Thin-Layer Chromatography

Thin-layer chromatography (TLC) is used as a tool for the determination of compound identity and purity. Most commonly, this technique is used to monitor the progress of a chemical reaction or to assay fractions collected from a larger chromatographic separation (e.g., column chromatography). TLC is typically performed using commercially available glass plates which are coated with a thin layer of adsorbent (TLC plates). Silica gel and alumina are polar adsorbents commonly used to separate compounds of low to medium polarity. Highly polar compounds will "stick" to polar adsorbents and may be purified alternatively using C18 reversed-phase TLC plates (glass plates coated with a highly lipophilic adsorbent). In reversed-phase separations, nonpolar compounds will "stick" to the adsorbent, making it possible to separate highly polar compounds. Regardless of the adsorbent used, TLC can be used to run test separations in preparation for column chromatography.

This unit describes a method for spotting test compound onto a TLC plate and developing the plate in a suitable solvent system, as well as the various methods for visualizing the results, which are then used to calculate retention factor (R_f) values (see Basic Protocol). The Alternate Protocol describes co-spotting the plate with candidate compounds in order to identify the unknown sample without relying on R_f values. Instructions for cutting TLC plates are given in Support Protocol 1, and a procedure for preparing spotters is given in Support Protocol 2.

BASIC PROTOCOL

THIN-LAYER CHROMATOGRAPHY

The preparation of a TLC plate is accomplished by dissolving the mixture of compounds to be separated in a suitable solvent (e.g., acetone, chloroform, ethyl acetate), spotting the mixture onto a TLC plate, and allowing the solvent to evaporate. The TLC plate is then developed in a TLC chamber containing a suitable solvent system (Fig. A.3D.1). The solvent in the chamber is drawn upward onto the plate by capillary action and "carries" along the compounds present in the mixture. Separation of compounds occurs because each compound is retained differently on the adsorbent. When the solvent has eluted to the top of the plate, the TLC plate is removed from the chamber. Only colored compounds may be visualized by the naked eye following development of the TLC plate; as many compounds are not colored, an alternative method for visualizing the plate is necessary. There are numerous methods for visualizing TLC plates (see Zweig and Sherma, 1972, for more information). Three different methods: destructive, semidestructive, and nondestructive, are described here.

Following visualization, the retention factor of each spot is determined. The retention factor, or R_f value, measures how strongly each compound is retained on the adsorbent in a particular solvent system. Compounds with large R_f values migrate higher up the plate than compounds with small R_f values.

Materials

Test compound
Appropriate test compound volatile solvent
Appropriate solvent system (eluent)
50% to 98% sulfuric acid (destructive visualization)
Solid iodine (semidestructive visualization)

Thin-layer chromatography (TLC) plate, cut to size (see Support Protocol 1)

