Identification of Unknown Substances II
Forensic Investigation Kit

Introduction

Drug-related crimes are extremely common. Crime scene analysis often requires the identification of unknown substances that may be illegal drugs. Thin-layer chromatography (TLC) is one technique used to identify unknown drugs.

Concepts

- Chromatography
- $R_f$ value
- Polarity
- TLC
- Eluent
- Absorption

Background

There are many different types of chromatography (chromato = color + graphy = to write), but most work on the principle of adsorbance. A good adsorbent is usually a solid material that will attract and adsorb the components to be separated. Paper, silica gel and alumina are all very good adsorbents. The other key element in chromatography is the eluent. The eluent is the solvent that carries the materials to be separated through the adsorbent.

Chromatography works on the principle that the compounds to be separated are slightly soluble in the eluent and will spend some of the time in the eluent (or solvent) and some of the time in the adsorbent. When the compounds to be separated spend different amounts of the time being adsorbed, they are then separated from one another. The polarity of the molecules to be separated and the polarity of the eluent are very important in the separation process.

Thin-layer chromatography (TLC) is a form of chromatography that uses a thin layer of adsorbent material on an inert support. The TLC sheets used in this experiment contain a thin layer of silica gel on a plastic sheet. The eluent for the TLC is an organic solvent that travels up the TLC plate as it is absorbed by the silica gel.

TLC is frequently used as an analytical method to verify the presence of certain compounds. The use of “known” substances as a baseline for comparison is critical for making a strong case for the composition of unknowns. In order to compare substances collected over different time periods, a relative value called the $R_f$ value is calculated for a given substance for specific chromatographic conditions. Typically, an unknown substance is spotted on a TLC plate alongside of known reference substances. Then the solvent is allowed to travel up the TLC plate carrying the substance components up the plate to different levels. The distance a component moves compared to the distance the solvent moves is recorded as a ratio and is called the $R_f$ value.

$$R_f = \frac{\text{distance component moves}}{\text{distance solvent moves}}$$

Although illegal substances are more likely to be involved in crime investigations, common over-the-counter drugs can be used to illustrate the same identification principles. TLC will be used to identify the components contained in unknown painkillers. Over-the-counter painkillers contain one or more active ingredients. The most common active ingredients are aspirin, acetaminophen, and caffeine. The active ingredients contained in painkillers can be determined by comparing the $R_f$ values obtained from unknown components with those of known solutions of aspirin, acetaminophen, and caffeine.

Materials

- Development jar with lid
- TLC plate, 4” × 2”
- Aspirin standard solution, 6–10 drops
- Acetaminophen standard solution, 6–10 drops
- Caffeine standard solution, 6–10 drops
- “Unknown” solution
- UV light source
- Ethyl acetate, 25 mL
- Microcapillary tubes, 4
- Ruler
- Pencil
Safety Precautions

The known drug solutions (aspirin, acetaminophen, and caffeine) contain a mixture of petroleum ether and ethyl alcohol solvents and are extremely flammable. The solvents are volatile and have characteristic odors—work in a well-ventilated laboratory only. They are all irritating to eyes and skin and toxic by ingestion. Never look directly at a UV light as it can be harmful to the eyes. Please review current Material Safety Data Sheets for additional safety, handling, and disposal information. Wear chemical splash goggles, chemical-resistant gloves, and a chemical-resistant apron.

Procedure

1. Obtain a TLC plate. The plates are fairly fragile and, if cracked or soiled, will yield incorrect results. Handle the plates by the edges only. Do not touch the powdery side of the plate. Place the plate powdery-side up on a piece of clean paper.

2. Use a pencil and ruler to gently draw a faint line 1 cm from the bottom of the plate and make other light markings as shown in Figure 1. Do not dig into the white powder on the plate. Note: Do not use a pen!

   ![Figure 1. Initial TLC layout](image)

3. Obtain micro tubes containing solutions of aspirin, caffeine, acetaminophen, and an unknown solution.

4. Use the end of an open-ended microcapillary tube to place one small drop of aspirin solution on the plate at the aspirin location. Let the spot dry completely. Place another drop exactly on top of the first drop and let it dry. Repeat this dropping procedure until 6–8 drops have been placed one on top of each other. Be sure to allow drying time in between each drop application.

5. Spot the caffeine, acetaminophen, and unknown on their appropriate spots on the TLC plate just like the aspirin. Allow each drop to dry before adding another drop.

6. While the plates are drying completely, add enough ethyl acetate to a developing jar until it covers the bottom and reaches a depth of 0.5 cm. Do not use too much solvent.

