Chromatography Primer

Abstract: An minimalist overview of chromatography for the person who would conduct chromatographic experiments, but not design experiments.

At its heart, chromatography is a technique to physically separate mixtures. It does this using a mobile phase and a stationary phase. The *mobile phase* moves the mixture through the *stationary phase*. Each component of the mixture interacts differently with the stationary phase. When the substance is interacting with the stationary phase, it is not moving (stationary! ☺) Put another way, the stationary phase *retains* each substance in the mixture to a different extent. Therefore each substance moves through the stationary phase at a different rate and is, in this manner, separated.

There are several ways this experiment can be set up. In *classic column chromatography*, gravity moves the mobile phase through a solid or gel stationary phase packed in a vertical tube (See Figure 1). Each substance either exits the column at a different time or is eluted (moved off the stationary phase) by changing the composition of the mobile phase. In *thin layer chromatography(TLC)*, capillary action is used to move the mobile phase through the stationary phase. A spot of the sample is placed above the mobile phase liquid level and, as the mobile phase moves up through the stationary phase and the spot by capillary action, it takes the sample with it. (See Figure 2.) Each component of the sample will be left in a different location on the layer of stationary phase.

Figure 1. Classic Column Chromatography

Figure 2. Thin Layer Chromatography.

Instrumental versions of chromatography are classified as *gas chromatography (GC)* if a gas is used as the mobile phase or *high performance liquid chromatography (HPLC)* if a liquid is used as the mobile phase. A special case of liquid chromatography that is used to separate ions is called *ion chromatography (IC)*. In GC, the samples must also be in the gas phase (as a gas cannot effectively push a liquid). This can be accomplished by heating a volatile liquid sample. In LC, the sample must be a liquid or dissolved in a solvent and soluble in the mobile phase. The speed at which the mobile phase moves through the instrument is called the *flow rate*. The stationary phase is confined in narrow tubes called *columns*. As each component of the sample exits the column, a *detector* signals its presence with a signal that is proportional to the amount of substance.

In the case of gas chromatography, the pressure of the confined gaseous mobile phase is used to push the sample through a long column (1-100 m) containing stationary phase. (See Figure 3). Since the columns are very long, they are typically coiled to fit in a smaller space. There are two types of GC columns. A *capillary column* has a very narrow internal diameter (typically 0.10 mm) and stationary phase coated on the walls. A *packed column* has a wider diameter (typically 1/8 inch) that is packed with inert particles. Stationary phase coats both the walls and packed particles of in this type of column. Since gases are very sensitive to changes in temperature and more substances are gases at higher temperatures, columns in GC are place in ovens (temperature-controlled environments).

In the case of liquid chromatography, pumps are used to push the liquid mobile phase and sample through the column. (See Figure 4). Columns are shorter (5-30 cm)

and always packed (typical internal diameter is 4.6 mm). Newer instruments are working with shorter, narrower columns and higher pressures (called UPLC or UHPLC) to achieve better separation while using less mobile phase. While the column environment can be temperature controlled, it usually is not.

There are a variety of ways that the stationary phase and analyte can interact. In *partition chromatography*, the interaction is based on intermolecular forces and the "like dissolves like" principle. If the stationary phase is polar, it attracts polar samples so that those samples stay on the column longer. In liquid chromatography, use of a polar stationary phase and a nonpolar mobile phase is called *normal-phase chromatography*. . Typical columns used in normal phase chromatography include silica and alumina. Hexane is a typical mobile phase. Using a nonpolar stationary phase and a polar mobile phase is called *reverse-phase chromatography* Reverse-phase columns are typically designed with a "C" followed by a number describing the number of carbons in the change. For example, the common C18 column has a stationary phase that contains eighteen carbons. Mobile phases are aqueous solutions often modified (made less polar) by mixing with methanol and/or acetonitrile. Despite its name, reverse-phase chromatography is actually more common than normal phase since inexpensive aqueous solutions can be used as the mobile phase.

If an ion-exchange resin is used as the stationary phase, ions in the sample exchange with ions loosely held by the resin. Consequently, smaller and more highly charged ions tend to be retained longer. Some columns exchange anions and others exchange cations. The two types of stationary phase are sometimes used in sequence.

