainly for unsaturated compounds and alcohols; alkenes/alkynes/aromatics usually stain without heating while other oxidizable groups require heating; yields yellow spots on purple background	Dissolve $KMnO_4$ (1.5 g) and K_2CO_3 (10 g) in deionized water (200 ml). To this add 10% NaOH (1.25 ml) and stir to get a clear solution. It will take some time for the solution to clear.
Phosphomolybdic acid (PMA)	Good general reagent; heating required to stain the plate, yields blue–dark green spots	Dissolve 12 g phosphomolybdic acid in 250 ml ethanol.
Sulfuric acid	Heating required to stain the plate; permanent charred spots are produced	5% sulfuric acid in methanol
Vanillin	Good general reagent; heating required to stain the plate, yields a range of colors	To a cold (0°C) clear colorless solution of vanillin (15 g) in absolute ethanol (250 ml), slowly add sulfuric acid (2.5 ml). Warm the resulting clear solution to room temperature and use directly. Store the excess in a refrigerator.

[a]Chemical reactions on the TLC plate after separation, which help find and characterize a substance, are almost unlimited and have not been fully investigated. Some frequently used staining reagents, and their application and preparation, are summarized in this table (adapted from OChemOnline *http://www.ochemonline.com/TLC_stains*). Some TLC derivatization reagents (ready-to-use) are readily available from major suppliers such as Sigma-Aldrich (*http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=8659122*) and Machery-Nagel (*http://www.mn-net.com/chroma/TLCStart/Sprayreagents/TLCdetectionreactions/tabid/5578/language/en-US/Default.aspx*). Although in most cases we talk about dipping solutions, the same reagents are often applicable for spraying TLC plates.

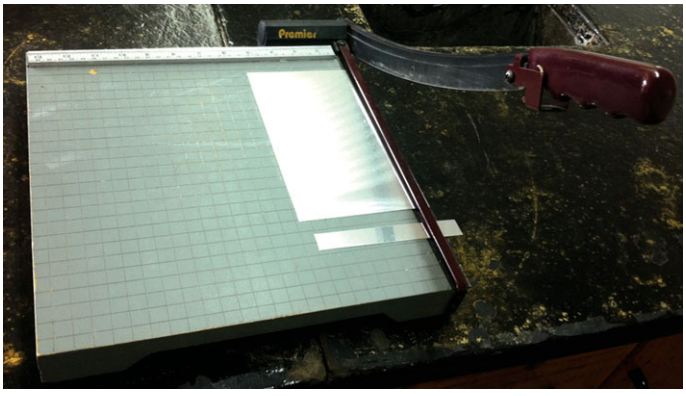


Figure 6.3.6 A paper trimmer for cutting silica-coated aluminum plates.

Figure 6.3.7 CAMAG smartCut plate cutter. Figure courtesy of CAMAG Scientific, Inc.

the aluminum-foil plates into desired sizes. Gently wipe the cut edges with a spatula to remove loose layer material.

A guillotine paper trimmer for office use is strongly recommended for cutting aluminum foil TLC plates into smaller sizes suitable for the intended TLC application. It is easy to handle and always generates well cut edges. Poorly cut edges may result a capillary gap between the stationary phase and the foil, which will cause spot deformation or skewed and distorted chromatogram lanes (also see Troubleshooting).

If a paper trimmer is not available, ordinary scissors can be used. When cutting the plate, the angle of the scissors is very important to avoid adsorbent flaking off the sides. Well cut edges can be obtained if the scissors are inclined slightly from left to right (NOT inclined to the left) during cutting (Hahn-Deinstrop, 2006).

1b. *For a glass TLC plate:* Use a simple diamond-tipped glass cutter to score TLC plates (e.g., 20 × 20 cm), using a ruler as a guide for scoring the glass. Once the entire plate is scored, break the glass into individual pieces (holding the plate in a paper towel).

This technique requires practice, and a "clean" break is seldom obtained, especially by beginners. For better and more reliable scoring, portable TLC plate cutters (Fig. 6.3.7) are available. The TLC plate cutter is usually equipped with a high-quality carbide scriber mounted into a movable plastic head (Fig. 6.3.7). It is designed to produce optimal scoring on glass TLC plates with a thickness up to 3 mm (glass plus coating). The plate cutter can be used to cut glass plates into small strips without damaging the coated layer.

1c. *For a plastic (PET) TLC plate:* Use sharp scissors or a paper trimmer as described in step 1a.

Sample application (spotting the TLC plate)

The most common technique for TLC sample application is to use a glass capillary spotter. The spotting capillaries are extremely small and easy to make from glass Pasteur pipets (steps 2 to 4, illustrated in Fig. 6.3.8).

