Chapter 21 Principles of Chromatography and Mass Spectrometry
Problems 1,2,3,4,8,9,11,13

21-1 What is Chromatography

Process to separate and purify compounds in a mixture by passing them trough a material that retains some compounds longer than others.

Figure 21-1

Start with material on top
Open stockcock and add fresh solvent
-More strongly adsorbed material stick to column and moves slowly
-Less strongly adsorbed material stays with solvent and washed through

Mobil phase, the moving phase, can be either gas or liquid
Stationary phase, Substance that stays fixed, can be a solid or a viscous liquid adsorbed on a solid
Compounds spending time in one phase over the other referred to as *partitioning*

If mobile phase is gas call this *Gas Chromatography*
If mobile phase is a liquid call it Liquid *chromatography*

The process of passing a fluid through a column is called *elution*, and
Fluid entering column is called *eluent*
Fluid leaving column called *eluate*

Type of Chromatography (Figure 21-2)

Adsorption Chromatography
- Solid stationary phase
- Liquid or gas mobile phase
- Solute is adsorbed on surface of solid particles
- Normal phase (Polar stationary phase- Non polar mobile phase)
- Reverse phase (Nonpolar stationary phase - Polar mobile phase)

Partition Chromatography
- Stationary phase is a solid coated with a liquid that also remains stationary
- Mobile phase is a gas (or immiscible liquid?)
- Solute is adsorbed into stationary liquid (and partitions between this and the mobile phase)

Ion Exchange Chromatography
- Has ionic groups covalently bonded to stationary phase
- Stationary phase is usually a plastic resin or polysaccharide
- Mobile phase is a liquid

Solute is attracted to staionary phase by charge/charge interactions

Molecular Exclusion Chromatography

(Also called gel exclusion or gel permeation)

-Stationary phase is a porous material containing pores that exclude molecules of a certain size or greater

-Mobile phase any compatible liquid

-Not true attraction between solute and stationary phase

-However smaller molecules enter pores and are retained for longer times, Larger molecules are excluded and come through column quickly

Affinity Chromatography

-Molecules that you and to purify are analyzed to find molecules that they bind specifically

- This binding molecules are covalently attached to a plastic resin or carbohydrate stationary phase

- Mobile phase is any compatible liquid

-Complex mixtures of molecules can be passed though the column, but only those molecules that bind the specific molecule are retained in the column

- Either pH, ionic strength, or a substance that competes for the binding site is added to knock the bound solute molecules

21-2 How we Describe a Chromatogram

Different chromatographic techniques described in subsequent chapters. Will look at basics that can be used to describe all chromatograms

Chromatogram - response of a detector vs time. - shows when various components come off a column

Retention time t_r - The time at which a component elutes from a column.

Theoretical Plates

Assume a chromatographic peak has a Gaussian shape

H is height of peak

$w_{1/2}$ is with at ½ height

(If true Gaussian $w_{1/2} = 2.35\sigma$

Where σ= standard deviation)

Width at baseline should be 4σ

Term ’theoretical plates’ comes from distillation; a distillation column could be divided into ‘plates’ where liquid and gas phase were in equilibrium. The more theoretical plates the better the distillation

For chromatography we retain the name Theoretical Plate, but calculate it using the retention time and the width at ½ height

$$N = \frac{5.55 t_r^2}{w_{1/2}^2}$$

N is number of Theoretical plates. Again the bigger the N the better, because that means the width of the peak is small compared to its retention time.

Practice calculation see attached chromatogram
T_r = 12.36 minutes
w = 12.57-12.13 = .44 min

N = $5.55(12.36)^2/.44^2$
= 789 plates

Both T_r and $w_{1/2}$ can be measured in units of time or elution volume. You have to make sure both are in the same units when you use this equation so the units cancel.

Sometimes when we want to change the size of a column a parameter called *plate height H* is useful.

H=L/N

So it is the linear distance along the column need for a theoretical plate

Continuing on with example. The column used for chromatogram was 150 mm long

H = 150/789
= .19 mm, or 190 µm

The smaller the plate height, the better the column
GC plate heights .1-1mm
HPLC 10 µm (.01mm)
capillary electrophoresis <.1µm (<.0001mm)

Resolution

How well does a column ‘resolve’ two closely spaced components
Δt = time between peaks (or peak separation)
w_{av} = average base width of the peaks

$$resolution = \frac{\Delta t_r}{w_{av}}$$

Scaling up a separation

In Analytical work you typically use small volume and very long skinny columns to give you the most resolution and theoretical plates. This give you great resolution, but you are limited to analyzing small amount of material.

In preparative work, where you must isolate significant amounts of material you frequently have to scale up what you were doing analytically.

