TLC SEPARATION OF AMINO ACIDS

LAB CHROM 7
Adapted from Laboratory Experiments for Organic and Biochemistry. Bettelheim & Landesberg

(PA Standards for Sci & Tech 3.1.12.D; 3.4.10.A; 3.7.12.B)

INTRODUCTION

Amino acids are the building blocks of peptides and proteins. They possess two functional groups—the carboxylic acid group gives the acidic character, and the amino group provides the basic character. The common structure of all amino acids is:

\[
\begin{align*}
\text{H} \\
\text{R-} \text{C-} \text{COOH} \\
\text{NH}_2
\end{align*}
\]

Amino Acid

The \text{R} represents the side chain that is different for each of the amino acids that are commonly found in proteins. However, all 20 amino acids have a free carboxylic acid group and a free amino (primary amine) group, except proline which has a cyclic side chain and a secondary amino group.

The properties provided by these groups are used to characterize the amino acids. The common carboxylic acid and amino groups provide the acid-base nature of the amino acids. The different side chains, and the solubilities provided by these side chains, affect their rate of migration in thin-layer chromatography (TLC).

Thin layer chromatography (TLC) is one type of chromatography; the stationary phase is a thin layer of adsorbent particles attached to a solid plate. A small amount of sample is applied (spotted) near the bottom of the plate and the plate is placed in the mobile phase. This solvent is drawn up by capillary action. Separation occurs as each component, being different in chemical and physical composition, interacts with the stationary and mobile phases to a different degree creating the individual bands on the plate. TLC can be utilized to identify the different amino acids such as aspartame, and artificial sweetener, and its hydrolysis products from certain foods.

\[
\begin{align*}
\text{O} \\
\text{O} \\
\text{NH}_2 \\
\text{CH}_3
\end{align*}
\]

Aspartame
Aspartame is the methyl ester of the dipeptide aspartylphenylalanine. Upon hydrolysis with HCl it yields aspartic acid, phenylalanine, and methyl alcohol. When this artificial sweetener was approved by the Food and Drug Administration, opponents of aspartame claimed that it is a health hazard, because aspartame would be hydrolyzed and would yield poisonous methyl alcohol in soft drinks that are stored over long periods of time. The Food and Drug Administration ruled, however, that aspartame is sufficiently stable and fit for human consumption. Only a warning must be put on the labels of foods containing aspartame. This warning is for patients suffering from phenylketonuria who cannot tolerate phenylalanine.

To run a thin-layer chromatography experiment, silica gel is used in a thin layer on a plastic or glass plate. The sample (aspartame or amino acids) is applied as a spot to a strip of a thin-layer plate. The plate is dipped into a mixture of solvents. The solvent moves up the thin gel by capillary action and carries the sample with it. Each amino acid may have a different migration rate depending on the solubility of the side chain in the solvent. Amino acids with similar side chains are expected to move with similar, though not identical, rates; those that have quite different side chains are expected to migrate with different velocities. Depending on the solvent system used, almost all amino acids and dipeptides can be separated from each other by thin-layer chromatography (TLC).

The rate of migration of an amino acid or a dipeptide is not actually measured, but rather, how far a particular amino acid travels in the thin silica gel layer relative to the migration of the solvent. This ratio is called the \( R_f \) value. In order to calculate the \( R_f \) values, one must be able to visualize the position of the amino acid or dipeptide. This is done by spraying the thin-layer silica gel plate with a ninhydrin solution that reacts with the amino group of the amino acid. A purple color is produced when the plate is heated. (The proline not have a primary amine gives a yellow color with ninhydrin.) For example, if the purple spot of an amino acid appears on the TLC plate 4.5 cm away from the origin and the solvent front migrates 9.0 cm (see figure below), the \( R_f \) value for the amino acid is calculated

\[
R_f = \frac{\text{distance traveled by the amino acid}}{\text{distance traveled by the solvent front}} = \frac{4.5 \text{ cm}}{9.0 \text{ cm}} = 0.50
\]
The aspartame that will be analyzed is actually a commercial sweetener, Equal by the NutraSweet Co., which contains silicon dioxide, glucose, cellulose, and calcium phosphate, in addition to the aspartame. None of these other ingredients of Equal will give a purple or any other colored spot with ninhydrin. Other generic aspartame sweeteners may contain other non-sweetening ingredients. Occasionally, some sweeteners may contain a small amount of leucine which can be detected by the ninhydrin test.