2. Heat the middle part of the Pasteur pipet over a Bunsen burner blue flame.

Hold both ends carefully, as they will get hot! Use heat-proof gloves if necessary!

3. When the middle part is hot enough, it will become pliable; pull both ends apart quickly and smoothly.

This will draw the middle part into a thin string-like tube.

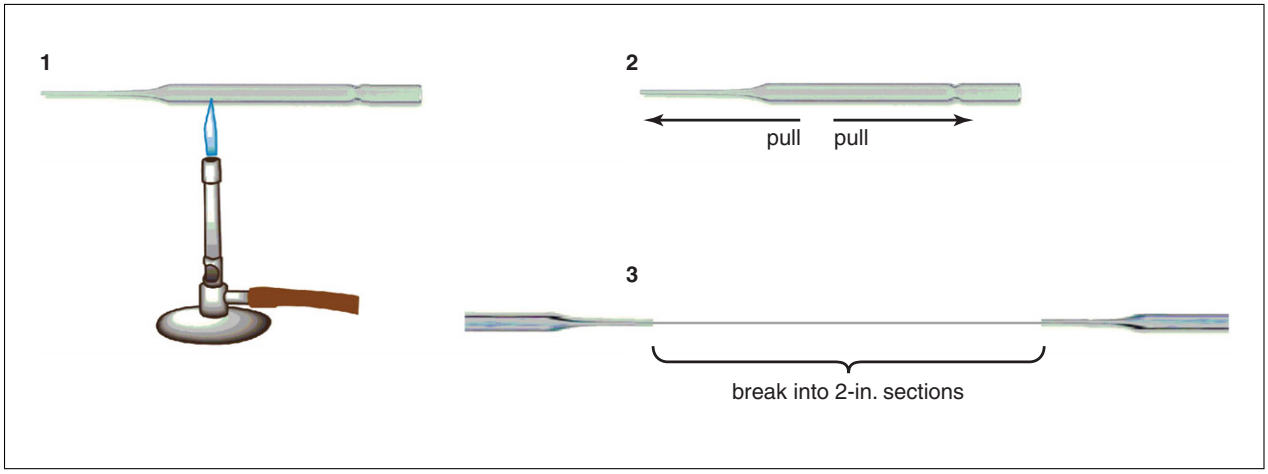


Figure 6.3.8 Making glass capillary spotter from Pasteur pipets.

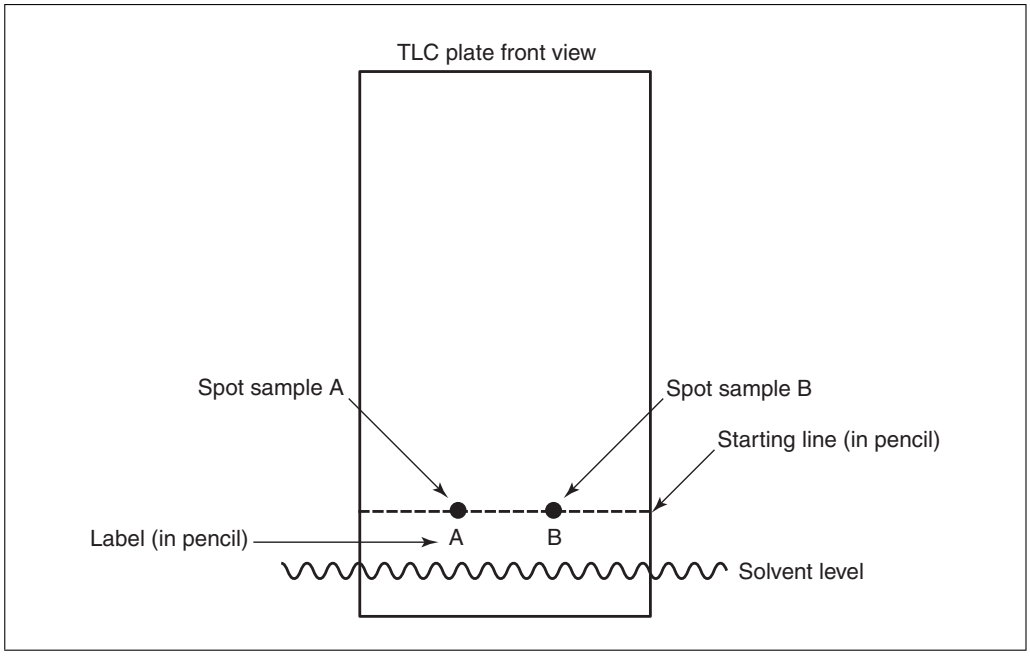


Figure 6.3.9 Spotting TLC plate.