7. Place the developing jar on a lab bench where it will not be disturbed for approximately 20 minutes.
8. A developing jar will be shared with another working group. The TLC plates will be placed in the developing jar in pairs to form a tent-like arrangement to support the plates (see Figure 2). The lid must be on the jar during the developing process.

![Figure 2. Two TLC plates in developing jar in tent-like support.](image)

9. Carefully open the jar without moving the liquid. Pick up one plate by the top and carefully lower it into the jar so the markings are on the bottom of the jar. Add the second plate into the jar so that the shiny, plastic sides of the plates are back to back toward the center.

10. Place the lid back on the jar carefully without disturbing the plates. Do not move the jar until the development is complete.

11. When the ethyl acetate has moved up the plates to within 1 cm of the top of the plates, remove the plates and place them on paper towels (plastic side down) until the plates are dry. Remove both plates at the same time.

12. When the plates are removed, use a pencil to mark the solvent front location on the TLC card.

13. View the plate with a UV light. Your instructor will help with the light and the interpretation of the various spots. *Do not look into the UV light directly.* Use a pencil to place a dot exactly in the center of each important spot.

14. Measure the distances in cm from the line at the bottom of the plate (starting line) to the solvent front near the top. Record this distance on the TLC Worksheet.

15. Measure the distance from the starting line to the center of each spot. Record the distances and calculate the $R_f$ value for each spot on the TLC Worksheet.

16. Answer the questions on the TLC Worksheet.

**Disposal**

Consult your instructor for appropriate disposal procedures.
Name: ______________________________

**TLC Worksheet**

<table>
<thead>
<tr>
<th></th>
<th>Distance Traveled</th>
<th>Calculations</th>
<th>$R_f$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unknown</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Which known substance moved the most? The least?

2. Are the spots well separated based upon their $R_f$ values? Do you think if you had a mixture of the three substances that you would be able to separate or identify all of them?

3. Compare the size of the spots after movement through the plate compared to the initial spots. Are they larger or smaller? Give possible reason.

4. What substance(s) were in the unknown painkiller?
Teacher’s Notes
Identification of Unknown Substances II

Materials Included in Kit
- Development jars with lids, 8
- TLC sheets, 20 cm × 20 cm, 2
- Aspirin, 5 g
- Acetaminophen, 5 g
- Caffeine, 5 g

- Ethyl alcohol, 100 mL
- Petroleum ether, 50 mL
- Ethyl acetate, 200 mL
- Microcapillary tubes, 30
- Microcentrifuge tubes, 75

Additional Materials Needed (for each lab group)
- UV light source (shared)*
- Ruler
- Pencil

- Graduated cylinder (preparation)
- Mortar and pestle (preparation)
- Scissors (preparation)

*See the Tips section.

Pre-Lab Preparation
The TLC sheets should be cut into 2” × 4” pieces before use.

Depending on class size, it may be necessary to make multiple batches of test mixtures. Adjust the number of batches to the number of students in the class. Make fresh solutions; do not store the solutions. Grind all tablets with a mortar and pestle prior to dissolving.

Aspirin Standard: Place approximately 0.5 g of aspirin (1 tablet) in a beaker. Add 20 mL of ethyl alcohol and stir or swirl the beaker for one minute to dissolve. All of the solid may not dissolve. Allow the mixture to settle and dispense the clear liquid solution into labeled microcentrifuge tubes. Close the top of each tube tightly.

Caffeine Standard: Place 0.5 g of caffeine in a beaker. Add 20 mL of ethyl alcohol and stir or swirl the beaker to dissolve (about one minute). All of the solid may not dissolve. Dispense the solution into labeled microcentrifuge tubes. Close the top of each tube tightly.

Acetaminophen Standard: Place 0.5 g acetaminophen in a beaker. Add 10 mL of ethyl alcohol and 10 mL of petroleum ether. Swirl the beaker for one minute. All of the solid may not dissolve. Dispense the solution into labeled microcentrifuge tubes. Close the top of each tube tightly.

Unknown Solutions: Unknowns can be made of aspirin, acetaminophen, caffeine or any combination of these three substances. Keep track of what is placed in the unknown solutions and mix them into solution following the same directions as above. Place the unknowns into microcentrifuge tubes and close tightly.

Safety Precautions
Petroleum ether, ethyl alcohol, and ethyl acetate are all flammable liquids and dangerous fire risks. They are all irritating to the eyes and skin and toxic by ingestion. Caffeine powder is very toxic! Never look directly into a UV light as it can be harmful to the eyes. Please review current Material Safety Data Sheets for additional safety, handling, and disposal information. Wear chemical splash goggles, chemical-resistant gloves, and a chemical-resistant apron.