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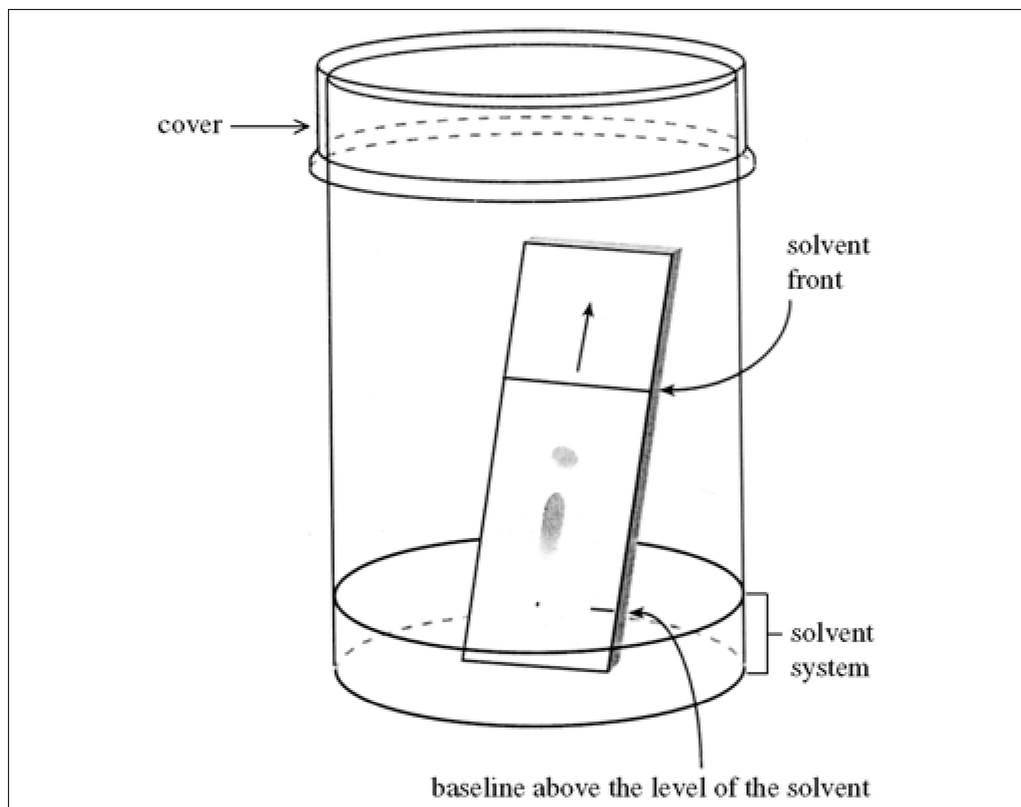

Figure A.3D.1 Diagram of a TLC developing chamber.

TLC spotter (see Support Protocol 2)
Wide-mouthed TLC chamber or beaker with lid (Fig. A.3D.1)
Filter paper
110°C oven (destructive visualization)
Hand-held UV light source (nondestructive visualization)

Spot a TLC plate

1. Using a pencil, draw a faint line at the edge of the plate ~1 cm from the bottom of a thin-layer chromatography (TLC) plate cut to an appropriate size.

   This is the baseline of the TLC plate. If spotting more than one compound along the bottom of the TLC plate, it may be helpful to make the appropriate number of notches (very faintly) on the baseline (~1 spot every 5 mm) to mark the position of each spot (Fig. A.3D.2).

   IMPORTANT NOTE: *Do not draw heavily on the TLC plate or the adsorbent will be scratched away.*

2. Dissolve the mixture of compounds to be analyzed in a volatile solvent (e.g., acetone, ethyl acetate, dichloromethane).

   If using a C18 reversed-phase TLC plate, the compound may dissolve only in water/organic solvent mixtures. Dissolve the compound in as little water as possible.

3. Draw a small amount of the dissolved mixture up into the tip of a TLC spotter and briefly touch the tip of the spotter onto the baseline of the TLC plate in the appropriate position to deposit a small amount of the sample in the form of a “spot.” Make the spot as small as possible (typically 2 to 3 mm).

   IMPORTANT NOTE: *The longer the tip of the spotter is held on the plate, the larger the spot will be.*

