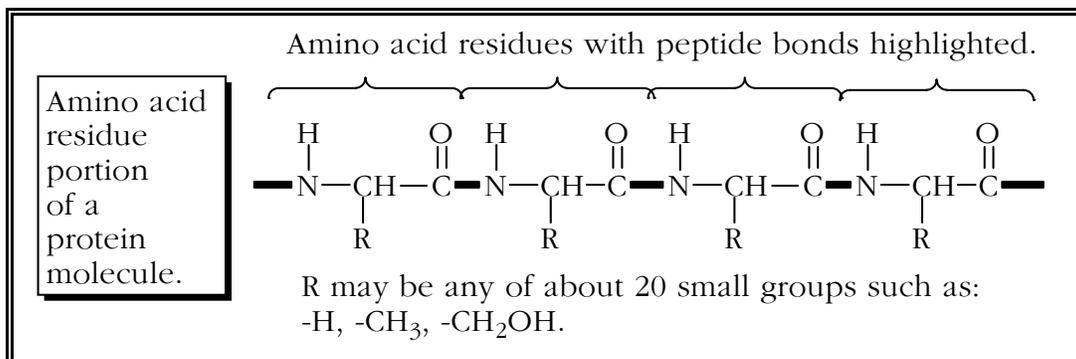


EXPERIMENT #11 – CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS

Introduction – Amino Acids

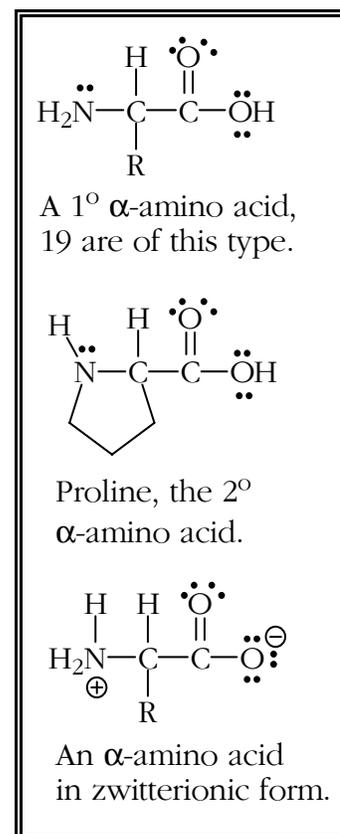
As you may recall from Experiment #4, protein molecules are composed largely, and in some cases exclusively, of *amino acid residues* that are joined to each other through a bond between a carbonyl carbon and an amine nitrogen known to biochemists as a *peptide bond*.



The amino acid residues that are found in proteins and their smaller siblings, the polypeptides, are derived from α -amino acids. Amino acids are compounds that contain the amino group and the carboxylic acid group. The carboxylic acid group was discussed in Experiment #10. The amino group consists of a nitrogen with three single bonds and an unshared pair of electrons. The amino nitrogen is a base in both the Lewis and Bronsted-Lowry senses. It can donate the unshared pair of electrons to form a bond, so it is a Lewis base. It can donate the unshared pair of electrons to form a bond to a proton, thus being a proton acceptor and hence a Bronsted-Lowry base. An α -amino acid is one in which the amino group and carboxylic acid group are both attached to the same carbon.

Amino acid residues from twenty different α -amino acids are commonly found in polypeptides and proteins. In 19 of these amino acids, the amino group is a *primary* (1°) one, meaning that there are two hydrogens and one carbon attached to the nitrogen; in one of these amino acids (proline) the amino group is *secondary* (2°), meaning that there is one hydrogen and two carbons attached to the nitrogen.

In each of these amino acids there is at least one hydrogen bonded to the carbon that holds the amino and carboxyl groups; the "R" group is also bonded there. The R group may be a hydrogen (glycine) or a small carbon containing group.



Since an amino acid contains the acidic carboxyl group and the basic amino group, the proton from the carboxyl group can be transferred to the amino group. When this happens the amino acid is said to be a *zwitterion* or in *zwitterionic form*. A zwitterion is not an ion; it has no net charge. Rather, it has a positive charge in one part of the molecule and a negative charge in another. Since the carboxyl group is a stronger acid than the protonated amine group, and the amine group is a stronger base than the carboxylate group, the zwitterionic form usually predominates over the non-zwitterionic form. [This is another example of the weaker acid ($-\text{NH}_4^+$) and weaker base ($-\text{COO}^-$) predominating at equilibrium.]