4. After cooling for a few minutes, gently break the middle string-like part into 2- in. sections to provide good TLC capillary spotters.

   The end should be as flat as possible, as a jagged edge will lead to unsymmetrical spots or no spots on the TLC plate.

5. Fill the prepared capillary spotter by quickly dipping it into the organic sample solution.

   With aqueous solutions, filling will be much slower—an Eppendorf pipettor with 10-µl tip is thus recommended for applying aqueous sample solutions.

6. Place the capillary spotter at the starting line (labeled in pencil) on the coated side of the plate vertically and carefully, to allow capillary action to draw the solution onto the plate. If more than one sample is running at the same time, make sure to properly label the plate with a pencil (Fig. 6.3.9) and use a different capillary for each sample to avoid contamination. Blow with cold or hot air to facilitate solvent evaporation of the applied samples.

Figure 6.3.10 CAMAG Flat bottom TLC chamber. Figure courtesy of CAMAG Scientific, Inc.

To keep spots small and compact, it is better to apply a sample in several portions with intermediate drying (to avoid diffusing), instead of holding the capillary against the plate for a long time.

For beginners, it is best to practice spotting a few times on a spare TLC strip. In this way, you will discover the capillary emptying time and have better control over the application pressure to be used with capillaries (avoiding damage to the layer).

Development of TLC plates

In most cases, ascending TLC is applied in a TLC chamber as for development once with a single solvent system (single development). Commercial TLC chambers of different sizes are readily available (Fig. 6.3.10), but usually a small wide-mouth flat-bottom glass jar/bottle with a lid large enough to fit the TLC plate works just as well.

7. First, fill the chamber with development solvent to a depth no greater than 0.5 cm.
8. Use tweezers to place the TLC plate in the prepared development chamber with its back layer leaning against the chamber's inside wall, and immediately cover the chamber with the lid (Fig. 6.3.1).

   Make sure that the starting line is above the solvent level (Fig. 6.3.9).

   Saturation of the chamber atmosphere with solvent vapor is important for TLC. In most cases, lining the chamber with a piece of filter paper (wetted with the eluent) will promote the saturation and may improve the separation and reproducibility (Fig. 6.3.1). Thus, to maintain the atmosphere in the developing chamber, it must not be opened during the development. Observe the solvent front through the side wall while keeping the chamber closed!

9. Development starts once the TLC plate is immersed; when the solvent front has reached an appropriate level (usually within 0.7 cm of the top of the plate), quickly remove the lid, take out the plate with tweezers, and mark the solvent front with a pencil.

   Proper marking of the solvent front will be of benefit in the analysis of results (see Understanding Results).

10. Allow the plate to dry in a fume hood or with a heat gun (heat gently) before proceeding to the visualization step.

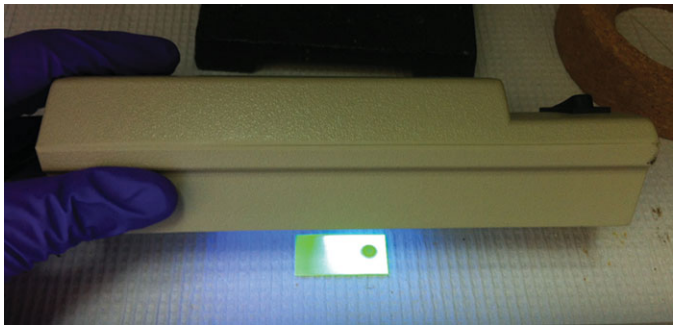


Figure 6.3.11 Visualization under a UV lamp (UVP 4-W compact UV lamp from UVP).

If certain separation cannot be achieved by a single development, multiple developments (with or without change of solvent system) may be performed: the TLC is developed two or more times with intermediate drying (also see Troubleshooting).

Visualization

Nondestructive visualization

11a. *For nondestructive visualization with the naked eye:* Simply put the plate under a UV lamp (Fig. 6.3.11), and the compounds become visible to the naked eye. Lightly circle the spots with a pencil, so that you will have a permanent record of their location for later qualitative assignment.

Only in a few cases is the sample a dye (colored) that can be seen with naked eye. Much more often, substance visualization can be achieved under UV light, since many substances have UV absorption.

TLC plates normally contain a fluorescent indicator that makes the TLC plate glow green under UV light of wavelength 254 nm (less frequently at 365 nm with a mercury lamp). Substances absorbing UV light in the respective region of wavelength will quench the green fluorescence, yielding dark purple or bluish spots on the plate (Fig. 6.3.11).