**PURPOSE**

In the present experiment the R_f values of three amino acids, phenylalanine, aspartic acid, and leucine, in addition to the artificial sweetener aspartame, will be determined. Aspartame will also be hydrolyzed using HCl as a catalyst to see if the hydrolysis products prove that the sweetener is truly aspartame. Finally, the analysis of Diet Coke will indicate if the aspartame was hydrolyzed at all during the processing and storing of the soft drink.

**EQUIPMENT/MATERIALS**

- TLC silica gel plates
- capillary tubes
- chromatography chambers
- butanol: acetic acid: water-solvent
- foil, ruler
- gloves
- 1% solutions of aspartic acid, phenylalanine, and leucine
- Beral pipets
- 3 M HCl
- Equal sweetener
- test tubes and rack

**SAFETY**

- Always wear an apron and goggles in the laboratory.
- Wash hands when lab experiment is complete.

**PROCEDURE**

1. Tare a weigh boat on a top-loading balance. Mass between 0.1 and 0.2 g of NutraSweet. Transfer the NutraSweet into a small test tube.

2. Using the Beral pipet labeled HCl, add 1 ml of 3 M HCl to the test tube. Place the test tube into a 150mL beaker that has been heating on a hot plat. Turn the hot plate up so that the NutraSweet/HCl solution boils for 30 seconds. Be sure that the liquid doesn’t completely evaporate.

3. Cool test tube and label it “Hydrolyzed Aspartame.”
4. Label five small test tubes, respectively, for aspartic acid, phenylalanine, leucine, aspartame, and Diet Coke. Using the labeled pipets, transfer about 0.5 ml of each solution into the matching test tube.

5. **Wearing gloves**, obtain two 14 x 6.5 cm TLC plates. With a pencil, lightly draw a line parallel to the 6.5 cm edge and about 1 cm from the edge. Mark the positions for the five spots on each plate, spaced equally, where the samples will be spotted. **Be sure not to touch the TLC plates without gloves because amino acids from the hand will contaminate the plate.**

6. Spot **Plate A** according to the following diagram:

   ![Diagram of Plate A]

   - 1 - PHENYLALANINE
   - 2 - ASPARTIC ACID
   - 3 - LEUCINE
   - 4 - ASPARTAME SOLUTION
   - 5 - HYDROLYZED ASPARTAME

7. For each sample spot use a separate capillary tube. Apply the sample to the plate until it spreads to a spot of 1 mm in diameter. Dry the spots with hair dryer if necessary.

8. Spot **Plate B** according to the following diagram:

   ![Diagram of Plate B]

   - 1 - DIET COKE
   - 2 - ASPARTIC ACID
   - 3 - ASPARTAME SOLUTION
   - 4 - DIET COKE
   - 5 - HYDROLYZED ASPARTAME

9. For the aspartic acid, hydrolyzed and non-hydrolyzed aspartame, use only one spot. For Diet Coke, multiple spotting is needed. Apply the capillary tube 10 times to the same spot, making certain that between each application, the previous sample has been dried. Try to control the spot size so that they don’t spread more than 2mm in diameter. Dry spot as before.
10. Measure about 15mL of solvent mixture (butanol: acetic acid: water) and pour it into a 1L beaker. Repeat for the second beaker. Place each spotted plate, sample end down, into a solvent filled beaker. Make certain that the spots applied to the plate are above the surface of the eluting solvent. Cover the beakers with aluminum foil to avoid the evaporation of the solvent. Allow 60 minutes for the solvent to advance.