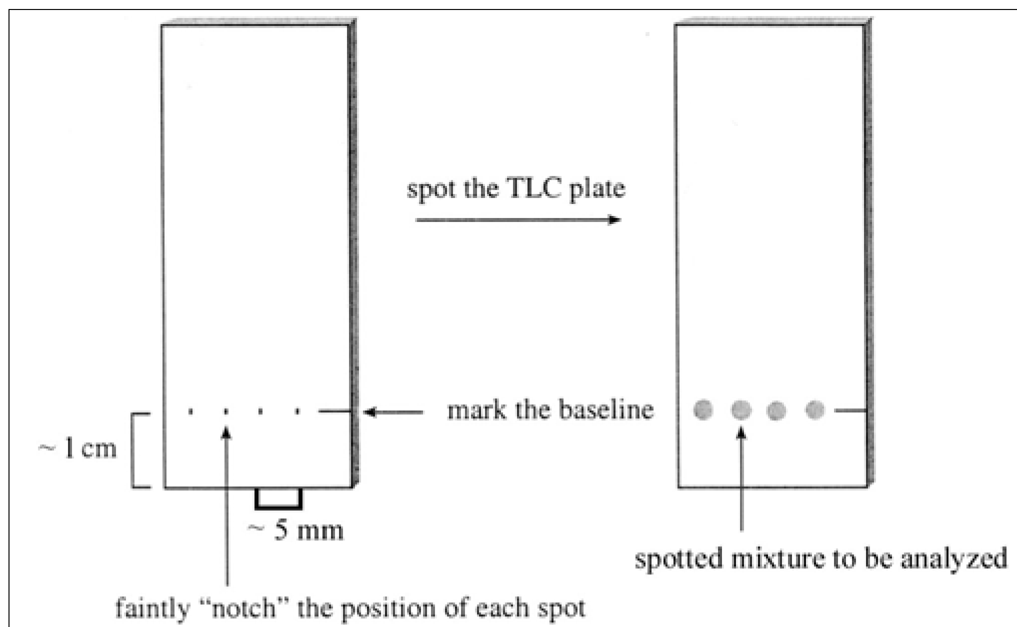

Figure A.3D.2 Spotting a TLC plate. The baseline is ~1 cm from the bottom and spots are ~5 mm apart.

4. Let the solvent in the spot evaporate and repeat the procedure (step 3) in the same place on the TLC plate in order to accumulate a sufficient amount of the compound at the baseline of the plate.

   IMPORTANT NOTE: *Be careful not to make the spots too concentrated, as mixtures of compounds that have been overloaded onto the TLC plate will appear "streaky" and unresolved when developed.*

   Compounds dissolved in water/organic solvent mixtures will take longer to dry.

   If the compound absorbs UV light, the plate can be visualized by UV absorption to determine whether enough compound has been loaded onto the plate (see step 9c).

5. Repeat step 4 along the baseline of the TLC plate until the desired number of mixtures to be analyzed have been spotted.

   IMPORTANT NOTE: *Do not spot mixtures too closely together along the baseline. Overlapping spots will "bleed" into each other's path of migration, making it difficult to identify the origin of each spot (see step 1).*

Develop plate

6. Choose an appropriate solvent system (eluent) to develop the TLC plate. Pour the chosen eluent into a wide-mouthed TLC chamber or beaker to a depth of no more than 5 mm, and cover the chamber to prevent evaporation. If necessary, line the inside edges of the chamber with filter paper in order to saturate the chamber atmosphere with the eluent vapors and prevent solvent evaporation.

   Choosing an eluent is often more complicated than it sounds. Each separation carried out on a TLC plate will require a different solvent system and will depend upon the adsorbent chosen for the separation. In general, organic solvent systems are used for separations carried out on silica gel or alumina, while water/acetonitrile or water/alcohol solvent mixtures are used for separations carried out on C18 reversed-phase medium. Refer to the CRC Handbook of Chromatography (Zweig and Sherma, 1972) for guidance in choosing a solvent system for a given separation.

7. Place the TLC plate into the chamber, making sure the baseline is above the level of the eluent in the chamber. Cover the chamber and allow the solvent to travel upward on the TLC plate (Fig. A.3D.1).

   Development of silica gel or alumina TLC plates may take only a few minutes, whereas the development of a C18 reversed-phase TLC plate may take considerably longer.

   Aqueous solvent systems elute up the TLC plate much more slowly than organic solvent systems.

8. When the eluent nears the top of the plate, remove the plate from the chamber, mark the position of the solvent front, and allow the eluent to evaporate in a fume hood.

   CAUTION: *Do not breathe solvent vapors.*

   C18 reversed-phase TLC plates developed with aqueous solvent systems will take several minutes to dry.

Visualize TLC plate

9a. *Destructive visualization:* Spray the TLC plate with 50% to 98% sulfuric acid, then heat the plate in a 110°C oven for several minutes.