11b. *For nondestructive visualization using iodine:* Use tweezers to insert the TLC plate into the prepared iodine chamber (described in Table 6.3.1) and remove it after it develops a light brown color over the entire plate. Cover the iodine-treated TLC plates with a clean glass plate, since the color stain will eventually fade.

You may also circle the observed spots with a pencil for documentation.

Iodine sublimes and will absorb to organic molecules. This method is therefore non-specific, but usually does not cause decomposition. The organic spots on the plate will turn brown and can be easily identified.

Dwell times for plates in a vaporization chamber can range from a few minutes for detection up to 20 hr for purity tests. It should be noted that, with long dwell times in iodine vapor, aluminum TLC plates may react with iodine and become impossible to evaluate.

Staining/derivatization

If the above methods (step 11a or 11b) do not allow localization of substances, chemical reactions can be applied on the TLC plate as in the subsequent steps. More reliable results can be obtained with specific reagents for spraying or dipping (or with subsequent heating), which form colored or fluorescent compounds with the substances to be detected.

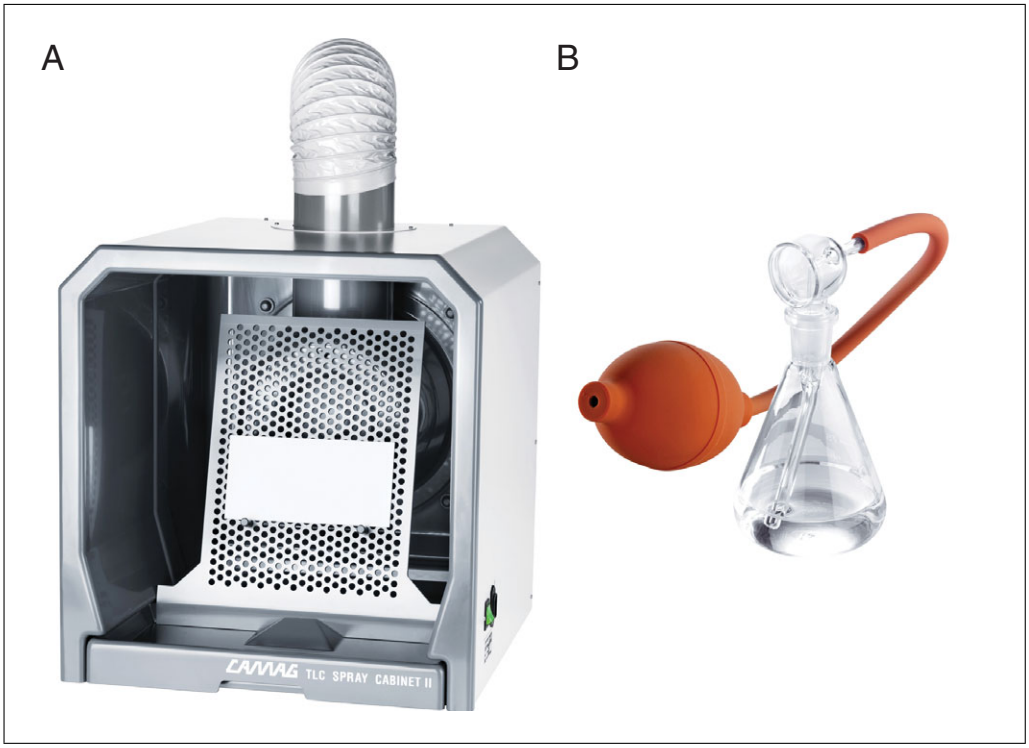


Figure 6.3.12 (**A**) CAMAG TLC Spray Cabinet equipped with a blower and (**B**) Glass Reagent Sprayer. The Spray Cabinet can also be used for drying plates after development. Figures courtesy of CAMAG Scientific, Inc.

For stepwise spraying technique

11c. Place the developed, dried TLC plate or sheet on a sheet of filter paper under a fume hood (or preferably in a TLC spray cabinet equipped with a radial fan, Fig. 6.3.12A).

12c. Fill the sprayer (Fig. 6.3.12B) with about 5 to 10 ml of solution. Spray from a distance of about 15 cm with the aid of a rubber ball/pump or a compressed air or nitrogen supply.

It is always better to spray a layer twice very thinly and evenly (with intermediate drying) than to saturate the layer with excessive spray reagent, which usually causes diffused spots.

For stepwise dipping technique

11d. Hold the edge of the developed plate (dried) with tweezers, quickly immerse the whole plate into the staining solution, and immediately take out the plate. Close the dipping chamber with a lid immediately.

