

## Separation of Amino Acids by Paper Chromatography

Chromatography is a common technique for separating chemical substances. The prefix “chroma,” which suggests “color,” comes from the fact that some of the earliest applications of chromatography were to separate components of the green pigment, chlorophyll. You may have already used this method to separate the colored components in ink. In this experiment you will use chromatography to separate and identify amino acids, the building blocks of proteins. The proteins of all living things are composed of 20 different amino acids, some of which are described below.

Chromatography is partially characterized by the medium on which the separation occurs. This medium is commonly identified as the “stationary phase”. Stationary phases that are typically used include paper (as in this experiment), thin plates coated with silica gel or alumina, or columns packed with the same substances. The “mobile phase” is the medium that accompanies the analyzed substance as it moves through the stationary phase. Both liquids and gases can be used as mobile phases depending on the type of separation desired. To refer to gas or liquid chromatography, chemists often use the abbreviations GC or LC, respectively. These abbreviations explicitly identify the phase of matter of the mobile phase. The term “paper chromatography” used in this experiment’s title identifies the composition of the stationary phase.

The compositions of the stationary and mobile phases define a specific chromatographic method. Indeed, many different combinations are possible. However, all of the methods are based on the rate at which the analyzed substances migrate while in simultaneous contact with the stationary and mobile phases. The relative affinity of a substance for each phase depends on properties such as molecular weight, structure and shape of the molecule, and the polarity of the molecule. The relationship between molecular shape and polarity will be discussed later in Chemistry 11 (Chapter 10 of your text).

In this experiment, very small volumes of solutions containing amino acids will be applied (this process is sometimes called “spotting”) at the bottom of a rectangular piece of filter paper. For ready comparison of each trial, it is vital that each solution be applied on the same starting line. After the solutions have been applied, the paper will be rolled into a cylinder and placed in a beaker that contains a few milliliters of the liquid mobile phase. For this separation, a solution containing *n*-propanol, water and ammonia is the optimum mobile phase. As soon as the paper is placed in the mobile phase, the solution (sometimes called the eluting solvent) will begin to rise up the paper. This phenomenon is called capillary action, a concept that is described in Chapter 12 of your text.

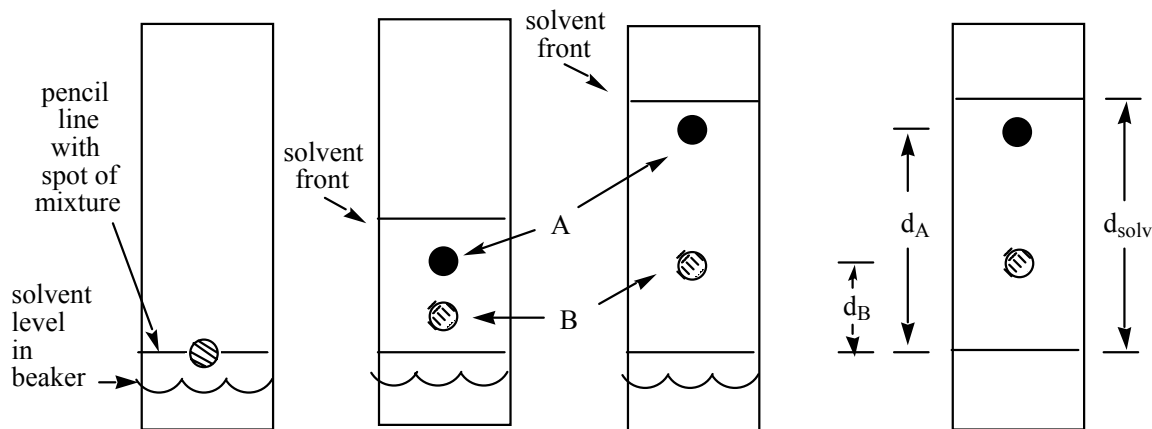
As the mobile phase rises on the paper it will eventually encounter the “spots” of amino acids. The fate of each amino acid in the mixture now depends on the affinity of each substance for the mobile and stationary phases. If an amino acid has a higher affinity for the mobile phase than the stationary phase, it will tend to travel with the solvent front and be relatively unimpeded by the filter paper. In contrast, if the amino acid has a higher affinity for the paper than the solvent, it will tend to “stick” to the paper and travel more slowly than the solvent front. It is these differences in the amino acid affinities that lead

to their separation on the paper. The affinities of these amino acids for the mobile phase can be correlated to the solubility of the different amino acids in the solvent (i.e., an amino acid that is highly soluble in the eluting solvent will have a higher affinity for the mobile phase than an amino acid that is less soluble in the solvent.).

When the solvent front comes near the top of the filter paper, the paper is removed from the beaker and allowed to dry. At this point, the various amino acids are invisible. The acids can be visualized by spraying the paper with a compound called ninhydrin. Ninhydrin reacts with amino acids to form a blue-violet compound. Therefore, the sprayed filter paper should show a number of spots, each one corresponding to an amino acid. The further the spot from the starting line, the higher the affinity of the amino acid for the mobile phase and the faster its migration.

