Max Shi  
CH 362  
Professor Attygalle  
I pledge my honor that I have abided by the Stevens Honor System.

**1) Title of Experiment:**

Separation of Reverse Phase Text Mixture and Determination of Caffeine Concentration in Commercial Beverage by High-Performance Liquid Chromatography

Date: November 14, 2020  
Name of Techinque: High-Performance Liquid Chromatography

**2) Technique:**

High-Performance Liquid Chromatography (HPLC) is a technique used for separation of compounds in mixtures, and can be used to determine the identity of compounds and their concentrations. HPLC is a type of chromatography, in which analytes are injected into a system and are separated based on each compounds’ ability to interact with the mobile and stationary phases in the system. In HPLC, the mobile phase is the solvent in which the analyte is dissolved in, usually water, acetonitrile, and/or methanol. The stationary phase is dependent on the type of HPLC being conducted: Normal Phase HPLC packs its columns with silica gel, a polar compound, while Reversed Phase HPLC packs its columns with octadecylsilyl coated silica, making the silica nonpolar. As the analyte passes through the column after being injected into the system, the different compounds in the analyte mixture interact with the stationary phase in different capacities, changing each component’s average velocity through the column, allowing them to separate and arrive at the detector at different times. The difference between HPLC and other types of chromatography involve the use of a liquid mobile phase and the use of a high-pressure system to push the liquid mobile phase through the column, as detailed in Figure 1.

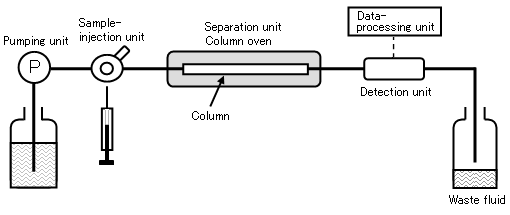


Figure 1: Diagram of HPLC Setup

Furthermore, the area of the peaks are dependent on the amount of analyte that arrives at the detector, thus relating this process to concentration of the different components in the mixture. Combining this with an internal standard will allow the recovery of the unknown concentration of a component of the analyte.

**3) Application of the Technique to My Experiment:**

For the first part of this experiment, HPLC will be used to separate a Reverse Phase Test Mixture made out of uracil, acetophenone, methyl benzoate, toluene, and naphthalene. Before the HPLC machine is used, the column parameters, solvent flow rate and pressure, the dimensions of the injector loop, and the ambient temperature of the lab will be recorded. Then, the HPLC machine will be set up to run a 50:50 water:acetonitrile isocratic run. Next, 10 µL of the test mixture will be loaded into the injection loop with a syringe, and the sample will be injected with the injection loop. A chromatogram will be recorded at an absorption wavelength of 254 nm. Then, after viewing the chromatogram and the UV spectra of all components, the injection will be repeated two more times. Using the chromatograms from all three trials, each peak area will be integrated and recorded. Then, the experiment will be repeated three more times, except this time, injecting 40 µL instead of 10 µL. Again, the peak areas will be integrated on each chromatogram. From each UV spectra, the chromatographic peaks can be assigned to each compound, and the peak areas between the 10 µL and 40 µL can be compared.

For the second part of this experiment, HPLC will be used to determine the caffeine concentration in a commercial beverage. To begin, 5.00 mL each of 1.000 mM benzophenone, 1.000 mM caffeine, and 1.000 mM benzoic acid will be mixed together, and 30 µL will be injected into the HPLC injection loop, and a chromatogram will be taken. This chromatogram will be used to determine the relative molar response ration of caffeine and benzoic acid to benzophenone, which will act as the internal standard for this part of the experiment. Next, to create a calibration curve, four 25 mL calibration solutions of 0.2, 0.4, 0.6, and 0.8 mM caffeine will be prepared from the stock 1.0 mM solution. Then, after creating another solution of 0.2 mM benzophenone, 10.0 mL each of the 0.2 mM benzophenone and each calibration solution separately will be combined to create four calibration mixtures. 30 µL of each mixture will be injected into the loop and chromatograms will be taken of each mixture. Then, 10.0 mL of the commercial beverage and 10.0 mL of the 0.2 mM benzophenone will be mixed, and 30 µL of this mixture will be injected and a chromatogram of this mixture will be recorded, representing the unknown. From these chromatograms, the peaks of benzophenone and caffeine can be identified, the peak areas can be integrated, and plotting a calibration graph of in accordance to Equation 1 can recover an RRF (Relative Response Factor) value, which can be used to determine the concentration of caffeine in the commercial beverage.