Introduction – Chromatography

You will be separating compounds using paper chromatography. Paper chromatography is one of several chromatographic methods. Fortunately, they all operate in essentially the same way, and the underlying principle is quite simple.

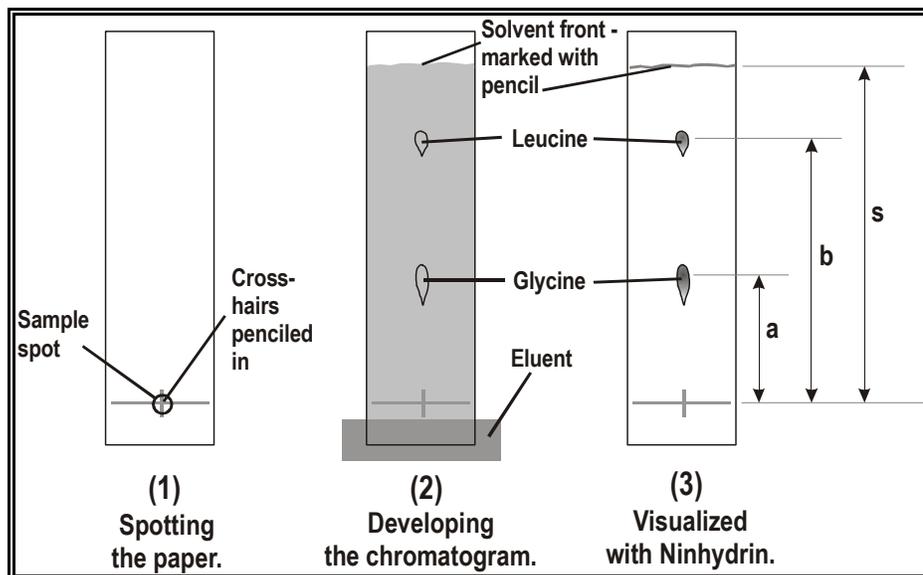
Chromatography is a method of separation. Originally, it was used to separate colored materials (separations of colored materials are easy to observe) – hence, the name. But, today, chromatography is used to separate materials whether they are colored or not.

The physical setup in chromatographic techniques involves a *mobile phase* and a *stationary phase*. The mobile phase may be a gas or a liquid. The stationary phase may be a solid or a liquid deposited on the surface of a solid. [Note that the term *phase* here means something like *material* or *substance*. It does not have anything to do with temporal phenomena as in “the phase of the moon” or “Joey bit Jennifer because he’s going through a phase.”]

The mixture to be separated into its components is dissolved in a small portion – a “slug” – of the mobile phase. The mobile phase, including the slug containing the components to be separated, is then caused to pass by the surface(s) of the stationary phase, always moving in the same direction. For example, in paper chromatography it is common to use a rectangle of filter paper (cellulose) as the stationary (also stationery! ☺☺☺) phase. A solvent or mixture of solvents, called the *eluent*, would be the mobile phase. The mixture to be separated would be dissolved in an appropriate solvent and one or more drops of the solution would be “spotted” onto the dry paper near the bottom edge, and the solvent would be allowed to evaporate so the compounds in the mixture have impregnated the paper. (See figure on page 3.) The bottom edge of the paper is then placed in the eluent keeping the “spot” above the solvent surface (so it does not dissolve off the paper). The eluent will rise up the paper by capillary action and as it passes the spot it will carry the components of the spot with it (the “slug”). The components of the mixture then partition themselves between the mobile phase and stationary phase. That is to say, the stationary and mobile phases compete to attract molecules of each component in the mixture. Each chemical component in the mixture (ideally) has at least a little different response to these attractions. Consequently, one component will spend relatively more of its time attached to the stationary phase (the paper) while another will spend relatively more of its time in the mobile phase (the eluent). Since the mobile phase is moving and the stationary phase is stationary, a

component that spends more of its time in the mobile phase will move up the paper faster than a component that spends less of its time there. Consequently, the compounds will be separated from each other forming a column of spots in the direction that the eluent moved.

