Thin Layer Chromatography

Chromatography is a word used to encompass a range of techniques in which mixtures of pure substances are separated into the individual substances by using a mobile phase (usually a liquid or gas) to push the mixture along a stationary phase (usually a solid or liquid coated on a solid). Because the individual substances have different molecular structures, they interact differently with both the stationary and mobile phases, and consequently are "pushed" at different rates by the mobile phase. A number of chromatographic techniques are summarized in Table 1.

Table 1: Types of Chromatography

Type	Stationary phase	Mobile phase
Gas chromatography (GC)	Polar or non-polar liquid	Helium gas
High Performance liquid Chromatography (HPLC)	Solid	Liquid
Gel Permeation Chromatography (GPC)		
Thin Layer Chromatography (TLC)	Solid on glass or plastic plate	Liquid

Thin-Layer Chromatography (TLC) is a simple and inexpensive technique that is often used to judge the purity of a synthesized compound or to indicate the extent of progress of a chemical reaction. In this technique, a small quantity of a solution of the mixture to be analyzed is deposited as a small spot on a TLC plate, which consists of a thin layer

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of silica gel (SiO_2) or alumina (Al_2O_3) coated on a glass or plastic sheet. The plate constitutes the stationary phase. The sheet is then placed in a chamber containing a small amount of solvent, which is the mobile phase. The solvent gradually moves up the plate via capillary action, and it carries the deposited substances along with it at different rates. The desired result is that each component of the deposited mixture is moved a different distance up the plate by the solvent. The components then appear as a series of spots at different locations up the plate. Substances can be identified from their so-called R_f values. The R_f value for a substance is the ratio of the distance that the substance travels to the distance that the solvent travels up the plate. For example, an R_f value of 0.5 means that the spot corresponding to the substance travels exactly half as far as the solvent travels along the plate.

The Process of TLC. Performing a TLC analysis consists of a number of steps: preparing a spotting capillary; marking the TLC plate; spotting the TLC plate; developing the TLC plate; drying the plate; visualizing the substance spots, and measuring the R_f values. We consider these steps in turn.

Preparing a spotting capillary. Glass capillaries used for spotting TLC plates are commercially available. However, it is occasionally necessary to make your own capillaries. To accomplish this, light a Bunsen burner and adjust for a medium flame. Hold a melting point capillary in the flame until it just begins to soften, then quickly pull the two ends of the capillary in opposite directions. The central, soft part of the glass will elongate and thin down to a capillary with very small diameter. Break the two pieces apart at the center of the thin portion to obtain two TLC spotting capillaries.

Marking the TLC Plate. Obtain a silica gel TLC plate that is approximately 2 cm wide and 5 cm long. Mark the TLC plate as follows using a pencil (pencil must be used rather than pen because inks are moved by many developing solvents). First, LIGHTLY draw a straight line parallel to the short dimension of the plate, about 1 cm from one end of the plate. Don't gouge the silica gel or make a trough with the pencil. Second, LIGHTLY make two small marks perpendicular to this line to divide the line into thirds. This subdivided line will serve as a guide for placing the substance spots, and as a point from which to measure R_f values. Third, LIGHTLY draw a second line parallel to the first line and about 1 cm from the other end of the plate. When you develop the plate, you will allow the solvent front to rise to this second line.

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Activating the TLC plate. Place your marked TLC plate in an oven at 50-60°C for 15-20 minutes to "activate" it. Activation involves driving off water molecules that bond to the polar sites on the plate.

Spotting the TLC plate. Place the narrow end of one of your capillaries into a vial containing a solution of the substance to be analyzed, and allow the solution to rise in the capillary; this will happen spontaneously. Once the capillary is loaded, hold it vertically just above the subdivided pencil line on the plate and centered in one of the three sections. Lower it until the narrow end of the capillary just touches the plate right on the pencil line. You will observe that some of the solution leaves the capillary and deposits on the plate. Leave the capillary in contact with the plate only briefly so that the spot is no larger than 1 mm in diameter, then raise the capillary. Allow the solvent to completely evaporate from the spot (if the solvent is water, evaporation will be slow; you can hasten it by putting the plate in the oven for 5 minutes). Then, if desired, make a second deposit on the same spot with the capillary. Again allow the solvent to completely evaporate.

