

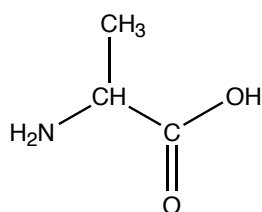
Experiment 4: Thin Layer Chromatography (TLC)

In *TOC*, read the introduction to Part 4, Chromatography, on pp 219-220, then read pp 221-235, Technique 17. View the videos: Thin layer chromatography (tlc) and How to visualize tlc spots.

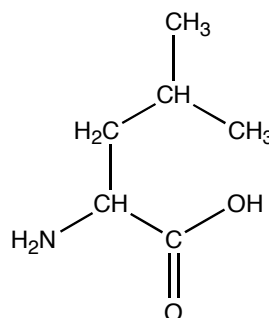
Thin layer chromatography (TLC) is an easy, convenient and inexpensive way to determine how many components are in a mixture and, in many instances, can be used to identify the components as well. In today's experiments, you will gain experience with both paper and thin layer chromatography, you will work with a variety of developing solvents, and you will use several different techniques to visualize the spots of a chromatogram.

Paper chromatography, which will be used to separate amino acids, is actually a form of partition chromatography (see *TOC* page 220). Water, a component of the developing solvent, forms hydrogen bonds with the fibers of the paper and serves as the stationary phase. The organic liquids that are also present in the developing solvent serve as the mobile phase. The components of the mixture are drawn up the paper to different heights, depending on their solubility in the mobile phase. The compounds that are more soluble in the organic liquid remain dissolved in the mobile phase longer than those that are less soluble and thus travel further up the paper.

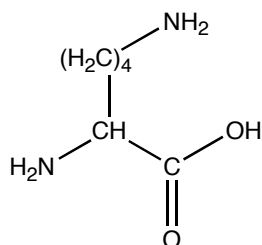
Proteins, large molecules found in all living organisms, serve a variety of functions in metabolism, such as catalysis, transport, storage, control of growth and immune protection. Amino acids are the building blocks of proteins. Every amino acid has an amino group, a carboxyl group and a distinctive side-chain. Nature uses twenty different amino acids to synthesize proteins. The four amino acids that you will separate by paper chromatography are alanine, leucine, lysine, and valine. Their structures are shown below.



