Preface:

2005 was the first year I was able to give an Instrumental Analysis course. In that year I had to come up with new labs for each instrument we tried to use. The labs in this manual represent these first attempts at creating labs that showed the strengths and weakness of the various instruments.

As this is the first time these labs have been written up, I do not regard this manual as a final, polished product. In fact, I regard my students as the best copy editors I can find, so your job is to find the errors and point them out to me. Also if you don't understand what I mean in some section, ask for a clarification. It was probably clear to me when I wrote it up, but if it isn't clear to you now, then I need to re-write it.
Computer Experiment 1 Noise. ........................................ 4

Computer Experiment 2
   Computer lab on Wave superposition (interference). .......... 9

Computer Lab 3
   SPIA & Basic FT Theory. ....................................... 11

Experimental Lab 1.
   Atomic Absorption and Emission. .............................. 18

Experimental Lab 2
   An Introduction to Measurements Involving Light. .......... 29

Experimental Lab 3
   UV/Visible Spectrophotometer lab. .......................... 38

Experimental Lab 4
   Fluorescence. .................................................. 48

Experimental Lab 5
   IR Spectroscopy lab. .......................................... 54

Experimental Lab 6
   NMR ............................................................ 62

Experimental Lab 7
   Mass Spectrometry
   Hot & Spicy lab I. ............................................ 72

Experimental Lab 8
   Chromatography
   or Hot & Spicy Lab II. ........................................ 78

Experimental Lab 9
   Caffeine in Popular Drinks II. ................................ 80
Noise reduction during data acquisition

Built into the signal on any analog instrument is an inherent noise level, that is, small positive and negative deviations from the true value. On the digital balance or pH meter the noise is the + or - uncertainty in the last digit you can read. On the spectrophotometer or the IR machine, generally the noise is so low that you never really notice it. In today’s lab I have taken data from the Circular Dichroism (CD) machine that is inherently very noisy to help illustrate many of the features of noise that are mentioned in your text.

Use excel to open the file Noise1. The first 5 lines of this file are instrument parameters that you don’t need to worry about. After the first 5 lines there are 2 columns of data; the first column is the raw signal in volts, the second column is the wavelength the instrument was on when it recorded the signal. Plot this data with the signal on the Y-axis and the wavelength on the X-axis. (You will have to manipulate the excel file a bit to do this.)

Assume that the data between 250 and 260 nm has no signal, and the variation in the signal comes entirely from the noise in the instrument. Using excel find the highest and lowest values in this region (peak-to-peak noise) and to calculate the standard deviation of the noise in this region. (See page 111 of your text) How close is the standard deviation you would get based on the peak-to-peak noise equation to the actual standard deviation?

Now use excel to calculate the average value for this region. Since you should have zero signal here, any difference between your average and 0.00 represents the bias in the machine. (See page 970 of the text). The bias here is called a baseline offset and, once you have measured it, is easily compensated for by simply subtracting it from all other voltage readings derived from this instrument.

Do that now. Subtract the bias, or baseline offset, from all the points in the data file and replot the data.

The maximum signal should occur at 292.5 nm. What is the average voltage in this region of the spectrum (use your bias corrected data, not the raw data)?

One of the most meaningful measurements of noise in an instrument is the signal-to-noise ratio or S/N. (Page 110 of text). Calculate the S/N for this experiment. If you look at this data carefully you will see that the noise at the 350 end of the spectrum is actually a bit lower than the noise at the 220, so the noise is a function of the wavelength. However, let’s ignore that detail and get on with the analysis.

Chapter 5 of your text covers where noise comes from and how it can be minimized with proper design in an instrument. I assume that this machine has been designed as carefully as possible considering it’s 1970’s technology, so the S/N you have measured is as good as it gets.

One of the most common methods for improving the S/N of an instrument is called Analog Filtering (Page 115 of text). Here the circuits of the instrument are designed with a low-pass filter; essentially electronics that remove the high frequency noise but let the low frequency signal get recorded. Almost all instruments that have a button that reduces the noise use this kind of filtering method. Analog filters can be very effective, but, if used in excess, they can degrade your signal and ruin your data.
Use excel to bring up the data files noise2, noise3, and noise4. Look at the data headers at the top of these files that we ignored before. One cell has the word ‘Time Constant’ in it. For noise1 the time constant or TC is .25 seconds, for Noise2, 3 and 4 it is 1, 4, and 16 seconds respectively. The Time Constant refers to the analog filtering circuit that is removing the noise. This feature is often called either ‘Time Constant’ or the ‘Period’ in other machines. You can think of the analog filter or Time Constant as an electronic circuit that slows the instrument response down. As the response gets slower and slower the machine simply can’t respond to the noise as quickly, so the intensity of the noise gets smaller and smaller. While this works great to remove noise, it can also destroy signal.

Let’s look, now, at using the analog filter to remove noise, and, in excess, to ruin signal.

Now plot Noise1, 2, 3, and 4 on the same plot. (Some of these data sets contain a few more data points than others. Just delete the extra points) Again remove the bias and plot signal on the Y axis and wavelength on the X axis, and plot only the region from 220-350 nm.

Calculate the S/N for each data set (based on the noise between 250 and 260, and the signal at 292.3).

Which data set has the best S/N, the worst S/N?
Which data set has data that has obviously been destroyed by the analog filter?

Why does altering the time constant, a circuit that affects how quickly the machine responds to a rapidly changing signal, affect our data that is a function of wavelength? When you run this machine you change the wavelength as a function of time, thus while it looks like the data that you are working with is signal as a function of wavelength, the instrument actually acquired the data as a function of time.

Now that you know the data is really a function of time, the degradation of the signal can now be explained. I said earlier that the higher time constant is a circuit that slows down the response of the instrument. What has happened in the bad data set is that the instrument response has been slowed down so much, that it can’t respond to the signal properly. If you think of the X axis, not as wavelength, but as time you can see how the response of the machine has slowed down. The machine is trying to respond to the change in signal, but because it has slowed down the left hand side of the peak is lower than expected because the machine can’t move to the higher values as it should. On the right hand side of the peak the machine has responded to the high values and is now trying to return to low values. Again the response is too slow, so the data gets distorted.

Now that you know what to look for, can you see smaller, more subtle distortions in any of the other data sets? Of all the data sets, which has the best S/N and no distortion?

From the above example you can see that there is an optimum setting for the analog filter that will eliminate as much noise as possible, yet not degrade your signal. This is something you always have to be aware of when you are using a device that has ‘time constant’ or ‘period’ or ‘noise filter’ setting built into the hardware. If you are lucky, you have plenty of signal and little noise so you don’t have to worry about these filters. But anytime you have a noisy signal and you want to get rid of the noise with these filters, you have to be careful to find this optimum setting for your set of conditions.

Now for the next question. Assuming you have chosen one of the above data sets as the optimum instrument setting, what could you do to further reduce the noise without distorting the signal?

There are actually two different ways you could further reduce the noise. The first and simplest is to go with the higher filter setting that kills the noise and distorts the signal, but slow down the machine so it can keep up with the signal. I don’t have an example here to show you, but take my word for it. We could use the 16
second time constant setting, and get great noise free data. The catch is that you would also need to slow the machine down by a factor of 16 so it could keep up. The net effect is that the experiment would also take 16x longer to do.

The other method is to take more than one data set and average the data sets together. This is called **ensemble averaging or coaddition**. (See page 117 of text). Find data set Noise5. This data set is a second run under the same conditions as noise3. Use excel to average the data from noise3 and noise5 together at every wavelength. **Plot noise3, noise5 and the average data set on the same plot. (Remember to remove the bias)** Can you see a difference in the noise level? Is there any distortion of the data? Calculate the S/N for the average data set (Noise from 250-260, signal at 292.3). Is the S/N of the average data set lower than the S/N of noise3 and noise5?

Theory predicts that ensemble averaging improves the S/N by the square root of the number of data sets in the average. Thus your average is based on 2 data sets, and the square root of 2 is 1.4, so the average data set should have an S/N that is 1.4x better than any of the individual data sets. **Is it?** This leads to a general rule of thumb that if you want to improve your S/N by a factor of 2, you need to average 4 \(2^2\) data sets together.

It is interesting to note that if you run four data sets, then your experiment will take 4 times longer to run. If your experiment takes 4 times longer to run, then you have a choice. You can either do the 4 independent experiments using one time constant, or you could do one experiment that takes the same total length of time, but you can turn the time constant or Analog filtering up and reduce your noise this way. In the end you end up with about the same signal to noise either way!

**Noise reduction after data acquisition or post-acquisition noise reduction**

The first part of the lab shows you how to set up an instrument for optimal noise reduction as you acquire the data. You now have an appreciation that if you mess this up, you can screw up your data, and you have to do the whole experiment over. Now we will look at noise reduction techniques that you can do after the experiment is over. These techniques have a major advantage that if you do them wrong, you can always go back to your original data set, so you can’t permanently screw up your data (unless you forget to save your original data file).

Technically the ensemble averaging is a post-acquisition method because you do this after the experiment is over. On the other hand, you can set up many instruments to acquire and do ensemble averaging as the machine acquires your data, so you can argue this point either way.

The simplest post-acquisition noise reduction technique is called **Boxcar Averaging** (page 119 of your text). In boxcar averaging you average several points together and use this average to replace an original data point.

Bring up data set Noise6 and remove any bias from this data set. Let’s try a 5 point Boxcar Average. Insert a new column in this excel file right next to your Raw Voltage column called Boxcar. Now calculate the average of the 220.575, 221.575, 222.575, 223.575, 224.575 voltages and put this into the cell that corresponds to the Boxcar value for 222.575 nm. Once you have figured out how to do this, pull that definition for the rest of the table so you have all the values filled in for the Boxcar column. Note how you cannot get values for either the first 2 or the last 2 points because you don’t have any data points to use in your average. (Excel may give you values, but they aren’t real) This is one potential weakness of boxcar averaging, you cannot do the points at the very end. However, since most of the time you don’t use the data at the ends of the plots, this is hardly ever a real problem.
Now set up 9, and 19 point boxcar averages of this data set. What is the S/N for the 5, 9, and 19 point boxcar average data sets (signal at 292.3, noise at 250-260)? Your S/N should improve every time. Just like our analog filtering, however, you can have too much of a good thing. Plot all 3 of these averages and the original data set on one plot. Now carefully examine the resulting data. Can you see any data distortions? What is the highest boxcar average you can apply to this data without distorting it?

A more sophisticated method that is closely related to boxcar smoothing Savitzky-Golay smoothing (text page 122 and reference). In this method you replace each point, not with the average of the points around it, but by fitting the points around it with a linear least square line and find the point at the middle of the line. Intuitively I think you can see why this would be superior to the simple boxcar smoothing, but computationally it sounds like a real nightmare. In Savitzky and Golay’s paper however, they proved that this linear least square fit can be done by simply taking each point, multiplying it by a weighting factor, summing these numbers together and dividing by an appropriate normalizing factor. They compiled and published all the needed factors so all you have to do is look up the factors in a table!

So let’s say your first 5 points are A, B, C, D, and E. According to their paper the middle of the least square best fit line to these 5 points can be found with the following calculation:

\[-3(A) + 12(B) + 17(C) +12(D) + -3(E)/35.\]  

Now it’s your turn. Figure out how to use excel to apply this function to all the points in the noise6 data set. There are two things to notice here: 

1.) Just like boxcar averaging you cannot do the first or last 2 points. 
2.) You have to be careful with your spreadsheet because you always apply the calculation to the RAW data, you cannot smooth a point, and then use the smoothed value in the calculation of another point.

Once you have a 5-point Savitsky-Golay smooth of the noise6 data set calculate the S/N of this data set. How does it compare with the S/N of the original data? Of the 5 point boxcar smooth? When you plot the data can you see any distortion?

Now try 9 and 19 point Savitsky Golay smooths using the coefficients given below. Again, what is the S/N of the resulting data set, how does this compare to the S/N of the boxcar average of the same size, and can you see any distortion of the data when you plot the data.

9 point coefficients 
-21,14,39,54,59,54,39,14,-21.  
Overall normalization factor: 231

19 point coefficients  
-136, -51, 24, 89, 144, 189, 224, 249, 264, 269, 264, 249, 224, 189, 144, 89, 24, -51, -136 
Overall normalizing factor: 2261

There are several other applications of the Savitsky-Golay smoothing algorithm that are mentioned in your text, but I think that is enough for one lab.

What to hand in 
1. Anything given in the text of the lab in bold. This includes plot, numbers, calculations and short answers. Please include enough dialog with your answer so I know what your answer means 
2. A printed copy of this lab with all corrections to the text in some color other than black.
Computer Lab 2
Computer lab on Wave superposition (interference)

Answers to questions in bold need to be handed in.

The equation for sinusoidal wave form is given in your text on page 120 equation 6-5 is

\[ y = A \sin(2\pi vt + \phi) \]  
Eqn (1)

- \( y \) is the displacement of the wave,
- \( v \) is the frequency of the wave (in cycles/second)
- \( \phi \) is the phase angle of the wave

Today you are going to use a math applet (a web based math application/animation) to explore what happens to waveforms as they add together (are superimposed) to create interference effects.

Start the web browser on your computer and go to the site http://math.hws.edu/javamath/config_applets/MultiGraph.html
This is an introductory page to using this program. If you want the details, go ahead and read it. If you want to get on with the lab, find a button in the middle of the page that says launch multigraph and click on it with your mouse.

You should now have a window in front of you that has a plot of three different functions in three different colors. Along the right hand side of the graph are windows to change the limits of the graph, and along the bottom are some spaces where you can plug in your own functions. Why don’t you start there. Click in the white space to the right of each function and hit the backspace or delete key until there is nothing in the function region. Next hit the enter key on your keyboard. One by one you should be able to remove each of the function from the graph.

Now lets load a waveform into function 1. Click in the f1(x) window and type in the following function:

\[ 3\sin(6.28*2*(x+0)) \]
and hit the enter key.

Hopefully you now have a sin function displayed in your function window. Note that the sin function isn’t perfectly smooth. That is because the graphing function doesn’t graph enough points to make it smooth, not because there is something wrong with your function. You can try playing with the limits a bit to see if you can get a nicer looking function if you lower some of the limits to display a smaller region of the plot.

Now let’s compare the function you have typed in with the ‘book’ equation, Eqn 1. The ‘3’ you have typed in corresponds to the A in Eqn 1. You know that the sine function naturally varies from +1 to -1, so if you want the function any larger or smaller you need to multiply everything by the A or amplitude term to get it to the size you want. Click on the equation in multigraph and change the amplitude from 3 to 5 and then to .2. What happens to your plotted function? (Remember to hit an enter on your keyboard after each change to see the changes effect). As you can see Amplitude refers to the overall size in the Y dimension of the waveform.
X in the equation you are typing, is supposed to be the time in units of seconds, so you can look at the rise and fall of Y and think of it in terms of the rise and fall of a function over a few second time period. Since the sine is a function of angle or arc within a circle, how do we get from a time in seconds to an angle?

Eqn 1 uses two steps to do this. First we have the frequency, \( \nu \). Since the frequency (\( \nu \)) is how many cycles there are in a second, \( \nu \) times \( t \) (\( \nu t \)) is how many cycles you have passed through. Your typed in equation has a frequency of 2 cycles/second. Prove this to yourself by setting \( \text{xmin} \) to 0 and \( \text{xmax} \) to 2 and counting the number of cycles there are in a 1 second time period.

So that explains the \( 2 \times X \), where does the 6.28 come from? The mathematical unit for an angle is radians, and there are \( 2\pi \) radians in a complete circle or cycle. \( 6.28 = 2(3.14) \), so the 6.28 is simply a conversion factor to get you from cycles to radians.

Notice the equation I had you plug into Multigraph is a bit different than Eqn 1. In Eqn 1, \( 2\pi \) is used to multiply \( \nu t \) only and then a \( \phi \) term is added before you take the sin. In your equation 6.28 multiplies \( \nu (t + 0) \) before you take the sin. What is going on here? What we are dealing with here is called the ‘phase’ of the function, or a shift either forward or backward along the X axis. In Eqn 1 the \( \phi \) function assumes that you shift the waveform in units of radians. In the equation on the computer I wanted you to think of the phase shift in terms of seconds. Look at the waveform carefully; note what the waveform is doing when \( X = .1 \) Now make \( F2(x) = 3*\sin(6.28*2*(x+.1)) \) Do you see how you have shifted the waveform by .1 seconds? The new wave is exactly the old one, it is just out of synch by .1 seconds.

Okay. Now you are at home with multigraph and the sine waveform, let’s move on to interference. Interference occurs when two waveforms are superimposed on each other. The superposition of two waveforms is achieved mathematically by adding the two waveforms together. Lets start by looking at the superposition of waves that have the same frequency but different phases. Set \( F1(x) = 3*\sin(6.28*1*(x+0)) \) and \( F2(x)=3*\sin(6.28*1*(x+.1)) \) if they aren’t already there. Now lets look at what happens when these two waveforms are superimposed on each other. Make \( F3(x)=3*\sin(6.28*1*(x+0)) + 3*\sin(6.28*1*(x+.1)) \)

What can you say about the amplitude, frequency and phase of the new waveform? Is this an example of constructive or destructive interference? Change the phase difference between the two waveforms from .1 to .2 to .3...

At what point do you have maximal constructive interference, when does destructive interference begin, where do you have maximum destructive interference? Now change the frequency from 2 to 3 and repeat the experiment. Does the maximum interference occur at the same time? Why or why not. Make a general statement or two about what happens when two sin waves with the same frequency but different phases are mixed together.

Now let’s try interference between two waves with the same phase and different frequencies. \( F1(x) = 3*\sin(6.28*1*(x+0)) \) and \( F2(x)=3*\sin(6.28*1.1*(x+0)) \) See how these two functions are exactly aligned at 0, but get further and further out of sync(or of phase) as you move away from zero? What do you think will happen as you add these two functions together? Try it (\( F3(x)=3*\sin(6.28*1*(x+0))+3*\sin(6.28*1.1*(x+0)) \)
Did you guess correctly? Try some other sets of frequencies. Make some general statements about what happens when you mix two sin waves with different frequencies together.
After the break we are going to be doing IR and NMR. Both of these machines use the Fourier Transform to take the raw data and transform it into something more useful. As you get further along in science you will see the Fourier Transform being used in many different applications because it is an incredibly useful tool. To really understand the Fourier Transform and its properties requires a fair amount of math and several hours of a graduate level class, thus, its nothing we can do in a three hour undergraduate lab. However, what I want to do here is lay some of the groundwork, expose you to some of the math, and try to give you an intuitive feel to some of the properties of the transform, so you can better appreciate what is going on inside the computer when we are working with IR and NMR data.

The book introduces the Fourier Transform in section 7I, pages 204-211. This is a section that we skipped over in the lecture. You can try reading that section now, but the approach I am using here is much different than your text, so it may not help.

Equations that may be useful:

\[ e^{i\alpha} = \cos(\alpha) + i \sin(\alpha) \]

\[ \sin(\alpha) = \frac{e^{i\alpha} - e^{-i\alpha}}{2i} \]

\[ \cos(\alpha) = \frac{e^{i\alpha} + e^{-i\alpha}}{2} \]

The Fourier Transform equation:

\[ H(f) = \int_{-\infty}^{\infty} h(t) e^{-2\pi if t} \, dt \]

The Inverse Fourier Transform equation:

\[ h(t) = \int_{-\infty}^{\infty} H(f) e^{2\pi if t} \, df \]
I. INTRODUCTION
   A. TRANSFORMS

   A transform is a mathematical process that changes the appearance of information or data, taking it from one form and putting into another. In this transformation no information is gained or lost, but the appearance of the data may be much different. Generally the data in one form is more suited for certain kinds of mathematical operations than the other form. Thus the point of the transform is to make the data into a form that can be more easily manipulated and interpreted by you, the user.

   Taking a logarithm is considered a mathematical transform. Can anybody here calculate BY HAND the logarithms of 17867 and 193267? While you probably can’t, your calculator can, so you are already using transforms in Chemistry without really thinking about it.