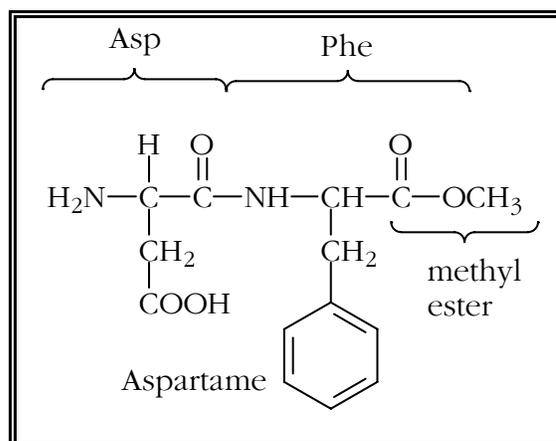
For example, suppose we wanted to separate a mixture that contained some amino acids and we wished to identify them. We would dissolve the mixture in some solvent and spot the solution onto the filter paper as shown in the figure to the right (1). After allowing the spot to dry we would place the chromatogram into



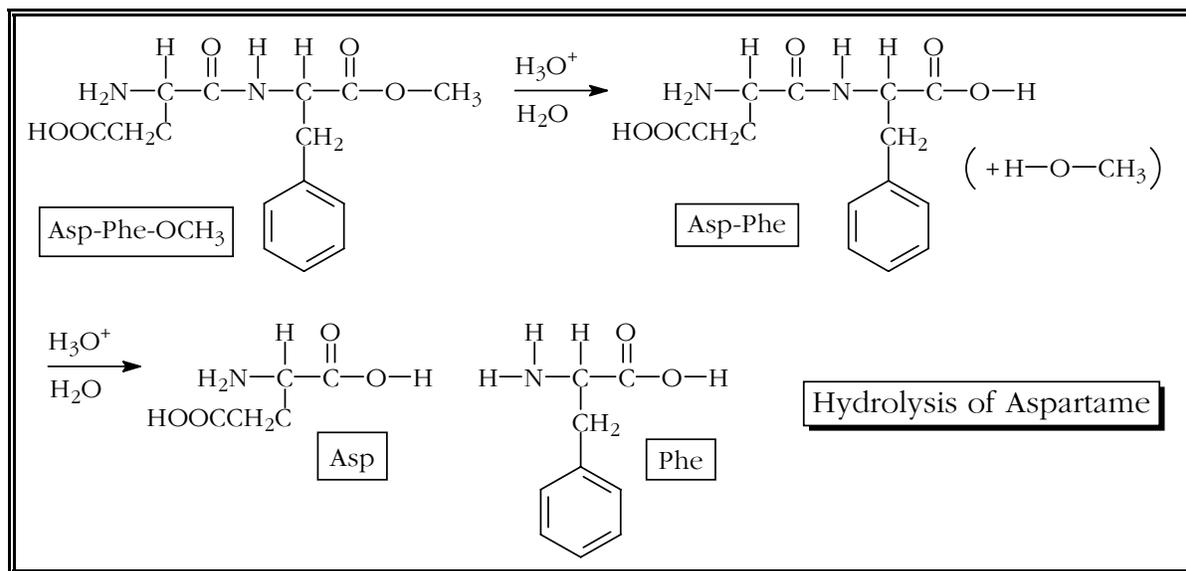
a developing chamber that contains the eluent (mobile phase solvent) so that the bottom of the paper is in the eluent but the spot is above its surface. By capillary action the eluent is drawn up the paper. The chamber is covered so eluent does not evaporate from the paper. The amino acids (let's assume that only glycine and leucine are present in the sample) also migrate up the paper, but at different rates (2). When the solvent front reaches a some point (near the top of the paper) we remove the paper from the chamber and, with a pencil, mark where the solvent front is located before the solvent evaporates from the paper. After the solvent evaporates from the paper we spray the paper with *ninhydrin* which reacts with the amino acids to produce a purple color, allowing us to see where the amino acids are located. We are now able to measure the *retardation factors* (R_f) of the amino acids. R_f (amino acid "a") = a/s . R_f (amino acid "b") = b/s . Under identical conditions (same eluent, same type of paper, paper stored at the same humidity) the R_f will be a constant for a given amino acid. So, by running authentic samples of all 20 amino acids and determining their R_f values, we could figure out that purple spot "a" corresponds to glycine and "b" to leucine.

Background for the Experiment

In this experiment you will use paper chromatography to identify aspartame and its hydrolysis products. You will also try to identify these compounds in Diet Pepsi®. Aspartame is the methyl ester of the dipeptide aspartylphenylalanine, or Asp-Phe-OCH₃.



The aspartame that you will analyze is a major component of the commercial sweeteners Nutrasweet® and Equal®. Equal contains silicon dioxide, glucose, cellulose, and calcium phosphate in addition to the aspartame.