Developing the TLC plate. Pour the desired developing solvent into a small wide-mouth glass or plastic bottle to a depth of about 4-5 mm. Using tweezers, pick up the TLC plate at the top, which is the end opposite where the subdivided pencil line is drawn. Place it carefully in the developing bottle so that it stands somewhat but not excessively tilted--that is, the bottom of the slide should be somewhat away from the wall of the bottle, whilc the top of the slide rests against the wall of the bottle. It is important that you not allow the TLC slide to tilt too much when in the developing bottle. If the slide is excessively tilted, solvent will not advance uniformly along the plate and development will not take place properly. Similarly, if the bottom of the slide is against the wall of the bottle, solvent will advance more rapidly up the edges of the slide than in the middle, causing the substances to be pushed toward the center of the slide as they move up. Leave the slide in the chamber until solvent has advanced to the top pencil line on the slide. Development normally requires at least 30 minutes. When the solvent front has advanced to the top pencil line, use the tweezers to withdraw the slide from the chamber.

Drying the Plate. Place the plate flat on a clean dry surface and allow the solvent to completely evaporate. If the solvent is not highly volatile, this can be facilitated by placing the slide on a flat surface in an oven at a temperature of 50-60 ^{o}C (higher temperatures will melt the plastic substrate material). When the plate is completely dry, it is ready for visualization.

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Visualization of the TLC Plate. If the substances being separated are colored, the spots can be seen without any further effort. Using a pencil, draw a boundary around each spot that matches the shape of the spot. Many substances are colorless (white) and do not show up on the white silica gel unless steps are taken to make them visible. There are a number of techniques for doing this. *First* is the technique of iodination. The dry plate is placed in a chamber containing a few crystals of iodine. The iodine vapor in the chamber oxidizes the substances in the various spots, making them visible to the eye. Once the spots are visible, they may be outlined with a pencil before the iodine coloration fades. *Second* is the ninhydrin technique, which is particularly effective for visualizing amino acid spots. In this method, a solution containing 0.2% ninhydrin in ethanol is sprayed on the dry plate. Alternately, the plate can be dipped in ninhydrin solution. In contact with an amino acid, ninhydrin displays a purple coloration that is easily seen. It usually takes a few minutes for this color to develop, so after the plate is sprayed, it is allowed to sit for several minutes. Placing it in a 50 ^{o}C oven will hasten the appearance of the purple color. Once this appears, the spots may be circled in pencil to permanently mark their positions. NOTE: NINHYDRIN STAINS THE SKIN PURPLE, TOO, DUE TO THE PRESENCE OF PROTEIN (AMINO ACIDS!). TO AVOID THIS, WEAR GLOVES WHEN HANDLING IT. *Third*, one may use TLC plates that have been loaded with a fluorescent substance that is uniformly distributed in the silica gel. Substances moving up the plate block this fluorescence at their locations. When the dried plate is viewed using a special UV light, the substance spots are visible for their lack of fluorescence on an otherwise uniformly fluorescing field. The spots may be outlined with pencil while being viewed in the light.

Measurement of R_f. The distance between the 2 horizontal pencil lines is the distance of solvent advance. The distance from the bottom pencil line to the center of a substance spot is the distance of advance of the substance. The ratio of substance advance distance to solvent advance distance is the R_f value for the substance. The figure shows a developed plate for a mixture consisting of 4 components with Rf values of about 0.05, 0.2, 0.5, and 0.9

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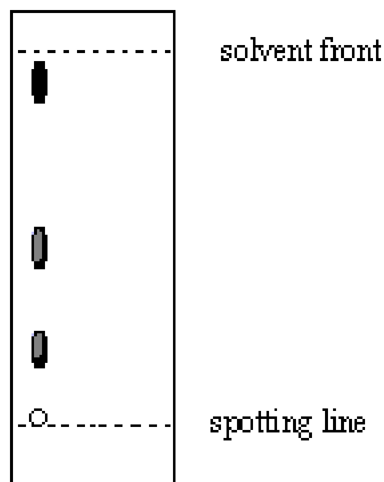