   What kinds of mathematical manipulations are easier to do with logarithms than with the original number? Well, multiplication come to mind. While I would hate to multiply 17867 x 193267 by hand, you can use the properties of logs to add the logs of these number together instead of multiplying. Thus the logarithms 17867 and 193267 are 4.25205 and 5.28616 respectively, and, when I add these two numbers together I get 9.53821. The inverse log transform of this number is $3.453 \times 10^9$ the same number I would get if I multiplied the two original numbers together.

   The Fourier Transform itself is usually introduced in context of reproducing a square wave function from of a series of sine or cosine functions. Here the Fourier Transform is used to find all the frequencies of all the sine waves that compose the square wave.

   This is a good example where of a PERIODIC function of time is transformed into a series of periodic frequency functions. Thus we have changes from a variable as a function of time to a variable as a function of frequency.

   It can also be shown that APERIODIC functions like pulses or delta functions can also be transformed, so the Fourier Transform also works with discrete functions as well as continuous functions.

   While some time will be spent on a few derivations at the beginning, and this approach seems mathematical, it will by no means be rigorous. I have put the emphasis on trying to give you an intuitive feel for the transform, so you will be interested enough to come back at a later to get the mathematics.
This day is spent on the FT because it is important in many fields like X-ray Crystallography, NMR, IR, electronics, quantum physics, noise and signal encoding to name a few. A little understanding of the math behind the FT can go a long way to demystifying some of the properties that can be observed and utilized in these different fields.

II. THE FOURIER TRANSFORM

Let's start by looking at the equation for the Fourier integral

\[ H(f) = \int h(t)e^{-2\pi if t} dt \]

In the equation you use a function that is a function of time \( h(t) \). You multiply it by the exponential term and find the total integral. This function will now be a function of frequency \( H(f) \). We will do a single example of this transform in a minute, but let's cover some more details first.

\( H(f) \) is generally complex ie.

\[ H(f) = R(f) + iI(f) \]

\[ H(f) = \text{amplitude} = \sqrt{R^2(f) + I^2(f)} \]

For those of you who don't remember complex numbers don't get all worried. Think of the complex numbers as being yet another dimension, orthogonal to our present dimension. Just as we can describe a point in space in terms of orthogonal axes \( X \) and \( Y \), we can describe a point in the universe in terms of real and imaginary dimensions, and the relationship is exactly like a set of points of equal length described by the equation

\[ A^2 = X^2 + Y^2 \]

(The equation of a circle \( A \) is the amplitude and is constant, but the component in \( X \) or \( Y \) changes with position.

For the sake of completeness I will do one complete Fourier transform here, just so you can see how it is done. After that you will use the SPIA program to do the transforms so you don't have to worry about the math.

I will use a simple exponential decay for my transform. An exponential decay is important because this is how an NMR signal decays, and it is easy to calculate.

\[ h(t) = \beta e^{-\alpha t}, t \geq 0 \]
\[ h(t) = 0, t < 0 \]

\[ H(f) = \int h(t)e^{-2\pi if t} dt \]
This expression contains both real and imaginary parts. Let’s separate it into two terms, one purely real, and a second purely imaginary. We can do this by multiplying by the term:

\[
H(f) = \frac{\beta}{\alpha + i 2\pi f}
\]

This expression contains both real and imaginary parts. Let’s separate it into two terms, one purely real, and a second purely imaginary. We can do this by multiplying by the term:

\[
\frac{\alpha - i 2\pi f}{\alpha + i 2\pi f}
\]

Where the real term is the lorentzian line shape you see in the real portion of a spectrum, while the imaginary part is the dispersive curve associated with the out of phase component of the spectrum.
This lab uses a program called SPIA to do some basic mathematical transforms. This is an ancient DOS program, so it is pretty primitive, but it works so let's use it. First read over the user's guide and get a feel for the program. This lab manual will give you a guide on getting started, but as you progress into the exercise the manual gives you less and less instructions, and you are to provide more and more inspiration. Feel free to take off on tangents at any time and try anything you want to. The worst you can do is crash the program and have to start again.

Exercise 1. Get SPIA running
On the NMR computer
Click on the My Computer icon
Click on the Hard Drive C: icon
Click on the SPIA folder
Click on the RunSpia.bat file folder
This starts the program
    you will now get a few messages, hit enter at the appropriate places
2. GENERATE A RECTANGULAR PULSE AND FIND ITS FT
A=RECT gets a rectangular function and loads it into the A block
C=A Copies A into C for future reference
A=FT(A) Do a fourier transform of A. This takes a minute or two so have patience. You now have a square function in C and its FT in A. Does this resemble FT pair given in the in class handout?
LET'S CHECK THE IMAGINARY PART OF THE TRANSFORM
B=A Save A in B for future reference
IA Display the Imaginary part of A. Is there any?
3. NOW LETS DO A SIMPLE COSINE FUNCTION
A=COS5X
C=A
A=FT(A)
B=A
IA
The FT of a cosine function has two peaks equidistant from the middle. Can you explain why? Is there an imaginary part to this transform? Now try it with COS2X can you explain the changes in the source function and in the FT?
        Now try the function SIN5X. What do the reals look like? The imaginaries? Do they make sense?
        Now try some other functions like EXPMX, LOREN, and GAUSS, use addition and multiplication functions to make more involved functions. Compare your results with those in the FT pair table. Sketch some of your results.
II. PROPERTIES OF FOURIER TRANSFORMS

1. Confirm the additivity property
Let A=COS3X and B=SIN9X generate the function C=A+B, take the FT of C and save it. Go back to A and B. take their individual transforms, and sum those functions, are they the same?

2. Confirm the time shifting property
Let B=SIN9X use the SHIFT function to shift the time frame by 10% Now examine both the reals and the imaginaries of the spectrum. What did you get?

3. Even functions give real and odd functions give imaginary transforms. Look over the work you have done so far. Which functions are EVEN. Were their transforms real or imaginary? How about the ODD functions? Can you use some other SPIA functions to generate more even and odd functions for additional confirmations?

III. CONVOLUTION
An NMR signal may be thought of as a sine or cosine frequency function multiplied by an exponential decay function. Lets try multiplying these functions together to synthesize an NMR like signal. Lets also try taking the Ft of the individual functions and then convoluting them to see if this gives an equivalent function.

1. Generate a single exponential decay
   A=EXPMX
   b=HEAVY
   a=a*b
generate the cosine signal
   b=cos8x
   a=a*b
   a=ft(a)
   b=a
   ia
The original cosine function was purely real. What about the new function? can you explain why?

   Now lets try it the other way
   generate the exponential decay in b and the cosine in a, take the ft for both functions, now try A=A CONV B. What do you get.

   Hint: CONV works only for real functions FTs of A and B generate real and imaginary data sets. You must tell the convolution function to ignore the imaginary portions of the data set
   COMPLEX OFF

IV. GETTING OUT OF THE PROGRAM
type EXIT
hit a control c like the window tells you
Close the SPIA window

What to hand in:
Nothing for this lab
Experimental Lab 1.  
Atomic Absorption and Emission

Last semester you determined the amount of Ca\(^{2+}\) and Mg\(^{2+}\) in my well water using EDTA titration and atomic emission. Most people didn’t like the titration because the endpoint was hard to see, and for the emission experiment I set it up and you wrote down the numbers. Over the next two weeks you will use both atomic absorption and emission, and you will make up the standards and run the machine by yourself. Wait till you see the sample.

You can schedule this as you like. You can do emission one week and absorption the next, or you can do Mg one week and Ca the next, whatever. Since you have already seen it done, I think I would start with emission.

Be aware that this is another experiment that has never been done at BHSU before, so I am guessing at the concentrations for the standards. They may be way off, so be prepared to adjust as we go.

Let’s try to make 1 set of standards that everybody can share and we will keep the standards for both weeks.

**Mg standard** (100 ppm)

Accurately weigh about 0.1g of Mg ribbon and place in a 1 l volumetric flask
add 10 ml of DI water
add HCl **one drop at a time** until the Mg dissolves
(Watch out, this might be a strong reaction)

Fill a separate beaker with 1000ml of DI water and add 10 ml of HCl to make a 1% HCl solution.

Fill the volumetric containing Mg to the mark using this 1% HCl solution.

**Ca Standard** (50 ppm)

Accurately weigh about .125g of CaCO\(_3\) and place in a 1 l volumetric flask
add 50 ml of DI water
add HCl **one drop at a time** until the Ca dissolves
(This reaction will not be as strong as the Mg reaction)

Dilutions

**Mg Absorption Standards** - Now get five 100ml volumetric flasks. Place 0.5 ml of the dilute Mg standard into the first flask, .25 ml into the second, .10 in the third, .05 in the fourth and .02 in the 5\(^{th}\). Next using the Repipet filled with alcohol, add 5.1 ml of alcohol to each flask. Finally fill all of the flasks to the 100 ml mark with water. Mix these flasks, then calculate the concentration of Mg in each of these flasks, clearly mark these dilute magnesium standards

**Mg Emission Standards** - Now get five 100ml volumetric flasks. Place 50 ml of the dilute Mg standard into the first flask, 25 ml into the second, 10 in the third, 5 in the fourth and 2 in the 5\(^{th}\). Next using the Repipet filled with alcohol, add 5.1 ml of alcohol to each flask. Finally fill all of the flasks to the 100 ml mark with water. Mix these flasks, then calculate the concentration of Mg in each of these flasks, clearly mark these dilute magnesium standards

**Ca Standard** (50 ppm)

Accurately weigh about .125g of CaCO\(_3\) and place in a 1 l volumetric flask
add 50 ml of DI water
add HCl **one drop at a time** until the Ca dissolves
(This reaction will not be as strong as the Mg reaction)

Fill the volumetric to the mark with DI water
Dilutions

*Ca Emission and Absorption Standard* Now get five 100ml volumetric flasks. Place 5 ml of the dilute Ca standard into the first flask, 2 ml into the second, 1 in the third, 0.5 in the fourth and 0.25 in the fifth. Next place using the Repipet filled with alcohol, add 5.1 mls of alcohol in each flask. Finally, fill all of the flasks to the 100 ml mark with water. Mix the flasks then calculate the amount of Ca in each flask and clearly mark the flasks accordingly.

Sample Preparation

Since it is impossible to know what the concentration of Mg and Ca is in the sample before we analyze it, it is best to prepare several different dilutions of the sample so you have something that is in the right range to analyze with the above standards.

- **1:5** 5 ml of sample in a 25 ml volumetric.
- **1:10** 2.5 ml of sample in a 25 ml volumetric.
- **1:40** 2.5 ml of sample in a 100 ml volumetric.
- **1:100** 1.0 ml of sample in a 100 ml volumetric.
- **1:400** 0.25 ml of sample in a 100 ml volumetric.
- **1:1000** 0.1 ml of sample in a 100 ml volumetric.

Add 1.28 ml of ethanol to the each 25 ml volumetrics and 5.1 ml of ethanol to each 100 ml volumetrics, then fill all volumetrics to the line with deionized water.
Experiment

Day 1?
Set up the instrument for Ca absorption. Calculate the concentration Ca in all the Ca diluted standards. Place the most concentrated Ca sample in the instrument and set instrument to read whatever concentration you calculated for that standard. Without recalibrating, place all other standards and a water blank in the instrument and read their values. Run through all standards including the blank at least five times so you can calculate a standard deviation for each measurement. Now try your various dilutions of the sample. Find the one or two dilutions that appear on scale and eliminate any dilutions that appear offscale. Again run the sample 5 times so you can get a standard deviation for your readings.

Switch the instrument over for Ca emission. Place the most concentrated Ca sample in the instrument and set instrument to read whatever concentration you calculated for that standard. Without recalibrating, place all other standards and a water blank in the instrument and read their values. Run through all standards including the blank at least five times so you can calculate a standard deviation for each measurement. Now try your various dilutions of the sample. Find the one or two dilutions that appear on scale and eliminate any dilutions that appear offscale. Again run the sample 5 times so you can get a standard deviation for your readings.

Day 2?
Set up the instrument for Mg absorption. Calculate the concentration Mg in all the Mg absorption standards. Place the most concentrated Mg sample in the instrument and set instrument to read whatever concentration you calculated for that standard. Without recalibrating, place all other standards and a water blank in the instrument and read their values. Run through all standards including the blank at least five times so you can calculate a standard deviation for each measurement. Now try your various dilutions of the sample. Find the one or two dilutions that appear on scale and eliminate any dilutions that appear offscale. Again run the sample 5 times so you can get a standard deviation for your readings.

Switch the instrument over for Mg emission. Calculate the concentration Mg in all the Mg emission standards. Place the most concentrated Mg sample in the instrument and set instrument to read whatever concentration you calculated for that standard. Without recalibrating, place all other standards and a water blank in the instrument and read their values. Run through all standards including the blank at least five times so you can calculate a standard deviation for each measurement. Now try your various dilutions of the sample. Find the one or two dilutions that appear on scale and eliminate any dilutions that appear offscale. Again run the sample 5 times so you can get a standard deviation for your readings.
Instrument directions

Part I. Atomic Absorption and Emission of Ca

Experiment I.A Atomic Absorption of Ca

While Emission is actually easier to set up, the book starts with absorption, so that is where we will start. Before you can measure absorption you must first have a light source of the proper wavelength. Thus we need to start by installing a Ca lamp in the spectrometer, and tuning the machine to have its best response for that lamp.

✓ Instruction

_____ Turn Machine power on
_____ Turn on exhaust fan (Switch on right side of door frame)
_____ Turn on far hood (Part of complete exhaust fan system)

_____ Turn Lamp1 & Gain knobs down to lowest value (fully counter-clockwise)

• Put in lamp
  • Open access cover
  • Unscrew two set screws to unfasten lamp holder
  • Find the appropriate lamp, remove lamp from box. A sticker on the lamp indicates the optimum and maximum currents, write these values down.
  • Mount lamp on lamp holder
  • Place lamp holder back in machine and fasten down
  __ Plug lamp in!

_____ Close lamp access door

_____ Turn slit to 0.7 and wavelength to 422.7Å

_____ Turn Signal knob to Lamp 1

_____ Turn Mode knob to Hold

_____ Turn Lamp 1 knob clockwise until you get a reading of 5 on the Lamp Energy Display
  (5 was the optimum current for the lamp!)

_____ Place a piece of tape on this knob so you can’t change its setting

_____ Turn Signal knob to ABS

_____ Turn Gain clockwise until you have a reading of about 50 for the Lamp Energy Display
  Tweak the wavelength slightly up an down until the Lamp Energy Display reads highest possible value. (If the number goes above 99 you will see an EE displayed. If this happens turn the gain down to about 50 and then continue to optimize Lamp Display to its highest value)

_____ Adjust the lamp position to give the display its highest possible value
  • move lamp forward and back inside the springs
  • move lamp side-to-side an up and down using adjusting screws

_____ When lamp position is optimized close lamp access panel

_____ Adjust Gain knob to get a reading of 75 on the Lamp Energy

_____ Turn Mode Knob to CONT.
You should now be ready to work with a sample

**In the following instructions, make sure you turn air on first and then acetylene; if you mix this up you will have a small explosion that will blow the burner off the machine!**

1. Make sure the air compressor in next room is on. If it is not already on, plug it in and wait 5 minutes for the tank to fill with compressed air before you continue.
2. Locate the panel to the left of the burner that controls the gases
3. Turn switch at the bottom of this panel so it points to AIR
4. Turn Acetylene on full at main valve. Adjust regulator so the low pressure dial reads about 10 psi
5. Flip fuel toggle up
6. The ignition switch doesn’t work, so light the burner with a match

If you have a flame, you should be ready to go

1. Change the **Signal knob** to **Conc**
2. Start aspirating distilled water into the flame
3. Hit the **AZ button** to Auto Zero the machine

1. Replace the DI water with you most concentrated standard
2. **Enter the concentration of this standard on the keypad**
3. Press S1 twice

You can calibrate with up to 3 standards, S1, S2, and S3. If you are doing multiple standards you must calibrate in order from lowest concentration to highest concentration. If you make a mistake, or want clear your calibration curve hit the CE key to cancel.

However, I think it is better technique to simply set your highest concentration to read whatever its concentration should be, and then record your values for all other standards and make your own calibration curve and not rely on the machine to do this for you.

If this signal bounces up and down too much you can change the Mode knob to hold, then hit the read button, and the machine will average points for several seconds together to give you a less noisy signal.

Note: for all readings including the blank, either record 3-5 independent readings so you can calculate a standard deviation, or record extreme high and low as well as an ‘average’ reading.

Replace the standard with the other samples you wish to run, and simply read their values off the display

Once you have read all of your standards, try your sample. Try your various dilutions until you find one or two that are in range. Make sure you do your dilutions accurately and that you keep track of your dilutions in your lab notebook.
Experiment 1.B. Emission of Ca

Since the machine is all tuned up for the Ca line, let’s switch the machine over to emission and obtain the same data you did above using the Spectrometer as an emission machine.

_____ Turn Lamp1 & Gain knobs down to lowest value (fully counter-clockwise)
- Open access cover
- Unplug Ca lamp
- Unscrew two set screws to unfasten lamp holder
- Remove Ca lamp, and put it back in its box
- Close lamp access panel
_____ Turn Slit to 0.2 nm
_____ Change from air to nitrous oxide for the oxidizer
- Make sure the nitrous regulator is plugged in (This is a heater so the regulator doesn’t freeze up!)
- Open the main valve on the nitrous tank, set the regulator so you have about 25psi
- With the flame on, smoothly (quickly) flip the toggle switch from air, thru off, and onto N₂O
_____ Change Signal knob from Conc to EM

You should now be switched over to emission mode.
_____ Place your most concentrated sample in the flame. Adjust gain to get a signal of about 90 on the Lamp energy
_____ Start aspirating distilled water into the flame
_____ Hit the AZ button to Auto Zero the machine

_____ Replace the DI water with your most concentrated standard
_____
Enter the concentration of this standard on the keypad
_____ Press S1 twice

As before, you can calibrate with up to 3 standards, S1, S2, and S3, if you want, but for this experiment simply set the highest concentration to its proper concentration and make your own calibration curve. If you make a mistake, or want clear your calibration curve hit the CE key to cancel.

Finally run your sample dilutions.

When you are through, switch the flame from nitrous back to air by smoothly (quickly) turning the toggle from N₂O back to Air

If you are going to do the Mg experiments today, go on to the next page. If you are going to do the Mg experiments on another day, go forward a few pages and find the section on SHUTDOWN.
Part II Atomic Absorption and Emission of Mg

Experiment II.A Atomic Absorption of Mg

Now we need to switch the machine over to work with Mg by replacing the Ca lamp with the Mg lamp. You do not need to turn off the flame, but please be careful that you don’t actually stick anything important in the flame while you are working with the lamp.

_____ Turn **Lamp1 & Gain knobs** down to lowest value (fully counter-clockwise)
• Open access cover
• Unplug Ca lamp
• Unscrew two set screws to unfasten lamp holder
• Find the appropriate lamp, remove lamp from box. A sticker on the lamp indicates the optimum and maximum currents, **write these values down.**
• Mount lamp on lamp holder
• Place lamp holder back in machine and fasten down
Plug lamp in!

_____ Close Lamp access door.
_____ Turn **slit** to **0.7** and **wavelength** to **285.2**
_____ Turn **Signal knob** to **Lamp 1**
_____ Turn **Mode knob** to **Hold**
_____ Turn Lamp 1 knob clockwise until you get a reading of 4 on the Lamp Energy Display
(4 was the optimum current for the lamp!)
**Place a piece of tape on this knob so you can’t change its setting**

_____ Turn **Gain** clockwise until you have a reading of about 50 for the Lamp Energy Display

_____ Tweak the wavelength slightly up or down until the Lamp Energy Display reads highest possible value. (If the number goes above 99 you will see an EE displayed. If this happens turn the gain down to about 50 and then continue to optimize Lamp Display to its highest value)

_____ Adjust the lamp position to give the display its highest possible value
• move lamp forward and back inside the springs
• move lamp side-to-side and up and down using adjusting screws

When lamp position is optimized
_____ Adjust **Gain knob** to get a reading of **75 on the Lamp Energy**
_____ Turn **Mode Knob** to **CONT.**

If you have a flame, you should be ready to go
_____ Turn the **Signal knob** to **Conc.**
_____ Start aspirating distilled water into the flame
_____ Hit the **AZ button** to Auto Zero the machine

_____ Replace the DI water with your most concentrated standard
_____ Enter the concentration of this standard on the keypad
_____ Press SI twice

Replace the standard with the other samples you wish to run, and simply read their values off the directly. As before for all readings including the blank, either record 3-5 independent readings so you can calculate a standard deviation, or record extreme high and low as well as an ‘average’ reading.
Experiment II.B Emission of Mg

Since the machine is all tuned up for the Mg line, let’s switch the machine over to emission.