Chromatographic techniques using this stationary phase are called either *ion-exchange chromatography* or *ion chromatography*.

When the stationary phase separates the sample based on size (molecular weight) the technique can be called *size-exclusion chromatography*, *molecular-exclusion chromatography*, *gel filtration* or *gel permeation*. If the samples adhere to the surface of a solid stationary phase, the technique is called *adsorption chromatography*. If the interaction is very specific, like an enzyme-antigen interaction, it is called *affinity chromatography*.

Results of a chromatographic experiment are generally presented in a graph called a chromatogram. (See Figure 5.) A chromatogram is a graph of signal (y-axis) versus time (x-axis). The time starts (time zero) when the sample is injected into the mobile phase stream. When an analyte reaches the detector (after passing through the column), the detector produces a signal that appears as a peak on the chromatogram. Therefore, each substance in the sample produces a peak.

The measurement of the time between injection and the time the sample reaches the detector is called the *retention time* (t_R). Since it is not uncommon for the sample to spread during the separation, peaks can be wide and sometimes unsymmetrical. The retention time is measured at the highest part of the peak. If a component of the sample does not interact with the stationary phase, it will still have a nonzero retention time as it still must traverse the distance from the injector to the detector. The time it takes for an unretained species to reach the detector is called the *dead time*(t_M). This is usually measured as the first signal of any sort, even a negative signal, produced by the detector.

The adjusted retention time (t_R') is the difference between the retention time and dead time. It reflects the time the analyte spends in the stationary phase.

How long it take the substance to reach the detector depends on a variety of factors including the actual distance between the injector and detector, flow rate, identity of the mobile and stationary phases, temperature and, most importantly, the identity of the sample. If experimental conditions are kept constant, the retention time is *characteristic* of the substance's identity. It is possible that more than one substance will have the same retention time. Substances might not interact differently with the stationary phase or the differences might be so small that overlapping peaks appear as one. Consequently, retention times are a good way to eliminate as possible analytes, but cannot be used to absolutely confirm a substance's identity. Also, because instrumental parameters tend to vary slightly, even with the same model instrument under the same conditions, retention times obtained by one instrument should not be used as absolute retention times in another. Retention times also have some intrinsic variability. With very large numbers of molecules passing through the system and interacting many times with the stationary phase, there is the usual statistical variation in where the average particle elutes. There may also be experimental variation, particularly if manual injection is used.

A signal, proportional to the amount of substance, is given off by the detector as the substance passes by the detector. Not all particles pass by the detector at exactly the same time. There was a finite time to move the sample into the mobile phase stream, and not every molecule interacts in exactly the same way with the stationary phase. Some might linger longer or interact more often. Consequently it will take a measurable

amount of time for the substance to pass by the detector and the signal appears as a peak rather than a line. To account for the entire sample and because the width of the peak varies, the area of the peak is the best measurement to determine the amount of sample. *Peak area* is generally determined by having the computer collecting the data integrate the peak. In rare cases, where the peaks are so narrow as to appear as lines, *peak height*, a peak's highest signal, can be used.

Typical table of chromatographic results include: retention time, peak height, peak area, percent height and percent area (See Table 1). As already noted, the retention time is used for information about the identity of the substance associated with that peak. Peak area is a better measure of amount that peak height. The percent area is determined using the sum of the peak areas as the denominator in the percentage calculation. While percent area can be useful for an approximation of amount of substance it should **NOT** be used for precise analytical measurements (the same applies to percent height). Percent area assumes that the proportionality constant between amount and signal is the same for each substance. This is NOT the case. It also assumes that all substances have been detected by the detector, which is also often not true.

To correctly determine the relationship between amount of substance and peak area, a *calibration curve* should be used. If there is more than one substance of interest in a mixture, there should be a calibration curve prepared for *each* analyte. Typical chromatographic calibration curves graph peak area (at the retention time appropriate for the substance) versus amount (usually as concentration although some detectors are mass-sensitive). *Standard addition* and *internal standard* calibration methods are also

commonly used in chromatography and also use peak area as the signal. For details on calibration methods, consult your text.