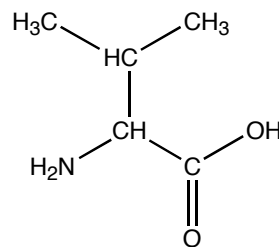
Alanine



Leucine

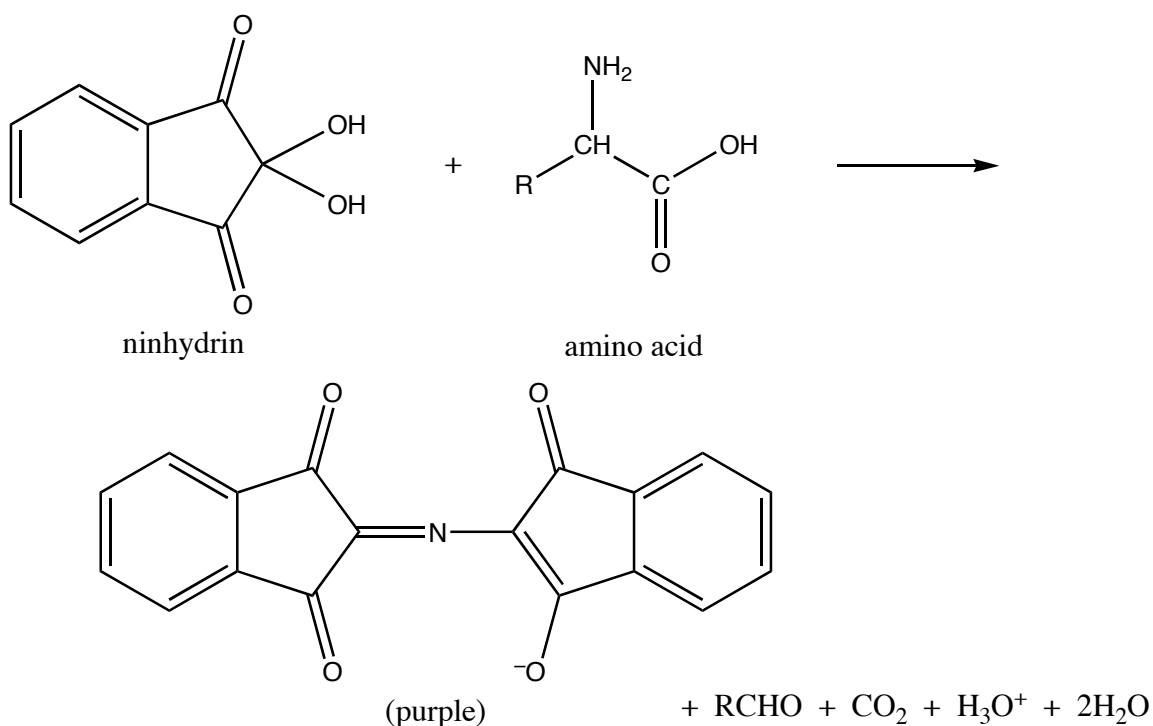


Lysine



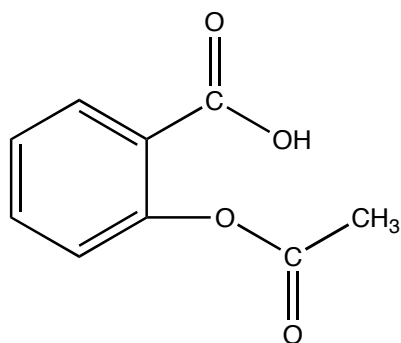
Valine

Amino acids are colorless compounds. In order to see the spots on the chromatogram, you will apply a solution of ninhydrin to the paper. Ninhydrin will react with the amino acid to produce a purple compound.

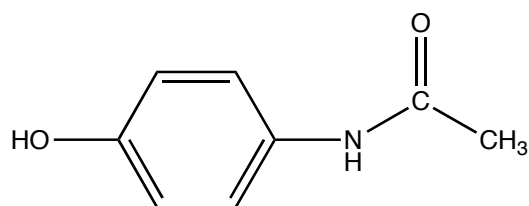


Silica gel will serve as the stationary phase in the thin layer chromatography procedures. Finding a solvent or mixture of solvents that serves as an effective mobile phase is the most difficult part of TLC. Often several different combinations of solvents are tested before one is found that will separate the compounds of interest successfully. You will observe the effect that different solvent systems have on the separation of the pigments in food dyes.

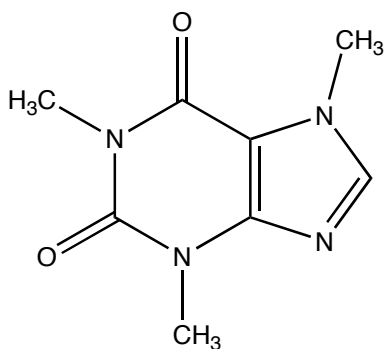
You will also use TLC to identify the active ingredient in an over-the-counter medicine tablet. The tablet you will test contains one of the following: acetylsalicylic acid (aspirin), acetaminophen (the active ingredient in Tylenol), caffeine, or diphenhydramine hydrochloride (the active ingredient in Benadryl). The structures of these four drugs are shown on the top of the next page. Like the amino acids, the medicine tablet ingredients will not be visible after the plate has been developed. The spots will be illuminated when viewed under short-wave ultraviolet light. Some of the spots will also change color when exposed to iodine vapors.