11. The solvent front **must not** advance up to or beyond the edge of the plates. When the solvent front nears the edge of the plates, about 1-2 cm from the edges, remove the plates from the beakers. **Immediately** mark with a pencil the position of the solvent front.

12. Under a hood dry the plates with the aid of a hair dryer. Also under the hood and wearing gloves, spray the dry plates with ninhydrin solution. Ninhydrin will discolor bare hands (which contain amino acids) for several days.

13. Place sprayed plates in a drying oven set at 105-110°C for 2-3 minutes. Remove the plates from the oven. Mark the center of the spots and calculate the Rf values of each spot. Record values and observations on the data table.

14. If the spots on the chromatogram are faded, they can be visualized by exposing the chromatogram to iodine vapor. Place the chromatogram into a wide-mouthed jar containing a few iodine crystals. Cap the jar and warm it slightly on a hot plate under the hood to enhance the sublimation of iodine. The iodine vapor will interact with the faded pigment spots and make them visible. After a few minutes' exposure to iodine vapor, remove the chromatogram and mark the spots **immediately** with a pencil. The spots will fade again with exposure to air. Measure the distance of the center of the spots from the origin and calculate the Rf values.
### TLC Separation of Amino Acids

#### Data Table

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLATE A</strong></td>
<td>DISTANCE Component TRAVELED (mm)</td>
<td>DISTANCE SOLVENT FRONT TRAVELED (mm)</td>
<td>R_f VALUE</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td></td>
<td></td>
<td>See equation on next page</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartame</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolyzed Aspartame</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLATE B</strong></td>
<td>DISTANCE Component TRAVELED (mm)</td>
<td>DISTANCE SOLVENT FRONT TRAVELED (mm)</td>
<td>R_f VALUE</td>
</tr>
<tr>
<td>Diet Coke</td>
<td></td>
<td></td>
<td>See equation on next page</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartame</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet Coke</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TLC Separation of Amino Acids

Hydrolyzed Aspartame

CALCULATIONS

Calculate the R_f value for each sample. Record on table above.

\[
R_f = \frac{\text{distance component moved from origin (spotting line)}}{\text{distance mobile phase moved from origin (spotting line)}}
\]

QUESTIONS

1. Name the amino acids found in the hydrolysate of Equal.

2. How many spots were stained with ninhydrin in (a) Equal and in (b) Diet coke?
   a. ____________
   b. ____________

3. In testing the hydrolysate of aspartame, if the position of the solvent front were not marked on the TLC plate, could the number of amino acids in the aspartame be determined? (a.) Could the amino acids be identified? (b)
   a. ____________
   b. ____________

4. Is there any evidence that the aspartame was hydrolyzed during the processing and storage of the Diet Coke?

5. The difference between aspartic acid and phenylalanine is twofold. Aspartic acid has a polar, acidic side chain, while phenylalanine has nonpolar side chain. The molecular weight for aspartic acid is smaller than the molecular weight of phenylalanine. Based on the R_f values obtained for these two amino acids in the solvent employed, which property influenced them?
TLC SEPARATION OF AMINO ACIDS

TEACHER NOTES

Lab Time: minutes

Answers to Questions:
1. Name the amino acids found in the hydrolysate of Equal.

2. How many spots were stained with ninhydrin in (a) Equal and in (b) Diet coke?
   a. ____________
   b. ____________

3. In testing the hydrolysate of aspartame, if the position of the solvent front were not marked on the TLC plate, could the number of amino acids in the aspartame be determined? (a.) Could the amino acids be identified? (b)
   a. ____________
   b. ____________

4. Is there any evidence that the aspartame was hydrolyzed during the processing and storage of the Diet Coke?

5. The difference between aspartic acid and phenylalanine is twofold. Aspartic acid has a polar, acidic side chain, while phenylalanine has nonpolar side chain. The molecular weight for aspartic acid is smaller than the molecular weight of phenylalanine. Based on the Rf values obtained for these two amino acids in the solvent employed, which property influenced them?