The relative extent to which solute molecules move in a chromatography experiment is indicated by  $R_f$  values. The  $R_f$  value for a component is defined as the ratio of the distance moved by that particular component divided by the distance moved by the solvent. Figure 1 represents the migration of two components. Measurements are made from the line on which the original samples were applied to the center of the migrated spot. In the figure,  $d_A$  is the distance traveled by component A,  $d_B$  is the distance traveled by component B, and  $d_{\text{solv}}$  is the distance traveled by the eluting solution. In all three cases, the travel time is the same.

Figure 1: Paper chromatography - migration of two components.



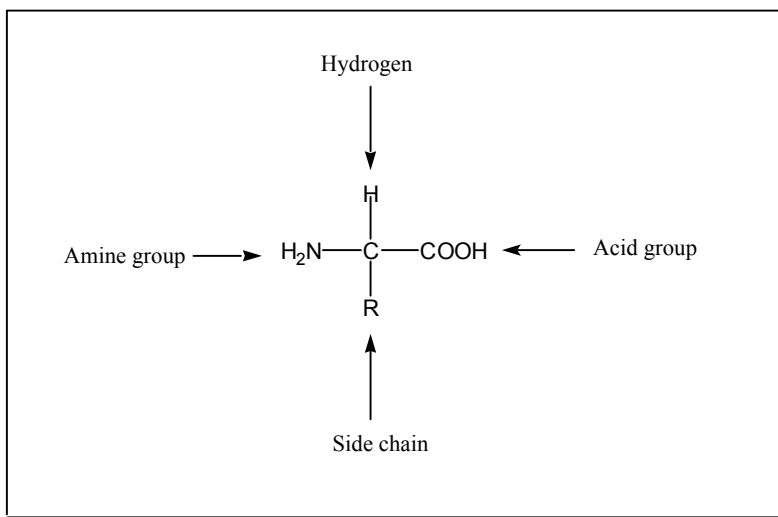
Thus the  $R_f$  values for components A and B are

$$R_f(A) = d_A/d_{\text{solv}}$$

$$R_f(B) = d_B/d_{\text{solv}}$$

Note that  $R_f$  values can range from 0 to 1. In this example,  $R_f(A)$  is obviously larger than  $R_f(B)$ . Although  $R_f$  values are not exactly reproducible, they are reasonably good guides for identifying the various amino acids. Paper chromatography is most effective for the identification of unknown substances when known samples are run on the same paper chromatograph with unknowns.

To best understand why different amino acids have unique  $R_f$  values, it is important to understand the structural features of these molecules. As the name suggests, each amino acid contains an amino group,  $-NH_2$ , and a carboxylic acid group,  $-COOH$ . The molecular structure of a generic amino acid is provided below:

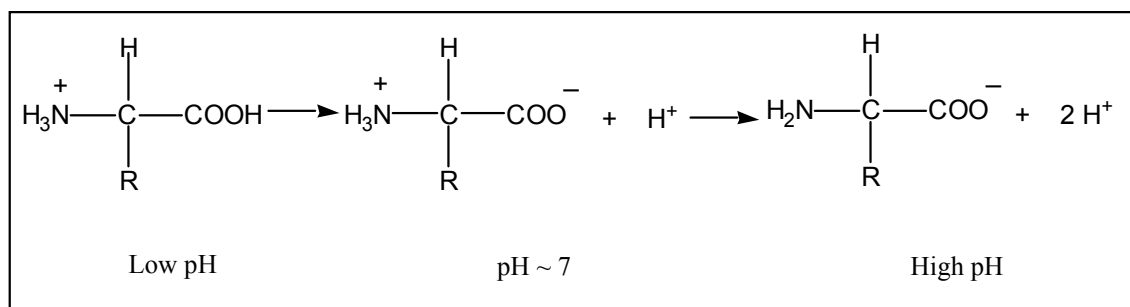


The 20 different amino acids that make up our proteins, and those of most other living things, differ in the identity of the side chain R. In glycine, the simplest amino acid, R is a hydrogen atom. Eight amino acids have R groups that consist of carbon atoms with attached hydrogen atoms. Two examples are valine for which R is  $-\text{CH}(\text{CH}_3)\text{CH}_3$ , and phenylalanine, which contains a benzene ring with R equal to  $-\text{CH}_2(\text{C}_6\text{H}_5)$ . These nonpolar hydrocarbon side chains are hydrophobic or “water-hating.” Hence, they tend to lower the water solubility of the corresponding amino acids. Six amino acids have polar but neutral R groups that tend to promote water solubility. For example, for serine R is  $-\text{CH}_2\text{OH}$ . In two amino acids, glutamic acid and aspartic acid, the side chains carry carboxylic acid groups. For example, in glutamic acid, R is  $-\text{CH}_2\text{CH}_2\text{COOH}$ . Finally, three amino acids have basic R groups. One of these is lysine, for which R is  $-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$ . Both acidic and basic R groups tend to promote water solubility, though the solubility will be pH dependent.