Equation 1: Relationship of Calibration Variables for Regression Line

**4) Calculations:**

A 25.00 mL x mM solution requires 0.025x millimoles of solute. 1 mL of 1.0 mM solution contains 0.001 millimoles of solute.

Creating a 0.2 mM solution of benzophenone from a 1.0 mM stock solution requires 1 part solution to 4 parts deionized water.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Desired concentration of caffeine solution (mM) | Desired volume of caffeine solution (mL) | Required volume of stock 1.0 mM caffeine solution (mL) | Required volume of water | Concentration of benzophenone after mixture (mM) | Concentration of caffeine after mixture (mM) |
| 0.2 | 25.00 | 5 | 20 | 0.1 | 0.1 |
| 0.4 | 25.00 | 10 | 15 | 0.1 | 0.2 |
| 0.6 | 25.00 | 15 | 10 | 0.1 | 0.3 |
| 0.8 | 25.00 | 20 | 5 | 0.1 | 0.4 |

**5) References:**

1. Attygalle, A. Instrumental Analysis I Lecture and Laboratory Manual <https://sit.instructure.com/courses/38802/files/6982711?module_item_id=1042514> (accessed Nov 14, 2020).
2. Harris, D. C. *Quantitative Chemical Analysis*, 8th ed.; W.H. Freeman and Co: New York, 2010. Chapter 24.
3. lc\_course3\_01.jpg (PNG Image, 510 x 215 pixels) <https://www.hitachi-hightech.com/image/global/products/science/tech/ana/lc/basic/lc_course3_01.jpg> (accessed Nov 14, 2020).

**6) MSDS:**

**Uracil**

CAS No.: 66-22-8  
Molecular Weight: 112.09  
Chemical Formula: C4H4N2O2  
Appearance: off-white solid   
Lab Protective Equipment: Lab coat, goggles

**Health effects:**May cause skin and eye irritation, may cause respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with soap and plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. Get medical attention.  
Ingestion: Clean mouth with water. Get medical attention.

**Other hazards:**Fire: not known to be a fire hazard.  
Explosion: not known to be an explosion hazard.

**Acetophenone**

CAS No.: 98-86-2  
Molecular Weight: 120.15  
Chemical Formula: C8H8O  
Appearance: colorless, slightly oily liquid   
Lab Protective Equipment: Lab coat, goggles  
Combustible liquid and vapor.

**Health effects:**May cause skin irritation. Causes eye irritation, may cause respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Do not induce vomiting. Get medical attention.

**Other hazards:**Fire: combustible liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Methyl Benzoate**

CAS No.: 93-58-3  
Molecular Weight: 136.0548  
Chemical Formula: C8H8O2  
Appearance: clear to light yellow liquid   
Lab Protective Equipment: Lab coat, goggles  
Combustible liquid and vapor.

**Health effects:**May cause skin irritation and eye irritation, may cause respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration with oxygen and a suitable mechanical device. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Do not induce vomiting. If victim is conscious and alert, give 2-4 cupfuls of milk or water. Never give anything by mouth to an unconscious person. Get medical attention.

**Other hazards:**Fire: combustible liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Toluene**

CAS No.: 108-88-3  
Molecular Weight: 92.14  
Chemical Formula: C6H5CH3  
Appearance: colorless liquid   
Lab Protective Equipment: Lab coat, goggles  
Combustible liquid and vapor. Aspiration hazard.

**Health effects:**Causes eye and skin irritation, respiratory and digestive tract irritation. Can enter lungs and cause damage.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Do not induce vomiting. Potential for aspiration if swallowed. Get medical attention.

**Other hazards:**Fire: combustible liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Naphthalene**

CAS No.: 91-20-3  
Molecular Weight: 128.17  
Chemical Formula: C10H8  
Appearance: white solid  
Lab Protective Equipment: Lab coat, goggles  
Flammable solid.

**Health effects:**Causes skin irritation, eye irritation, respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Do not induce vomiting. Get medical attention.