— Turn Lamp1 & Gain knobs down to lowest value (fully counter-clockwise)
  • Open access cover
  • Unplug Ca lamp
  • Unscrew two set screws to unfasten lamp holder
  • Remove Ca lamp, and put it back in its box
  • Close lamp access panel
— Turn Slit to 0.2 nm

— Change from air to nitrous oxide for the oxidizer
  • Make sure the nitrous regulator is plugged in
    (This is a heater so the regulator doesn’t freeze up!)
  • Open the main valve on the nitrous tank, you shouldn’t have to touch the regulator
  • With the flame on, smoothly flip the toggle switch from air, thru off, and onto N₂O
— Change Signal knob from Conc to EM

You should now be switched over to emission mode.

— Place your most concentrated sample in the flame and adjust the gain to get a signal of about 90
— Start aspirating distilled water into the flame
— Hit the AZ button to Auto Zero the machine

— Replace the DI water with your most concentrated standard
— Enter the concentration of this standard on the keypad
— Press SI twice

Replace the standard with the other samples you wish to run, and simply read their values off the display. Again take several readings for each solution so you can calculate a standard deviation for each measurement.

When you are through, switch the flame from nitrous back to air by smoothly turning the toggle from N₂O back to Air
**Shutdown of Instrument**

Again, follow the order of fuel then air, if you don’t you can also get a small explosion.

1. If you are running N₂O, change the flame over to air by flipping the toggle from N₂O to Air.
2. Turn the N₂O valve off at the main valve on the tank.
3. Unplug the N₂O regulator.
   - Turn acetylene gas off **at the main valve on the tank**.
4. After the flame dies out, flip the fuel toggle down on the instrument.
5. Unplug the air compressor.
6. Flip the Air/N₂O toggle back and forth a few times to clear these gases out of the lines.
7. Turn **Lamp1 & Gain knobs** down to lowest value (fully counter-clockwise).
8. Remove lamp from the lamp compartment and put it away.
9. Turn off exhaust fan (right side of door frame).
10. Turn off fan on left hood.
11. Turn off power of AA.
12. If machine is cool, replace cover.
Data analysis

1. Linearity
For each of the four experiments, make a plot of recorded value (y axis) vs actual value(x axis). Was the data linear for all data sets? For each of the data sets determine a line of best fit. For each data point I told you to record the range of values you observed. Try to put this uncertainty into your plots so I can get a feel for the noise in your data. If you can’t do this by computer you may have to pencil it in by hand on your graphs.

2. Samples
Where do your samples fall on these curves? How do the methods compare? For an accurate analysis the sample you analyzed should fall between 2 values on your calibration curve. Did this happen with your data? Can you suggest a better set of standards for use in a calibration curve next year? Based on the uncertainty in your readings, what would you say is the uncertainty in your measured concentrations?

3. Detection limit
On page 100 of your text, you will find that you can estimate the standard deviation of a signal by dividing the total range of that signal by 5. Use this to estimate the standard deviation of the blank ($s_{bl}$). Assume you need to have a signal at least 3 standard deviations larger than the average blank value ($\bar{s}_{bl}$) and find the minimum signal for each of the 4 different experiments you did. (Use equation 1-4 page 13 with k=3). Use equation 1-5 (page 13) from your text to determine the detection limit ($c_{d}$) in each of your calibration curves.

4. Summary
Based on your data, what was the detection limit, limit of linearity, and dynamic range for Ca on this instrument?
Based on your data, what was the detection limit, limit of linearity, and dynamic range for Mg on this instrument?
Were the chosen calibration curves appropriate for these limits? If not, suggest the calibration curves that we should try next year to better test these limits on this machine.

5. For next year.
What did you like about this experiment? What did you dislike? Were any parts of the procedure unclear? Can you suggest a change in procedure, or a different sample that you might find more interesting?
This is what I will be grading:

Was the writeup or spreadsheet well organized and well written?

Proper calculation of Ca and Mg in all standards

Calibration curves (50 points):
Four Plots of standards: Mg Emission, Mg absorption, Ca Emission, Ca Absorption
Plots should include:
  Recorded value (Y) vs. actual concentration(X)
  axes clearly marked
  plot clearly labeled
  some indication of uncertainty in plotted values
  line of best fit
If a curve is missing, some explanation of why it isn’t plotted

Data (15 points)
actual data points in a table, or indicated on the above calibration curves
estimate of uncertainty of data
some analysis of how data fits with calibration data

Detection limit (30 points)
  Minimum signal and detection limit for each of the 4 experiments
  detection limit, limit of linearity and dynamic range for each of the 4 experiments
  Statement of appropriateness of calibration curves to the data

Any suggested changes for next year (5 points)

Extra experiments (Bonus)
Purpose:
- Introduce visible light spectroscopy
- Introduce %T and absorbance as way to measure light absorption
- Introduce the Lambert-Beer’s Law

Most of the time when light passes through a molecule, the light and the matter to not interact and nothing happens. Occasionally however, when the energy of the light matches some electronic transition within the molecule, the light can transfer a quantum of energy from the light beam into the molecule. When this happens we say the molecule has absorbed the light.

All chemicals absorb light at some wavelength, and the amount of light that is absorbed by the chemical can be related directly to the amount of that chemical that is in the path of the light. The light absorbed by a chemical is measured in an instrument called a spectrophotometer. The simplest spectrophotometer, shown in Diagram 1, has a light source, a cell holder in which we place our sample, and a detector to measure how much light passes through the sample. By measuring the light that hits the detector both with and without a sample in the sample holder we can measure the amount of light absorbed by our material.

Diagram 1. Block diagram of a simple spectrophotometer.

Section I. Familiarization with spectrometer and light.

The spectrophotometer you will use in this lab is the Red Tide USB650 spectrometer built by Ocean Optics. The entire spectrometer is the 3x3 in black box you see in front of you. Your sample holder is the rectangular hole in the smaller of the two rectangles that make the spectrophotometer. Go ahead and pick up the spectrometer and move it around. Just don’t drop it or unplug it. Right now the light source is turned off so you won’t see any light coming out of it. Once you are through playing with it put the spectrometer on a flat surface with the name up so you can see it.
Starting the Computer.

Start the computer to run the spectrometer the way you would any Windows program.

Move the mouse to:

- Start
- Programs
- Vernier Software
- Logger Pro (left mouse click)

The Logger Pro software is designed to run many different instruments, so the first thing you have to do is to tell it what it is supposed to do. Locate the ‘Experiment’ tab on the upper line of command tabs in the Logger Pro Window. Click on ‘Experiment’, move your mouse to the ‘Connect Interface’ and ‘Spectrometer’ drop down menus, then click on ‘Scan for Spectrometers’

You won’t see anything on the computer to tell you that you have connected, but you should now see your light source turned on if you look down the sample holder of the spectrometer.

Now you want the computer to calibrate the spectrometer

- Experiment
- Calibrate
  - Spectrometer:1 (click)

A window should now pop up that says it needs to warm up for a minute, so let it warm up. Once it has warmed up the print ‘Place a blank cuvette in the device’ shows in black. For this experiment you are not going to use a cuvette, so simply click on the ‘Finish Calibration’ button, let it finish its calibration, then click on the ‘OK’ button.

The simplest way of measuring how much light a sample absorbs is using a number called % Transmittance. Let’s switch the spectrometer over to read in these units

- Experiment
- Change Units
  - Spectrometer:1
    - % Transmittance (Click)

When you calibrated the spectrometer the computer measured how much light was passing through the sample chamber with nothing in it. Now in % transmittance mode, when you put something in the sample chamber measure it with the spectrometer it will tell you what % of that original light actually gets through the sample.

Let’s try this. To start measuring % transmittance click the Green ‘Start’ button on the right hand end of the first row of icons in the Logger Pro Window.

Let’s now talk about the rest of the display. As far as spectrometers go, this is a pretty fancy one, so it is measuring the absorbance at all wavelengths from 380 nm to 950 nm in about a second. See how the numbers on the left and the graph on the right change slightly about every second as the computer discards the old data and finds new data?

The table on the left hand side of the display gives you the wavelength and the % transmittance number in table form, and the display on the right hand side plots this graphically. What I really like about this display is the rainbow in the middle of it. What this rainbow represents is the color at each wavelength. I can never remember that green light has a wavelength of 500-550 nm, but with this display you don’t have to remember, its right there in front of you.
Notice that the red line representing the actual spectrum does not go beyond the right hand end of the rainbow, and this tells you that the spectrometer can’t measure light at these wavelengths <380 nm. **What does the fact that the spectrum line goes past the right hand end of the rainbow up to 950 nm mean?**

Now that you are familiar with the display, what should it mean. If you have followed the instructions, there should be nothing in the path of the light, so what should your % transmittance read? (100% for all wavelengths because there is nothing there to absorb the light).

What you see for most of your spectrum is line near 100% T but with some variations just under 100%. This is called ‘noise’ and represents small inaccuracies between the initial calibration and each individual measurement. In general we will ignore this noise.

What about the Left hand end of the spectrum. Does this drop down to 0% T at 380 nm? This shows you that some components within the spectrometer actually don’t work right at these wavelengths, so you should ignore data in this region. If you look at the table display on the left you will see the values of 100% T start about 400 nm. This says you can’t trust any data obtained with this instrument below this wavelength. **What is the range of wavelengths that you should trust using this instrument?**

Colors and % Transmittance

Obtain colored plastic sheets in red, blue, green and yellow. The red sheet appear to be red because it transmits red light and absorbs light of other colors. With this in mind, fill in the following prediction table BEFORE you do the experiment.

**Prediction**

<table>
<thead>
<tr>
<th>Color of Plastic</th>
<th>% Transmittance (H for high &gt;90%, L for low &lt; 10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>Blue region</td>
</tr>
<tr>
<td>Green</td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td></td>
</tr>
</tbody>
</table>
Now do the actual experiment. One at a time, cut out a piece of each color plastic and place it in the spectrometer. Make sure that the film lies between the light source and the detector on the opposite side of the cell holder.

Once it the film is in the spectrometer, the display instantly changes to show the % transmittance spectrum of that film. Stop the spectrometer by clicking on the red stop button on in the icon row so you can look in detail at a single spectrum. Print this spectrum for your lab notebook in the usual way by hitting the File-Print buttons. A window will come up and ask if you want to include things like your name and comment in the print material. Go ahead it is always good to have this information printed with the spectrum so you can identify it at a later time.

If you are computer literate, and want to store this spectrum in an excel file so you can use excel to plot and analyze this data at a later time do the following steps. 1.) Open excel in a separate window. 2.) Cut and paste both columns of data from the left hand data table into your excel window. 3.) Save the data in excel in the usual way. OK now move on to the next film. Take the current film out of the spectrometer, insert the next film. You will need to start the spectrometer taking data again so click on the green collect button. A window will come up to ask if you want to erase the previous data before you collect the new data. Click on the ‘Erase and Continue’ button. Collect data for all four films.

Record your data in the table below (and in a similar table in your lab notebook).

<table>
<thead>
<tr>
<th>Color of Plastic</th>
<th>% Transmittance (H for high &gt;90%, L for low &lt; 10 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blue region</td>
</tr>
<tr>
<td>Blue</td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td></td>
</tr>
<tr>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td></td>
</tr>
</tbody>
</table>

Explain any difference between your experimental data and your predicted data.

II. Lambert-Beer law for pathlength
Pathlength refers to the thickness of the material in the light beam. As you might expect, the thicker the material, the more light that is absorbed. Let’s test this.

Use a micrometer and measure the thickness of the film. Let’s say this film transmitted 80% of the light that hit it. Now what would happen if you placed a second piece of this film in the light beam as shown in the diagram below:

100% of light → 1<sup>st</sup> film → 80% of light → 2<sup>nd</sup> film → ? % of light.
With the above diagram in mind fill in the prediction table below.

<table>
<thead>
<tr>
<th># of films</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>total thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Transmittance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Now do the experiment. Before we actually start acquiring data it is best to recalibrate the spectrometer. Make sure the spectrometer's stopped, then follow the same steps you did before to calibrate the instrument. This time you can cancel the warmup because the spectrometer has been on for a while, and you will again not put in a cuvette in the last step of the calibration.

Based on your previous spectrum of this film locate a single wavelength that has a transmittance of about 80%. Use the slider in the left hand data table to locate the cell that corresponds to this wavelength. Now start adding films between the light and the detector to fill in the data table below.

<table>
<thead>
<tr>
<th># of films</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>total thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Transmittance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Use excel or a piece of graph paper and plot the above data. Is %transmittance directly or inversely related to pathlength. Is this a linear relationship or is there some curve to the line?

If you can plot data and see a straight line you have a powerful tool to use to analyze the data. You can plot the data, use the computer to find a line of best fit, then use the equation of the line ($Y = mX + b$) to predict $X$ given a $Y$ value or vice-versa. Unfortunately, if you did the above experiment right, you did not get a straight line, so you can’t do the with % transmittance data. Let’s try to transform the data to get a straight line.
If you think back to your prediction table you should have filled in the table with the following logic:

1st thickness 1 unit of light x .8 of light transmitted = .8 (80%) of light transmitted
2nd thickness .8 of light x .8 for the next thickness = .8x.8 =.64 (64%) of light transmitted
3rd thickness .64 of light x .8 for the next thickness = .64x.8 =.512 (51.2%) of light transmitted
4th thickness .8 of light x .8 for the next thickness = .512x.8 =.41 (41%) of light transmitted
etc.

If you think about this .8x.8x8x8... you can see the general form of the equation is:

Fraction of light for n thicknesses = 1 x (fraction of light for 1 thickness)^n

and the above exponent tell you that this is an exponential function. That curved line you observed for the plot of % Transmittance vs pathlength is a typical exponential decay curve.

In mathematics the function that cancels out the exponent is the logarithm function. So let's see if what that does to our data. Fill in the following table by converting your % transmittance values first to fraction of light transmitted, and then to log (fraction of light). Finally since your log values are all negative, and I don't like plotting negative data, take the negative of the log.

<table>
<thead>
<tr>
<th># of films</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>total thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Transmittance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction of light</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log(fraction of light)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-(log(fraction of light))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Now make a plot with thickness in the X dimension and -(log(fraction of light)) in the Y dimension. What is the relationship between thickness and -(log(fraction of light))? Is this a linear relationship?

Hopefully you have found that pathlength and -(log(fraction of light)) are directly, linearly related, this is a powerful relationship, and it is the first half of the Lambert-Beer law.
Writing the term \(-(\log(\text{fraction of light}))\) is long and cumbersome, so let’s call this mathematical transformation the **Absorbance** of the light. If the light hitting the sample is \(I_0\) and the light that actually makes it through the sample is \(I\), then:

\[
\text{Fraction of light} = \frac{I}{I_0}
\]

and

\[
\text{Absorbance} = A = -\log\left(\frac{I}{I_0}\right)
\]

Most of the time the spectrometers are set up to record absorbance for you so you don’t have to worry about finding \(I\) and \(I_0\) to do this calculation. Occasionally however, you do have to convert between absorbance and % transmittance and that equation is:

\[
\text{Absorbance} = A = -\log\left(\frac{\% \ T}{100\%}\right)
\]

You have just seen that absorbance is a powerful measurement because it can be directly and linearly related to pathlength. Just as important is its relationship with concentration. When you doubled the pathlength of the film what you physically did was to double the number of absorbing molecules in the path of the light. A second way to double the number of absorbing molecules is to have the same film thickness, but to double the concentration of molecules in that film.

With that in mind let’s try another prediction. Say you had a film with an 80% Transmittance when it has a concentration of absorbing molecule 1M. Using the above transformation 80% \(T = .097A\) fill in the following prediction table:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Transmittance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorbance (A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Now do the experiment. Obtain 11 dry, clean cuvettes. Fill one cuvette with water and a second cuvette with solution 10. Since it has been a few minutes since you last used the spectrometer it needs to be recalibrated. In this recalibration you can again skip the warm-up, but this time, place the cuvette filled with water into the sample holder in the final calibration step. Two things to check when you do this. 1.) Have all of the plastic films been removed? And 2.) Does the light pass through the longest and clearest portion of the cuvette?
Since you have now seen that Absorbance is a better way to measure light interactions than % transmittance, switch the spectrometer over to absorbance mode so it does the \(-\log(I/I_0)\) calculation for you. To do this use the mouse to:

Experiment
  Change Units
  Spectrometer
  Absorbance

Now place the cuvette with solution 10 in the light with cuvette in the correct orientation and observe the absorbance spectrum of this solution. After you have obtained a spectrum in absorbance mode, stop the spectrometer, then toggle between absorbance and % Transmittance mode a few times.

**What is the relationship between peaks in one spectrum and valleys in the other? Does this make sense? If a molecule absorbs a lot of light it has a high or low % Transmittance? And a high or low Absorbance?**

In your spectrum try to find a peak that has an absorbance value of about 1. **What is this wavelength?**

Now obtain absorbance readings for all other solutions at this wavelength and fill in the following data table:

<table>
<thead>
<tr>
<th>Soln.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Plot absorbance (Y) vs concentration (X). What is the relationship between concentration and absorbance?**

Hopefully you just found that absorbance is directly and linearly related to concentration. Thus in the last two experiment you have found the mathematical relationship that

\[ A \propto \text{pathlength} \times \text{concentration} \]

Expressed as a mathematical equation you could write

\[ A = K \times \text{pathlength} \times \text{concentration} \]

Chemists usually use the symbol \( c \) for concentration and use the units of molarity (M) and to make a convenient instrument we usually use a 1 cm pathlength and use the symbol \( b \) for pathlength. (Why \( b \)? Don’t ask me)
Thus

\[ A = K \times b \times c \]

Let's talk about units. To get the A we took the \(-\log(I/I_0)\) The units of I and I₀ are the same, so the units cancel out, so A is a unitless number. The units of K then have to be M⁻¹ cm⁻¹ to cancel out the units in the b and c variables. When K has units of M⁻¹ cm⁻¹ we give it a special name, the \textit{molar absorbtivity coefficient}, and give it the symbol \(\varepsilon\).

Thus you final mathematical statement of the Lambert-Beer law is:

\[ A = \varepsilon \times b \times c \]

And you have proved this law with your own two hands.

Your final task is to take your plot of absorbance vs concentration and calculate the \(\varepsilon\) value for this molecule at this wavelength. Will \(\varepsilon\) be the same at a different wavelength? For a different molecule at the same wavelength?
Experimental Lab 3
UV/Visible Spectrophotometer lab

At this point in your career you have used the single beam Spec 20's several times, the double beam Lambda 2 a few times and you have probably used the photodiode array instrument once or twice. In using these machines you have gone with the default parameters and acquired reasonable data, so you are probably pretty comfortable with the machines.

The focus of this lab is not on using these instruments for yet another spectrophotometric method, but to look at some of the default parameters you have used and to try to push the limits of the machine to see if we can determine just how accurate it is.

Section I - The Cuvettes or cells
(Can be done with either spectrometer)

Part I.A Evaluating cells
Before you can even start to use any spectrophotometer, you need to have an appropriate cell to put your sample in. Since the choice of appropriate cells is one of those things that people often forget about, let's start there. This spectrometer scans from 1100 nm (near Infrared) down to 190nm (UV). The ideal cell should be completely transparent throughout this entire wavelength range. Let's see if the cells we have in the lab are ideal.