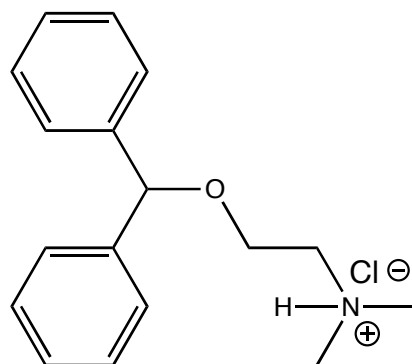
Acetylsalicylic acid



Acetaminophen



Caffeine



Diphenhydramine hydrochloride

Procedures

Separating amino acids using paper chromatography

Work with a partner.

Obtain a sheet of 13 x 18.5 cm Whatman no. 1 chromatography paper. When you handle this paper, hold it only on one of the long (18.5 cm) sides, which will be considered the “top” of the sheet. The amino acids from your fingers will contaminate the paper and lead to erroneous results if it is touched on the “bottom”. Lay the sheet of chromatography paper on a piece of notebook paper, and draw a line *in pencil, not pen*, 1.5 cm above the bottom. Make small marks along the line using the dimensions given in [Figure 1 on the Figs. 1, Expt. 4](#) page (you may bring the figure pages to lab). Write labels at the top as shown in the figure.

Use the small capillary tubes provided to make four spots, one of each amino acid (alanine, leucine, lysine and valine), along the pencil line. Follow the labels written at the top of the sheet. Your TA will assign each of you an “unknown” sample that contains one or more of these four amino acids. Spot this solution on the paper as well. For every spot you make, touch the capillary to the surface of the paper *quickly and lightly* so that the spot is approximately 2-3 mm in diameter. Allow the spot to dry, then re-apply the solution at the exact same place, again touching the paper quickly and lightly. Allow the spot to dry, and repeat one more time. After all six solutions have been applied to the paper in this manner, allow the spots to dry for five minutes. Working together, roll the paper into a cylinder with the spots on the outside, then staple it so that the edges do not overlap or touch. See [Figure 2 on the Figs. 1, Expt. 4](#) page.

Pour 50 mL of the amino acid developing solution into a 1000 mL beaker. The developing solvent is comprised of a four-to-one mixture of 1-butanol and glacial acetic acid that has been saturated with water. Position the cylinder inside the beaker with the bottom edge immersed in the solvent. Make sure the paper does not touch the glass. Place a piece of aluminum foil over the mouth of the beaker. Allow the chromatogram to develop undisturbed for 60 to 75 minutes. **Do not move the beaker while the chromatogram is developing!** When you remove the paper from the beaker, mark the solvent front with a pencil. Set the cylinder on notebook paper, and allow it to dry.

When the chromatogram is completely dry, remove the staples, and hang it from the clips in the fume hood. Wearing gloves, evenly coat the paper using the ninhydrin spray. Do not allow the paper to become dripping wet. Place the chromatogram in an oven set at 80° for about 5 minutes. Circle the spots with a pencil. Measure the distance from the origin to the center of each spot and the distance from the origin to the solvent front. Later, you will attach this chromatogram to your Results and Calculations sheet.

Optimizing the developing solvent for the separation of food dye pigments

Work on your own.

Obtain two TLC plates with the dimensions of 5 cm x 6.7 cm. Draw a line *in pencil, not pen*, 1 cm from the bottom along the short (5 cm) side of each plate. Be careful not to disturb the silica gel as you draw these lines! Use the small capillary tube provided to spot four spots, one of each color, along the line drawn on each of the plates (see [Figure 3 on the Figs. 2, Expt. 4](#) page). When spotting a TLC plate, touch the capillary to the surface of the plate *quickly and lightly* so the spot is very small. The spots will be highly colored since the food dye solutions are quite concentrated. Oftentimes it is necessary to re-apply a dilute solution to the spot (allowing the spot to dry in between applications) until the spots are highly colored, however this will not be necessary in this case.