One of the advantages of chromatography is that multiple analytes in the same sample can be detected with one experiment. This is also an advantage in the construction of calibration curves as you can do one standard for each analyte with one experiment. When designing such experiments, it is helpful to vary the amount of each standard in a different way (See Table 2). This has two advantages. First, you can confirm the retention time of each analyte by noting which peak increases in each trial and which decreases. Secondly, as the total amount of analytes stays relatively constant, you can use smaller overall (total) volumes.

One disadvantage of chromatography is the time it takes to do an experiment. Because the separation occurs in the time dimension, this is unavoidable. Typical experiments may take anywhere from five to thirty minutes. The chromatographer is always in a battle to optimize the balance between time and separation.

Because chromatography is a powerful separation tool, it is often combined with other powerful instrumental techniques to get the advantages of both. Commonly, chromatography is combined with mass spectrometry (GCMS or LCMS). When combined, the chromatographic technique separates the mixture into its components. Mass spectrometry analyzes each of the components to determine the identity, often definitively.

Chromatography is common and powerful technique used to separate mixtures. With this technique, retention time is characteristic of the identity of the separated substance and peak area is proportional to the amount of substance. Thus with one

technique the analyst can obtain both qualitative and quantitative information. It is an essential tool in the analytical chemist's toolbox.

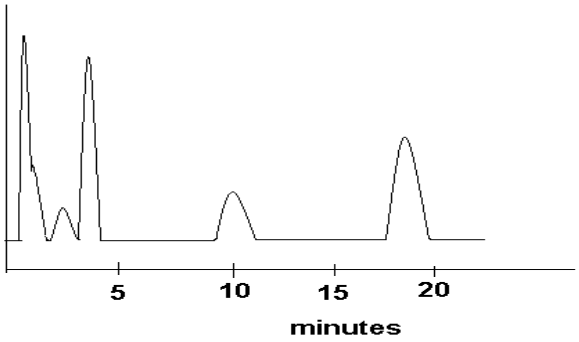
Chromatography Problems

1. A separation on an affinity column produced the chromatogram shown below. Overtop of the chromatogram, sketch show what it would look like if a second sample with more (higher concentration) of analyte was run.

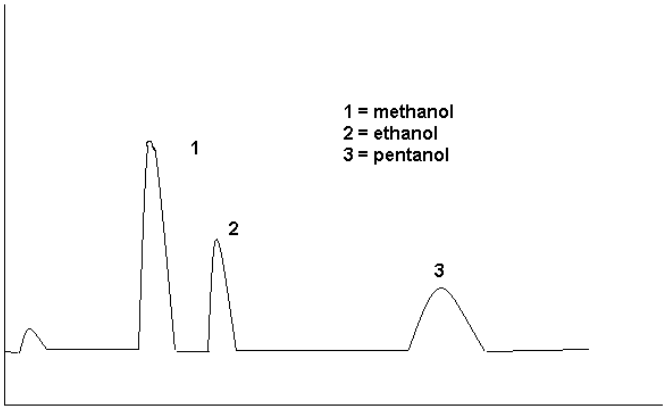
2. Are the following changes in conditions likely to increase or decrease retention time?
   a. faster flow rate
   b. longer column
   c. longer connector between column and detector
   d. higher analyte concentration
   e. higher temperature
   f. larger sample size
   g. using a less polar column while doing a reverse-phase separation
   h. using a less polar mobile phase while doing a reverse-phase separation

3. In what order will the following substances elute?
   In reverse-phase liquid chromatography:
      a. butylamine, 2-butylene, ethylamine, ethylene
      b. benzene, chlorophenol, phenol, trichlorophenol
   In gas chromatography with a polar column:
      c. butanol, methanol, water
   In gas chromatography with a nonpolar column:
      d. butanol, methanol, water
   In ion chromatography:
      e. bromide, fluoride, sulfide

4. For the following chromatogram, calculate retention time and adjusted retention time for each peak.



5. The following chromatogram was obtained using a C18 column and a methanol:water mobile phase.



How would the chromatogram change if

a. more ethanol were added to the sample
b. butanol was added to the sample
c. water was added to the sample
d. an unretained species was added to the sample