TLC of Inks. Before attempting to apply TLC to the challenging problem of separating and identifying amino acids, it is advisable to learn and practice the technique by applying it to mixtures that are easily visualized and separated. Inks provide an ideal practice vehicle for TLC because they normally contain several colored components that separate nicely in common solvents such as ethanol, acetone, or chloroform. Spotting the plate is also easy: it may be done simply by making a VERY small mark on the plate with the tip of a pen, just above the pencil line drawn across the bottom edge of the plate. Inks are of different types and colors, of course. Some are washable (water-soluble), others are permanent. Different types require different solvents for development. Common sources of ink are ball point pens, felt-tip markers, and roller ball pens. Bottled ink is still available for people using fountain pens. Particularly good ink sources are bottled inks by Parker, Sheaffer, and Mont Blanc; Sharpie marker inks (black, red, blue, orange, brown, yellow, green); Marks-a-Lot Stay sharp markers; and Sanford calligraphy pens.You should select inks from at least 2 different sources available in the lab and should find a solvent or mixture of solvents that separates each ink into its component colors.

Experimental Procedure. You will be assigned two ink samples to examine. Obtain four TLC plates, four TLC spotting capillaries, and 3 developing tanks (snap-top plastic vials). Mark your TLC plates with a narrow pencil line about 1cm from the bottom. Prepare spotting solutions of your ink samples in 1-dram vials by diluting 1 drop of ink with 9 drops of ethanol (this is called a 1-to-10 dilution). Insert one end of a spotting capillary into the first ink solution. You should see the ink solution rise in the capillary. Withdraw the capillary when the liquid has risen to a height of 1-2 cm. Hold the capillary vertically and briefly touch the filled end to the pencil line, about 5 mm from the left edge of the

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TLC plate. Liquid should flow from the capillary to the plate to form a spot. THIS HAPPENS RAPIDLY. If possible, lift the capillary before the spot gets any bigger than 1 mm in diameter. Allow the ethanol to evaporate. Touch the capillary to a Kimwipe to empty it of ink solution. Use the other end of the same capillary to draw up your second ink solution, and spot the plate about 5 mm from the right edge. Discard the capillary in the broken glass receptacle. Allow the ethanol to evaporate from the plate.

Into your first developing tank, pour ethanol to a depth of about 5 mm. Using tweezers to hold the top end of the spotted plate, lower the plate into the tank. Allow the bottom of the plate to rest on the bottom of the tank, with the top of the plate leaning against a tank sidewall. NOTE: IT IS VERY IMPORTANT THAT THE START LINE AND SPOTS BE ABOVE THE LEVEL OF SOLVENT. Close the tank and monitor the movement of solvent up the plate. When solvent has advanced to the top pencil line, remove the plate from the tank, and allow all solvent to evaporate. Use a pencil to outline each observed spot on the plate, preserving the shape of the spot.

If ethanol did not effect a separation of the ink into components, try another solvent. If you found that the ink moved along with or close to the solvent front, try a less polar solvent. If you found that the ink did not move at all with ethanol, try a more polar solvent (e.g., methanol). Your goal is to cleanly separate each of your ink samples into its constituents, each constituent producing a single TLC spot. Be aware that some inks contain only one component!

TLC of Amino Acids. TLC of amino acids is more difficult than TLC of inks, because amino acids are colorless. Therefore, not only can you not monitor their progress up the plate, but you cannot see the spots with the naked eye once the plate is fully developed and dried. To see the spots, it is necessary to use either the ninhydrin or the black-light visualization techniques. Of course, the latter works only if you use fluorescent TLC plates, which are available in the lab. Until you see the spots, you will not know whether or not a chosen solvent system has been effective in moving an amino acid or in separating a mixture. Therefore the process of finding an effective solvent system can be long and painstaking. For this reason, we will specify the solvent system that you are to use. As points of general information, amino acids are quite polar and tend to move on silica gel plates with polar solvents. They have R_f values close to 1 when water or concentrated ammonia is used as the developing solvent, probably because of their high solubility in water. Diluting a polar solvent with a less polar one results in smaller R_f values, roughly in proportion to the amount of less polar solvent used, Thus, alanine, glycine, threonine, and proline all have R_f values of around 0.60 when developed with a 50/50 mixture of water and n-propanol, and around 0.40 when developed with a 30/70

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mixture of concentrated NH_3 and n-propanol. The following procedure assumes the use of 50/50 water/n-propanol as the developing solvent, but you are free to try other polar/non-polar combinations.

