Experiment 3

Using Internal Standards in High-Performance Liquid Chromatography (HPLC)

(Modified "Using an Internal Standard in High-Performance Liquid Chromatography" from "Exploring Analytical Chemistry" by D. Harris)

Theory

Chromatography is an important analytical tool for separating and quantifying components in a complex liquid mixture. In this physical separation method the components of the mixture are partitioned between two phases. One of the phases stays in place and is called a stationery phase, whereas the other moves and is called mobile phase. According to the type of the mobile phase we distinguish between gas chromatography (GC), supercritical fluid chromatography (SFC), and liquid chromatography (LC). The separation is based on the different partition coefficients of the sample components between the two phases. In liquid chromatography (LC) and gas chromatography (GC) the sample is introduced rapidly into a mobile phase – a liquid or gas respectively. As the analyte traverses through the column components of the sample are separated owing to its specific partitioning between the liquid mobile phase and the stationary phase. Using the language of the separation processes we can divide the chromatographic column into hypothetical zones – theoretical plates. Within each plate new partition equilibrium is established between components of the sample in the two phases (stationary and mobile). Since performance of a separation process is enhanced by providing more theoretical plates the more equilibrium processes can take place within a column of a given length the narrower theoretical plate will allow more demanding separation processes to be solved.

The components of the analyte that have been separated are detected by measuring changes in some chemical and physical properties. The most common detectors register the changes in conduction of electrical current, light absorption, or the ability to conduct heat.

The number of theoretical plates increases with the smaller size of the particles of the stationery phase, therefore the high-performance liquid chromatography (HPLC) uses micron-size particles for stationary phases (10 μm or less). However, such fine column packings results in high flow resistance which makes it necessary to use pumps to force analyte through a column of the stationary phase. The main advantage of the HPLC over the gravity liquid chromatography is that samples can be separated more quickly. It also allows separation of samples which would thermally decompose or which are not volatile and because of that cannot be separated using gas chromatography (GC).

The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The retention time depends

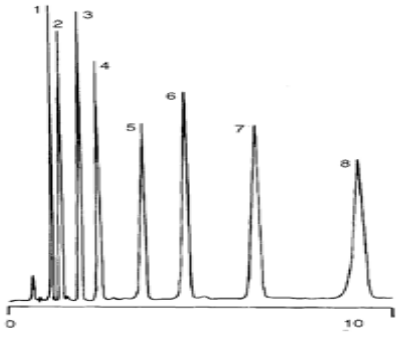

Figure 1. Example of a chromatogram. Each peak corresponds to a different substance eluted from the column. Retention time (x-axis) is the time needed after injection for an individual solute to reach the detector.

on the nature of the analyte, stationary phase and mobile phase compositions, and mobile phase flow rate. The different types of interaction of the solute with the stationary phase give rise to classification of HPLC techniques as: adsorption, partition, ion-exchange, molecular exclusion, and affinity chromatography. In this experiment we will be concerned with the *partition chromatography* in which the solute partitions between the liquid mobile phase and a second, immiscible liquid that is coated on or bonded to surface of solid support. Compounds that partition more strongly into stationary phase are retained longer in the column. If the stationary phase is more polar than the mobile phase this type of chromatography is referred to as *normal phase*, the opposite; when the mobile phase is more polar than the stationary phase is called *reverse phase* chromatography. Liquid-liquid partition chromatography is commonly conducted with a bonded stationary phase covalently attached to silanol groups on the silica surface. The octadecyl (C_{18}) stationary phase is by far the most common in reverse phase HPLC. Columns packed with this material are very non-polar; they are referred to as C_{18} column. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Therefore, the retention Time (t_R) of a non-polar analyte is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent.