As an alternative derivatization technique, brief dipping of the TLC plates in staining reagent solutions is being used increasingly for many reasons: (1) the application of the reagent solution to the TLC layer is more homogeneous and uniform; (2) this technique gives improved reproducibility; (3) the consumption of reagent is low; (4) complex spraying equipment is not needed; (5) contamination of the workplace with reagent is minimized.

Dipping chambers/jars vary in size and shape, and dipping solutions are generally less concentrated than spraying solutions. Under certain circumstances, the dwell time in the dipping chamber should be determined experimentally.

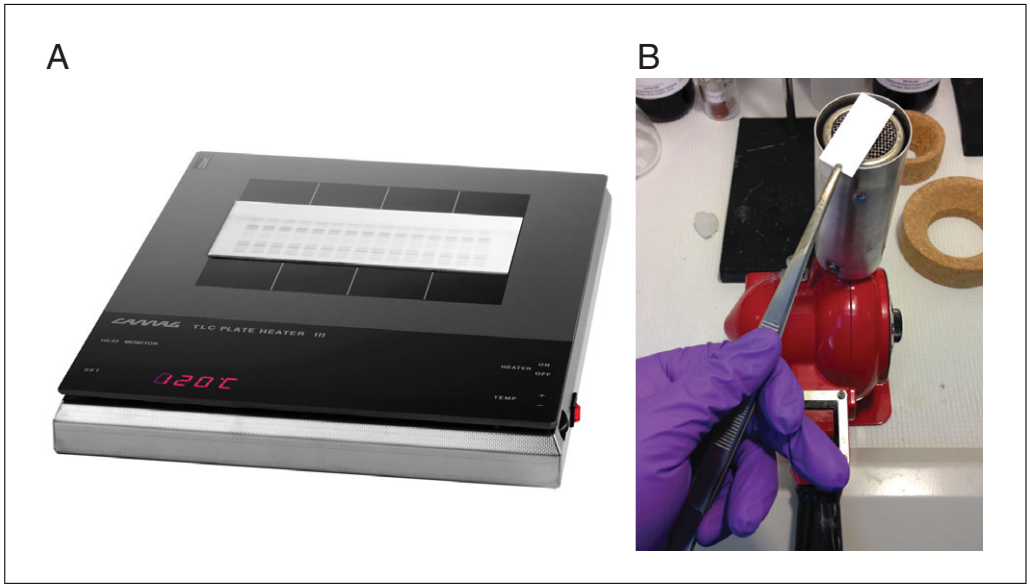


Figure 6.3.13 (**A**) CAMAG TLC Plate Heater. Figure courtesy of CAMAG Scientific Inc. (**B**) Heating TLC plate with a heat gun.

12d. After the dipping operation (especially before heating), carefully wipe the back of the plate with a paper towel that has been wetted with the staining solution.

The dipping chamber should always be closed with a lid after dipping to prevent evaporation of solvent.

13. If heating is necessary, perform this on a hotplate (or a TLC plate heater, Fig. 6.3.13A), or with a heat gun (Fig. 6.3.13B).

Make sure to remove the TLC plate from the heat source once the spots are visible and before the background color obscures the spots.

When glass plates are used, the spots can sometimes be seen more clearly from the glass side of the plate.

COMMENTARY

Understanding Results

Retention factor (R_f)

The analysis of TLC depends on the purpose. For most qualitative determinations, localization of substances is sufficient. This can be easily achieved by parallel runs with reference compounds (e.g., authentic sample, starting material, compound with similar structure, etc.). A practical example of a TLC analysis for monitoring the progress of a reaction is illustrated in Figure 6.3.14 (adapted from Zhao et al., 2010). The starting material, *N*-acetylglucosamine (Lane A), and the product, *N*-acetylglucosamine-1-phosphate authentic sample (Lane B), were used as controls by parallel runs with the reaction mixture (Lane C). The disappearance of the starting material spot and generation of the new product spot in the reaction mixture clearly indicate complete consumption of the starting material and completion of the reaction.

An important parameter often used for qualitative analysis of TLC is the R_f value (retention factor, Fig. 6.3.15). If two spots travel the same distance or have the same R_f value, then it might be concluded that the two components are the same molecule. However, identical R_f values do not necessarily mean identical compounds. For R_f value comparisons to be valid and reproducible R_f values to be obtained, TLC plates must be run under the same exact conditions with respect to chamber saturation, composition of solvents, temperature, etc. As shown in Figure 6.3.14, the authentic sample of *N*-acetylglucosamine-1-phosphate (Lane B) and *N*-acetylglucosamine-1-phosphate product from the reaction mixture (Lane C) have the same R_f value, providing evidence that the desired product was obtained.