Set up the machine to perform three scans from 1100 to 190nm. (If you are using the Lambda 2 see section III - Set instrument to do 3 scans and set scan rate to maximum.) For a baseline in this experiment, run the machine completely empty, i.e. no cell in either the sample or the reference half of the sample chamber. Then, for each of the next three runs (one scan per cuvette) put an empty cuvette of each of the following types in the sample part of the machine, leaving the reference side empty.
1. A large volume disposable cuvette
2. A small volume ‘UV’ disposable cuvette
3. A large volume quartz cuvette

Analysis I.A (to be handed in)Plot all three of these spectra. Assuming we don’t want the cuvette to have an absorbance of >.1 OD, what are the wavelength ranges that we can safely use these three different cuvettes?

Part I.B Cell Cleanliness
Under ordinary circumstances I would assume that the disposable cuvettes have not been used before, so they are as clean as they will get. On the other hand the glass or quartz cuvette has certainly been used before and may be dirty.

Analysis I.B (To be handed in) Do you see anything in the spectrum of the quartz cell that suggests it might be dirty? How could you check?
Part I.C Matched Cells
When you use a double beam instrument you should use a pair of matched cuvettes. To be a matched pair of cuvettes, the two cuvettes cannot have more than a 1% difference in their transmittance values over the entire wavelength range you are working in. Find a pair of cuvettes that you think are matched (preferably glass or quartz). Set up the spectrometer to record transmittance, then obtain the transmittance spectra for each of these two cells.

**Analysis I.C (to be handed in)** Are they well matched? If they aren’t matched, then what are the wavelengths where they aren’t matched. Do you think this can be corrected?

Section II - The Perkin Elmer Lambda 2 double beam spectrophotometer

Part II.A Scan rate
Now that you have critically evaluated the cuvettes let’s look at the spectrometer. The specs on the Lambda II are as follows:
- Scan speed 7.5 to 2880 nm/min
- Measured with 2 sec response time
  - accuracy +/- .005A at 1A
  - range -3A to +3A
  - stability <.0003A/hr drift (1 sec response time with warmup)
  - baseline +/- .001A 200-110 nm 2 s response time 240 nm scan
  - noise <.0003A (at 0A 500nm 2 sec response time)

The first thing I noticed about the specs is that this machine can scan as fast as 2880 nm/min. The default scanning speed is 240nm/min at 3.8 minutes to scan the entire range of the instrument. By setting the rate to its maximum you can reduce this time to 19 seconds.

Now if you have been paying attention in the noise lab you should realize that all instruments have some built in response time, and if you take in data faster than that response time you are asking for trouble. Since I can’t see any way to set the instrument response time I am instantly suspicious that setting the scan rate to its maximum value may be a fubar. **Set the instrument to scan between 650 and 190 nm in absorbance mode and set the scan rate to its maximum speed. Run a blank with a water sample in both reference and sample beams, and then obtain a spectrum with water in the reference beam and Sample 1 in the sample beam. Repeat using the default scan speed of 240.**

**Analysis II.A (to be handed in)** Use excel to put all these two spectra on the same plot. Can you seen any signs of error from scanning too fast? How would you determine the maximum scan rate for a given sample?

The only other scanning parameter I can see to adjust is the smooth. It should be set to 0, meaning there is no smoothing. The S/N on this machine is so great, I can’t really see a use for smoothing, so let’s not mess with this.

Section II.B Dynamic Range
The next thing I want to look at is the dynamic range. The upper end of the dynamic range is set by the LOL or limit of linearity. The lower end is set by the LOQ limit of quantitation.

The LOQ (page 124 of text) should be 10x the standard deviation of the blank, and, since the specs say the noise level is about .0003A the LOQ should be about .003.
The LOL is the upper limit of linearity. The specifications say that the instrument will work accurately up to an absorbance of 3. So let’s see if it really does.

**Set the machine back to its default scanning speed. Look at your spectrum for Sample 1. What is the largest wavelength where this sample has an absorbance > .01** Set the machine to scan from this wavelength down to 190 nm. Set up the machine to do 4 scans under these conditions. Again do the blank run with water in both the sample and reference cuvettes. Use solutions 1, 2, 3, and 4 respectively for the four scans. Make sure you rinse the cuvette several times with the solution you are going to scan so there is no dilution or contamination of one sample with the next. The relationship between these solutions is as follows: Solution 2 is 10x the concentration of Solution 1; Solution 3 is .1 the concentration of Solution 1; and Solution 4 is .01 the concentration of concentration of Solution 1.

**Analysis II.B (to be handed in)** Since solution 2 is 10x more concentrated than Solution 1, all values recorded for Solution 2 should be exactly 10x larger than those for Solution 1. Probably the easiest way to see this is to load both data sets into excel, then use excel to multiply data set 1 by 10 at all wavelengths. Now plot data set 2 and 10x data set 1 on the same plot. Do the plot coincide? At what wavelength are they roughly equal? At what wavelengths do they widely diverge. Now go back to the plot of data set 2 and place a big red X in the region where the two plots diverge. This tells you that you cannot trust any of the data in the region. Looking at this can you make any generalities about when you can trust this instrument? What is the LOL of this method?

Now lets look in the other direction. Compare data set 1 with data set 3 and data set 3 with data set 4 in a similar manner. Do you trust this instrument down to very low A values? Where would you set the LOQ for this instrument? What is the dynamic range of this instrument?

**Section II.C Noise**

From my point of view this instrument is too good. The S/N is so large that, ordinarily, it is hard to see any noise in any signal you work with. However maybe I can push on the machine a bit to get something for you to look at. Look at the spectrum for Solution 2 and find wavelengths where the A for this solution is 1, 2, and 3. If you think back to your definition of absorbance and transmittance you should realize that with an absorbance of 1, 1/10 of the light is getting through the sample, with an absorbance of 2 the light is down to 1/100, and with an absorbance of 3 the light is 1/1000 of its original value. With less and less light getting through there should be more and more noise. Let’s see if you can observe this

**Set the machine up to do a time dependent measurement at the wavelength that has an absorbance of 1.0. Use the button with the clock on it to set up the instrument for a timed acquisition. Use the Timed tab to set up the wavelength, time and interval of data acquisition.** Let’s set it for a total time of 60 seconds, and to take data with a time interval of 1.0 second. Now go to the instrument tab and set the instrument response to its lowest value. Put water in the reference side and Sample 2 in the sample beam and acquire a set of data. Repeat the experiment with the response of the instrument set to the middle and highest response values.

Now repeat this experiment completely at the wavelengths corresponding to 2.0A and 3.0A
II.C Analysis (to be handed in) What is the S/N for each of the above experiments. What is the effect of the response time on the noise. If we had a signal that actually changed with time, how would the response time affect this signal, and why?

Section III. Instructions on using UVWin lab to run the Lambda 2

Setting up a scan
Click on the menus:
Applications
  Scan
-Or-

Click on the scan icon (looks like a tiny spectrum with 3 peaks)

Parameters that can be changed
  Changing Scan Wavelengths
    Under the Scan tab - Set Start and Stop wavelengths as desired
  Changing data Interval
    Under the Scan tab - Set Scan Interval as desired
  Turning autosave on or off
    Under the Scan tab - Set autosave button on or off
      Autosave automatically saves your data.
  Changing the max and min on the plot
    Under the Scan tab - Set ordinate max and min to desired values
  Changing the display between A and %T
    Under the Instrument tab - Set Ordinate mode to desired value
  Changing Scan Speed
    Under the Instrument tab - Set Scan Speed as desired
      (Default is 240)
      May need to set Data Interval (Under the Scan Tab) to a lower for fastest speed.
  Changing Instrument to do multiple scans with one baseline
    Under the Sample tab - Set Number of Scans as desired
      (Will do a single blank follow by scans)

You cannot change response time in Scan mode. Perhaps the software selects the best time?

When all parameters are set to their desired values hit the green START button
Setting up a timed acquisition

Click on the menus:
Applications
Timed Acquisition

- Or -
Click on the Timed Acquisition icon (has a small clock)

Parameters that can be changed
Setting the Wavelength
Under the Timed tab – Set wavelength at desired value

Setting the total acquisition time
Under the Timed tab – Set total time at desired value

Setting the interval between time points
Under the Timed tab – Set interval at desired value

Turning autosave on or off
Under the Timed tab - Set autosave button on or off
Autosave automatically saves your data.

Changing the max and min on the plot
Under the Timed tab - Set ordinate max and min to desired values

Changing the display between A and %T
Under the Instrument tab - Set Ordinate mode to desired value

Setting the Instrument Response time
Under the Instrument tab – Set Response time to desired value
(Default is 10, but you can go down to .1)

When all parameters are set to their desired values hit the green START button
Retrieving Stored data in an Excel compatible format

Step 1: Loading the data

Click on File
Open

Find the spectra you want (File type *.sp)
-or-
Find the Time Drive data you want (File type *.td)
You can click on one or several files at this point

Click on OK
The computer will load all your files into the display window.

Step 2: Writing the data

Click on File
Save as
The computer will now display the ‘Save as’ window with one of your files loaded and ready to be saved.
To save this file you must first change it to ACSII format
File type = ASCII
To keep from accidentally overwriting the original file with this file, I always change the file name to .ASC at this point.
Now click into the directory you want to store the data in under the directory part of this window.

Finally click OK
The file is saved

At this point, if you opened multiple data files, the ‘Save As’ window will come up with the next file name from the list. Repeat all steps until all the files are stored. Note: Before you can load another set of data using the File-Open command, you must first clear all the spectra off the display window by clicking on ‘View’ - ‘Remove all curves’.

Section IV. The Ocean Optics Photodiode array spectrometer

Section IV.A Instrumental parameters

The Lambda 2 was a scanning type instrument; there was a single detector and the machine had to physically move the monochrometer ‘shine’ different wavelengths of light on this detector. The Ocean Optics spectrometer is a photodiode array machine. It has a single detector that contains about 2000 photodiodes that are arranged to detect light between 200 and 850 nm. While you may set it up the display to examine different regions of the spectrum, you are actually getting a complete spectrum from 200 to 850 every time this computer looks at the instrument, so we can’t play the scanning games you did with the Lambda2.

While we can’t do things like setting the scan rate or the response time, there are three other critical parameters that you need to set, that I never told you about before because I didn’t want you messing with them.

Make sure the sample holder is empty and turn on the lamp (toggle switch on the front of the lamp/sample module). Bring up the OOI software. It takes a minute or two to initialize, but once it is up, you should see a spectrum in red that flickers every fraction of a second as the machine acquires a new set of data. On the user bar across the top of the spectrum you should see a button with a blue S, in the ‘on’ position, showing you that the machine is in scope mode (raw data mode). In this mode you are
not seeing either transmittance or absorbance, but the raw signal hitting the computer. The first three parameters, Integration time, Average, and Boxcar, displayed across the top of the spectrum are the only ones we need to worry about with this machine, the other parameters have to do with fancier instruments.

Integration time. Right now it should be set to ~40 msec. This says that the signal you are observing is the total number of photons each of the photodiodes has hitting it in a 40 msec (.004second) period. Change this parameter to a lower value, and watch the display values go down, because less photons hit the detectors in a shorter period of time. Now set the integration to value higher than 40 msec. What happens at about 60 msec? Do you see the main peak start to flatten as the top hits the 4000 count limit? The way the electronics are designed, the counter or each diode can only count up to a maximum of 4000 counts. If more photons hit the detector it overflows and the data is lost. Thus you should set this instrument up so this value does not overflow. I have set it to 40 to give me a relatively large signal, but not at the absolute maximum because this signal can drift up and down as the lamp intensity changes from day-to-day, or hour-to-hour. You can override my default of 40 and set it to a higher value if you want, but keep it at least one notch away from the maximum so you have a little safety zone.

Average. This corresponds to ensemble averaging. Right now the average should be set to 3. This means that the display you see is actually the average of three different 16 msec accumulations. Set the average to 1 and carefully watch the signal. You should see small changes from one scan to the next as the lamp and the signal drift and change. Longer averages will give you a lower noise levels, and shorter averages will give you a higher noise level. However, since this is a single beam machine, you are going to see a fair amount of noise and drift anyway. I have a feeling that changing this value is not terribly critical so you can change it to whatever you want. Boxcar. In the average we were reducing noise by averaging data at a single wavelength over a number of independent experiments. Boxcar refers to the boxcar averaging we looked at in the last lab, where you average several points from a range of wavelengths together in a single spectrum to remove noise. The reason for this is not obvious at first, but try this. Adjust the boxcar to its lowest value, and observe the spectrum. What do you see? You should begin to see many very sharp spectral lines appearing in the spectrum. These lines represent lines that are emission lines in the excitation lamp. By applying the boxcar average we smooth out this data and get rid of these sharp lines which might cause artifacts in the final spectrum. The value I use is the one, that to my eye, gets rid of the worst of the lines, but doesn’t overly smear data points together. Again, I think you could change this and you probably won’t see a big change in your final spectrum.

Analysis III.A What are the values you are going to use for the above parameters?

Section IV.B Dynamic Range
Now that you have an idea what these parameters mean, lets try to set up an experiment. Again we will use a matched set of cuvettes. Even though this is a less precise instrument, it is probably more important that you have a set of matched cuvettes here than when we were using the double beam instrument. Why? Remember in the lambda 2 you always ran a blank first, where the reference cell and sample cell were compared with each other and the differences electronically removed before you started the actual run. Here we can never do this, you either have one cell in the machine or the other, and you never have a chance to compare them and compensate for their differences.

Get your matched set of cuvettes; fill one with water, and the other with solution1. Also locate the solid black aluminum block that looks like a solid cuvette because you will need that as well.
Put the water blank in the sample holder and modify the parameters we talked about on the previous page to where you want them. Once these are set, put the solid block in the machine and hit the button that looks like a burned out light bulb. Since you have something blocking the light beam, 0% of the light is getting through, and hitting the black lamp tells the computer that the data it is reading now corresponds to 0% transmittance. Now put your blank in the sample container and hit the button with the yellow light bulb. You are now telling the computer that it is recording data for the 100% transmittance levels. Once the machine has the 0 and 100% transmittance levels set, you can hit the A button and the display will shift from raw scope values to actual absorbance values based on the 0 and 100% levels you have stored. (Note: these values can be updated any time by putting the appropriate sample in the beam and hitting the right buttons. I advise you to do this frequently)

So let’s do the same comparison for Dynamic range that we did for the double beam instrument. Obtain spectra for test solutions 1, 2, 3, and 4 using this instrument. For the highest accuracy make sure you reset your 0 and 100% transmittance before each different run. To actually save the absorption spectrum to a disk, hit the following buttons:

File
Save
Processed
Click on the folder ‘spectrum’ and give it a good name
(You can also plug in a USB drive and send it directly to your drive)

If you stored the spectrum on the computer and not on your USB drive, it will be in C:Program Files\Ocean Optics\ OOI\Base32\Spectrum

Analysis IV.B (to be handed in) Repeat analysis II.B using the data obtained with this instrument

Section IV.C Noise

Now let’s test the noise.

Set the zero and 100% transmittance and put in sample2 again. By eye, find three wavelengths where this solution has absorbance values of 1.0, 2.0 and 3.0. With this machine we can actually follow the noise at thee three wavelengths simultaneously. Let’s set up the machine to do this. Find the words ‘Time acquisition’ along the top command line, and click this button

On the drop down menu go to the ‘Configure’-‘Configure Time Acquisition’ button.

Check the box Stream Data to disk, and fill in an appropriate filename
Then check the box ‘Show Values in Status Bar’
All other boxes should be unchecked
Leave the initial delay at 10 msec
Set the frequency to 1 second
Set the Duration to 1 minute
hit OK

Now go to time acquisition and use the ‘Configure Time Channels’ drop down menu.
Under the channel A tab
Check both the Enabled and Plotted boxes
Set the wavelength to the wavelength that has a 1.0 A value
Now under Channel B and C tab do the same thing, but set the wavelength to the 2.0A wavelength for channel B and 3.0 wavelength for channel C (Note: when I did this I didn’t have any Absorbance values at 3.0 that I trusted so I used 2.5 instead of 3)
Finally hit the OK button on the bottom
You are now ready to start the run
Time Acquisition
   Activate Time Acquisition
Time Acquisition
   Start

Along the bottom of the screen you should see information saying that it is acquiring various data points.
At the end of the acquisition time this display will stop. You will find your data in the default directory ‘C:Program Files\Ocean Optics\ OOI\Base32\Time Acquisitions’ if you have not redefined the program to store your data in some other directory.
Note that the computer has played a little fast and loose with your parameters. It did not record 1 data point exactly every second, but it got close.
With this instrument we don’t have a time constant or a response parameter to change to try to lower our noise levels. But if you think back to our set of 3 parameters that we first set when we started using this instrument, the ‘Average’ parameter let us average any number of runs together, and this should work to reduce noise. Try this experiment again with the average set to 1, 5 and 10 and acquire your data

Analysis IV.C (To be handed in) What are the S/N levels at these wavelengths when you use these different averages?

Section IV.D Drift
Since this is a single beam instrument, and is more subject to drift. Lets try one more experiment to see if we can actually see this drift. Set the 0 and 100% and put solution1 back in the sample chamber. Set the time acquisition to take 1 sample every 10 seconds for 10 minutes. Go ahead and also set the average parameter up to about 10 so we have a limited amount of noise. Set the machine up for a single channel, and set the wavelength of this channel to a value that will give an absorbance of about 1.0. Now let the machine acquire this long term data.

Analysis IV.D (To be handed in) First let’s get a feel for the noise. Randomly choose a 1 minute period in the data set and calculate the standard deviation of the signal in that time period. Now let’s look at the drift. To get a look at the drift you first need to get rid of the noise. Go back to your noise lab, and determine the most efficient way to remove noise from this signal. Use this method to remove as much noise as possible from this data. Plot both the original and smoothed data to see if they correspond properly.
What are the highest and lowest values in the smoothed data? This represents the range of your drift. What is this range? Is this range larger or smaller than the standard deviation of the noise? So would you say the drift is significant or not significant?
I would like to measure the drift as $\Delta A/\Delta t$. What was the time between the high and low drift points? Divide the total drift range by the time and you now have a number for drift that is change in absorbance/minute. What is this number?

Analysis V. Summary. Now that you have some numbers, make a comparison of these two instruments based on the various measurements you have made in this lab.
What to hand in: You should answer any questions in bold in the text of the lab. Here is what I will be grading.

Was your write-up or spreadsheet well organized and easy to read?

I.A Plots of A vs λ for 3 cells (quartz, Plastic small and large volume) assuming A not > .1 analysis of what ranges can be used

I.B Plots of A vs λ for 1 quartz cell looking for evidence of dirt

I.C Plots of T vs λ for 2 quartz cells to see if they are matched to with 1% Are they matched?

II.A Plots of sample 1 from 190-1100 nm at different rates can all rates be achieved? Do all track so we can use the max speed?

II.B Dynamic range
   Plot of 1 vs 2 to see LOL comment on what LOL actually is
   Plot of 1 vs 3 to see if can go the other way
   Plot of 3 vs 4 to try to go to even lower
   Some comment on above results, LOL and LOQ

II.C Determination of S/N at absorbance of 1, 2 and 3
   Determination of S/N with different response times
   comments on above results

IV.A Chosen values for Integration time
   Average
   Boxcar

IV.B Dynamic range
   Plot of 1 vs 2 to see LOL comment on what LOL actually is
   Plot of 1 vs 3 to see if can go the other way
   Plot of 3 vs 4 to try to go to even lower
   Some comment on above results, LOQ and LOL

IV.C Determination of S/N at absorbance of 1, 2 and 2.5?
   Determination of S/N with different averages
   comments on above results

IV.D Drift
   What was the ΔA/Δt for your instrument

V. Summary comparison of two instruments
Experimental Lab 4
Fluorescence

Purpose
In this lab you will learn how to use a simple fluorimeter, investigate the fluorescence properties of naphthalene or anthracene, and construct two calibration curves that could be used to quantitate the amount of naphthalene anthracene in an unknown sample.

Solutions to prepare
1. One group will prepare a concentrated naphthalene stock (Solution A), the other group will make an anthracene stock (Solution A'). In both cases the directions are the same: Accurately weigh about .0010 g of the solid (About one crystal) and place it in a 100 ml volumetric flask. Add HPLC grade methanol to fill the flask and shake until the material dissolves. If you are in a hurry you may warm the anthracene solution slightly.