   This method is termed destructive visualization because any compound present on the plate will decompose to give a dark spot. This is not the only destructive visualization method available. There are numerous "sprays" and "dips" that can be used to visualize compounds containing certain functional groups (Zweig and Sherma, 1972).

9b. *Semidestructive visualization:* Assemble a wide-mouthed, covered chamber containing a few crystals of iodine. Place the TLC plate inside the chamber and let it stand for several minutes. Remove the plate and circle the colored spots with a pencil.

   Most, but not all, compounds present on the plate will absorb iodine vapors and change color. Over time, the spots will disappear.

9c. *Nondestructive visualization:* Shine either long-wave UV light (dark spots against a green background) or short-wave UV light (glowing spots against a dark background) with a hand-held UV light source to visualize compounds.

   CAUTION: *UV light is damaging to the eyes and exposed skin. Protective eyewear should be worn at all times while using a UV light source.*

   UV light can be used to visualize certain compounds on TLC plates. This method is termed nondestructive visualization because non-photolabile compounds generally do not decompose under these conditions. Commercially available TLC plates contain a fluorescent additive that glows bright green when the plate is placed under long-wave UV light. Compounds containing a UV-absorbing chromophore are termed UV-active and can be visualized using this method. In general, these compounds will appear as dark spots on a fluorescent green background when viewed under long-wave UV light. When viewed under short-wave UV light, the plates remain dark and the compounds glow. In either case, visible spots should be circled with a pencil for future reference. Hand-held UV lamps with both long-wave and short-wave UV light are available commercially for this purpose.

Determine R_f value

10. Measure the distance from the baseline to the center of the spot, which is the distance the spot traveled on the TLC plate.

11. Measure the distance from the baseline to the solvent front, which is the distance the solvent traveled on the TLC plate.

12. Divide the distance the spot traveled (step 10) by the distance the solvent traveled (step 11). This ratio is the R_f value and should be in the range of 0.0 to 1.0.

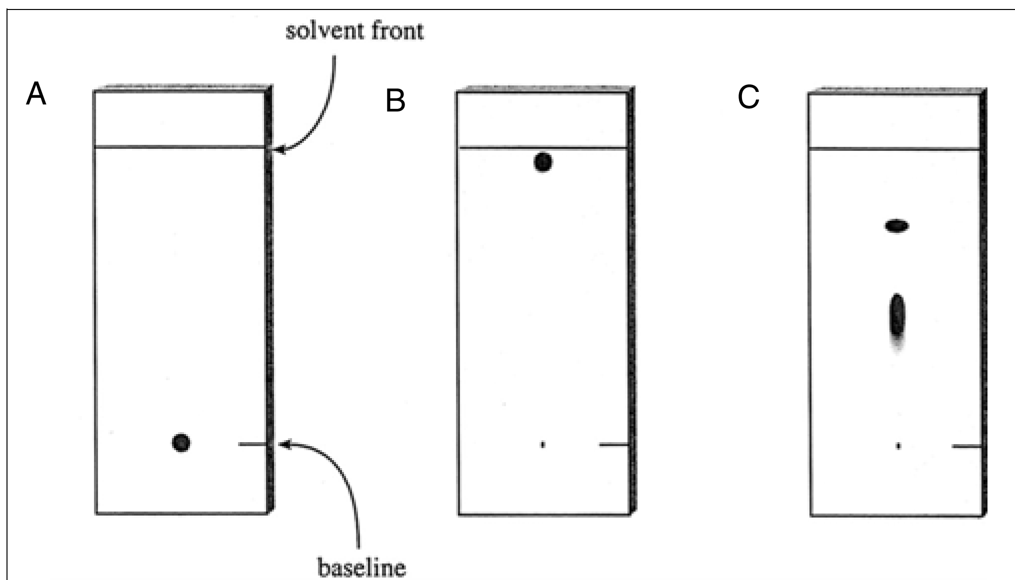

Figure A.3D.3 Results of TLC. **(A)** The spot has a R_f of 0.0. For silica gel adsorbent, this means that the eluent is not polar enough. For C18 reversed-phase adsorbent, this means that eluent is too polar (water content too high). **(B)** The spot has a R_f of 0.94. For silica gel adsorbent, this means that the eluent is too polar. For C18 reversed-phase adsorbent, this means that the eluent is not polar enough (organic content too high). **(C)** The mixture actually contains two compounds, as is made apparent when the TLC plate is developed using a suitable eluent. The spots have R_f values of 0.29 and 0.67, respectively.