Most straightforward scale up procedure is to keep the column length the same, but to increase the cross-sectional area in proportion to material amount.

$$Scaling\ Equation = \frac{l\arg e\ load\,(g)}{small\ load\,(g)} = \left(\frac{l\arg e\ column\ radius}{small\ column\ radius}\right)^2$$

Say our small load was 2 mg and our small column radius was .5 cm

How large a column would we need to purify 20 mg of material?

$20/2 = (X/.5)^2$
$10 = X^2/.25$
$2.5 = X^2$
$X = 1.58$ = radius
diameter = 3.16

When you increase the column crossection, you also have to increase the flow rate and the flow volume proportionately.

Here where the area of the large column is 10X larger than the small column
The volume you dissolve the solution in must by 10 X greater
and the flow rate (ml/min) must also be 10X larger

This easy scale up calculation sometimes doesn't work. As you increase the width of a column you frequently lose resolution, so this is only a first guess, you may have to make the column longer as well.

21-3 Why do Bands Spread

Ideally we would like the width of the sample to remain as narrow as possible. But one of the facts of life of chromatography a that as the material runs through the column, the bands spread out. Let's see if we can understand why.

Band Diffusion

If you put an infinitely narrow band in the middle of a column, it would be at a high concentration, within the band, but at a 0 concentration everywhere else. You've seen this enough to recognize that under these conditions all molecules will diffuse, and try to spread out to have a low uniform concentration. This inescapable process is called *longitudinal diffusion* and will occur for as long as a material is on a column.

The only way to avoid longitudinal diffusion is to get the material on and off the column as quickly as possible so, in general, you always try to minimize the separation time.

Not if u is the flow rate, then

Broadening due to longitudinal diffusion $\propto 1/u$

Mass Transfer

We are assuming that we are going slow enough that we have a true equilibrium between our molecule of interest and the two different phases. However if we go to fast, we don't reach equilibrium, and this also broadens our peaks

Broadening due to finite rate of transfer $\propto \mu$

Multiple Paths

As a solute molecule wanders through a column it can randomly take any path it wants. Some paths are shore, some are long (figure 21-10). This also spreads a width of a peak apart, but it is dependent of the flow rate so we have a final factor

Broadening due to multipel paths $\propto$ K (independent of u)

The van Deemter equation

Taking all three of these factors together we see that we can't run the column too slowly, or logitudinal diffusion will spread the bands. We can't tun it too fast, or mass transfer will broaden the bands. The bottom line is that we need to find a sweet spot where we have minimal diffusion. This relationship was captured by ven Deemter in the equation

$$H = A + B/u + Cu$$

Which has a plot like figure 21-9

H here is plate height. What was plate height? (Plates/length of column)

How does this relate to band broadening? (Calculated plates as $t_r^2/w_{1/2}^2$, narrower width, more plates, more plates/length, plate height goes down, - **So want plate height kept to a minimum**)

The based on first principles, the van Deemter equation can be derived for simple systems like gas chromotography columns. In fact our GC system has a program built into it so you can simply plug in flow rate, temperature, flow gas, and column dimensions, and it spits out the optimum flow rate. For more involve systems like the HPLC, you can derive the van Deemeter equation directly, but you can run experiments to find your sweet spot empirically.

Move discussion of open tubular and packed GC columns to section on GC!

Peak Shapes

At the beginning of this discussion said that peaks should have a Gaussian shape. This often is not true. We often observe either 'fronting' or 'tailing' Figure 21-12 where the peak shape is distorted from the Gaussian. The causes of these artifacts are widely varied, but here are a couple of common problems associated with these shapes

Fronting is often caused when the column is overloaded by too much of the solute material. Essentially the solute tended to dissolve in itself, rather than adsorbing to the stationary phase, hence it come through faster than expected

Tailing often occurs when there are two different kinds of interactions occuring between the solute and the stationary phase, the normal binding that gives a normal peak shape and an additional strong binding that tends to retain the sample longer than it should. In nonpolar columns like reverse phase columns, this additional binding is usually due to the presence of some polar OH groups in the column material. Thsi can be corrected by silanization a chemical process in which the OH groups is derivatized to $OSi(CH_3)_3$ a non polar group.

21-4 Mass Spectrometry

Really this should be a chapter all by itself, But Harris has included it here because the GC/MS instrument uses gas chromatography to separate compounds, and then a mass spec to identify the compounds. So you can't understand this instrument unless you include both.

The mass spec , also called mass sensitive detector, on our GC/MS looks like figure 21-13 . The gas coming out of the GC enters the ionization chamber on the left. This ionizes the molecules and the ions are sent into the quadropolar mass separator. I an ion makes it through the separator, the nit is detected using the electron multiplier ion detector. Let's discuss these components in this order.

Ionization

There two common ways to ionize molecules, Electron Ionization and chemical Ionization. The most common method is electron ionization.