2. Dilutions of Stock
   **Solution B or B'**
   Make a 1:100 dilution of the above stock by pipeting 1 ml of the above solution into a 100ml volumetric flask, and adding HPLC grade methanol to fill the flask to the line.

3. From this stock make 4 additional dilutions:
   **Solution C or C’** - 10 ml of Solution B or B’ in a 25 ml volumetric, fill to line with methanol.
   **Solution D or D’** - 5 ml of Solution B or B’ in a 25 ml volumetric, fill to line with methanol.
   **Solution E or E’** - 1 ml of Solution B or B’ in a 25 ml volumetric, fill to line with methanol.
   **Solution F or F’** - 0.1 ml of solution B or B’ in a 25 ml volumetric, fill to line with methanol.

Procedure

**Part I. UV absorption**
Obtain a UV spectrum of the concentrated stock (Solution A) in the region from 350-200 nm for naphthalene and 400-200 nm for anthracene.

**Analysis:**
Where are the absorbance maxima in this plot? What are the ε values for this material at these maxima? Based on the work you have done previously with the Lambda 2 what do you think is the lowest concentration of this material you would be able to quantitate using UV absorption?

**Part II. Fluorescence spectra**
In theory Fluorescence is more sensitive than absorption, so we should be able to work at much lower concentrations. However with our fairly primitive machine it is much easier to calibrate with a high concentration solution, and then move to lower concentrations for the actual analysis, so that is what we will do here.
Part II.A. Determination of excitation maximum
First, we want to find out what wavelength in your sample elicits the most fluorescent signal, so we will scan all excitation wavelengths to find the wavelength that give the most fluorescence. In this experiment we will use the machine’s excitation monochrometer to scan all excitation wavelengths, and set the analyzer to zero, so it lets all wavelengths of emission light hit the detector.

Part II.A.1 Instrument Setup
Turn on Fluorescence machine
1. Turn Power on - Xenon Power supply
   Wait 30 seconds
2. Press White Lamp State Button
3. Press Power button - lower right Spectrofluorimeter
4. Turn on Computer
5. After Windows comes up, click on the Logger Pro 3.5.0 icon and start this program. On the lower left side of the screen you should see a window reading “Potential”.
6. For this experiment put solution A in position 1 of the carousel, and a methanol blank in position number 2. Then place the carousel in the fluorimeter. Be cautious with the cells, I can see some hairline cracks in them already, and they run about $100 apiece!

Preliminary instrument Settings
Close the sample chamber and use the lever outside the chamber to put sample 1 (Solution A) into reading position

<table>
<thead>
<tr>
<th></th>
<th>Naphthalene</th>
<th>Anthracene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplifier gain selector to:</td>
<td>x1</td>
<td>x1</td>
</tr>
<tr>
<td>Sensitivity to:</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Set exciter λ to</td>
<td>280</td>
<td>350</td>
</tr>
<tr>
<td>Set analyzer λ to</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Look at the Potential window on the computer.
With the shutter up (closed)
Adjust the 0 setting (outer of the right most upper knob on the fluorimeter) so the potential reads as close to 0.0 as possible.
With the shutter down (open)
Adjust the 100% setting (inner of the rightmost knob on the fluorimeter) so the potential reads -.60 V

If you can’t get the setting knob to adjust the voltage to -.60, either increase the sensitivity or decrease the sensitivity and try again. If you change the sensitivity setting, you will have to go back and readjust the 0 setting again with the shutter closed.

Once this is properly adjusted you can move on to the next part of the experiment
Part II.A.2 Acquiring the Excitation Spectrum

A. Set up the computer to acquire data.
   1. Click on the button (Upper right) that has a clock on it.
      A. Set the length to 560 seconds
      B. Set the Sampling rate to 1 second/sample
      C. Click the Done button at the bottom of this window.
   2. On the main display window change the maximum of the graph to 0, and the minimum to -1.0 by clicking on the numbers and changing them.

B. Set up the Instrument
   1. Change excitation λ slightly less than 220
   2. Engage the exciter scan drive (move lever on bottom of fluorimeter to the left)
   3. Turn scan motor on. (Toggle switch on bottom of fluorimeter)
      Watch the excitation λ
      When it lines up on 220 nm, hit start ‘Collect’ on the computer
      At this point the computer acquires 1 data point every second, and the excitation wavelength changes by 1 nm every second so each data point represents a different λ.
      When the excitation λ reaches 650 nm you can stop.
      (You don’t have to go all the way to 650, but go at least 50 nm past the longest wavelength you observed in your UV spectrum)
      1. Stop the computer,
      2. Turn the scan drive off on the fluorimeter (toggle switch)
      3. Disengage the scan drive (move lever to middle position)

Copy and paste your data table into Excel.
(Logger Pro:Edit- select all, Edit - copy, Excel:paste)
You should have 2 columns of numbers, the first is the data point, and the second is the Voltage at that point. Since the first data point was at 220 nm, change this value from 1 to 220. Since each data point represents data at the next larger wavelength (2=221nm, etc), figure out a way to change all the data in column 1 to the actual nm at that point. Also change all the negative voltages into positive values.

If you plot the data at this point you may be mislead because the signal is a composite of both the fluorescence of your material, and any scattering artifacts from the solvent. Thus what we need to do next is to acquire a solvent blank. Minimize this window and return to the Logger Pro interface window.

Use the lever on the sample chamber to swing sample 2 (your solvent blank) into the reading position. Without changing any settings on the machine except the wavelength, now do a scan of this blank just like you did your scan of the sample. In this case, hit the ‘Collect’ button BEFORE you start the wavelength drive, because the computer will pause and ask you what you want to do with the previous set of data.
Now start the wavelength drive, and when it gets to 220 nm hit the ‘Erase and Continue’ button to start acquiring data. When you have finished this scan, cut it from the Logger Pro window and paste it into Excel right next to your previous data. Since you already know the wavelengths for each data point you can actually delete the column that contains the number of the data point. Now use Excel to subtract the blank from the sample (remember to change the sign of the potentials before you do this).
Analysis:
Now plot all three data sets, sample, blank, and (sample-blank). Which peaks on these curves are due to the scattering of the solvent itself, and which are due to the fluorescence of the naphthalene? What is the wavelength of the excitation maximum? Does this correspond to any peak you saw in your absorption spectrum?

At this point it might be good to save this spreadsheet to a disk so you don’t mess it up accidentally.

Now that you have identified the excitation maximum for your material, your next step is to find the emission spectrum of the material when it is excited by light of this wavelength.

**Part II.A.3 Acquiring the Emission Spectrum**

With the excitation wavelength set to the maximum you found in the previous section, acquire the emission spectrum in a manner similar to the previous spectra. This time you will start with the analyzer just below 220 nm, and move the drive lever to the right to make the analyzer wavelength change instead of the excitation wavelength. Again you will need to acquire both sample and solvent spectra and save them to Excel as you go.

Analysis???
What do the sample, blank, and (sample-blank) emission spectra look like? If your run is like mine, you should be scratching your head because there should be a region of about 100 nm where you have a reading of -1.1. In case you haven’t figured it out, the -1.1 reading is the maximum this machine can record, so this means that the fluorescence was so strong that the machine was off scale. You will have to go back and reset the machine to get this region on scale. Before you do this however, you need to figure out where the middle of this off-scale region is so we can try to calibrate the machine at this wavelength to bring it on-scale.

**Part II.A.4 Acquiring the Emission Spectrum - Try 2**

Setting up for the second Emission spectrum
- Rotate the sample back into position.
- Leave your excitation wavelength alone
- Set the analyzer wavelength to the middle of your off-scale region

Look at the Potential window
- With the shutter up (closed)
  - Adjust the 0 setting (outer of the right most upper knob on the fluorimeter) so the computer voltage window reads as close to 0.0 as possible.
- With the shutter down (open)
  - Start changing the sensitivity control to lower and lower numbers, until the voltage reads about -.25 volts.
  - Adjust the 100% setting (inner of the rightmost knob on the fluorimeter) so the computer voltage reads exactly -.25 V.
  - Depending on the position of the inner knob you may have to go up or down one notch on the sensitivity knob to get this exactly right.
  - Double check that you have a good zero with the shutter up (closed) and readjust the outer knob as necessary. If you do change this, then
Now that things are set up a little better, let’s try the emission spectrum again. To save time on this scan, don’t do the entire range from 220-650, instead just scan the region that was off scale before, since you know the maximum must be somewhere in this region. Again scan both the sample and the blank and subtract the two using excel.

Analysis:
Plot the sample, blank and (sample-blank) spectra in this region. Where is your emission maximum? What can you say about the relative wavelengths of the excitation and emission?

**Part III.A Quantitative Analysis - Creation of a first calibration curve**

Leave the excitation wavelength right where it is, but set the analyzer to the wavelength of the emission maximum. The instrument should now be set for the maximum sensitivity. Load the sample carousel with your methanol blank in position 1, Solution B in 2, solution C in 3 and solution D in position 4. Place the carousel in the machine and move solution B (position 2) into the analyzer. Adjust the machine as you did earlier so this more dilute sample will have a reading of -1.00 V when the shutter is open (down) and 0.0 when the shutter is closed (up). Notice the machine has a fairly slow response time, so you should wait several seconds each time you take a reading. Once the machine is properly calibrated and data table is set, take fluorescence readings on the blank and the three samples, letting the computer accumulate the data for about 30 seconds. Cut and paste this data into Excel and calculate the mean and standard deviation of each data set. Make sure you wait 5-10 second between samples so the slow response time doesn’t mess up your readings.

**Analysis:**
Calculate the concentration of material in each of these samples. Plot the fluorescence reading vs concentration. Use your line of best fit spreadsheet from last semester and calculate the line of best fit to this data. If you had a sample with a fluorescence reading of -.5V, what concentration would that correspond to, and what would the uncertainty in the concentration be?
Do you think you could quantitate a sample of this concentration using UV absorption? Why or why not?

**Part III.B Second calibration curve**

Load the sample carousel with your methanol blank in position 1, Solution D in 2, solution E in 3 and solution F in position 4. Place the carousel in the machine and move solution D (position 2) into the analyzer. Adjust the machine as you did earlier so this more dilute sample will have a reading of -1.00 V when the shutter is open (down) and 0 when the shutter is closed (up). Once the machine is properly calibrated take fluorescence readings for 30 seconds on the blank and the three samples and record the mean and standard deviation of these readings.
**Analysis:**

Calculate the concentration of naphthalene in each of these samples. Plot the fluorescence reading vs concentration. Use your line of best fit spreadsheet from last semester and calculate the line of best fit to this data. If you had a sample with a fluorescence reading of -.5V, what concentration would that correspond to, and what would the uncertainty in the concentration be? Do you think you could quantitate a sample of this concentration using UV absorption? Why or why not?

**What to hand in:** You should answer any questions in bold in the text of the lab. Here is what I will be grading.

Was the write-up or spreadsheet well organized and easy to read?

Concentration calculations

I. UV absorption
   - Plot of UV spectrum from 350-200
   - \( \varepsilon \) values for maximima
   - Estimate of lowest concentration detectable in UV

II A. Excitation
   - Plot sample, blank and (sample minus blank), identifying excitation max,
   - Additional identification of peaks due to solvent, and peaks due to naphthalene would be nice.
   - Some kind of comparison with original UV spectrum would be nice.

II B. Emission
   - Plot of sample, blank, and (sample minus blank) for final good run
   - Identification of emission maximum

III Quantitative analysis
   - Calibration curve for solutions B, C, D
     - Concentration of a sample that reads .5 V and uncertainty
       - Could this sample be quantitated with UV?
   - Calibration curve for solutions D, E, F
     - Concentration of a sample that reads .5 V and uncertainty
       - Could this sample be quantitated with UV?
Experimental Lab 5
IR Spectroscopy lab

**Purpose:**
To familiarize the student with several different aspects of IR spectroscopy.

**Part 1 - Instrument familiarization**

- **Step 1** - Turn on IR Machine (look for green LED on the front of the machine. If it isn’t lit, then you need to turn the machine on. The switch is on the back of the machine about 4 inches from the left hand side.
- **Step 2** - Turn on the computer, click on the EZ-Omnic icon to start the program.

**Tutorials**
There are a couple of nice tutorials built into the EZ-Omnic software that runs this machine. One describes the software itself, and the other describes how the FT-IR machine works. To access these tutorials go to either:
- Help
  - Getting started
    - Getting started with Omnic
  - Help
    - Getting started
      - Beginners guide to FTIR

Take a look at these aids, use them if you want, ignore them if you want.

**Actual Lab**

**Sample compartment** - Let’s start by getting a feel for the instrument. Slide the sample compartment cover assembly to the right to completely expose the sample compartment. Normally we won’t use this slide mechanism because you don’t have to open the chamber up so completely unless you are removing the sample holder. You can see there is plenty of room in this sample chamber for different sample holders. We will use some different ones over the course of this lab. For now there should be a simple single upright stand in the middle of the chamber that you will slide different adapters in from the top. Slide cover assembly back in place and open the door on the top of the assembly by pressing down and letting the door pop open. Using this door gives more limited access to just the sample holder, and is how we will normally change samples in this machine.

**Optics** - Lift off the sample preparation tray (rectangular piece about 4x6 inches toward the back of the top of the machine). Once the tray is out of the way you can see a clear piece of plastic covering the optical bench. Locate the moving mirror part of the interferometer. (This is the only moving piece in the entire machine!). The mirror should be shifting back and forth with a steady rhythm. The red light you see bouncing off the mirror is the ruby laser that is used to keep track of the mirror position. Just to the left of the moving mirror you can see a small orange disk. This is actually the end of a tube of glass. The other end of the tube is located on the IR source. This is used to visually tell if the IR source is actually on.
Experiment I - Obtaining the Interferogram

In lecture we have discussed how the true IR signal is an interferogram that records the intensity of IR light hitting the detector as one moving mirror is moved relative to a fixed mirror. This interferogram is then transformed into useful data using a Fourier Transform. Ordinarily the software does this all automatically so you never see the real raw data, but for this experiment we will have the computer display the interferogram so you can see it.

Along the upper set of command tabs. Find the:
Collect
Experiment Setup
Under ‘Final Format’, find and highlight ‘Interferogram’ (its at the top of the list).
Under ‘Background handling’ click on Collect Background after every sample
Then hit the OK button at the bottom of the experiment setup window

The Computer will now display the raw interferogram instead of the Fourier transformed IR data.

You will now run an experiment with nothing in the chamber to see this.
Collect Sample – OK
Hit OK a second time when the Confirmation window pops up.
Hit ‘Yes’ when the next Confirmation window pops up and asks if you want to add to Window 1.

What you see in front of you is the first 3620 points of the 8191 point interferogram. See the white bar near the bottom of this window? Click on this bar with your left mouse button and move it to the back and forth. See what happens in the main display? Wiggle this around until the highest peak in the interferogram is centered in the main display. Notice how the center of the interferogram is off scale? Let's get this on scale by clicking on the ‘View’ then ‘Full Scale’ on the drop down menus. The full scale of the interferogram is now displayed. Hopefully it runs from about –5 volts to +5 volts. If it doesn't, then either the IR source is getting weak, or the interferometer isn’t working right. At this point either print the interferogram or cut and paste it into a word document for your lab report

Now hit the ‘Clear’ button to clear the interferogram off the display. Now we will collect the data, but let the software do the Fourier Transform and look at what this data looks like as a function of frequency.

Reset the IR to record the % transmittance instead of the interferogram.
(Collect – Experiment Setup – Final Format = % Transmittance – OK)
and Collect a sample of the empty chamber again.

When the Confirmation window comes up asking if you want to run a background, click on Cancel, and do the same for the second Confirmation window asking if you want to collect more scans. But click on ‘Yes’ for the Confirmation window asking if you want to display in Window 1.

What you should see now in the main window is the Fourier Transform of the interferogram. It is the IR spectrum of the air inside the sample chamber. Notice that this background it not nice and flat like a background you would get in a UV spectrometer if it was empty. Why isn’t it flat? The big double dip near the center of the spectrum is absorbance of IR by CO₂ in the air. The really fine structure to the right and left are absorptions due to vibrations in water molecules in the air. So your background, even where there is nothing in the sample chamber, is very busy from
absorption of the gases in the air itself!. That is why when you use this machine you always need to run a background of some kind.

At this point either print this background spectrum or cut and paste it into a word document for your lab report. Finally hit the ‘Clear’ button to clear the spectrum off the display.

Experiment II - Resolution
First you need to be sure that the IR is set up in a normal manner. Click on the Collect’ - ‘Experiment’ tabs again, and check that the instrument is set for:
- Number of scans = 8
- Resolution = 4
- Final Format = % Transmittance
- Correction = None

Along the right hand side of the window you can see a set of buttons to collect sample and background spectra in several different ways. When you are doing lots of routine samples you only have to take a background every hour or so. However, with the different kinds of things we will be doing today, the best setting for now is to collect a background, then sample. Click this button now. Later in the lab you may have to change this.

For this experiment we could use any sample, but I wanted to start off with something that should give reproducible results. The sample we will use here is a simple isotactic polymer film containing a self-adhesive substance. In other words, a piece of tape.

Open sample compartment and insert an empty sample holder for a background run.

Hit the clear icon near the top center of the screen to clear out any data left by a previous user.

Hit the ‘Col Smp’ icon to record a spectrum. It will ask you for a name. You can either accept the time and date that is has for a sample name, or replace the time and date with something that has more meaning for you. After you hit OK it will then ask to collect the background. You should be ready to go, so hit OK. It will take a few seconds to set up the machine and the it should begin to collect the data. You will see a bar on the lower left hand side, this keeps track of how far you are on collecting your eight sample runs.

When it finishes the runs it will halt and ask if you are ready to run the sample. Before you do that, let’s look at the display in front of you so you understand what you see.

First note that the machine does not display the interferogram. You will never see the raw interferogram again (That is why I had you look at it earlier). The Fourier transform is so fast, and the interferogram so hard to interpret, that the computer automatically process the data into a spectrum and displays that information for you. This is what is currently on the screen. Since this is a blank run it is equivalent to a single beam run from a double beam instrument. The highest peaks correspond to the most light hitting the detector, and the low regions represent low light. You can see many non-uniformities in the light energy as it passes through the machine and the response is very non-linear. It is low at the ends and high in the middle, showing the IR source had its peak intensity near the middle of the spectrum. Next note the double peak near the center of the display. This is an absorbance of the light beam due to CO₂ in the air. The various fine peaks in the 3500-4000 and 1000-2000 cm⁻¹ regions are due to water in the air. All of these imperfections will also occur in your sample spectrum, but the computer will subtract this background from your sample, so if it all works properly, you will never see these again.
Let’s move on to taking the spectrum. Open the sample compartment and remove the sample holder. Take a piece of tape from the roll and tape it over the opening that the IR light passes through. Now place the sample holder back in the machine and tell the computer to acquire the sample spectrum. When it has finished it will ask if you want to add the spectrum to window 1. Hit OK.

At this point you have several options for keeping this spectrum for your records. If you hit the print button the spectrum will get printed out on the printer next to the NMR machine.

I would also hit the ‘File’ – ‘Save as’ button to save this spectrum so you can come back later if you need to. A third option is to open up a window running Word on the computer, then hit the ‘copy’ icon to copy the spectrum into the copy/paste buffer. Then pop into your word document and paste you spectrum into the document. You can keep this word document open and paste added spectra as you do this experiment, then come back later and add text and turn the document into your lab report.

The right hand end of the spectrum tends to have narrower peaks than the left hand end. This is a common property of the IR spectra for most compounds. The broad bands on the left hand end can usually be associated with various bends and stretches of functional groups in a compound, while the fine structure on the right hand end is due to complex interactions between groups, so they are harder to identify. They are, however, distinctive for each compound, so this right hand region is called the ‘fingerprint’ region because it can be used to uniquely identify a compound.

There are a couple of software tricks that can be used to expand the narrow peaks and contract the wide ones so you can see the differences more clearly. Find the ‘View’ button along the top edge and click on it. Now click on the ‘display setup’ button.