Disposal
Please consult your current Flinn Scientific Catalog/Reference Manual for general guidelines and specific procedures, and review all federal, state and local regulations that may apply, before proceeding. Residual over-the-counter drug mixtures may be disposed of according to Flinn Suggested Disposal Method #18a and #26b. Ethyl alcohol may be disposed of according to Suggested Method #26b. Save and recycle the developing solvent (ethyl acetate) for future use.
Connecting to the National Standards

This laboratory activity relates to the following National Science Education Standards (1996):

Unifying Concepts and Processes: Grades K–12
- Evidence, models, and explanation

Content Standards: Grades 9–12
- Content Standard A: Science as Inquiry
- Content Standard B: Physical Science, structure and properties of matter
- Content Standard F: Science in Personal and Social Perspectives; science and technology in local, national, and global challenges

Tips

• Enough materials are provided in this kit for 30 students working in pairs, or for 15 groups of students. The laboratory activity can be completed in one 50-minute class period.

• Visualization of the compounds on TLC plates requires a short-wavelength UV light source—a black light is not high enough energy.

• White powders (aspirin, acetaminophen, or caffeine) can be placed in your simulated crime scenes. They can be carefully collected and dissolved similarly to the Pre-Lab Preparation directions and then TLC can be used to analyze the substance’s components.

• The TLC sheets contain a fluorescent powder that glows bright green when placed under a short-wave UV lamp. The spots that contain separated compounds will not glow green and will frequently be dark. Carefully mark the location of the spots using a pencil while the TLC plate is under the UV lamp. Trace an outline of the spots on the plate so a record of the TLC can be kept.

• When spotting the TLC sheet, keep the spot as small as possible and make sure the spot will be above the solvent line in the developing jar. Briefly and gently touch the tip of the micropipet tube to the TLC surface. Let the solvent evaporate before touching the capillary tube to the TLC plate again. Touch the capillary to the same spot again. Remove the capillary and gently blow on the spot to evaporate the solvent. The spot should not be more than 2 mm in diameter when completed.

• Carefully place the TLC sheet in the developing jar, making sure that the sample end is down but the spots are above the solvent. The TLC run is stopped when the solvent goes about 80% up the plate.

• Good TLC technique is required for students to see well-separated, little spots on their sheets. Some of the common causes of poor separations are:
  1. Wrong solvent mixture applied to the TLC sheet.
  2. Too much sample applied to the TLC sheet.
  3. Initial spot too large.
  4. Initial spot is below the solvent level in the developing jar.

• Allow enough time for the development of the TLC sheets. The sheet must be left in the jar long enough for the solvent to be drawn up to the end of the sheet. Do not stop the development until the solvent front nears the top of the plate. Typical time for development is 10–20 minutes.
Sample Data

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<tr>
<th>Substance</th>
<th>Distance Traveled</th>
<th>Calculations</th>
<th>( R_f ) Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>To solvent front: 7.5 cm, To spot: 6.5 cm</td>
<td>( R_f = \frac{6.5, \text{cm}}{7.5, \text{cm}} )</td>
<td>0.87</td>
</tr>
<tr>
<td>Caffeine</td>
<td>To solvent front: 7.5 cm, To spot: 1.7 cm</td>
<td>( R_f = \frac{1.7, \text{cm}}{7.5, \text{cm}} )</td>
<td>0.23</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>To solvent front: 7.5 cm, To spot: 4.8 cm</td>
<td>( R_f = \frac{4.8, \text{cm}}{7.5, \text{cm}} )</td>
<td>0.64</td>
</tr>
<tr>
<td>Unknown</td>
<td>Results will vary.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Answers to Post-Lab Questions

1. Which known substance moved the most? The least?
   Aspirin moved the most and caffeine the least.

2. Are the spots well separated based upon their \( R_f \) values? Do you think if you had a mixture of the three substances that you would be able to separate or identify all of them?
   The spots were well separated and easily identifiable.

3. Compare the size of the spots after movement through the plate compared to the initial spots. Are they larger or smaller? Give possible reason.
   If initial spots are allowed to dry, the initial spots will be smaller than the spots after dissolving in the solvents.

4. What substance(s) were in the unknown painkiller?
   Answers will vary.

The Identification of Unknown Substances II—Forensic Investigation Kit is available from Flinn Scientific, Inc.

<table>
<thead>
<tr>
<th>Catalog No.</th>
<th>Description</th>
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<td>Identification of Unknown Substances II—Forensic Investigation Kit</td>
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<tr>
<td>AP5261</td>
<td>Ultraviolet Lamp, Long Wave/Short Wave, Versalume Model</td>
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