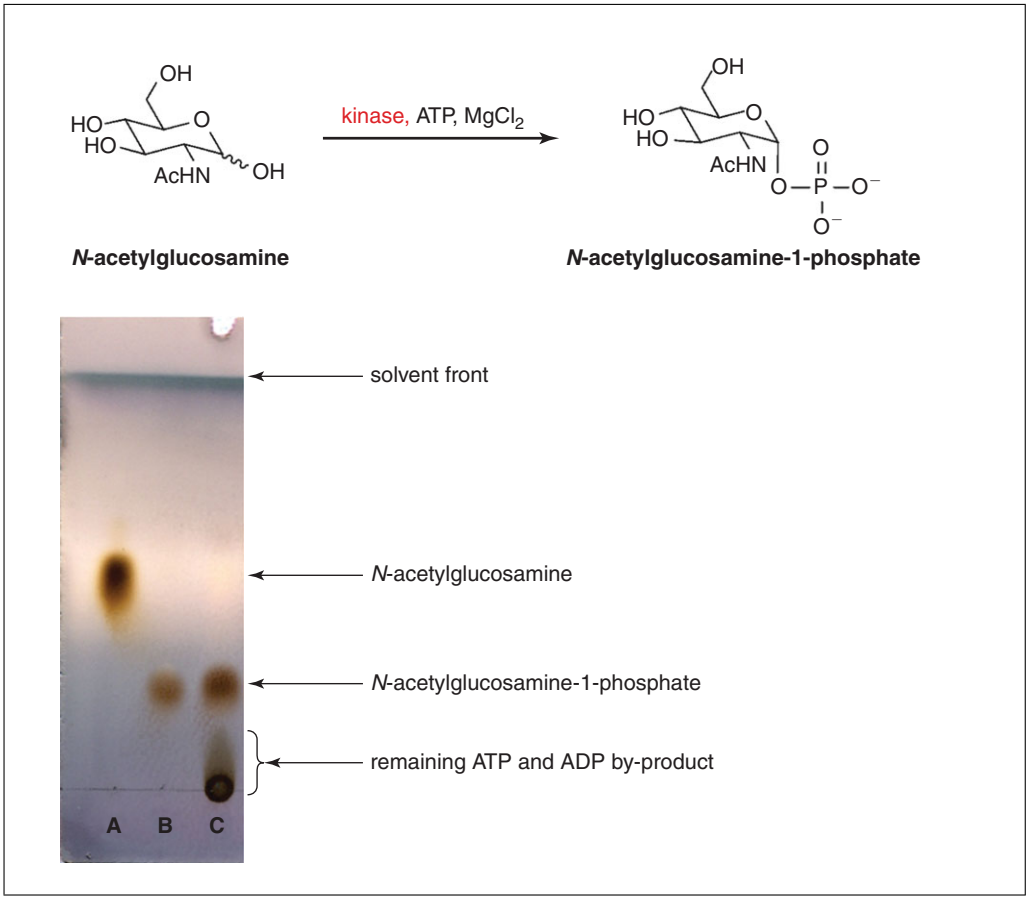


Figure 6.3.14 Use of TLC to monitor the progress of an enzymatic synthesis reaction, adapted from Zhao et al. (2010). The developing solvent used was 2:1:1 (v/v/v) *n*-butanol:acetic acid:water for highly polar carbohydrates. The developed plate was stained with *p*-anisaldehyde stain. Lane: A, *N*-acetylglucosamine; B, *N*-acetylglucosamine-1-phosphate authentic sample; C, reaction mixture of *N*-acetylglucosamine and ATP using a kinase. The *N*-acetylglucosamine and *N*-acetylglucosamine-1-phosphate show dark/light brown on the TLC plates. The authentic sample of *N*-acetylglucosamine-1-phosphate and the *N*-acetylglucosamine-1-phosphate product have the same R_f value.

Further techniques such as switching the solvent system, co-spotting, and multiple development are helpful to make the final conclusion when two compounds have very similar R_f's (also see Troubleshooting).

Digital imaging systems for TLC

The unique visual impression provided by a TLC plate (as a color image) makes it perfectly suitable for digital acquisition and evaluation. Similar techniques of digital imaging have already been widely used for photographing gels and for gel-based quantitation of protein and nucleic acids (see *UNITS 2.2*, *7.4*, and *7.5*). TLC visualization systems equipped with illumination units and digital cameras have thus been designed. For the CAMAG TLC Visualizer (*http://www.camag.com/en/tlc_hptlc/products/evaluation_documentation_tlc-ms_biolumi nenscence/tlc_visualizer.cfm*), the camera captures visible polychromatic light emitted either by fluorescent substances or from the TLC layer background under various illumination modes such as 254-nm UV, 366-nm UV, visible/white light, or white transmitted light. Compatible software (CAMAG winCATS) function as powerful tools for background correction, image archiving, data analysis, and multiple images/plates comparison.