This gives you a window that you can use to manipulate the way the spectrum is displayed. Locate the ‘X-axis format’ option near the bottom left hand side of this window. Use the arrow button to find the different formats available. Normal is where it should be right now, try the ‘split at 2000’ or the ‘split between 220 and 1000’. These alternate displays can be used to blow up the fine structure in this right hand region to help you see more details. Play with any of the other display options you want. Finally record or print the final spectrum you want to keep.

At this point I don’t know what the tape is made of, so I’m not going to do much with analyzing what the peaks in your spectrum actually mean. There is one interesting spectral feature that I do want you to look at. Do you see a sawtooth pattern between 1800 and 2400 cm⁻¹? These ‘interference fringes’ arise from interference of light as it passes through the tape. You can use this pattern to calculate the thickness of the tape, as shown in text on page 458. This can also be used to determine the pathlength of 50 and 100 μm cells sometimes used for UV experiments. **Make sure you save this spectrum because you will be asked to determine the thickness of the tape using these fringes in your lab write-up.**

Now let’s mess with the instrument a bit. Before you go any further, lift off the sample preparation tray again, and look at the moving mirror. Make a rough estimate of how fast the mirror is moving, and how far it is moving. Leave this open for a bit.

Go back to the ‘Collect’ – ‘Experiment setup’, and change the resolution of the machine from 4 cm⁻¹ to 16 cm⁻¹ and collect and new background and sample just as you did before. As you do this **don’t** hit the clear button. That way this low resolution run, and the previous medium resolution run will both be displayed in window 1.

As you are doing this low resolution spectrum also watch the movement of the moving mirror. Is it still running at the same speed as it was before? Is it moving the same distance? Does it take a longer or shorter time to acquire your spectrum? (Note these answers down in your lab notebook, they will be part of the lab write-up) Save these two spectra for analysis later.
You just saw what low resolution does, let’s go the other direction.
Change the resolution to 1 cm\(^{-1}\).
Before you start accumulating this spectrum hit the clear button once. This
should clear off just the most recently acquired low resolution spectrum. This will allow
you to overlay the medium and the high resolution spectra on top of each other for
further analysis. (Save and plot) As before, note what happens to the moving mirror as
you do the high resolution experiment. Is it moving at the same speed? The same
distance? Does the entire spectrum take a longer or shorter time to acquire?

That is it for this experiment. Set the resolution back to the default of 4 cm\(^{-1}\), and
remove and discard the tape.

**Experiment III - IR spectrum of an unknown liquid**

Find the appropriate holder for the 32 mm NaCl window, the liquid unknown, and
two 32mm NaCl discs (located in the desiccator).
Please note that these are, indeed, NaCl windows, and they will dissolve in
water, so avoid contact with water (or the sweat on your fingers!)
Since the machine is still set up to take a background first, place the empty cell
holder in the sample compartment and take a background spectrum. Once this is
complete. Remove the cell holder and prepare the sample. Place two or three drops of
the unknown on one of the NaCl discs. Place the other disc on top and squeeze the
discs together slightly. Place the discs into the holder, place the holder in the machine
and obtain an IR spectrum of the unknown material.

The intensity of this spectrum will depend on the pathlength, and that will depend
on how hard you squeezed the plates together. Try to get a spectrum that has
Transmittance of about 40-60 for the peak near 3000 cm\(^{-1}\) and the peaks in the 1000-
2000 range should be between 1 and 10%, but not bottomed out at 0%. You may have
to try a second time to get the intensity about right.

Once you have a good spectrum, let’s first try to characterize the bands you have
observed. In the old days you would do this by going to a large table of frequencies
(Figure 15-5, pages 412-413 of your text) and trying to associate each peak in your
spectrum with a particular frequency. The EZOmnic program has this table built in so it
is easy to use.
Hit ‘analyze’
IR spectral interpretation’

This brings up a color coded display of the functional groups the computer thinks
you have in your spectrum. Use the + button and the eyeglasses to get more
information about each of the possible groups and frequencies it has found in your
spectrum.
When you are though you can hit the ‘close’ button to return you your original
spectrum. (You may want to print these pages before you close the window)

Now let’s try to actually find your unknown in the computer’s data base

Hit ‘Analyze’
Library setup’

You now have access to several different IR libraries. I think you will find the most
entries in the ‘Nicolet standard’ library, but just to be sure, let’s use all the libraries, so
click on all the entries, and then and hit the ‘add’ button
Then hit OK

Then ‘Analyze’
Search’
You should get a window with several IR spectra displayed. Your spectra is on top, and the possible matches are shown underneath. The names of the matches are in a table on the lower half of the page. Hit the ‘print’ button to record what you got. When I did this, I got 8 matches between 67 and 82%. I didn’t feel that any of these matches really matched the spectrum all that well, and, indeed, the actual compound wasn’t on the list. At this point I don’t know it if the data base simply doesn’t have the unknown, or if it simply didn’t get a good match from my spectrum. It will be interesting to see if any of you do better.

**Experiment IV - Getting IR of solid samples**

There are three different ways to analyze solid samples in the IR. Each has its good points and bad points. Let’s try them all.

For this portion of the lab you have your choice of several different solid materials to analyze: Caffeine, benzoic acid, acetyl salicylic acid (aspirin) or serine (an amino acid). Choose one compound and use it in all your experiments so you can compare your results for the different methods.

A. Mull

In this method you simply suspend the solid crystals in an oil. You can use either crude mineral oil or ‘nujol’ a refined mineral oil. Because you are obtaining your spectrum in the presence of oil, and oil has its own IR absorption, you first need obtain a spectrum of the pure oil itself. Using the same technique you used for your unknown liquid, obtain a spectrum for a thin sample of mineral oil.

Now weigh out 2-5 mg of you chosen solid sample and place this sample in the agate mortar and pestle and grind it into a fine powder. Next add 2-3 drops of oil to the pestle and continue to grind some more. Now obtain the IR spectrum of this oil/solid suspension, just like you did for the pure oil. The difference between the suspension and the pure oil spectrum is the spectrum of your compound. You can either simply look at the peaks in your spectrum that are not obscured by the oil peaks, or you can try to get the computer to subtract one spectrum from the other using the subtract spectra icon.

Once you have a suitable spectra, try to analyze it the way you did your liquid unknown. This may or may not work because the oil peaks in your spectrum may foul-up the computer matching program.

B. KBr pellet

At this point it will be useful to change the setup on the IR machine so it takes the spectrum first and the background second. Do this now.

In this method your sample is dispersed in KBr, and then the KBr is compressed until it flows to make a ‘window’ that is (hopefully) IR transparent. Carefully clean the mortar and pestle to remove all traces of mineral oil. (See Dr. Z for some acetone if you need it.)

Place about 1 mg of your solid sample and 100 mg of KBr and the mortar and grind with the pestle until the two compounds are thoroughly mixed into a fine powder. Screw one of the bolts all the way into the KBr press, and place the hex head of the bolt into the hex holder on the desktop. Then use a small spatula to transfer your KBr-sample mixture into the press on top of the bolt. Screw the other bolt down by hand until you meet resistance. Use the tool to tighten the upper bolt and use moderately strong pressure to tighten the bolts together and hold the pressure for about 5 seconds.

Gently unscrew both bolts and look into the die. If you see a somewhat clear window, you are done, it worked. If the window collapses, or looks totally white and opaque, try again.

Once you have a good window, place the sample in the IR using an appropriate sample holder, and obtain a spectrum. When you have finished and the machine asks for a background, simply take the die out of the machine for a second, use the spatula to poke your window out of the die, and return the die with the window removed to obtain the background spectrum. Because KBr is ionic, it has no absorbance in the
mid-IR region, so your spectrum should be free of interferences. Try to analyze this spectrum like you did for your liquid, both for characteristic functional groups, and to see if there is a match for it in the computer’s data base.

C. ATR

See the discussion of ATR in your book on page 472 and look of the EZOmnic under ‘Help’ ‘Sampling techniques’ ‘ATR sampling techniques’

Have Dr. Z help you remove the standard sample holder and replace it with the ATR accessory.

Grind about .5 g of your solid sample into a fine powder using a normal mortar and pestle.
Set up the machine for 4 cm⁻¹ resolution and at least 8 scans (You may want 16 to improve S/N) and display ‘% reflectance’ for the Y value. Also have to run a reference first, then sample.
Obtain a reference spectrum with the ATR accessory empty.
Place your entire sample on the ATR stage and close the clamp to press your sample against the ATR crystal. Now obtain your sample spectrum.
How does this compare with your other spectra?

Experiment V - IR Spectrum of a Gas

We also have a gas cell if you want to try sampling a gas. However this lab may be long enough as it is, so I won’t require you to run this sample. If you want try it just for fun, see Dr. Z and he can set you up to run either CO₂ or CO. (This will probably will take about ½ to 1 hour more)

What to hand in:

Interferogram
A plot of the interferogram and corresponding spectrum for the air in an empty chamber.

Resolution Experiment
1. Using the data obtained at 4 cm⁻¹ resolution, determine the thickness of the tape.

2. Spectra of the tape at 1, 4, and 16 cm⁻¹ resolution. Preferably 1 and 4 overlaid on top of each other on one sheet and 4 and 16 overlaid on another. A written analysis of the differences you see on these spectra. A summary of what happened to the moving mirror as you changed the resolution and how long it takes to acquire the spectra at different resolutions. In your opinion, is the default resolution of 4 cm⁻¹ a reasonable value?

Liquid unknown
A spectrum of the liquid unknown. An analysis of this spectrum identifying the major group frequencies. In particular, identify the CH stretch frequency. Once this frequency is identified, calculate the force constant (k) of the C-H bond using equation 16-9 from your text. Finally, try to identify this unknown by matching it against other spectra in the various data bases on the IR computer.
Solid sample
Spectra of your solid using the oil mull, the KBr pellet, and the ATR. Analysis of one or all of these spectra for functional groups, and a comparison with the Computer data base to try to find a match.
Written conclusions. Which technique was the easiest? The hardest? What artifacts did you observe? Did they even work at all? If you had a new unknown solid which technique would you try first? Why? Etc.

Here is how I will be grading:

15 points:  Was the write-up or spreadsheet well organized and easy to read?
15 points:  IR spectrum of tape and Fringe analysis to determine thickness of tape
15 points: Spectra at different resolutions and comments on how resolution changes the acquisition speed or mirror movement
15 points:
   IR spectrum of unknown liquid
   Best match found by computer
   Group frequencies by hand or by computer
15 points:  Force Constant of the CH bond
15 points:
   IR spectrum of solid
   By Mull
   By KBr
   By ATR
   Comments on how easy/difficult the different methods were to use
   Comments on which method gave the most reasonable results
10 points:
   Analysis of best solid spectrum
   Best match by computer
   Group frequency analysis
Experimental Lab 6
NMR

Overview

In this lab you will first learn how to obtain your best NMR signal by ‘Shiming’ the magnetic field to make it a uniform as possible. Next, you will do several standard NMR experiments, first on a known, already prepared sample, and then on an unknown that you have to prepare yourself. The first and most basic experiment is to obtain a one-dimensional proton or $^1$H spectrum. Next you do a two-dimensional proton experiment called the COSY that tell you which protons are coupled to each other. You then obtain a one-dimensional $^{13}$C spectrum, and finally you do a two-dimensional HETCOR experiment which correlates the proton in one dimension to the carbon nuclei in the other dimension.

Using the NMR is quite computer intensive. You use one program call WinPNMR to actually run the NMR itself and obtain your raw NMR data, and a second program called NUTS to process an manipulate this data into a useable form. Make sure you pay attention to what tasks you are doing with which program.

Some of the commands for using these programs are, as usual, imbedded in the text of the lab. Many of the more standard procedures, however I have placed together toward the end of the lab so they can be clipped out and used as a general ‘cookbook’ for anyone who wants to use the NMR. Finally there are one or two procedures that I tell you where to find in the actual NMR manual, so you get used to the idea of looking them up by yourself, rather than using a prepared manual.

Section 1: Shimming

Shimming refers to making the magnetic field around the sample as homogenous as possible. The reason we do this is that each proton in the magnet resonates at a certain frequency because it is at a certain magnetic field. If the same proton in a different part of your sample ‘sees’ a different magnetic field, it will resonate at a different frequency, so your peak gets broader when the magnetic field is not uniform across the sample. In the ancient days (before my time) this was done by literally putting metal shims around the poles of the magnet, hence the name shimming.

What is done now is to put coils of wire around the poles of the magnet, and then run DC current through the wires to create small electromagnets. These electromagnets then allow you to adjust the magnetic field electronically instead of mechanically. When I learned NMR you had a box with 6 or 8 dials that you would have to turn to adjust these electromagnets. You would literally watch the signal, and then twist a dial until the signal got to its best value. Once you got one dial to its best value, then you would try the next dial. The problem was, once you changed the second dial, you would have to go to the previous dial because each dial had a feed-back into the other dials. Trying to get a good shim could literally take hours as you tried and adjusted different sets of dials.

On this machine the process has evolved one step further. The computer watches the signal, and the computer adjusts all the shims at once to find the global optimum value. Thus all you have to do is to type ‘shim’ and walk away for a few minutes, the computer does the rest.

Let’s start by making sure the machine is badly shimmed. Have Dr. Z come in and put a set of bad shims onto the machine. Find the factory sealed n-propyl benzoate and put it into the machine. Double check that it is spinning properly. Now obtain a $^1$H spectrum of this material.
Go to Procedure A Obtaining a $^1$H spectrum

Next do a Fourier transform of the NMR data and do the first couple of processing steps.

Go to Procedure B Processing $^1$H spectra - steps 1-4 only

You should now be in the ‘Nuts’ program with an NMR spectrum in from of you. Zoom in on the rightmost peak in your spectrum.

Click on the ‘zo’ icon

Move the mouse to the left hand side of the peak and click the left mouse button and hold it down while you move the mouse to the right hand side of the peak. Now release the mouse button. There should now be a red ‘zone’ around the peak that you want to blow up. Move the mouse to anywhere inside this red zone and click the right mouse button one time. Your red region should now get blown up to fill the screen. Notice as you move the mouse you have a cross and the letters zo at the cursor position. This means you are still in the zoom mode, and the computer is waiting for more commands to zoom in more. If you want to zoom in closer, go ahead, otherwise hit the return button on the keyboard to get out of zoom mode.

How does your peak look at this point? Is it a nice clean single peak? Is it symmetric? Any deviation from a perfectly symmetrical Lorenzian peak says that you have bad shims. Even if you have a perfect peak, it still might be wider than it could be, so let’s actually measure the width of the peak.

Type lf (for line fit)
- The computer will now try to fit a red peak to your actual data.

Type o (for optimize)
- The computer will now try to find the best fit of this peak to your data

Type i (for information)
- The computer will now display the information for this line. Note down the line width (in Hz) and the Fraction Lorenzian in your lab notebook.

Now let’s try to improve things by tuning up the spectrometer. Go back to WinPNMR and type

shim

This starts a routine to try to homogenize the magnetic field around your sample. This may take a few minutes. Note as this routine starts to run you will have to adjust some parameters. Do you see the information box appear in the upper right hand corner? The only thing you should have to worry about is to use <Ctrl+G> to adjust the receiver gain to the proper value. Once this is accomplished use <Ctrl+Q> to start the actual shim routine. As it shims watch the display. What you are looking for is to have the yellow display of the data to expand as far across the screen as it can, and to get the largest area (Green letters in bottom left corner) possible.

Once it has finished shimming, obtain a new spectrum and examine the shape and width of the TMS peak to see if it has improved. Note these new values down in your lab notebook.

If you get your peak nice and sharp, then you may see some small peaks on both sides of your main peak, these are called ‘spinning side-bands’. The shimming procedure to get rid of these is a bit more extensive, so we won’t worry about them today.
Shimming the magnet is something that should be done every time you put a new sample in the machine. (Measuring the line widths before and after is not ordinarily done, I have just added it so you could actually measure what you have accomplished).

Section 2: Manipulating a \(^1\)H spectrum

There are several things that are done routinely with proton spectra that you need to learn how to do. Let’s try them with the spectrum of n-propyl benzoate you have just acquired.

**A. Adjusting the TMS to read 0.00 ppm**

The magnetic field in the NMR slowly drifts up and down, so the position of the internal standard, TMS slowly shifts off from the proper position of 0.00 ppm. Take another look at your spectrum. Is the right most peak exactly at 0.00 ppm, or is it a bit off? There are two ways to fix this. One is to make an adjustment in how the computer actually acquires the spectrum through the WinPNMR. If you take this route you have to adjust the machine and then take an entirely new spectrum. I will show you how to do that later. The other quick fix is to simply adjust the scale displayed using the winNUTS program. Do that now

*Go to Procedure B Processing \(^1\)H NMR data - steps 5 & 6*

Print a copy of this adjusted spectrum for your lab report.

**B. Labeling all peaks in the NMR spectrum**

Often you want to know the exact position of the peaks in the NMR spectrum. The Nuts program has a nice command to automatically label all the peaks in your spectrum above a certain threshold.

*Go to Procedure B Processing \(^1\)H NMR data step 7*

Print a copy of this labeled spectrum for your lab report.

**C. Integrating peaks to count the number of protons**

The intensity of each peak in your proton spectrum is directly proportional to the number of protons at that frequency. For instance a CH peak will have an intensity of 1, a CH\(_2\) an intensity of 2 and a CH\(_3\) an intensity of 3. This intensity is hard to judge, however, because the peaks are frequently split into different kinds of multiplets due to coupling between resonances. The way around this is to integrate the total area within a multiplet peak. This is another series of commands done in the WiNUTS routine.

*Go to Procedure B Processing \(^1\)H NMR data step 8*

Print a copy of this integrated spectrum for your lab report.

Finally as long as you have a good spectrum worked up, why don’t you save this processed spectrum in case you want to call it up later

*File*

*Save as...*

Save this in the same data\chem 434 directory but give it a slightly different name so you can distinguish it from the raw data file.

Now that you have done all of the above for the n-propyl benzoate spectrum lets look at the structure of this molecule and see if the NMR spectrum makes sense.
The structure of this compound is shown at the right. There are 7 aliphatic protons that should appear in the 1 to 5 ppm range; 3 from the terminal CH$_3$, 2 from the middle CH$_2$, and 2 from the CH$_2$ next to the O of the ester linkage. Of these protons the terminal CH$_3$ should have the smallest ppm value because it is the most shielded, and the CH$_2$ next to the ester should have the highest value because it is the least shielded. Also, when integrated, the CH$_3$ should have an intensity that is 3/2 larger than the intensity of the other CH$_2$ peaks. Check this in your spectrum. Set the rightmost peak to have an integral of 3. Do the other aliphatic resonances have integrals of about 2 each?

There are 5 aromatic protons in the 7-9 ppm range. The two protons ortho- to the benzoate group will have one frequency; the 2 meta- protons will have another, and the para- proton a third. It is very likely that the meta- and para- protons will have about the same frequency so you may only see 2 peaks, one with an intensity of 2 for the ortho-protons and one peak with an intensity of 3 for the meta- and para- protons.

Now that you have a feel for the proton spectrum of this compound in one dimension, let’s go to a two dimensional experiment. One of the most useful 2-D Proton experiments is called a COSY. COSY stands for Correlated Spectroscopy. In this experiment you will have the standard 1D spectrum plotted along the diagonal of a square, and any peaks that are off the diagonal represent a coupling interaction. In the Proton spectrum the strongest coupling occurs when 2 protons are adjacent to each other in the structure, so you can use a COSY to identify individual protons by ‘walking’ from one resonance to the next.

Before we do the COSY there is one last detail we need to take care of. Remember how the TMS peak didn’t exactly match the 0.00 ppm on our display scale? Before we simply reset the display scale to match. Before we do anything in two dimensions, it is best to adjust the NMR spectrometer so it is actually running at the right frequency so we don’t have to try to adjust the peak positions in two dimensions.

**Go to Procedure C - Adjusting the Field Offset**

After you have adjusted the field offset, acquire, transform, and store a new proton spectrum of your sample.