The final result of a chromatography experiment is a *chromatogram* (Figure 1) which shows detector response as a function of retention time. The most common detector in HPLC is an ultraviolet detector with a flow cell. Simple systems employ a fixed wavelength, the intense 254-nm emission of a mercury lamp for example. In more sophisticated systems, the UV-vis absorption spectrum of each solute can be recorded with a photodiode array. A problem with these detectors is that, although most compounds absorbs the UV wavelengths, the degree of absorption, and therefore the sensitivity, varies significantly between different types of compounds. For example aromatics will absorb strongly, but paraffins will not. A more universal detector is the refractive index detector which measures the deflection of a light ray by the eluate. In general, a solute has a different refractive index from that of a solvent.

To judge the chromatogram it is necessary to calculate some data. Usually, this task is performed by the computer software which controls the HPLC instrument and allows analysis of the collected data. An ideal chromatographic peak has a Gaussian shape like that in Figure 2. As mentioned before, the retention of a compound on a column is described by the number of theoretical equilibration steps that occur between the injection and elution. We can think of a column as if it were divided into discrete sections (theoretical plates) in which a solute equilibrates between mobile and stationary phase. The more equilibration steps (theoretical plates), the narrower the bandwidth when a compound emerges. The number of theoretical plates, *N*, can be calculated from a chromatographic peak using one of the following relationships

$$N = 16\left(\frac{t_R}{w_b}\right)^2 = 5.55\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (1)$$

where t_R = retention time, w_b=peak width, $w_{1/2}$= peak width at half-high (measured at ½ peak height), see Figure 2 for description. Both t_R and $w_{1/2}$ must be expressed in the same units time or volume. Most factors that affect column efficiency are evaluated by their effect on *H*, the height of the theoretical plate which is determined by

$$H = \frac{L}{N} \qquad (2)$$

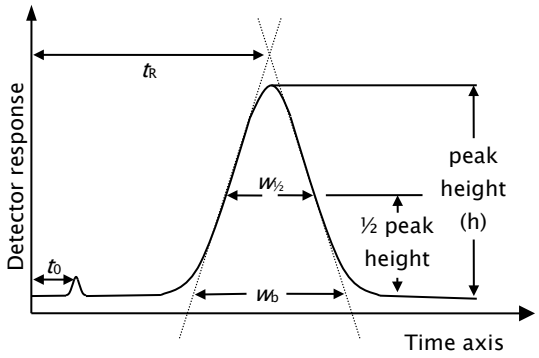

Figure 2. Schematic of an ideal chromatographic peak, showing measurement of retention time t_R, dead time of column t_0, width at the base w_b, and width at half-height $w_{½}$.

where L is the length of the column. The theoretical plate height is affected by the following experimental parameters:

- Diffusion coefficient of the solute in the mobile and stationary phases (depends on temperature and viscosity).
- Retention factor which is a measure of the retention of a peak. It depends on the phase systems (the types of mobile and stationary phase) and temperature.
- Diameter of the particles packing of the column.
- Thickness of the liquid coating on the stationary phase.
- Mobile phase flow rate.

As the last point indicates the performance of a column is not independent of the mobile phase flow rate. An optimum flow rate, u_{opt}, is observed where the performance is the highest as judged by the height of a theoretical plate. This relationship is described by the van Deemter curve (Figure 4) which plots the high of a theoretical plate , H, as a function of the linear flow velocity u_{opt}. At u_{opt} the plate height, H, is smallest which, according to the equation 2 gives the highest number of theoretical plates. The peaks are narrowest and the maximum possible resolution is reached. Any deviation from the optimum flow rate yields smaller peak heights and resolutions; yet the optimum velocity is not identical for all the compounds of a sample mixture.