**Other hazards:**Fire: flammable solid.  
Explosion: not known to be an explosion hazard.

**Methanol**

CAS No.: 67-56-1  
Molecular Weight: 32.04  
Chemical Formula: CH4O  
Appearance: clear, colorless liquid   
Lab Protective Equipment: Lab coat, goggles  
Flammable liquid and vapor. Poisonous

**Health effects:**May be fatal or cause blindness if swallowed. Causes eye, skin, and respiratory tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Potential for aspiration if swallowed. Do not induce vomiting. Get medical attention.

**Other hazards:**Fire: flammable liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Acetonitrile**

CAS No.: 75-05-8  
Molecular Weight: 41.05  
Chemical Formula: C2H3N  
Appearance: clear, colorless liquid  
Lab Protective Equipment: Lab coat, goggles  
Combustible liquid and vapor.

**Health effects:**May cause skin irritation, eye irritation, respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention.

**Other hazards:**Fire: combustible liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Benzophenone**

CAS No.: 119-61-9  
Molecular Weight: 182.0694  
Chemical Formula: C13H10O  
Appearance: white solid  
Lab Protective Equipment: Lab coat, goggles  
May cause eye and skin irritation, respiratory and digestive tract irritation.

**Health effects:**May cause eye and skin irritation, respiratory and digestive tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: If victim is conscious and alert, give 2-4 cupfuls of milk or water. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention.

**Other hazards:**Fire: not known to be a fire hazard.  
Explosion: not known to be an explosion hazard.

**Caffeine**

CAS No.: 58-08-2  
Molecular Weight: 194.0956  
Chemical Formula: C8H10N4O2  
Appearance: white solid  
Lab Protective Equipment: Lab coat, goggles

**Health effects:**May cause eye and skin irritation, respiratory and digestive tract irritation. Harmful if swallowed. May cause cardiac disturbances.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: Wash off immediately with plenty of water while removing all contaminated clothes and shoes. Obtain medical attention if irritation develops or persists.  
Inhalation: Move to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.  
Ingestion: Call a poison control center. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention.

**Other hazards:**Fire: combustible liquid and vapor.  
Explosion: not known to be an explosion hazard.

**Benzoic Acid**

CAS No.: 65-85-0  
Molecular Weight: 122.12  
Chemical Formula: C7H6O2  
Appearance: white powder   
Lab Protective Equipment: Lab coat, goggles

**Health effects:**Causes skin irritation and severe eye irritation. Harmful if swallowed and causes respiratory tract irritation.

**First Aid measures:**Eye contact: rinse immediately with water, especially under eyelids, for >15 minutes. Get medical attention.  
Skin contact: wash off immediately with plenty of water for >15 minutes. Remove contaminated clothing. Obtain medical attention.  
Inhalation: Move to fresh air. If breathing if difficult, give oxygen. If not breathing, give artificial respiration with a one-way valve. Get medical attention immediately if symptoms occur.  
Ingestion: Do not induce vomiting. Do not give anything by mouth to an unconscious person. Get medical aid. If conscious and alert, rinse mouth and give 2-4 cupfuls of milk or water.

**Other hazards:**Fire: flammable.  
Explosion: dust may form explosive mixtures with air.

**7) Pre Lab Questions:**

1. A sample loop is used because the loop in HPLC is under very high pressure, and to inject a sample at such a high pressure directly would involve a very expensive and complex injector. Instead, using a sample loop, a sample can be injected into the HPLC machine using a regular syringe, as the sample gets rotated and pressurized by rotating the sample loop. Not only does this eliminate the need for a complex injector, but it also does not require us to keep aliquots of a sample at high pressure, which could also be dangerous, depending on the compound. The sample loop pressurizes only the injected portion, which is a very small sample, on the magnitude of microliters.
2. By this chromatogram, it can be said that there are three components of the mixture, as there were three peaks. Because peak area is directly proportional to concentration, and by Equation 1, those quantities are related to each other, it can be said that the concentration of the second compound is two times the concentration of the first compound, and the third compound is five times as concentrated as the first. Furthermore, depending on the type of HPLC conducted, the polarity can be compared. For example, if this was Reverse Phase, the first compound could be said to be the most polar out of the three compounds.