In fact, the water solubility of all amino acids varies with the acidity of the solution, i.e. the  $\text{H}^+$  ion concentration that is commonly communicated via pH values. This is because all amino acids, even those with neutral side chains, contain an acidic  $-\text{COOH}$  group and a basic  $-\text{NH}_2$  group. The most prevalent ionic form of an amino acid in solution therefore depends on the pH of the solution.

As the equation below suggests, in solutions of low pH (high  $\text{H}^+$  concentration), the amino and acid groups are both protonated and this contributes a net plus charge. Near the neutral pH of 7, an  $\text{H}^+$  has dissociated from the carboxylic acid group and the positive and negative charges balance each other. In solutions of still higher pH (low  $\text{H}^+$  concentration), the amino group is in the  $-\text{NH}_2$  form and the net charge is negative because of the  $-\text{COO}^-$ . This means that the rate of migration of an amino acid will

depend on the pH of the mobile phase, and that the details of this dependence will vary from amino acid to amino acid. The presence of an acidic or basic R group further complicates this pH dependence.



The acidic group of one amino acid can react with the basic group of another to form what is called a peptide bond, with the elimination of a water molecule. This process can be repeated to form polypeptides or proteins. Many proteins contain well above 100 amino acids. When a protein is heated in the presence of acid or base, it is hydrolyzed, the peptide bonds are broken, and the constituent amino acids are released. One of the samples for study in this experiment is such a hydrolyzed mixture.

### Experimental Procedure

Obtain a sheet of filter paper, and draw a faint pencil line about 1 to 2 cm from one of the long edges and parallel to that edge. This will be the bottom of the chromatogram. Mark off seven equally spaced points along this line. (They should be separated by about 2 cm). Your samples will be applied to these spots. The laboratory contains solutions of four identified amino acids and a sample of a hydrolyzed protein. In addition, you will be given a numbered unknown that will contain one or more of the known amino acids.

The samples can be applied to the paper by using a narrow capillary tube. The procedure is pretty simple, but it is a good idea to practice making sample spots on a separate sheet of filter paper before you start on your chromatographic paper. Dip the open end of a clean capillary into the solution to draw up a small volume of the solution into the tube. Lightly and briefly touch the tube to the paper and allow the sample to transfer. The spot should be about 2-3 mm in diameter. Once you have mastered the technique, place one spot of each of the four known amino acids on the separate points that you previously marked on the filter paper. In addition, apply samples of your unknown to two of the points, and the hydrolyzed protein to another. Be careful not to contaminate either the solutions or the spots. Label each spot (with pencil and below the starting line) to indicate its identity. Finally, it's a good idea to avoid getting fingerprints on the chromatographic paper.

When you have finished spotting your paper, allow it to dry by waving it in the air or using a heat lamp or hair dryer. (Don't get it too hot.) Meanwhile, in the hood, pour about 15-20 mL of the eluting solution (*n*-propanol and concentrated aqueous ammonia) into a clean, dry 600 mL beaker and cover the beaker with a watch glass or plastic wrap.

When the sample spots have dried, roll the paper into a cylinder, with the short sides almost touching. Use a bit of “Scotch” tape along the top of the paper to hold the cylinder together. Evenly lower the paper cylinder, sample side down, into the beaker. The solvent will wet the paper, but the sample spots should not be immersed. In addition, the paper should not touch the walls of the beaker.

At this point, cover the beaker with a watch glass or plastic wrap and place the beaker in the hood. When the solvent front gets within about 1 or 2 cm of the top of the paper (in perhaps about 2 hrs), remove the paper, use a pencil to mark the solvent front at several points, unroll the cylinder, and let the chromatography paper dry in the hood. When the paper is dry, spray it with ninhydrin reagent. Allow the paper to dry, perhaps using the hair dryer, heat lamp, or an oven at about 100°C, but don't overcook it!

When the chromatographic paper has fully dried, outline the spots, mark the centers of each of the spots, and note their colors. (Not all amino acids give the same color with ninhydrin). Measure and record the distances the solvent and each of the amino acids traveled from the origin. Use these distances to calculate  $R_f$  values for each sample. Comparison of the spots should enable you to identify the amino acid(s) present in your unknown sample. Also note whether the known amino acids are present in the hydrolyzed protein.

## **Report**

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Your report should include the following experimental data and conclusions:

1. Your marked chromatograph
2.  $R_f$  calculations and results
3. The identification of the amino acid(s) in your group's unknown and support for your conclusions
4. Conclusions about the amino acids present in the hydrolyzed protein

In addition, your report should include the following information or answers:

1. The formulas for the four amino acids you chromatographed
2. The most prevalent ionic form (structure) of these amino acids in the very basic eluting solution
3. Explanations for the observed differences in  $R_f$  values for these four amino acids
4. An explanation for the difference in appearance of the chromatograph for the hydrolysed protein and for the pure amino acids
5. Why are you advised to mark the paper with pencil and warned not to get fingerprints on it?