[Figure 4 on the Figs. 2, Expt. 4](#) page shows the proper set-up for developing a TLC plate. Line a 250 mL beaker with a piece of filter paper. Place a small amount of the 3:1 isopropanol: concentrated ammonia developing solvent in the beaker. The liquid should cover the bottom of the beaker to a depth of about 0.5 cm; however, the level of the liquid *must* be below the line when the plate is placed in the jar (that is, less than 1 cm in depth). The filter paper lining will saturate the atmosphere within the beaker with solvent fumes. Fit a piece of aluminum foil over the mouth of the beaker. Place one of the plates that you have spotted in the beaker, cover it with the foil and allow the solvent front to move up the plate until it is approximately 1 cm from the top (one hour maximum). **Do not disturb the beaker while the chromatogram is developing!** Remove the plate and mark the solvent front with a pencil. Allow the plate to dry for a few minutes, then circle any visible spots with a pencil.

Into a filter paper lined 400 mL beaker, pour *either* pure isopropanol *or* 1:1 isopropanol: concentrated ammonia to a depth of about 0.5 cm. Develop the second plate in the same manner as the first using the chosen solvent. Mark the solvent front and circle the spots. Observe the chromatogram of someone who used the other solvent for his or her second plate.

Sketch diagrams of all three chromatograms in your notebook. Measure the distance from the origin to the center of each spot and the distance from the origin to the solvent front for each of the three chromatograms.

Analyzing a non-prescription medicine tablet with TLC

Work on your own.

Obtain a silica gel TLC plate with the dimensions of 6 cm x 6.7 cm. Draw a line *in pencil* 1 cm from the bottom along the short (6 cm) side of the plate. Be careful not to disturb the silica gel as you draw the line! Use the small capillary tube provided to spot 4 spots, one of each active ingredient solution (acetylsalicylic acid, acetaminophen, caffeine and diphenhydramine hydrochloride), along the line. Leave room for a fifth spot. Your TA will assign a non-prescription medicine tablet for you to analyze. Spot your TLC plate with the supernatant from a mixture of this crushed tablet and methanol. When applying these solutions to the plate, touch the capillary to the surface of the silica gel *quickly and lightly* so the spot is very small. In each case, reapply the spot, allowing it to dry in between applications, two more times.

Place a small amount of the ethyl acetate developing solvent in a 400 mL beaker. The liquid should cover the bottom of the beaker to a depth of about 0.5 cm. Line the beaker with a piece of filter paper to saturate the atmosphere within (see Figure 4 on Figs. 2, Expt. 4). Fit a piece of aluminum foil over the mouth of the beaker. Place the plate that you have spotted in the beaker, cover it with the foil, and allow the solvent front to move up the plate until it is approximately 1 cm from the top. **Do not disturb the beaker while the chromatogram is developing!** In this case, the solvent will travel up the silica gel plate very quickly and will reach the top in two to three minutes.

Remove the plate and mark the solvent front with a pencil. Allow the plate to dry for a few minutes, then observe it under *short-wave* ultra-violet light. With a pencil, circle any spots that are illuminated.

Write your initials in a corner of the plate, and place it in an iodine chamber. Position the plate so that the silica gel surface is completely exposed to the iodine vapors and is not covered by other plates in the chamber. Leave it there for 5-10 minutes. After removing the plate from the chamber, record in your notebook whether or not any colored spots appeared as a result of exposure to the iodine vapors. If new spots appear, circle them with a pencil.

Sketch a diagram of the chromatogram in your notebook. Measure the distance from the origin to the center of each spot and the distance from the origin to the solvent front.

Name _____ Date _____

T. A. _____ Lab period _____

Results and Calculations (to be handed in at the next lab period)

Determine the R_f values for all spots seen on the amino acid chromatogram (see pp 229-230 in *TOC*).

Attach the paper chromatogram or a photocopy of it.

Identify the amino acid(s) contained in the unknown solution that *you* tested:

Unknown code number _____

Determine the R_f values for all spots seen on the food dye chromatograms. Indicate the color of the spot that corresponds to each R_f .

Determine the R_f values for all spots seen on the drug chromatogram. Indicate which spots (if any) appeared after reaction with iodine vapor.

Identify the drug contained in the tablet that you tested:

Unknown code number _____