Determination of Caffeine by HPLC

Introduction
It was a long history before real high performance liquid chromatography (HPLC) had evolved. The very first indication of a chromatographic separation was introduced in 1903 by the Russian botanist M. S. Tswett on the separation of plant pigments using powdered calcium carbonate. However, it was not before 1965 that modern HPLC was developed. The main reason was a technological one were high pressure pumps, well packed columns and sensitive detectors with extremely low volume flow cells were necessary.

Chromatography versus Extraction
It is a common practice to start with simple extraction process as an introduction to chromatography.

When a solute is distributed between an organic phase and an aqueous phase, the distribution constant (K) can be defined as:

\[ K = \frac{C_0}{C_{aq}} \]  

(1)

Where \( C_0 \) is the concentration of solute in the organic phase and \( C_{aq} \) is its concentration in the aqueous phase.

It is clear that in order to decrease the concentration of solute in the aqueous phase to zero, multiple extractions with new fresh organic phase should be conducted.

Another constant can be defined which is known as capacity factor. This factor gives the ratio between the amounts of solute in the organic phase to that in the aqueous phase

\[ K' = \frac{C_0 \cdot V_0}{C_{aq} \cdot V_{aq}} \]  

(2)

\( K' \) is the capacity factor. \( V_0 \) and \( V_{aq} \) are volumes of the organic and aqueous phases, respectively.

Note that \( C \cdot V \) give the number of moles.

From equation 1 and 2 it can be shown that

\[ K' = KV_{r} \]  

(3)

Where \( V_{r} \) is the phase volume ratio.
A third separation constant can now be defined which is known as the selectivity factor. Assume that two solutes are present in solution, the selectivity factor (\( \alpha \)) can be written as

\[
\alpha = \frac{K_1}{K_2} = \frac{K_1'}{K_2'}
\]

There will be no possible separation if \( \alpha = 1 \). As \( \alpha \) is different from 1 it is possible to separate the two solutes by extraction, and thus by chromatography.

Chromatography is very similar to situations mentioned above, i.e. the presence of the two phases. However, in chromatography one phase is fixed and called the **stationary phase** and the other is moving and called the **mobile phase**. Since one phase is fixed and the other is moving, the separation process in chromatography is a dynamic one. Solutes interact with both phases which is the main reason for selective separation because different solutes interact differently.

**Reversed-Phase Liquid Chromatography (RPLC)**

The mobile phase, in this case, is more polar than the stationary phase. This mode of chromatographic separations is the most important and the most widely used. Detailed theoretical background of chromatographic separation concepts will be developed for this type of separation techniques.

**Retention in Chromatography**

Differential migration of solutes through the chromatographic column is the basis of separation. Solutes tend to migrate in different rates through the column due to different distribution constants. It is always a good practice to relate migration to affinity of a solute towards stationary or mobile phase. When a specific solute has higher affinity towards the stationary phase than the mobile phase, the solute will be retained in the column for a longer time than a solute of lower affinity towards the stationary phase. Figure 1 shows a chromatogram of one solute.

![Chromatogram](image)

**Figure 1:** Retention time
Retention time = \( t_R - t_o \) \hspace{1cm} (5)

\( t_R \) is the retention time of solute at the peak maximum and \( t_o \) is the retention time of unretained solute and is called dead or void time.

The efficiency of separation can be calculated as:

\[ N = 5.54 \left( \frac{t_R}{W_{1/2}} \right)^2 \]

The efficiency calculated per meter of column length is: \( N \times 100 / \text{(column length)} \)

**Instrumentation**

Description of HPLC instrumentation is very simple. A good high pressure pump equipped with good pulse dampers is used for mobile phase delivery. A loop valve injector is used for sample introduction into the chromatographic column (the heart of HPLC system) where separation takes place. The effluents pass through a microflow cell placed inside a suitable detector. Detectors can be UV, Vis, fluorescence, electrochemical, refractive index, or a photodiode array (our detector in the system we have). Figure 2 shows a schematic of a typical HPLC system.