Experimental Procedure. In the hood, prepare 10 mL of a mixture consisting of 50% 1-propanol and 50% water by volume, and pour about half of this into a clean developing tank. Make sure that the level of liquid in the tank is no higher than 5 mm, and close the lid. In a 1-dram vial, prepare a solution of about 0.001 g of your amino acid in 0.2 mL of water. Dissolve the acid, then draw some solution up in a spotting capillary and double-spot a properly marked and activated TLC plate. Allow the plate to dry for 5 minutes, then use tweezers to carefully lower the plate into the developing tank so that its bottom is submerged in the developing solvent. NOTE: IT IS VERY IMPORTANT THAT THE START LINE AND SPOTS BE ABOVE THE LEVEL OF SOLVENT. Close the lid, and allow the plate to develop until solvent has risen to the pencil line at the top of the plate. Remove the plate from the tank and place it in an oven at 50 ^{o}C to dry. When the plate is dry, visualize it using ninhydrin spray or iodination. Circle the amino acid spots with pencil, and calculate R_f values. Compare the measured R_f values with the values posted for the amino acids.

On this basis, you can narrow down the identity of your amino acid. In combination with other data that you obtain, this information will help you unambiguously identify your amino acid. Suppose that your amino acid has an R_f value similar to that of, say, alanine. You should then prepare a small amount of alanine solution and spot it alongside your amino acid on a new TLC plate. Develop, dry, and visualize the plate to confirm that your amino acid indeed has exactly the same R_f value as alanine, and that the spot is the same shape and color.

Finally, it is very important to be observant of detail in doing TLC. In addition to the R_f value for a substance, the shape of the spot produced by a particular developing solvent and the shade of color produced by iodine or ninhydrin can be characteristic of the substance. Please note all of these things. For example, when alanine, glycine, threonine, and proline are spotted side-by-side on a plate and developed with 70% n-propanol/30% conc NH_3, the following observations can be made:

Amino Acid	**Solvent**	**Spot Color**	**Spot Color**	**R_f**	**Spot**

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		after Iodination	with Ninhydrin	Value	Shape
alanine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	purple		elongated oval
alanine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.65	circle
glycine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	pink	0.25	elongated oval
glycine	50/50 water/n-propanol	white on brown bkgrnd	pink	0.55	circle
threonine	30/70 conc NH_3/n-propanol	white on brown bkgrnd	purple		elongated oval
threonine	50/50 water/n-propanol	white on brown bkgrnd	purple	0.57	circle
proline	30/70 conc NH_3/n-propanol	dark brown on brown bkgrnd	yellow with pink border		elongated oval
proline	50/50 water/n-propanol	white on brown bkgrnd	yellow with pink border	0.65	circle

Problems in TLC.

Over-large Spots. Sample spots made using TLC capillaries should be no larger than 1-2 mm in diameter, because component spots in the developed plate will be no smaller than, and will usually be larger than, the size of the initial spot. If the initial spot is larger than 2 mm in diameter, then components with similar R_f values may not be resolved

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because their spots will be so large that they will overlap considerably and may appear to be one large spot. Small initial spots, on the other hand, maximize the potential of complete separation of components.

Uneven Advance of Solvent Front. A common problem in TLC is uneven advance of solvent along the plate. Instead of a straight line, the solvent front may appear to bow either up or down in the center. Uneven advance of solvent leads to uneven advance of substance spots, and inaccurate R_f values result. A frequent cause of uneven solvent advance is the use of a developing chamber that does not have a flat bottom. Glass bottles usually have bottoms that curve upward from the edges to the center. If the bottom of the TLC plate is placed on this curved surface, the shape of the solvent advance line may mirror the shape of the container bottom. It is therefore important to use flat-bottomed developing tanks in TLC. A bowed solvent front may also result if too little developing solvent is placed in the chamber; if the plate is cut improperly, so that the sides are not exactly perpendicular to the bottom edge; and if the slide is excessively tilted in the chamber. Care in choosing and using a developing chamber is the best defense against curved solvent fronts.

Water is seldom used as a developing solvent because it has a tendency to produce a dramatically curved front. This may be due to its unusually high surface tension.

Streaking. Sometimes a substance will move along a TLC plate as a long streak, rather than as a single discrete spot. This is the result of spotting the plate with too much substance, more than the moving solvent can handle. The solvent moves as much substance as it can, but a substantial amount of substance is left behind. The substance is dragged along by the solvent leaving a trail of substance that may sometimes span the entire distance between the starting line and the solvent front. Streaking can be eliminated by systematically diluting the spotting solution until development and visualization show the substances moving as single spots, rather than elongated streaks.

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