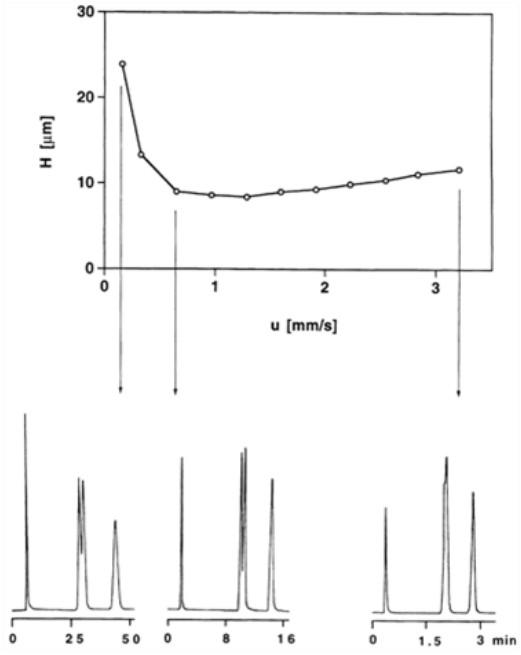

Figure 4. A plot of the plate height (H) as a function of the linear flow velocity (top) and corresponding chromatograms.

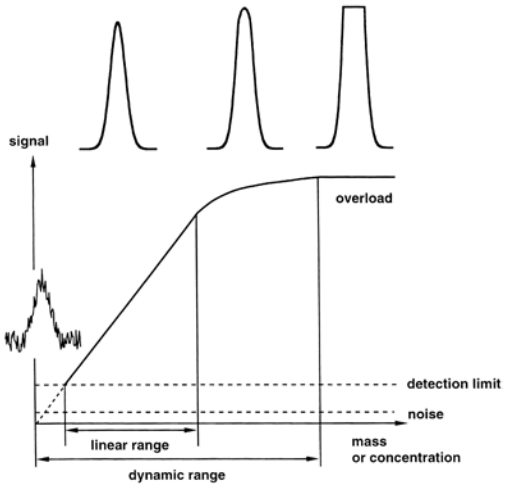

Figure 3. A schematic drawing showing the detector response signal (y axis) as a function of mass or concentrations (bottom of the picture) and the corresponding peak shapes.

The resolution, R_s, between two closely spaced peaks A and B is determined by their separation and their widths

$$R_S = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}} = \frac{1.18(t_{R2} - t_{R1})}{\left((w_{1/2})_{b1} + (w_{1/2})_{b2}\right)} \quad (3)$$

where the indices 1 and 2 designate the two peaks. AT resolution R_S = 1.0 the baseline between the peaks is not reached. Complete resolution is obtained at R = 1.5 or higher depending on the height ratio of the peaks. The smaller a peak compared with its large neighbor the greater is the resolution necessary to resolve the two peaks.

We expect the detectors to yield signal which is proportional to the amount of sample injected over a wide range of mass or concentration. This so-called *linear range* is represented by a straight line in a plot of signal as a function of mass or concentration. Within the

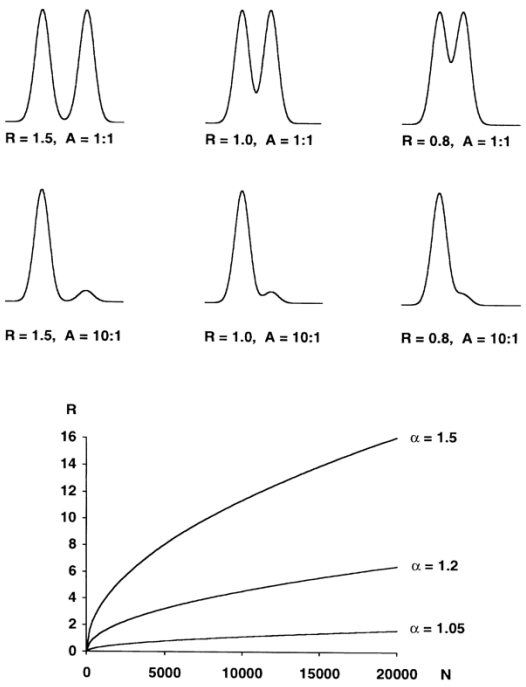

Figure 5. The upper figure presents several pairs of peaks separated with varying resolution (R). The graph below demonstrates how the resolution increases with increasing plate number (N) for three different separation numbers ()

linear range the peaks are recorded with their true shape. If more sample is injected than is allowed by the linear range the signal is too flat. Although, there is still a dependence of signal on mass or concentration this relationship is weak and accurate quantitative analysis is not possible. If the sample amount is even larger than the dynamic range the signal becomes saturated. Under such circumstances the UV detector does not register any light and the peaks are cut off horizontally. A calibration curve must be confined within the linear range and it is wrong to extrapolate a calibration curve beyond the linear range. In order to be sure that samples of high concentration are analyzed accurately the upper end of the linear range must be known. If necessary the samples can be diluted to avoid this problems.