Electron ionization

Looking back at figure 21-13 our GC gas enters a chamber. At one end of the chamber is a hot filament like a light bulb filament. Not only is light and heat coming off this filament, but also lots of electrons. If we make the chamber + charged with respect to the electrode, these electrons are accelerated into the chamber and have an energy of 70 electons volts. (Note there is also an electron collector electrode at the bottom of the chamber to make sure the electrons flow out.)

As these electron with 70 ev of energy stream past our analyte, a few (~.01%) of the molecules get ionized as the electrons filment electrons get close enough to kick electrons out of the molecule

M + 70 eV filament electron

$\rightarrow M^+$ + ~55eV filament electron + ~.1 ev molecular electron

M^+ is called a molecular ion

And usually the ion has such a high energy that it flies apart into many smaller pieces

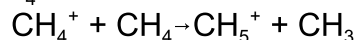
Now look at diagram 21-13 again, and notice the 15V potential between the chamber and the quadrupole mass separator. Any un ionized gases are simply vacuumed out of the ionization chamber, but any + ions see this 15V potential, and move into the separate chamber with a 15V acceleration

Chemical Ionization

Chemical ionization works just about the same way, only we fill the ionization chamber with a second gas, like methane. We put this second gas into the chamber so it is at least 100x more concentrated than out molecule of interest. In this case it is the methane that gets ionized instead of the molecule on interest

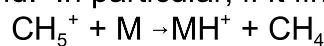
CH_4 + High E electron $\rightarrow CH_4^+ + 2e^-$

The CH_4^+ then reacts with other CH_4's



$CH_4^+ + CH_4 \rightarrow CH_5^+ + CH_3$

And this CH_5^+ ties to pass off its extra proton onto any other molecule it can find. In particular, if it find our molecule



$CH_5^+ + M \rightarrow MH^+ + CH_4$

Since this is proton exchange is a much lower energy event than the electron collision, out molecule gets positively ionized, and usually doesn't fragment. Singe ti s + ionized, we need to reverse the polarity of our ionization chamber, and again we get the molecular ion accelerated into the mass separator.

Mass separator
This chamber is kept at a high vacuum, so our ions don't hit anything on their way though the chamber. Notice there are 4 parallel rods (Hence the name quadrupole) The voltage across these rod is varied at radio frequencies. These frequencies make the ions dodge back and forth between the rods as the ions move through the chamber. The frequencies are then tuned so at one particular frequency only an ions of one particular mass can make it through the chamber without hitting one of the rods. When an ion hits the detector, we know we have ions of that mass in our sample. The machine then changes the frequency and looks or ions of a different mass. In fact the electronics are so fast that you can scan all the masses between 1 and say 500 amu in a fraction of a second!
(More details in Instrumental)

Electron multiplier
The final detector is similar to the photomultiplier tube we talked about in UV/visible spectroscopy, only we don't need a photoemissive element in the first stage. When the ion hits the first element, it throws off several electrons, and then we use several dynodes to multiply the electrons signal until we can use our electronics to count the number of ions hitting the tube.

21-5 Information in a Mass Spectrum

So now we have a machine hooked on our Gas Chromatography machine that changes molecules into ions (often many different ions) and can tell you the mass of each ion. So what?

Total ion and Selection Ion Chromatograms

Selected Ion Chromatogram - Well if we know that our molecule typically ionizes to make one particular ion, we can plot the appearance of that ion vs elution time, and figure out when our molecule of interest come off the column.

Total Ion Chromatogram - On the other hand, most of the time we want to know what all the different compound in our sample are and how much there is of each compound. In this case we let the computer sum up all the ions of any size that it sees at any particular time, and plot this 'Total ion' chromatogram to see where and how much of everything we have

The total Ion chromatogram is useful for seeing when or where things are coming off the column, but we have thrown away all the interesting information that would identify the individual components in the chromatogram. The real power of this technique is that there is so much information in a mass spectrum that we can usually use it to identify every single component in our chromatogram. Lets focus now on that. Using the Mass spectrum to identify a compound.

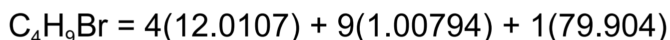
Before we do that there are some definitions to get to.

Dalton - The unit of atomic mass - 1/12 the mass of ^{12}C.

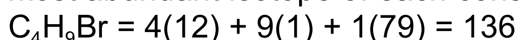
Atomic Mass - Mass of a mole of a particular element
Since most elements are a mixture of isotopes, the atomic mass is and average that reflects the natural abundances of these isotopes. For instance

Sulfur	94.93%	^{32}S	31.97207 Da
	0.76%	^{33}S	32.97146 Da
	4.29%	^{34}S	33.96787
	.02	^{36}S	35.96708

Molecular mass - Sum of atomic masses of all the atoms in a molecule


C_4H_9Br = 4(12.0107) + 9(1.00794) + 1(79.904)

Nominal mass (This is the new one) - INTEGER mass of all the species with the most abundant isotope of each constituent atoms.