R_f value = (distance the spot traveled)/(distance the solvent traveled) = 0.0 to 1.0

13. Evaluate the R_f value.

Values less than 0.2 or greater than 0.8 are not particularly informative. These spots may contain mixtures of compounds that were not separated during the development of the plate (Fig. A.3D.3). Spots with R_f values of <0.2 or >0.8 should be reevaluated by adjusting the polarity of the eluent to obtain R_f values within the range of 0.2 to 0.8.

CO-SPOTTING A TLC PLATE

ALTERNATE PROTOCOL

Although the R_f value of a particular spot may give some information about the identity of a compound, it is not always exactly reproducible. For example, an R_f value may vary depending upon the amount of sample spotted on the plate. For this reason, an R_f value alone cannot be used as a certain indication of compound identity. However, the identity of a spot can be determined by co-spotting the unknown compound being analyzed with standards of what the compound may be, or “authentic” compounds. In this protocol, a method for determining the identity of an unknown compound as either authentic compound A or B is described (Fig. A.3D.4).

Additional Materials *(also see Basic Protocol)*

Unknown compound
Authentic compounds A and B
2 × 5–cm TLC plate (see Support Protocol 1)

1. Cut a TLC plate that is wide enough to hold 4 spots at the baseline (~1 spot every 5 mm; see Support Protocol 1).

The first three positions will be used to spot each compound by itself. The fourth position will be used to spot all three compounds together.

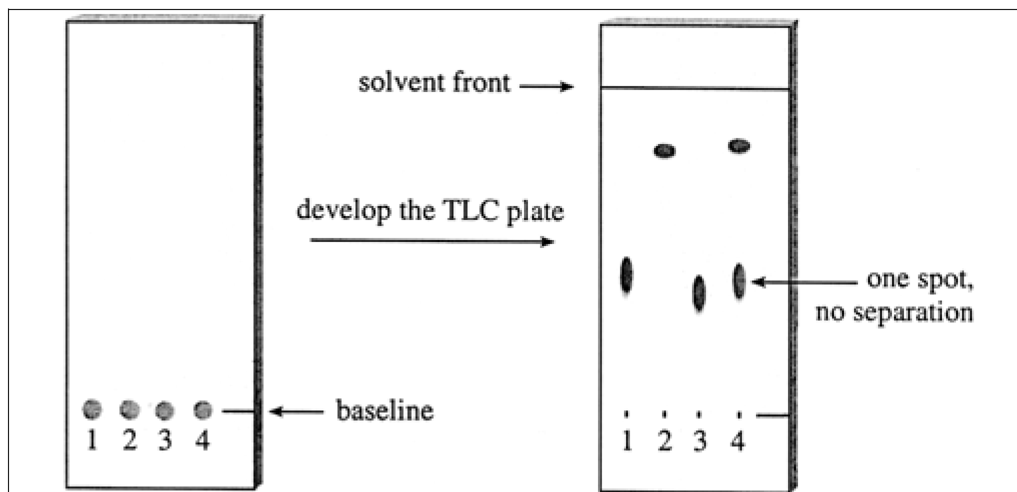

Figure A.3D.4 Co-spotting a TLC plate (see Alternate Protocol). Lane (1) unknown compound. Lane (2) authentic compound A. Lane (3) authentic compound B. Lane (4) mixture of authentic compounds A and B and unknown compound. Notice that the unknown (lane 1) and authentic compound B (lane 3) have similar R_f values (0.44 and 0.40, respectively) when run side-by-side on the TLC plate. Upon closer examination of lane 4, it is shown that, in fact, the unknown and authentic compound B are identical.