Since the area under each peak is proportional to the concentration of that component in the original mixture. Hence, HPLC can be also used in quantitative analysis. Internal standards are commonly used for this purpose in HPLC. An internal standard is a known amount of a compound, different from analyte, which is added to the unknown. As long as the concentration of standard is known, the concentration of the analyte can be derived. This approach is especially useful for analyses in which the quantity of sample analyzed or the instrument response varies slightly from run to run. The detector generally has a different response to analyte and standards; however, the relative response to each component is usually constant over wide range of conditions and can be calculated from

$$\frac{A_X}{[X]} = F\left(\frac{A_S}{[S]}\right) \qquad (4)$$

If the concentrations of the analyte and standard, [X] and [S], respectively are the same and the area under the analyte peak, A_x, is 10 times greater than the area under the standard peak A_S, we say that the **response factor**, F, is 10 times greater for analyte than for standard.

A typical high-performance liquid chromatograph consists of container of mobile phase, a pump capable of high pressures up to 4000 psi or greater, a valve for injecting the sample, the column, a detector with associated electronics, and a recorder (computer). Figure 6 shows the components of our Agilent HPLC with all the above-mentioned components. This instrument is fully controlled by a personal computer.

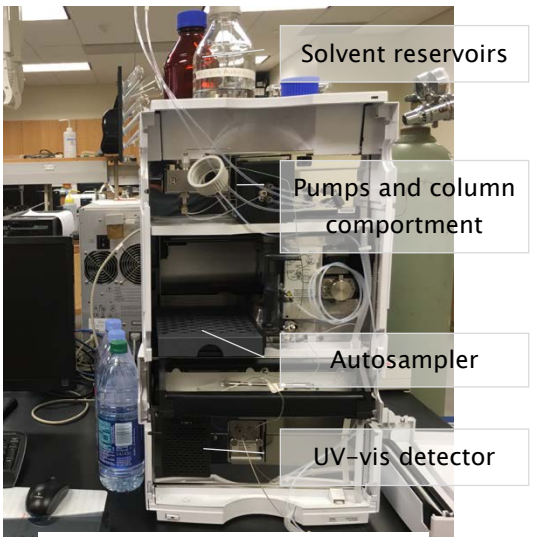

Figure 6. Agilent chromatograph, instrument components.

Experimental

You will receive an unknown solution containing two compounds from the set biphenyl, naphthalene, and 2-naphthol. The goal of this experiment is to identify the components in your unknown using high-performance liquid chromatography and to measure the concentration of one component using an internal standard method.

Solutions

To be prepared:

- ✓ Standard solutions. Biphenyl (1 mg/mL), naphthalene (1 mg/mL), and 2-naphthol (1 mg/mL) in HPLC grade methanol

Provided:

- ✓ HPLC solvent: Methanol (HPLC grade).
- ✓ Unknown mixture of any two of the standard compounds dissolved in HPLC solvent.

Equipment

- ✓ Agilent HPLC system
- ✓ Reverse phase Ascentis C_{18} HPLC column (10 cm long)
- ✓ 10 mL volumetric flasks (5)
- ✓ 1mL pipet
- ✓ 1.5 mL HPLC vials
- ✓ 5 mL vials
- ✓ Analytical balance

Notes on use of HPLC instrument:

Your instructor will show you how to use the equipment and the software. Condensed instructions are provided in step 3 of the procedure.