6. Chromatography was performed on the following solutions, where the total volume of each solution is 100.0 mL:

solution	**volume 100 ppm X (mL)**	**volume 50 ppm Y (mL)**	**volume 50 ppm Z (mL)**
1	5	20	10
2	10	15	20
3	15	10	5
4	20	5	15

This resulted in 4 chromatograms with the following results:

	peak A		**peak B**		**peak C**	
Chromat-ogram	time (min)	Area (x 10^4)	time (min)	Area (x 10^5)	time (min)	Area (x 10^4)
1	6.44	0.68	8.00	4.10	9.72	7.99
2	6.54	1.98	7.96	8.30	9.68	7.63
3	6.34	2.18	8.03	2.53	9.66	7.27
4	6.49	3.02	8.12	6.29	9.58	6.93

a. Which peak corresponds to which analyte?

b. Make a calibration curve for each analyte

c. A unknown solution was analyzed with the same method. Based on the results below, what is the composition of the sample?

retention time (min)	peak area
6.59	33419
8.05	554193
9.68	76416

7. A calibration curve obtained from a chromatographic experiment resulting in a line with the equation

$$\text{Peak area} = (139997 \pm 5949\ \text{ppm}^{-1})[\text{analyte}] + 913 \pm 554$$

a. If sample had a resulted in a peak area of 568336, what is the concentration of analyte, with error?

b. In another experiment, 10.00 µL of sample was diluted with water to make a 1.00 mL solution. The resulting analyte peak had an area of 2765422. What is the concentration of analyte in the original solution?

c. In another experiment, 1.00 L of sample was processed through a solid phase extraction cartridge. The analyte stayed on the cartridge. Then 5.00 mL of methanol was used to elute the analyte from the cartridge. When the eluted solution was chromatographically separated, the analyte peak area was 6729661. What was the concentration of analyte in the original sample?

8. *Standard Addition*. The chromatographic analysis of Sample A resulted in a peak at 8.95 minutes and a peak area of 24237396. When 10.0μL of Sample A was mixed with 5.00 μL of 20.0 ppm standard, chromatographic analysis of the resulting solution resulted in a peak with a retention time of 9.12 minutes and a peak area of 121220801. What is the concentration of analyte in the original sample?

9. *Standard Addition.* A chromatographic analysis of Sample B produced the following results:

Time	Peak area	%area	Height	%height
5.726	764499	39.41	62503	35.89
7.207	241332	12.44	46947	26.96
10.735	934148	48.15	64691	37.15

a. What is a likely reason that the percent areas and percent heights are different?

b. When 4.34 g of Sample B are mixed with 3.98 g of pure analyte and the mixture analyzed, the following results were obtained. What is the % analyte in the mixture?

Time	Peak area	%area	Height	%height
5.739	528045	29.50	43954	28.90
7.554	162697	9.089	33037	21.72
10.686	1099110	61.41	75114	49.38

10. *Graphical Standard Addition.* The following solutions were made and analyzed with ion chromatography--the results included in the table. Create a standard addition graph and determine the concentration of fluoride in the original sample, with error.

Note: all solutions were made with a total volume of 10.00 mL and a fluoride standard with a concentration of 35.21 ppm.

Vol sample (mL)	Volume std (mL)	Retention time (min)	Peak area
5.00	0.00	2.50	135235
5.00	0.50	2.80	396313
5.00	1.00	2.15	764095
5.00	1.50	2.46	1050673
5.00	2.00	2.41	1523699

11. *Graphical Internal Standard.* The following solutions were made and analyzed with ion chromatography using 81.7 ppm fluoride ion as an internal standard and 64.4 ppm sulfate ion as an analytical standard. What is the concentration of sulfate in the sample, with error?

Total volume of solution = 10.00 mL; last row is sample not standard

Vol SO_4^{2-} (mL)	**Vol F^- (mL)**	**t_R peak 1 (min)**	**Area peak 1**	**t_R peak 2 (min)**	**Area peak 2**
1.00	3.00	3.09	62817	18.30	96234
2.00	3.00	2.93	68075	18.37	204377
3.00	3.00	3.20	61262	18.54	274731
4.00	3.00	3.03	67054	18.47	402935
5.00 (sample)	***3.00***	***2.97***	***63281***	***18.39***	***278748***