C_4H_9Br = 4(12) + 9(1) + 1(79) = 136

(Note this wasn't a simple roundoff)

Now we have that taken care of, let's return to our mass spec machine itself. I mentioned earlier that there are 2 way to ionize your compound, electron ionization and chemical ionization. Because chemical ionization is gentler, it usually give you a fairly simple spectrum, the MH^+ ion and one or two other ions, so it is usually pretty easy to interpret to figure out if your molecule of interest was in your chromatogram.

The hard part of doing the interpretation comes if you use electron ionization, because in this case your molecule has been smashed into dozens of pieces, and you have to figure out how to put them back together to come up with the original compound. Your book talks a little about fragmentation, and this is a long subject that I think is more appropriate to talk about in Instrumental analysis.

Instead we will take the practical way around. Fragmentation under standard conditions have been done on thousands of compounds, and libraries of compounds and their fragments are available. In fact they are already downloaded into the software of our machine. When you get a peak in your chromatogram you tell the computer to search its library to find a 'best' match and you use that match to identify

your compound.

Information included in this chapter in a previous edition that talked more about chromatography.

Chromatography is a powerful analytical technique because it can be use both qualitatively and quantitatively. That is, it can be used both to identify different compounds in a mixture, and to determine the exact amount of each compound as well.

How do you identify a compound?

1. Compare retention time of unknown and a standard, if they are the same, then they are likely the same compound.

2. Since the retention time can very from one run to the next, a surer way to identify a compound is to try 2 runs of your material, one alone and a second of your material with an added spike of a known. If the spike increases size of one of your peaks, then there is added proof that you have correctly identified your unknown.

3. At his point it is still possible that you might have to different compound that just happen to co-elute at the same time. To be absolutely certain you have identified your compound you should run 2 or 3 other independent chromatographic methods. If the unknown and your proposed standard co-elute under all conditions, then you have probably properly identified the compound.

4. There are kinds of detectors that respond to every compound in a unique manner. These are IR and Mass Spec detectors. These will be studied in more depth in Instrumental Analysis, but for now what you need to know is that when you detect one of your peaks with one of these detectors, it can absolutely identify your compound in a single run, and no further checking and cross checking is necessary.

20-5 Internal Standards

Quantitating what is coming through the column has its difficulties as well. There are literally hundreds of different detectors, that can be used in chromatography, and each detector is going to respond to a compound in a different way. Further the same detector can respond to different compounds in different manners, so you have to be very careful how you analyze your results.

One of most common way of quantitating the results of a chromatographic technique is called an internal standard. You create an internal standard by adding a known amount of some compound, different from your analyte, to your sample. You then compare the size of the peak of the standard to the size of the peak of your analyte to calculate the amount of analyte in your sample. Of course you have to run some experiments first to determine the response of your particular detector to both the standard and the analyte This is called the response factor (F)

The equation you use much of the time is:

$$\frac{Area_X}{[X]} = F\frac{Area_S}{[S]}$$

There are many advantages to using an internal standard. First , it you add the internal standard to your sample as the first step in your purification, it can act as a control to remove uncertainly doe to your preparation method, since every step you do to your sample is also done to that standard at the same time. Further it removes much of the uncertainty from your chromatographic method as well, since the sample and standard are injected at the same time , there is no uncertainty in the amount injected, and there is no uncertainly in other parameters like the temperature or flow rate of mobile phase on the column that disrupt your quantitation of the unknown.

Final example, use of internal standard
1st we have to calibrate the standard is a preliminary experiment

Well assume that the material we want to quantitiate is called X, and the standard is called S. I will prepare a sample that is .0837M X and .0666M S. When we run this we get two peaks, and the computer integrates the X peak to be 423 units and the S peak to be 347 units. What is the response factor?

423/.0837=R 347/.0666
R = .0666(423) / .0837(347) = .970

Now we get to the real experiment. We are going to start with 10 ml of sample in a 25 ml volumetric, and add to it 10 ml of .146M S and then bring the volumetric to the mark with water . When we inject this on the machine, the area of peak X is 553 and the areas of S is 582. What is the concentration of X in the sample?

First note that we have done some dilutions here. What is the concentration of S in the sample we put on the machine?

MV = MV .146(10) = S(25)
S = .146 X (10/25) = .0584

Now we can use our response facto equation

553/X=.97 X 582/.0584

X = 553(.0584) / .97(582)
X = .0572M
But this was a dilutes sample, what was the original concentration?

MV=MV
X(10) = .0572(25)
X = .0572(25/10)
X=.143M