Procedure

1. CAUTION: All organic solvents should be handled in a fume hood. Vessels should be sealed whenever possible to minimize evaporation of methanol. If solvent evaporates from the solutions you use for quantitative analysis, the analysis becomes inaccurate.
2. *Prepare standards.* If not prepared beforehand, prepare 10 mL of biphenyl, naphthalene, and 2-naphthol in HPLC grade methanol by dissolving the appropriate amount of each standard in HPLC grade methanol to obtain accurate concentrations of ~1 mg/mL in fresh volumetric flasks. Filter the solutions through a 0.22 μm syringe filter. Exact concentrations should be labeled.
3. *Establish separation:* If a method is not already established, set up the instrument to inject 10 μL of the unknown into a C_{18}-silica column and elute it at 0.75 mL/min with 60 vol % methanol in water. Monitor the eluate with an ultraviolet detector at 254 nm wavelength. If the peaks are too intense, inject a smaller volume or dilute the unknown as necessary to bring them on scale. If you make a dilution you must know the exact dilution factor. If peaks overlap, try reducing the percentage of methanol in the mobile phase (to, say, 55 %) or use a lower flow rate. If there is a good separation you could try increasing the flow rate or the percentage of methanol in the mobile phase to reduce the run time. Do not exceed the allowed pressure limit for your chromatograph.

   To run the samples: click "Control", "Single Run". In the Single Run dialogue box, enter a sample ID and data file name and save the data in your own folder (result path). Make sure to change the "vial" number to the corresponding position of the vial in the autosampler tray. Press "start" to begin the run. Once the chromatographic run is finished, click "Reports", "Print", "Area %" and print the chromatogram with integrated peak areas. If the integration is incorrect, use Manual Peak or Manual Baseline icons at the bottom of the screen to make your own baseline and the program will reintegrate the peak and generate a new report that can be found in your folder.
4. *Identification of unknown by co-chromatography.* Prepare three samples for co-chromatography by mixing 6 drops of each pure standard with 30 drops of unknown in a vial. Using the same column conditions and injection volumes as in step 3, run

the three samples. By observing which peaks grow or whether new peaks appear you should be able to identify the compound that corresponds to each peak in your unknown. It may be necessary to use ratios other than 1:5 to produce reasonable effects in the chromatogram. Identify the two components in your unknown.

5. *Quantitative analysis with an internal standard.* Choose one component, designated X, of the unknown to measure. X should not overlap with other components. Select the compound which is not part of your unknown to be the internal standard, designated S. S must be also separated from other peaks in the chromatogram.
6. By trial and error, prepare a mixture of the unknown and the internal standard (S) such that the peak areas of components X and S are within a factor of 2. Use volumetric pipets or syringes to make accurately known mixtures of S and your unknown in tightly capped vials. Keep track of the exact volumes added when making the mixtures as you will need this information later on. When you mix standards and unknowns in this experiment it is assumed that volumes are additive. That is, if you mix 0.1mL of S with 1.000 mL of unknown, assume that the volume is 1.100 mL. (Additivity is not true for concentrated solutions.)
7. By trial and error and using the standard solutions prepared in step 2, prepare a known mixture of pure component X and pure component S that has approximately the same ratio of peak areas as the mixture in step 6. Prepare two more solutions in which the concentration ratio of [X]/[S] is approximately half as great and twice as great as in the first known mixture. Keep track of the exact volumes added when making the mixtures as you will need this information later on.

Calculations:

- ✓ *Chromatographic parameters.* For each component of the unknown, measure the retention time, the number of plates, and the plate height from the chromatogram in step 3.
- ✓ From the known concentrations and measured peak areas from step 7 calculate the mean response factor using Equation 4. The response factor from all three solutions in step 7 should be the same (within some small experimental uncertainty) if the analysis is within the linear response range.
- ✓ Using the calculated mean response factor and the peak areas of X and S from step 6, calculate the concentration of compound X in your original unknown solution. (Note that the concentration of component X in the original unknown solution is not the same as its concentration in step 6, since the concentration of X has been diluted by the addition of the internal standard to make the mixture in step 6.)