**Instructions for the COSY experiment are found in the EFT manual. In the section marked ‘Guides’ on page 11.** Follow the directions as given, with the following notes:

- Just as the instructions say, relaxation delay of 2 works well.
- This sample has a great signal to noise ratio so set number of scans to 1
  (With a poorer sample you can use 4 or some multiple of 4, but the experiment takes longer)

When the experiment starts your signal will be red, indicating the receiver gain is too high. This is because the default receiver gain written into this experiment is 40 and is about 4 times too high for this sample. Hit <Ctrl+G> and adjust the gain until the signal is showing yellow. Once the signal is good, then hit <Ctrl+Q> to actually start the experiment. The window in the upper right hand corner will tell you how long the experiment will take. Sit back and get a coffee or a soda and wait until you get a message that the experiment is finished.
After the experiment has finished enter the NUTS program (Instruction 9) When you hit <Ctrl +F5> NUTS asks you what file you want to process. Once you have found the file, it will then open a window that displays the acquisition information about the experiment, and you can go in and add comments to be recorded with the raw data. If you don’t have any comments you can simply hit OK at the bottom of the window. Once the window clears, the computer does the 2D Fourier transform and plots your data.

You should now have a square in front of you. Black marks on the square indicate where peaks are.

Note Instruction 12, where you add borders. You do not type in the border command, it is one of the buttons on the upper left hand corner of the screen. When it asks to pick the top spectrum, use the processed 1D spectrum that you did in the previous section.

Finish with all of the instructions and print your COSY spectrum. When you have finished plotting, make sure you go to the file button and click on the exit ip command. This takes you out of the 2-D mode. If you don’t do this, the next person who tries to process a 1-D spectrum in Nuts will get all sorts of error messages and things won’t work.

Now what does the COSY mean? Well locate your diagonal that runs from the bottom left to the upper right. This diagonal contains all the peaks that are in your 1D spectrum. Can you see mark on the diagonal that corresponds to each peak on the 1D spectrum?

The off-diagonal peak represents proton peaks that are coupled to each other. You can see that there is one off-diagonal peak connected the resonance at 1 with those at 2. You have already identified the peak at 1 as the terminal CH₃ in the propanol group. This cross peak unambiguously says that the resonance at 2 ppm must be the middle CH₂ in the propyl group because it is the only set of protons that could couple to the end group. You then see another crosspeak between the 2 ppm and the 4 ppm peak that says these protons are coupled to each other and this fits with the 4 ppm being the peak of the CH₂ next to the O. Can you make sense of the crosspeak between the aromatic protons?

At this point you have now assigned every proton in your 1D spectrum to a particular proton in the n-propyl benzoate structure. Now let’s go to the next step, and try to identify all the ¹³C resonances as well.

Section 3: ¹³C spectra

The second most useful nucleus for NMR is the ¹³C nucleus. It is useful because all organic compounds have C in them, and the ppm range for C goes from 0 to about 200, so there is a much larger range of chemical shifts to work with. C is a bit of a pain, however for two reasons; first its magnetogyreric ratio is lower than a proton, so its internal 'magnet' is only about 1/4 the strength of the proton, so its signal is about 1/4 that of a proton. Second, the natural abundance of the ¹³C is less than 1%, so your signal is an additional 100X smaller. The net result is that Carbon NMR is inherently a lot noisier, and you need to have lots more sample to get an experiment to work.
Let’s start with a simple 1D carbon spectrum.

**Instructions for the $^{13}$C experiment are found in the EFT manual. In the section marked ‘Guides’ on page 8.** Follow the instructions as given, with the following notes:

- **Step 3** - Nu c13- changes all the spectrometer electronics so it will look at C instead of protons. You can see that all the parameters on the page change when you execute this command.

- **Optional step after 3** - Shim - Since you need all the signal you can get, the command ‘shim’ shims the magnet one more time.

- **Steps 4, 5 and 6.** The command ‘zgh’ runs a proton spectrum so you can double check the field offset. Follow the written commands and reset the field offset as needed.

- The computer uses this information to calculate the proper field offset for the carbon spectrum as well as the proton spectrum.

- **Optional step after 6** - Eventually you will do a 2D HETCOR experiment. This step allows you to take a good proton spectrum (zgh) process the spectrum (Switch to NUTS and process with the a2 command) and store the processed spectrum (sa command in NUTS) so you can use it later as a border in the HETCOR.

- **Step 7** - verify that the default parameters are OK. Every time I have done this neither the SI nor the RD are set right. To change SI simply type ‘si’ a window will come up, type the correct value in the window. Similarly to reset RD simply type ‘rd’ and put the correct value in the window that appears.

- **Step 8** - Actually acquire the spectrum. As usual the computer will ask for a file name to keep the data in. Notice what the first scan looks like. Lots of noise. Also watch what happens as each additional scan gets added in. Do you see how a pattern that is large on the left and tapers to the right, emerges, as well as some regular modulations within this signal seem to appear out of the noise? This is your actual data.

- **Steps 9 & 10** - Transfer to NUTS and process the data. Because the C has so much noise, an extra process step is added to remove noise using the LB function. The 0.5 value given in the text works well. If you want to find out how this gets rid of noise see Dr. Z. It is a math transform that involves the convolution of your data with an exponential function, and draws on concepts that you had in that first FT computer lab.

- Do the optional step after 10 to pick the peaks, then plot the spectrum (Step 11)

- Do the step 12 to save this transformed data as well.

Great, you have a C-13 spectrum, what does it mean. Well, like the proton spectrum it is time to try to assign every peak you observed to a C atom in the molecule. You can use Figure 19-32 from your text to take a stab at making your assignments. Pretty tough, isn’t it. Let’s try a short-cut.

For the protons the shortcut was to do a 2D COSY experiment that would tell you when the protons were next to each other. That same trick doesn’t work for Carbon. Can you guess why? Remember that the natural abundance of $^{13}$C is less than 1%. Now calculate the chance of finding two $^{13}$C atoms next to each other. Pretty small, right? That is why the equivalent of a COSY doesn’t work at natural abundance for C. Let’s try something else. Most of the Carbons have a H attached to them, and you are probably pretty confident about you H assignments, so why don’t we try to couple our H’s and our C’s together. The experiment that does this is called the HETCOR for Heteronuclear Correlation.
Instructions for the HETCOR experiment are found in the EFT manual. In the section marked ‘Guides’ on page 11. Follow the instructions as given, with the following notes:

These instructions are the same ones that you used for the COSY, with a few changes given in the manual.

Step 3 - You should still be in C13 mode, so the prompt in the WinPNMR should be C13>. If this is what you have type het to do the hetcor experiment. As usual, the first step in the process is to give the computer a file name to save the data under. Again the 2 second relaxation delay works pretty well. 4 scans per experiment should also work for this sample. Once the experiment starts it will tell you how long you have to wait. Go ahead and get another soda while you wait, but don’t bring it in the lab.

Step 10 - Go to NUTS and run the command <Ctrl+F6> for the hetcor.

Step 12 - Remember that you click the border command from the buttons on the upper right of the screen. For the top border use the PROCESSED C-13 spectrum, for the right border use the PROCESSED H-1 spectrum.

In this 2D experiment the horizontal dimension is C while the vertical is Proton. Since these are two different sets of frequencies, there is no diagonal like there was in the COSY. Each peak in this spectrum represents a C and its attached proton. Thus you should be able to pick out the C and assign all of the C that were attached to the hydrogens. Go back to your structure. Are there any carbons that didn’t have attached protons? Find these missing carbons in your 1D spectrum and take a guess at which is which.

Make a table that gives the chemical shifts of all C and H atoms in this compound and identify where each of these atoms is in the molecules structure.

Section 4: Doing it for yourself

I have walked you through the process of making assignments for a known compound. Now it is time for you to try it on your own. At the present time there are 5 possible unknowns for this lab. 3-bromopropene, 2 chlorobutane, ethylchloroacetate, ethyl malonate and ethyl phthalate. Before you come to lab look up the structure of each of these compounds and predict what you think their ¹H and ¹³C spectra should look like.

Sample preparation
Choose an unknown. Place about 1 ml of you unknown in an Eppendorf tube, add about 0.1ml of TMS to be your internal standard. Mix well and place this sample in an NMR tube. You are now ready to go. Do all of the above experiments (1D proton, COSY, 1D carbon, hetcor) use these experiments to identify your compound and to assign all the resonances to each atom in the compounds structure.
Procedure A Obtaining a $^1$H spectrum

1. Make sure the computer is set on the WinPNMR program. It should have a screen with a blue background and white grid lines. If the blue background is not showing use the mouse to click on the WinPNMR button on the task bar at the bottom of the screen.

2. Type **ns 1** followed by an **enter**.
   - Ns stands for number of scans, thus you have told the computer to do 1 scan.

3. Type **rg 1** followed by an **enter**.
   - Rg stands for receiver gain, this command tells the computer how much amplification to put on the signal. Ns=1 means no amplification, ns=100 says amplify the signal 100 times. With this command you are trying to blow up the signal as much as possible, without making the signal so large that the computer can’t follow it. By setting ns=1 at this point you are doing no additional amplification, and then you will look at this signal and judge whether or not to change the ns in a later experiment.

4. Type **zg** followed by an **enter**.
   - Zg stands for zero go. In other words, zero out the computers memory and go ahead with the experiment.

5. A window will appear in the middle of the screen asking for where you want to store your data. If you don’t want to save this data simply **hit OK** to accept the default. If you do want to save the data, give it a file name you can remember. Start the name with the path ‘data\chem 434\yourname’ so all of the data files obtained in this class go to the same place.

6. It will take the computer about 10 seconds to set up the instrument and start the experiment. As the experiment runs you should see a yellow noisy trace in the middle of the screen that is your actual data. A window with instructions will also appear in the upper right hand corner of the screen.
   - If you get **RED** noise in the middle of the screen your receiver gain was too high go back to step 5 and make rg about half of what it was.
   - If you get **YELLOW** noise look at the start of the yellow pattern (the left hand side) does it go from about $\frac{1}{2}$ way down to about $\frac{1}{2}$ way up on the screen?
     - Yes - great- its all working, so go **back to step 4, set ns = 4 and run a set of 4 scans**.
     - No - you need to set your gain a bit higher. **Go back to step 5 and set rg to be about double what it was.**

   When the window at the upper right disappears your data is complete and ready for processing.
Procedure B Processing ¹H NMR data

1. It is now time to use the NUTS program to process this data into a form that you can understand. **Use the mouse to click on the WinNuts button** on the taskbar at the bottom of the screen.

2. You should now have a mostly WHITE screen in front of you. If you don’t, try the WinNuts button again or call for help.

3. Type **a2 (no return)**
   - The a2 command is a shortcut command that does the following things.
     - **zz** - import the data from the WinNuts program
     - **bc** - baseline correct the spectrum (Shift it up or down if it isn’t on the zero)
     - **ft** - Fourier Transform the data
     - **pc** - Phase correct the spectrum with recorded parameters
   
   You can either use the a2 command, or type in each of these commands just to watch what happens.

4. Type **qp (no return)**
   - qp stands for quick phase. The phase parameters recorded in the computer are probably a bit off, so you need try again to try to get the spectrum to look right.

5. You now have a nice looking spectrum, but your TMS internal standard probably isn’t at 0.0 ppm where it should be, let’s fix that.

6. **Hold down the LEFT mouse button, and move the crosshairs** you get so it is aligned right on top of the right most peak in your spectrum. This is a peak corresponding to a standard called TMS that is supposed to be located at 0.0 ppm on the X axis.

   **While still holding the left mouse button down type o** with your other hand
   - This brings up the OFFSET INFORMATION window that lets you set the offset.
   - Locate the bottom box on the left side of this window
   - This is the Horizontal dimension in PPM. **Set this to 0.0** and hit **OK**.

   You should now have your spectrum back, and the right most peak should be located right above 0.0. If not, try again.

7. Type **pp** (pick peaks)
   - This command will label all peaks in your spectrum over a certain threshold.
   - Print this spectrum with the peaks if you want. On the other hand, if this looks like too much clutter, you can clear the picked peaks from the top of the spectrum with the following steps. Click on ‘view’ then click on ‘show peak labels’

8. **Integration**
   - **type id** (For integral display)
   - Starting from left to right
   - for each multiplet peak
     - **left click** on left hand side of the peak
     - now **click and hold the left** mouse button down as you **drag the mouse across** the multiplet. Then **release the left mouse button on the right hand** side of the multiplet.
Finally do a **left click on the right hand border** of the region. A black integral line should appear on the spectrum over the region you chose.

- Now go to other multiplets and repeat until all have integrals for everybody (except the TMS peak)
- Now choose a peak that you think should have either 1, 2 or 3 protons in it.
- **Put the cursor on the integral for your chosen peak and hold the left mouse button down**
- Type `v` on the keyboard.
  A window should pop up so you can tell the computer how many protons this multiplet should contain. Once you fill this value, the window will disappear, and the computer will calculate how many protons are in all the other peaks based on this guess. Do the integrals look reasonable? Don’t be surprised if they are off by a couple of tenths, like 1.9 instead of 2 or 3.2 instead of 3. However if you have some values like 1.5 or 1.3, then maybe your standard wasn’t a good choice, and you need to try something else. Simply click on your next choice and type `v` again to try another time.
- Hit the print button to record your integral if you like it.
- Then hit the return button to get out of the integration routine (and remove the integrals from the spectrum.)

**Procedure C - Adjusting the Field Offset**

- Enter the WinPNMR program
- Acquire a proton spectrum of your sample (zg command)
- Enter the NUTS program
  - process the data with the `a2` command, phase if necessary with `qp` command
- Hold the left mouse button down and move the crosshairs on top of the TMS peak. Mark down the exact position of this peak (to three decimal places) including the sign.
- Go back to the WinPNMR program
- Type the command `fo` (for frequency offset)
  - The computer will ask where the current position of the reference is, give it the position you just found in NUTS
  - It will then ask for where the peak is supposed to be. Enter 0.00
  - The computer will then reset itself.
  - Finally just to check that everything worked, acquire a new proton spectrum, process it in Nuts, and double check that the TMS is now at zero.

Note that the magnet will slowly drift, so you may have to do this procedure every hour or so if you are doing very critical work.

**What I will be grading:**

Was the write-up or spreadsheet well organized and easy to read?

1. (20 points) Analysis of shape and linewidth of TMS peak in n-propylbenzoate before and after shimming.
   - Some comment on why you need to shim a sample.

2. (30 points) $^1$H, $^{13}$C, COSY and HETCOR experiments for n-propyl benzoate

3. (50 points) $^1$H, $^{13}$C, COSY and HETCOR experiments for unknown
   - Identification of unknown
   - Assignment of all $^1$H and $^{13}$C resonances for unknown
As you have seen in class, an EI type mass spectrometer needs to have samples that are easy to put into the vapor phase, and thus usually have a mass of <1,000 MW. Our mass spectrometer is part of a GC/MS system, so to get a sample onto the mass spec, it also needs to be relatively nonpolar, so it can be separated by the Gas Chromatograph before it is placed into the mass spec.

Many of the compounds that we use to spice foods fit into this category. Actually this makes sense, since you know most of our sensation of taste comes from compounds interacting with our olfactory nerves (nose) and if you are going to be able to smell something, it also needs to be able to go into the gas phase.

Thus the main point of this lab is to try to identify flavor compounds from several natural spices.

Sample preparation - Dried spices

To be able to inject a sample onto the Gas Chromatograph, it is best to have the sample dissolved in a low molecular weight solvent with a very low boiling point. Thus nonpolar solvents are best. This is actually a good match with our flavor compounds, since the flavor molecules tend to be nonpolar as well. (Again this makes sense, since polar compounds have higher boiling points, and are harder to get into the gas phase)

The solvent you will use today in all experiments is ethyl acetate.

I have prepared three different samples for you to try: Cinnamon, Black Pepper, and Red Pepper. I have kept preparation to a minimum here, all I did was to place ~0.1 g of the dried spice in an Eppendorf tube, added 1ml of ethyl acetate containing an internal standard, and let the solvent extract any nonpolar compounds from the spice for at least 24 hours.

I will also let you prepare a sample of your own. For this I have brought some dried peppers from my garden. The hot part of the pepper is the white core that holds the seeds. Find this part on one or two dried peppers and remove this core from the rest of the pepper. Accurately weigh about .02-.03 g of this material and place in a small glass homogenizing tube. Add 2 mls of the ethyl acetate/internal standard solution, then carefully insert the plunger and homogenize the solution.

Sample Injection on the GC/MS

Follow the directions. Nothing solid should be applied to the GC/MS. Make sure you centrifuge all Eppendorf tubes before you remove a sample, and only take your sample from the clear solvent layer at the top of the tube, not from the precipitate layer at the bottom of the tube.

Sample Analysis

Follow the directions. This is an entirely new lab, so I have no idea what you are going to find. In each chromatogram try to identify as many compounds as possible, and try to make a rough estimate of the relative amount of each compound in the extract. To do this, print out a table of the integrated areas of all peaks. Identify your cholesterol internal standard peak. You know its concentration. Now use the ratio of the integral of any unknown peak to the integral of the cholesterol peak, so get a rough estimate of the amount of material in the unknown peak.
There is a fair amount of information available for some of the compound you should expect to see. In fact the web literature on hot peppers is quite extensive. Here is some of the information I have pulled from the web and from the Merck Index. See if you have any of these compounds in your samples. Also note that natural products are very ‘messy’. Thus, don’t be surprised to see several compounds that may not be an exact match, but are somewhat related to the ones outlined here.

**Cinnamon.** - I could find no listing of the specific compounds found in cinnamon, but just from their names I am guessing that you should see Cinnamaldehyde (3-phenyl-2-propenal) and Cinnamic acid (or 3-Phenyl-2-propenoic acid)

![Cinnamaldehyde and Cinnamic Acid](image)

**Black Pepper** it comes from the dried ripe fruit from *Piper nigrum*. White pepper come from the same thing, but it is take from the ‘decoricated’ fruit

The principle hot component is called piperine.

![Piperine](image)

**Red Pepper (Cayenne Pepper)** - Capsicum name used for cayenne pepper extract used to make tabasco sauce. Capsicum contains various oils plus capsaicin and capsanthin

Capsanthin is a red pigment that may be also isolated from paprika. It is not clear to me if it has a hot flavor, or if it just adds color

![Capsanthin](image)
Hot Peppers
The principle ‘hot’ compound is Capsaicin

Capsaicin

\[
\text{CH}_3\text{O} - \text{C}_9\text{H}_9\text{N} - \text{CO}_2\text{H}
\]

This compound creates a potent burning taste, and can be detected by taste in concentrations as low as 1 part in 100,000. The hotness of a pepper can be evaluated empirically using the Scoville unit. (See attached Web page). Note that the Habanero pepper (the globular orange one) has about the highest natural concentration of capsaicin, with a score of about ~350,000 Scoville units. The Jalapeno (the longer green pepper) is somewhat lower, at only ~8,000 units. Don’t be mislead into thinking the Jalapeno is mild. I think you can get a reasonable ‘hot’ taste from a fruit with only 1000 Scoville unit score.

It is interesting to note that Capsaicin is used in salves used to ease pain. I believe that these salves essentially fire all the pain receptors, so there is nothing left to feel pain with.

Notes on running the GC/MS

When you get on the GC/MS machine locate the following four icons near the middle of the screen:
- Run_GCMS
- Class Logbook
- MS5973
- MS5973 Data Analysis

The Run_GCMS is a complete list of instructions for running the GC/MS machine. The instructions that I have included in this handout are a copy of this text with some relevant changes. Simply bring this up in a window and follow the directions.

Class Logbook is a Word File that I ask you to record the time you are on the machine, the names of any files you create, and any remarks about how the machine or the sample worked.

MS58973 is the program that actually runs the GC/MS.

MS5973 Data Analysis is the program that you use to analyze your data after you have acquired it.
Added notes:

- When you first fire up the MS5973 program make sure the banner on the top of the window reads: ‘MS5973-Spice1.M-EI’ this makes sure it has loaded the proper method for your run.

- Maximize this window so you can see everything.

- The machine has several interlocks to make sure the run doesn’t start until everything is ready. Find the oven button. There is a small box in the upper corner of this button. If it is green you are ready to go. If it is red, then something isn’t right. If you have fired up the MS5973 program for more than 10 minutes, and the box hasn’t turned green, come find me.