2. Spot the unknown compound at the first and fourth positions on the TLC plate. Allow the solvent to evaporate completely.
3. Spot authentic compound A in the second and fourth positions. Again, allow the solvent to evaporate completely.
4. Spot authentic compound B at the third and fourth positions.

   The TLC plate should be spotted as in Figure A.3D.4.

5. Develop the TLC plate using a suitable eluent chosen previously for the unknown (see Basic Protocol, steps 6 to 8).
6. Visualize the developed TLC plate (see Basic Protocol, step 9 to 13).

   The TLC plate should demonstrate the results shown in Figure A.3D.4. Notice that the unknown and authentic compound B have similar R_f values when run side-by-side on the TLC plate. Upon closer examination of the fourth position, it is shown that, in fact, the unknown and authentic compound B are identical.

SUPPORT PROTOCOL 1

CUTTING TLC PLATES

Precoated TLC plates (10 × 20 cm) are commercially available with a variety of adsorbents. Before performing TLC, the larger glass plates need to be cut down to a workable size. Prescored TLC plates are commercially available and can simply be broken at the right positions. Alternatively, cutting can be done manually using a cutting tool, as described here. This protocol produces approximately twenty 2 × 5–cm plates. Longer plates may be needed for applications with longer elution times, which are generally required for more difficult separations. The width of the plate can be adjusted to accommodate the number of spots that are to be loaded and the size of the chamber used to develop the plate.

Materials

10 × 20–cm TLC plates
Glass cutter: diamond cutters preferred

1. Place a 10 × 20–cm TLC plate adsorbent-side-down on a large piece of filter paper or paper towel.

   IMPORTANT NOTE: *Do not use printed paper as it contains dyes and chemicals that may be transferred to the adsorbent on the plate.*

   IMPORTANT NOTE: *When handling precoated glass plates, always wear gloves or be sure to handle with clean hands. Dirt or oil from the hands can be transferred to the adsorbent.*

2. To guide the cut, hold a ruler down the center of the plate lengthwise and score the plate using a glass cutter (diamond cutters work the best). Break the plate along the score, producing two ~5 × 20 cm plates.

   CAUTION: *Cut TLC plates are very sharp.*

   IMPORTANT NOTE: *C18 reversed-phase TLC plates may "flake" when broken along the scored line.*

3. Place each plate adsorbent-side-down on the filter paper. Holding the ruler as a guide, make a series of scores across each plate, ~2 cm apart. Break the plates along the scores to make approximately twenty 2 × 5–cm TLC plates.

   TLC plates of this size should be appropriate for 4 to 5 spots.

SUPPORT PROTOCOL 2

PREPARATION OF TLC SPOTTERS

The spotter is the apparatus used to load the mixture of compounds to be separated onto the TLC plate. It is appropriately named because it makes a "spot" on the plate. They are easily prepared from capillary tubes, as described here. Alternatively, commercially available, disposable micropipets can be used as spotters. These are sold in packages of 100 and are available from most scientific supply companies (e.g., 1 μL; Drummond).

Materials

~1 × 100–mm capillary tubes, open-ended

1. Put the center of the open-ended capillary tube into a small, blue Bunsen burner flame. Hold it in the flame until the center of the capillary tube begins to liquefy.

   IMPORTANT NOTE: *Do not leave the capillary tube in the flame too long or it will melt the edges of the tube together in the center.*

2. Immediately remove the capillary tube from the flame and pull the ends in opposite directions (Fig. A.3D.5).

3. Break the capillary tube in the center to make two spotters.

   Several spotters may be needed for loading different compounds onto the TLC plate.