Troubleshooting

Common problems involved in TLC experiments, their possible causes, and the corresponding tips and solutions are summarized in Table 6.3.2.

Variations

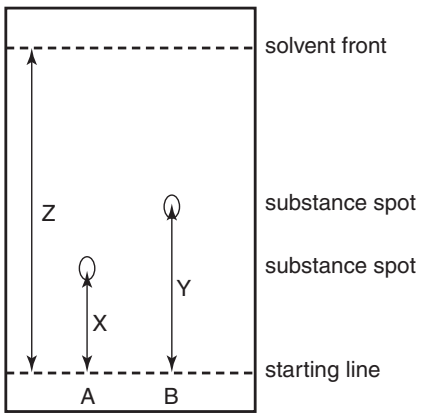
Preparative TLC

This technique is essentially the same as the analytical TLC described in this unit. However, a larger and more thickly coated plate

Table 6.3.2 Troubleshooting Guide for TLC

Problem	Possible cause	Solutions
TLC looks like a huge smear	Reaction is in a high-boiling-point solvent such as DMF, DMSO, pyridine, amine solvents	Spot the plate as usual and place it in a flask under high vacuum (oil pump) for a few minutes, then run it. Do a mini aqueous work-up[a] on a small amount of the reaction mixture in a small centrifuge tube (1.5 ml), then use the organic layer for TLC.
Reactant and product have very similar R_f's	Poor separation/resolution	Try co-spotting: if you are monitoring a reaction using TLC, make sure to spot the starting material, the reaction mixture, and a co-spot of both. Try multiple development with or without change of eluent. Change solvent systems. Try product- or reactant-specific stains.
Not sure if the compound is stable with respect to silica gel or not	Compounds may be acid sensitive	Run a 2-D TLC in a square plate using the same solvent system. If the sample is stable, all spots will appear on the diagonal.
Compound is unstable on silica	—	Use gas chromatography (GC) or run an NMR directly with a sample of the reaction mixture
For air-sensitive reaction, difficult to get a TLC sample without opening the reaction flask	—	Insert a capillary spotter into a disposable needle, and put the needle into the septum. Use the spotter to get your sample, and remove the needle. This procedure involves minimal exposure to air.
The reaction mixture clogs the capillary spotter and cannot be spotted onto a plate	Heterogeneous or viscous reaction mixture	Do a mini aqueous work-up[a] on a small amount of the reaction mixture in a small centrifuge tube, then use the organic layer for TLC
Compounds stay on the baseline of TLC	Very polar compounds	A. Try these solvent systems: ethyl acetate:2-propanol:water (for mono/disaccharides, Cai et al., 2009) $CH_2Cl_2/CH_3OH/NH_4OH$ (for amines/amides, Cai et al., 2007) *n*-butanol/acetic acid/H_2O (for carboxylate, phosphate, diphosphate, nucleotides, Zhao et al., 2010); B. Use reversed-phase silica gel [C_{18} (octadecyl) modified silica] plates.
TLC looks like a streak	Sample was overloaded or too many components in the sample	Try diluting the sample
Solvent front runs crookedly (skewed and distorted chromatogram lanes)	Either the adsorbent has flaked off the sides of the plate or the sides of the plate are touching the sides of the TLC chamber	Carefully cut another TLC plate and try obtaining good cut edges. You can also use a spatula to gently wipe the cut edges to remove loose layer material.
Many random spots are seen on the plate (or after visualization/staining)	Contaminants on TLC plate	TLC plates must only be touched with the finger tips on the outer cut edges. Do not leave your fingerprints on the plates! These can be visible after derivatization by some staining reagents.

[a]Steps for mini aqueous work-up: (1) withdraw a small amount of the reaction mixture (<0.2 ml) into a small 1.5-ml centrifuge tube; (2) dilute the reaction mixture with ~0.3 to 0.5 ml of appropriate organic solvent (e.g., CH_2Cl_2 or ethyl acetate); (3) add water or aqueous solutions (e.g., $NaHCO_3$, HCl, or brine) and then perform an extraction in the centrifuge tube (just like using separatory funnel); (4) discard the aqueous phase using a pipet and use the organic layer for TLC.



$$R_f \text{ for A} = \frac{\text{distance from starting line to middle of spot A}}{\text{distance from starting line to solvent front}} = \frac{X}{Z}$$

$$R_f \text{ for B} = \frac{\text{distance from starting line to middle of spot B}}{\text{distance from starting line to solvent front}} = \frac{Y}{Z}$$

Figure 6.3.15 Calculation of R_f value. This figure shows how R_f can be measured and calculated for each spot observed on a TLC plate: the ratio of distance start–substance zone to distance start–solvent front. The R_f values are between 0 and 1. However, the optimum separation of compounds by TLC is usually achieved when R_f values are between 0.3 to 0.5. For scale-up to preparative separations, the TLC solvent system's polarity must be decreased to lower the R_f between 0.15 and 0.3.