You will hydrolyze aspartame (Asp-Phe-OCH₃) to produce aspartic acid (Asp), phenylalanine (Phe), methanol and possibly aspartylphenylalanine (Asp-Phe). You will analyze this *hydrolysate* (the material that results from hydrolysis) by comparing the R_f values of the substances it contains with the R_f values from authentic samples of aspartic acid and phenylalanine. Since ester bonds are usually hydrolyzed more easily than peptide bonds, you will also analyze for aspartylphenylalanine (Asp-Phe) by comparison with an authentic sample. [The methanol that is produced in the aspartame hydrolysis is very volatile and will evaporate from the paper. It would not be detected by ninhydrin in any event.] You will also analyze current and expired samples of Diet Pepsi for aspartame, aspartic acid, phenylalanine and aspartylphenylalanine.

If you go to a busy supermarket and look at the expiration date for the Diet Pepsi on the shelves you will find that it is about 3 months in the future. If you check the expiration date on regular Pepsi it will be about 12 months in the future. The relatively short expiration date for the Diet Pepsi has to do with the fact that phosphoric acid is added as a flavor enhancer and the hydrolysis of aspartame is catalyzed by acid. When the aspartame is hydrolyzed the taste changes from sweet to unpleasant. So, the fresher the Diet Pepsi, the better the taste. The situation is different for regular Pepsi. It, too, contains phosphoric acid, but the sweetener is either sucrose or fructose. Fructose is not hydrolyzed by acid. Sucrose is hydrolyzed by acid, but the products of hydrolysis are fructose and glucose, both of which are sugars! The bottom line is that regular Pepsi will probably taste OK even a year beyond its expiration date, while Diet Pepsi loses its luster shortly after the date on the can.

Objectives

1. To learn a chromatographic technique.
2. To identify the amino acid residues in aspartame.
3. To see how aging affects the quality of aspartame in a soft drink.

Procedure

1. Place 4 drops of 0.5% aspartame and 4 drops of 6M hydrochloric acid in a 10x75mm (small) test tube. Cautiously heat the tube with a microburner, boiling the contents gently for about 90 seconds. Allow the contents to cool and label the test tube hydrolyzed aspartame.

2. Label six 10x75mm test tubes as follows: Asp (aspartic acid), Phe (phenylalanine), Asp-Phe-OCH₃ (aspartame), Asp-Phe (aspartylphenalanine), fresh Diet Pepsi, and expired Diet Pepsi. Place 2 drops of each material in the correspondingly labeled tube. The aspartic acid and phenylalanine are 0.2% aqueous solutions; the aspartame is a 0.5% aqueous solution; the aspartylphenalanine is a 0.4% aqueous solution.

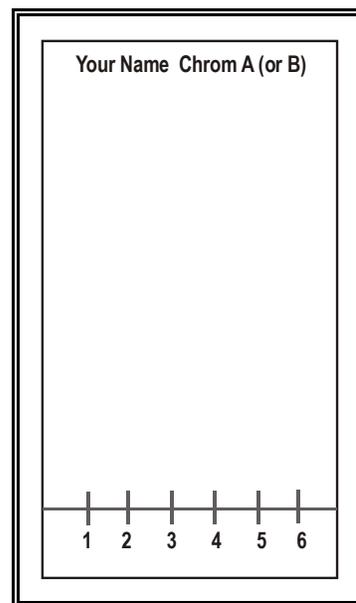
3. Wearing plastic gloves so you don't deposit amino acids from your hands onto the paper, obtain two 7.5x18 cm pieces of Whatman #1 chromatography paper. With a *pencil (not a pen)* lightly draw a line parallel to the 7.5 cm side and about 2 cm from the edge. Along this line, starting 1.25 cm from the 15 cm edge, place 6 tick marks at intervals of 1 cm. Write the numbers 1 through 6 between the tick marks and the closest 7.5 cm edge. Write your name near the 7.5 cm edge that is farthest from the tick marks and "Chrom A" to identify this sheet. See the figure to the right.

4. Repeat the process in #3, but label this sheet "Chrom B".

5. Using open-ended 1 mm diameter capillary tubes, draw out 8 spotting micro-pipettes. Your instructor will demonstrate how to do this. Practice spotting a scrap piece of the chromatography paper with water using one of the micro-pipettes. Fill the pipette by submerging the tip in water.

Then touch the tip of the micro-pipette to the paper until the sample spreads into a circle of approximately 3 mm diameter – about the size of this circle: ○. Repeat the spotting 2 or 3 times, trying to keep the wet spot from spreading. When you're confident you can apply spots consistently, proceed to step 6.