   It is good practice to use a different spotter for each sample; however, in some cases it may be necessary only to "wash" the spotter before using it again (e.g., for analyzing fractions collected during column chromatography). To wash a spotter, simply draw a suitable solvent (e.g., acetone) up into the thin end (tip) of the spotter. Release the acetone onto a paper towel by touching the tip of the spotter to the towel. Repeat several times to ensure that all of the previous sample has been removed from the spotter.

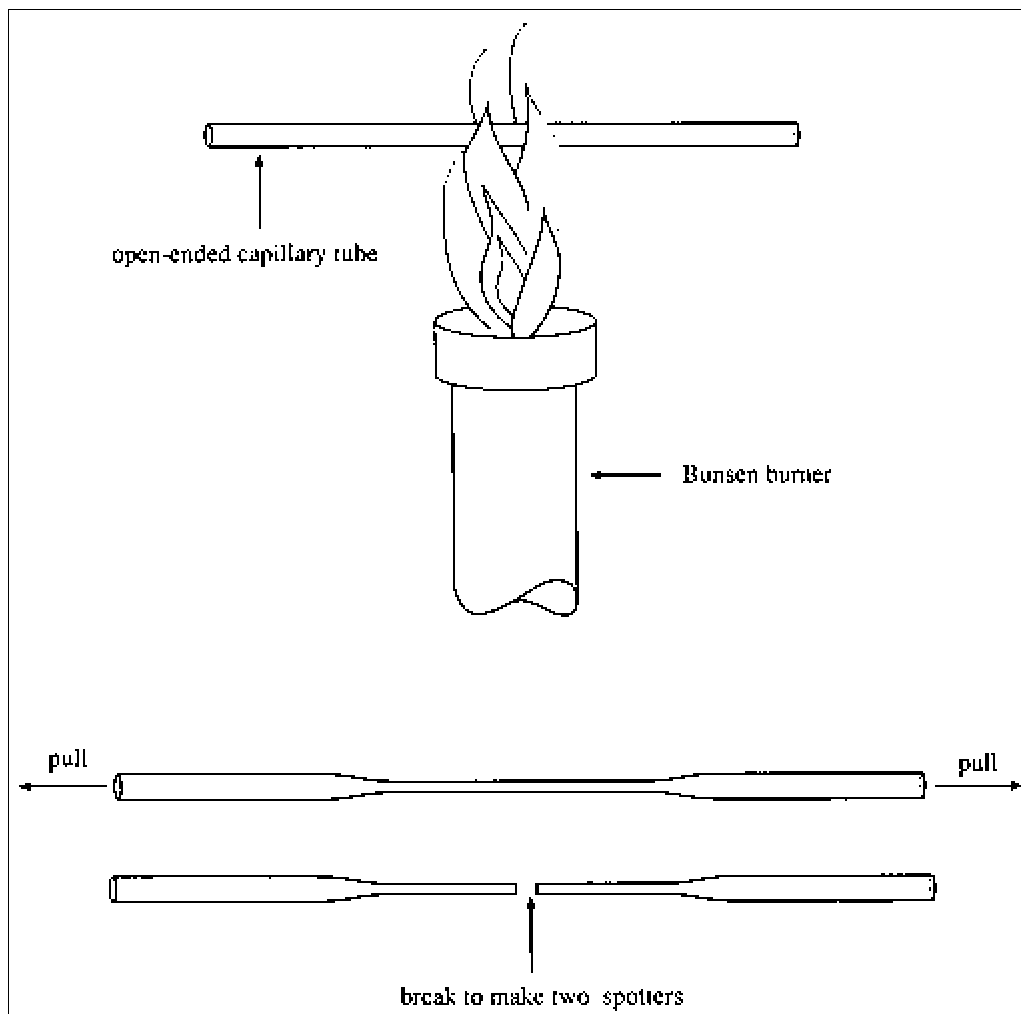

Figure A.3D.5 Preparation of TLC spotters.

LITERATURE CITED

Zweig, G. and Sherma, J. 1972. CRC Handbook of Chromatography, Volumes I and II. CRC Press, Cleveland, Ohio.

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