![Figure 2: A schematic of a typical HPLC system.](image)

Refractive index (RI) detectors find excellent applications. The most widely used indicators are UV and RI detectors while analytical columns that are 5, 10, 15 and 25 cm long and 4.6 mm in diameter are the most frequently used dimensions with the 15 and 25 cm long being the most widely used in analytical applications. Other dimensions are also available but of limited advantages and are used for some specific applications. A schematic of a loop valve injector is shown in Figure 3.
Figure 3: A schematic of a loop valve injector.

Qualitative and Quantitative Analysis

Qualitative analysis is possible by injection of standards and comparison of the retention times obtained. Usually, the components of a sample are known but separation of these components from each other and determination of their quantity is the most likely required information. Quantitative determination of a particular component can be achieved by several methods ranging from manual to digital integration procedures. Peak height or area is the most widely used factors for comparison with standards in order to determine the amount of an unknown component concentration. Peak area can be determined by multiplication of peak height times half the peak width at the base line or peak height times peak width at half height. Usually, precision exceeding 5% are achieved using any of the methods mentioned above. However, precisions better than 1% can be obtained when electronic digital integrators are used for calculation of peak area.

Background

Soft drinks usually contain appreciable amounts of saccharin (artificial sweetener), benzoic acid (preservative), and caffeine. Determination of all these species is possible by HPLC separation on a C18 column and UV detection at about 250 nm. In addition, many pharmaceutical formulations contain caffeine as well, and it is important to quantify this ingredient as well as principal ingredients. The method is relatively fast, simple, and produces excellent results. In this experiment, we will determine caffeine only at about 270 nm.

Chemicals and Reagents

a. Provided
1. Mobile phase containing 60:40 water/methanol (1.5% glacial acetic acid is added to water before mixing with methanol).
2. Caffeine standard solution (about 200 ppm).

b. Need Preparation
1. Degas your Cola soft drink in a sonicator or under vacuum. Transfer exactly 10 mL of the 0.2 μm filtered cola to a 50 mL volumetric flask and fill to mark using your provided mobile phase.
2. From your standard stock caffeine solution, prepare three dilute solutions by transferring 5, 10, and 15 mL to three 50 mL volumetric flasks and complete to mark using your mobile phase. Solution (Sample 1).

3. Accurately weigh a pharmaceutical tablet that contains caffeine (Paramol Plus contains about 65 mg of caffeine). Using a mortar and pestle, grind the tablet into a very fine powder and weigh out about 150 mg of the powder. Dissolve the accurately weighed powder in about 25 mL of mobile phase in water, transfer to a 50 mL volumetric flask and complete to mark with the mobile phase, after very good shaking. Filter about 15 mL of this solution using a 0.2 µm filter disc.

4. Transfer 5 mL of the 0.2 µm filtered preparation into a 50 mL volumetric flask and fill to mark using your provided mobile phase (Sample 2).

Procedure

1. Turn on your devices, and adjust your method and separation parameters as indicated in the standard operation procedure provided with the experiment. Select a flow rate of 1.5 mL/min and a wavelength of 270 nm. Make sure to include appropriate names for your runs.

2. Flush the column for about 10 min at 1.5 mL / min with the mobile phase, till a stable base line is obtained.

3. After you are ready and the baseline is satisfactory, inject your standards one by one and record the resulting chromatograms.

4. Inject your sample, and record the resulting chromatogram. All settings should be kept the same as in step 3.

5. Determine the concentration of Caffeine in either or both samples (as instructed) from the corresponding peak height and/or peak area, as compared to standards (you should use a calibration curve).

6. When you are done, flush the column with the 50 % methanol solution for 10 min (1.5 mL/min), and turn the system off as instructed.

7. Report your results as ppm caffeine in the original sample.

Note: Failure to flush the column may result in a clogged column. Please clean after each class.

Data/Calculations:

<table>
<thead>
<tr>
<th>Standard (mL)</th>
<th>Peak Area</th>
<th>Peak Height</th>
<th>Ret. Time</th>
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1. List retention times, height, and areas for the caffeine peak in your samples, and use peak height or area to determine the concentration of the caffeine.

2. Use the peak width at half height to calculate the separation efficiency for 1.00 m of the column, using the peak for the caffeine sample.