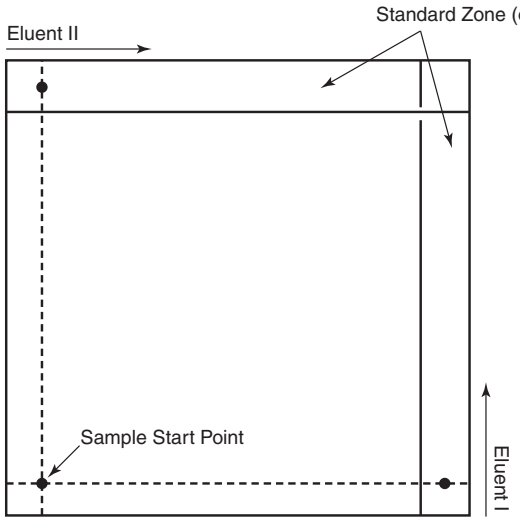


Figure 6.3.16 Schematic diagram of a 2-D TLC.

is used, and it is developed in a large TLC chamber (Fig. 6.3.10). The thickness of the analytical TLC layer is 0.25 mm, while a preparative layer is usually 0.5 to 1 mm (up to 2 mm). Compared with column chromatography, this preparative separation method is actually rather expensive considering its small separation scale. Nonetheless, it is extremely useful for separating valuable natural product extracts of small amounts of material (Gardner, 1968) or any components that are inseparable using column chromatography.

A glass TLC plate of dimensions 20 × 20 cm is often used for preparative TLC. Unlike analytical TLC samples, samples for preparative TLC should be loaded as a streak over the whole width of the plate (for devices and techniques for preparative sample application, see Kim, 1998). However, analytical TLC must be performed before preparative TLC to determine a suitable solvent system. After development and separation, mark the desired band(s) with a pencil as visualized by UV light. Then, use the edge of a spatula to scrape the band(s) off the plate. Finally, wash the desired compound(s) off the silica gel with a small fritted-glass funnel using a small amount of proper TLC eluent.

Two-dimensional TLC

2-D TLC is a very useful method for the identification of samples with a large number

of components, such as crude natural product extracts (Waksmundzka-Hajnos et al., 2006) and patient urine samples. In this technique, the spotted sample (on one edge of a square plate) is first developed in one direction; then, after drying, the plate is turned 90° and developed again with a different mobile phase (Fig. 6.3.16). Thus, it takes advantage of the different separation powers of two solvent systems.

If the same solvent system is used in two directions, theoretically all the substances will be visualized on the diagonal. Nonetheless, it becomes a useful approach to determine if a reaction or sample decomposition has occurred during TLC. Deviations from the diagonal line will be observed under such circumstance (also see Troubleshooting).

Acknowledgments

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Internet Resources

ftp://ftp.mn-net.com/english/Flyer_Catalogs/Chromatography/Catalog-en/KATEN200001-4TLC-www.pdf

MACHEREY-NAGEL: Basic Principles of TLC and product catalog.

http://courses.chem.psu.edu/chem36/Experiments/PDF's_for_techniques/TLC.pdf

TLC student experiments and the principles of separation.

http://www.discoverysciences.com/uploadedFiles/Home/ChromAccessories_TLC_PlatesNAcc_p188_198.pdf

Grace TLC catalog with brief introduction to TLC and solvent system selection.

http://www.ochemonline.com/TLC_stains

Useful TLC stains with illustrations of selected stained TLC plates.

http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=8659122

Commercial TLC derivatization reagents from Sigma-Aldrich. Some of these reagents can be easily prepared according to Table 6.3.1.

http://www.mn-net.com/chroma/TLCStart/Sprayreagents/TLCdetectionreactions/tabid/5578/language/en-US/Default.aspx

More TLC detection reagents from MACHEREY-NAGEL with preparation and application guide.

http://www.camag.com/en/tlc_hptlc/products/evaluation_documentation_tlc-ms_bioluminenscence/tlc_visualizer.cfm

CAMAG TLC Catalog with detailed introduction to the TLC visualizer.