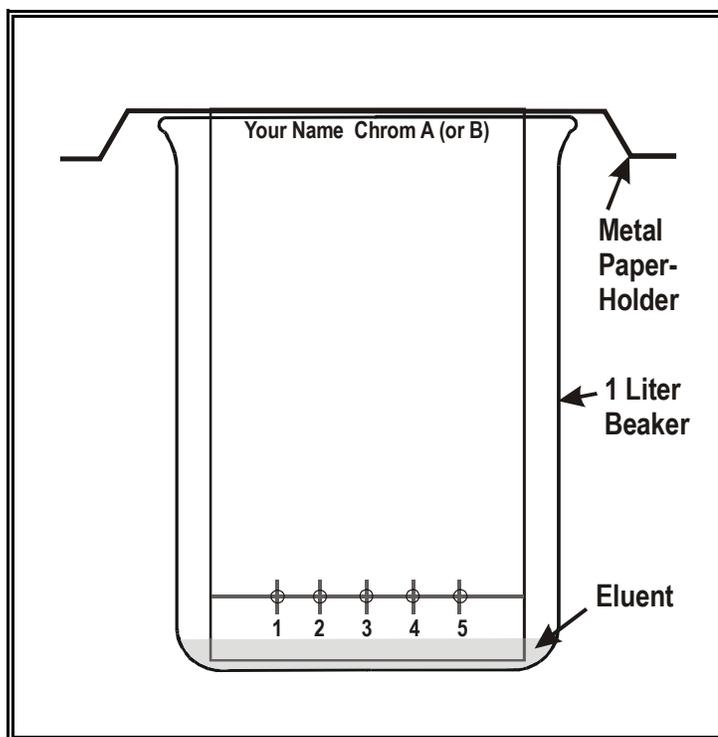
6. Spot sheet "Chrom A". For each sample use a separate micro-pipette to do the spotting. Dry the spots using either a heat lamp, heat gun, or the oven. Repeat this process for sheet "Chrom B". The table below indicates which sample you should spot at each position on each sheet.



Sheet	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6
Chrom A	Phe, phenyl- alanine	Asp, aspartic acid	Asp-Phe-OCH ₃ , aspartame	Asp-Phe, aspartyl- phenyl- alanine	hydrolyzed aspartame	
Chrom B	Phe, phenyl- alanine	Asp, aspartic acid	Asp-Phe-OCH ₃ , aspartame	Asp-Phe, aspartyl- phenyl- alanine	fresh Diet Pepsi*	expired Diet Pepsi*

* Since the aspartame is fairly dilute in the soda these samples have been prepared by concentrating the soda by a factor of about 30, i.e. a 12 ounce can of soda is reduced to about 10 milliliters.

7. Attach the Chrom A chromatography paper to the metal holder using tape so that the edge adjacent to the row of spots is just above the bottom of a 1 liter beaker with the metal holder supported by the rim of the beaker. The long edges of the paper should be parallel to the sides of the beaker, *but not touching those sides*. See the figure to the right. When you have things adjusted, lift the paper and holder away from the beaker and add 50 milliliters of the eluent (which contains 1-butanol, acetic acid, and distilled water in a ratio of 12:3:5 by volume) to the beaker. Replace the paper into the beaker as before, making sure that the long sides of the paper do not touch the sides of the beaker and that the bottom of the paper is in the eluent but the line of spots is not. Cover the beaker with plastic wrap or aluminum foil so the eluent cannot evaporate off the paper.



8. Repeat step #6 using the Chrom B paper and a second 1 liter beaker.

9. Allow the chromatograms to run for 1 to 1.5 hours. You should watch things for a few minutes to be sure the solvent is moving up the paper evenly. Then you're on your own for an hour. When you return, remove each of the chromatograms, Chrom A and Chrom B, from its beaker. *Be sure to mark the location of the solvent front with a pencil as soon as you remove the paper from the beaker.*

10. Dry the chromatograms with a heat lamp or heat gun. Using plastic gloves to protect your hands, spray the chromatograms with ninhydrin solution until they are wet, but not dripping. Dry the chromatograms with a heat gun or in the oven for a few minutes until you can see the purple spots. If you get ninhydrin on your skin it will turn purple and the color will take several days of wear off.
11. Outline the spots, using a pencil. The spots may fade; the penciled outline will let you know where they were.
12. Calculate the R_f values of the spots and record your observations on the report sheet.