- When you start the first run by clicking on the big green arrow, you will get a window for recording your data. For this lab I want the data stored in a separate file, so it will be easier to access later.
  The ‘Data File Path’ should read: C:\MSDCHEM\2\DATA
  The ‘Data File Name’ should read Siemens2.D

Change the name of the ‘Data File Name’ to
Chem434\yourfilename.D

This will tuck your file into the directory C:\MSDCHEM\2\DATA\Chem434
This change of directory will automatically be made on your next run, so on your second run the ‘Data File Path’ should read: C:\MSDCHEM\2\DATA\CHEM434, so when you give it the data file name, don’t give it a path just give it ‘yourfilename2.D

What to turn in.

Each group must do at least three different samples. If you want to do more for curiosity that would be fine. (You don’t need to do a detailed analysis on the extra samples, but it might be nice just to identify the 2 or 3 major components)

For each required sample, print a copy of the TIC (Total Ion Current) chromatogram.

Pick out 3-5 peaks on each chromatogram for further analysis, and indicate these peaks on the TIC (you may have to have plots of the TIC at a couple of different scales so I can see all the peaks you are analyzing)

If you need it, I have data from 2007, that indicates where certain peaks should be, but I want to see if you can find them for yourself before I give the answer away.

If you find cholesterol or a similar compound that is good. Plants do not make cholesterol, and should not contain this material. However it is the internal standard that I added to the ethyl acetate. I should have noted in the pre-lab material what the concentration of the cholesterol is. By comparing the size of your unknown peaks with the cholesterol peak you should be able to roughly quantify the amount of each peak in your TIC.

For each peak you analyze on the TIC, find one or more good matches for the compound. What is the name of the compound? What is the structure of the compound? How good is the match? Does it physically resemble any of the target compounds that were mentioned in the write-up? If you don’t get a match for any of the target compounds, you may have to search several peaks to see if you can find a target. Based on the intensity of the peak on the TIC, can you estimate how much of the compound is in the sample? I will leave a Merck Index in the Mass Spec room so you can look up extra information on the matched compounds
Finally for one compound in each sample, print a copy of the mass spectrum, and try to identify the five largest fragments in the mass spectrum. (I would go for a low molecular weight compound, it might be simpler. As an added aid, look through the software for help; I know in the NIST window there are some icons for fragment analysis, but I haven’t figured out how to use them yet)

This is what I will be grading:
Write-up or spreadsheet was well organized and easy to read
(10 points) TIC vs time plot for 3 different spice samples

For 3-5 peaks on each chromatogram an attempt to identify the peak using the mass spec
(Total of at least nine - 10 points apiece)
Include
Name
Structure
‘Goodness’ of match
Is it a target compound?
Rough estimate of % of total

For 1 compound each chromatogram (total of three -10 points apiece) An attempt to identify 5 fragments

Added information taken from: ‘What is a Scoville Heat unit?’ article at www.widegeek.com 4/9/07

What is a Scoville Heat Unit?

Scoville Heat Units are used to specify of the hotness of food, specifically chili peppers. In 1912 Wilbur Scoville, devised a system to determine how hot foods are. He used a panel of tasters to provide heat scores for different peppers. Although we still use Wilbur’s name for the Scoville Heat Units, the current method is much more scientific.

The sensation of heat that we experience from eating certain peppers is attributable to a chemical called capsaicin. The more capsaicin present in a pepper, the hotter it will seem. Although the Scoville scale spans from 0 to 16 million, the American Spice Traders Association (ATSA) set the standard for conversion from ppm (parts per million) of capsaicin to Scoville Heat Units as 1:15. This means that a sweet bell pepper has 0 Scoville units because there is no capsaicin present, and pure capsaicin crystals have 15,000,000 Scoville Heat Units (or 16 million, depending on who you ask.

As is evident from the table below, Scoville Heat Units vary widely from one species to the next. The hottest pepper ever grown is the Naga Jolokia from Assam, India which has a Scoville score of 855,000! There are also variations of heat from one pepper to the next within the same species; growing conditions, soil and other factors have an affect on the amount of capsaicin within a given pepper. The Scoville Heat Units listed below represent the average minimum amount detectable within the item in question, but keep in mind that the amount of capsaisin in any single type of pepper can vary greatly:
<table>
<thead>
<tr>
<th>Scoville Units</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>15,000,000</td>
<td>Pure Capsaicin</td>
</tr>
<tr>
<td>5,300,000</td>
<td>Police-Grade Pepper Spray</td>
</tr>
<tr>
<td>2,000,000</td>
<td>Common Pepper Spray</td>
</tr>
<tr>
<td>855,000</td>
<td>Naga Jolokia</td>
</tr>
<tr>
<td>580,000</td>
<td>Red Savina™ Habanero</td>
</tr>
<tr>
<td>350,000</td>
<td>Habanero Pepper</td>
</tr>
<tr>
<td>325,000</td>
<td>Scotch Bonnet Pepper</td>
</tr>
<tr>
<td>200,000</td>
<td>Jamaican Hot Pepper</td>
</tr>
<tr>
<td>100,000</td>
<td>Thai Pepper</td>
</tr>
<tr>
<td>50,000</td>
<td>Cayenne Pepper</td>
</tr>
<tr>
<td>30,000</td>
<td>Manzano Pepper</td>
</tr>
<tr>
<td>23,000</td>
<td>Serrano Pepper</td>
</tr>
<tr>
<td>10,000</td>
<td>Chipotle Pepper</td>
</tr>
<tr>
<td>8,000</td>
<td>Jalapeno Pepper</td>
</tr>
<tr>
<td>5,000</td>
<td>Tabasco™ Sauce</td>
</tr>
<tr>
<td>2,500</td>
<td>Rocotilla Pepper</td>
</tr>
<tr>
<td>2,000</td>
<td>Ancho Pepper</td>
</tr>
<tr>
<td>2,000</td>
<td>Poblano Pepper</td>
</tr>
<tr>
<td>1,000</td>
<td>Coronado Pepper</td>
</tr>
<tr>
<td>500</td>
<td>Pepperoncini Pepper</td>
</tr>
<tr>
<td>500</td>
<td>Pimento</td>
</tr>
<tr>
<td>0</td>
<td>Sweet Bell Pepper</td>
</tr>
</tbody>
</table>
How does changing the GC conditions change your chromatogram

Introduction:
Last week you used the GC/MS to separate and identify various components from several different spices. In that lab you focused on using the Mass Spec part of the GC/MS to identify compounds. This week you will focus on changing the GC conditions to see how they change the chromatographic behavior of the compounds.

Procedure:

Part 1. Column flow
Pick one sample that you ran last week. Run it again using the method called Spice2. (To run this method, simply start off like you did last week, but when the chromatographic window comes up, go to File...Load Method... and find and load Spice 2.) Method Spice2 is exactly like method Spice1 that you ran last week, except for one change. I have increased the flow rate from 2 ml/minute to 3 ml/minute of gas through the column.

Part 2. Oven Temperature
Using the same sample, now set up and run method Spice3. In this method I have returned the flow rate to the original value, but I have now changed the rate at which you heat the oven. In Spice1 the oven program was as follows:
- Hold the oven at 60°C for one minute
- Heat the oven at 7°C / minute until it reaches 150°C then
- Heat the oven at 10°C/min until it reaches 300°C.

In Spice3 I have increased the warming temperature to try to speed up the run. The Spic3 program looks like this:
- Hold the oven at 60°C for one minute
- Heat the oven at 10°C / minute until it reaches 150°C then
- Heat the oven at 13°C/min until it reaches 300°C.
Analysis (to be handed in):

Compare the chromatograms you got for Spice1 and Spice2. Try to plot both side-by-side and pick out several identical peaks in both chromatograms. Do the peaks come out in the same place? Do the peaks have the same resolution or band width? Are any peaks poorly resolved in one method, but better resolved in the other?

Pick one easily identified peak and calculate ‘Number of plates (N)’ and Plate height (H) for that peak under the two different chromatographic conditions. The length of the column is 30 meters.

Pick out two peaks that are close together in one of the chromatograms. Calculate the Resolution, R between these two peaks. What is the R between these peaks when you change the flow rate? Compare these values.

Summarize your results for increasing the flow rate of gas through the column.

Compare the chromatograms you got for Spice1 and Spice3. Try to plot both side-by-side and pick out several identical peaks in both chromatograms. Do the peaks come out in the same place? Do the peaks have the same resolution or band width? Are any peaks poorly resolved in one method, but better resolved in the other?

Pick one easily identified peak and calculate ‘Number of plates (N)’ and Plate height (H) for that peak under the two different chromatographic conditions. The length of the column is 30 meters.

Pick out two peaks that are close together in one of the chromatograms. Calculate the Resolution, R between these two peaks. What is the R between these peaks when you change the flow rate? Compare these values.

Summarize your results for increasing the temperature of the column.
Introduction:
Last semester we used a two-component analysis to simultaneously determine the concentration of benzoic acid and caffeine in different drinks. If you think back, the UV experiment was simple enough (getting absorbance readings at two different wavelengths) but the analysis was a little more difficult because you had to do some fancy algebra to get the answer. The algebra problems were compounded by some sampling problems. Some of the drinks were slightly cloudy, so there was a scattering artifact that made the absorbances higher than they should be, and some of the drinks contained other colored components that skewed the absorbance data as well.

In this experiment we return to the same samples and try to do the same analysis, but this time we will use HPLC to separate out the different components of the drink before attempt to quantify them. Further, we will use both UV absorbance and fluorescence to give us two different measures of caffeine levels.

Procedure:
Note - Since the HPLC has an autoinjector, once you load up the sample try and start it up, the HPLC computer system will take over. You don’t have to do anything but stop by occasionally and see how it is going. Thus each group can simultaneously run several samples in the HPLC and finish up the second half of the GC/MS lab at the same time.

Sample Preparation - Pipet 10.00 ml of each drink sample into a 100 ml volumetric flask. Fill the flask with water and mix.

Standard Preparation - I have a 100 mg/L stock solution of caffeine already made up. Put 25 mls of this into one 100 ml volumetric and 50 ml into another volumetric. Fill both volumetrics to the mark with water and mix.

Preparing sample for the autoinjector - All of the plumbing in the HPLC system consists of very narrow bore tubing. As a result, ANY particulate matter in the sample could potentially clog the system and make it inoperable. To prevent this ALL SAMPLES MUST BE FILTERED!!

Find a 1ml tuberculin syringe and fill it with your first sample. Attach a syringe filter to the syringe and filter the contents of the syringe into a sample vial for the autoinjector. Place a cap on the vial, then tap it a few times to make sure that no air bubbles are caught in the narrow end of the vial. Do this for all your samples as well as the two standards. Also make a blank that contains pure water. Finally place all your vials in the autoinjector tray, noting down which sample is in which position of the tray.

Place the tray in the autoinjector and start up the Millennium HPLC system. Use the method ‘Caffeine2 2007” to run these samples. Each run will take about 20 minutes, so you can calculate how long you have before you have to come back and check the system. Also note that the overall flow rate is set at ~0.6 ml/minute, and you use about .3 ml/minute for each solvent. Double check that you have enough solvent in both reservoirs for your entire experiment. If it even looks close, have Dr. Z. refill the solvent bottles so you don’t run out.
Detailed directions for running the HPLC using the Millennium\textsuperscript{32} system

First, turn on all the hardware. The switches are on the lower right-hand side. You should turn on five different instruments: The 474 Fluorescence detector, two 501 pumps, the 486 Absorbance detector and the 717 Autosampler. The 486 takes the most time. You will hear it grinding and clicking and ticking. Note that as it starts up it says ‘CAL’ in the display window to tell you it is calibrating itself. You will have to wait until the message disappears, and it starts displaying #’s for wavelength and absorbance values before you can start up the computer. While you are waiting you can open up the lower door on the autosampler and insert the carousel with your samples. (Did you already write down which sample was in which slot of the carousel?)

To start the program go to Start

\begin{verbatim}
Programs
\textbf{Millennium}\textsuperscript{32}
\textbf{Millennium}\textsuperscript{32} Login
\end{verbatim}

This should bring up the Millennium\textsuperscript{32} login window. Locate the box in the upper right hand corner that says ‘Run Samples’. Do a double click on this window. This should bring up a ‘Run Samples’ window - Simply hit OK on this widow to continue.

The complete Run Samples Window now comes up. Go ahead and maximize this window so you can see everything.

Find a box in the lower left hand of this window called ‘Instrument Method’. Click the arrow on the right side of this box to get a drop down menu of possible methods. Find the method ‘Caffeine2 2007’ and click on it. This is the instrument method you will be using. An instrument method is the controller for how all the individual instruments will be working during the run. While I don’t want to edit this method, go ahead and click on the edit button so you can look at how this method is set up.

Clicking on the Edit button takes you to the Instrument Method Editor. On the left side of this window is a list of all the instruments that are being used. As you click on each instrument, the right hand side of the window tells you what the instrument is doing.

For instance, The W747 is the fluorescence detector. You can see that it is set for an exitation wavelength of 295nm and an emission wavelength of 330 nm.

 PCM stands for Pump Control Module. The table here says that we are pumping using an isocratic procedure (the solvent composition does not change). In this procedure we will pump solvent A (Methanol) at the rate of .25 ml per minute and solvent B (water) as a rate of .37 ml per minute.

486 is the UV detector, and you can see it is set for 330 nm, a wavelength that should pick up both caffeine and benzoic acid

Now that you have seen how everything is set, hit the file-Exit or simply kill this window.

Now that you have seen how things are set, let’s start the run using these settings. Hit the ‘Setup’ button in the lower right hand portion of the window. Look for the message ‘Setting up’ at the middle bottom of the window. When this message changes to ‘Idle’ you are ready for the next step.
The pumps should now be going. The next step is to monitor both the Fluorescence and UV channels to see if the baseline is stable. Find and click on the 'Monitor' button. This will connect the output of these channels into your window so you can see what is happening. Note you can use the mouse to resize these windows if you need to make one smaller or the other larger. You are now going to wait until these baselines are relatively flat. Notice I said flat, not a reading of 0.00. While you are waiting you can start to fill in the data table with all the samples you are going to be running. So that is what we will work on next.

Use the mouse to click on the first cell in the data table. The Vial # is the position of the vial in the carousel. If you put your vials in, starting at #1 you don’t have to do anything with this value. If your vials started at some other position, set the Vial # to the position of your first vial.

- Next fill in a sample name.
- Change the injection volume from the default of 10 ul to 100ul
- Leave the # of injections at 1
- Leave the function set at ‘Inject Samples’

Use the slider on the bottom to access more cells to the right
- Click inside the 'Method Set/Report Method' column
- Find the method 'caffeine2 2007' in the drop down menu and click on it.
- Set the Run Time to 16 minutes
- Set the Next Injection Delay to 2 minutes
- All other columns can be left at their defaults

Now let’s go for the next sample. Click on any cell on the next row. Notice it copies all the entries from the first vial to the second, at the same time it increments the vial# so it automatically will inject the next sample. Thus, the only thing you should have to change is the sample name!

Fill in the rest of the table. You should have 7 lines in the table, one for each drink and two for the different dilutions of caffeine and one for the blank.

Now let’s add two additional experiments. Choose any sample. What I want you to do with the next run is to use a slower flow rate to see what happens to the sample elution time and peak width. Click on the next row.

- Set the vial # to the number to the appropriate number for your sample.
- Change the Method set/Report Method to ‘caffeine3 2007’ (this is a slower flow rate method). Now lets try to run all the experiments that are to be done with this solvent mixture.

Is the baseline stable yet?

If not, wait until it is. If it is, you can go to the next step

**The Next Step**

To stop monitoring the baseline, find the button with the red dot along the upper edge of this window. Click on this button. It should ‘gray’ out, and after a few seconds, a green dot will appear next to it. If you are ready to roll, click on the green dot.

This will bring up a Window that asks for a name for this set of samples. Give it a name and hit the ‘run’ button. The method will now start. You will see the first line in the table is now displayed in red, to indicate that it is running, and if you look in the middle of the bottom line of the window you will see a message, first that it is setting up, and then that it is waiting for an injection.
Because of software incompatibilities, the autoinjector does not talk to the computer that is trying to run the system, so we now have to manually start the autoinjector running. On the autoinjector press the button underneath the ‘Auto Page’ on the display. This brings up a table that you fill in to tell the injector what to inject and when. Use the arrows and the keypad to fill in this table. If samples are all in order in the carousel this will be easy. For step 1 tell it to go from vial #1 to #X, where X is the number of the last via. # inj = 1, Inj Vol = 100, and Run Time = 19. This tells it to do a single injection of 100 µl, and then wait 19 minutes before it does the next injection.

This timing is important. Remember when we set the computer we gave it a run time of 16 minutes, during which it acquires data, and then an injection delay of 2 minutes, during which it simply resets and waits and gets ready to inject the next sample. So 18 minutes after the first injection it hits a state where it waits for a signal the an injection has been made. Thus by telling the injector to inject every 19 minutes, the computer is reset and waiting for the injection to begin.

At the end of the regular samples you have one sample that you are going to rerun using Caffeine3 2007. To get this sample injected create a second line in the injection table, Step 2. This will run from Vial #Y to Vial #Y, where Y is the number of the vial you are taking the sample from, and the rest of the parameters should remain the same.

You are now ready to fire off the injector. Hit the button underneath the ‘Auto Start’ label and the system should take off. You will hear the Autosampler click and whine as it loads a sample. You might even watch to see in injection syringe move back an forth slowly as it loads and shoots the first sample. After the sample is loaded the windows with the UV and Fluorescence data will appear and you can see the data as it is recorded. Note that at the beginning this data looks pretty noisy. This is because the software makes the scale very sensitive. As you get real data peaks in, the scale will reset to a more reasonable value and it will look better.

Once the machine is started, there is nothing to do but sit back and watch. Do a quick calculation of total run time. In theory you don’t have to do anything else until that time has past. However, before you leave, make sure you have enough solvent. Assume you are running about ½ ml of Methanol and ½ ml of water each minute. What is the total amount of Methanol and Water you will use in this time period? Look at the solvent reservoirs and do a quick calculation to see if you will run out of solvent. If it looks like the solvent levels will dip below 150 mls see Dr. Z. to get more solvent.

Once it has finished these runs, there is one more run you have to do, the run using caffeine4 2007. Because this run changes the solvent composition, and it takes the column a few minutes to come to equilibrium once the solvent changes, we have a few more steps to do.

When the computer and the autoinjector both say that they have finished with the first set of samples, go to the computer and find the ‘Instrument Method’ box, within this box load in the ‘Caffeine4 2007’ method. Then hit the ‘Monitor’ Button. The computer will now change to this solvent mixture, and it will start the windows for the UV and the fluorescence detectors running so you can see what happens to the baseline as the new solvent hits the detectors. Watch this for about 15-20 minutes. Once the detectors have achieved a stable baseline you can set up the computer and the autoinjector to do a single run of the sample you want run under these conditions.
At the end of the run
If you are finished for the day, take the following steps
Close all Millennium Windows except the login window. (Note it has started up a few windows you didn’t see) Leave the window 'Fish as System/Administrator' window for last. This window closes all the data bases, and takes a long-long time to close (Several Minutes). Once these windows have closed, then you can hit the logout button on the login window, and ‘X’ out of the window.

Once Millennium is closed down -DO NOT LOG OUT- Leave the computer up and running for the next person.

The last step is to turn off each of the individual instruments. Under ordinary circumstances you should rinse the column out with pure Methanol before leaving it for a long time, but for today you can just leave it as-is for the next group.
Getting your data off the Millennium\textsuperscript{32} HPLC system

Start up the Millennium\textsuperscript{32} software

Start
Programs
Millennium\textsuperscript{32}
Millennium\textsuperscript{32} Login

Double click on the ‘Review Data’ Box
A Review Data Window will come up - click OK

The computer will now open a project data table that contains every run that had been done on this machine since 1990!

Below the ‘Sample Set’ Tab are three sub tabs
Sample Set Name - Sample Set Start Date - System Name
When you click on any of these sub-tabs the data will rearrange based on the order of data in that tab from high to low or, if you click a second time, from low to high.

Since your data was done in the past few days, click on the ‘Sample Set Start Date’ tab once or twice to bring the most recent data to the top of the table. Do you see your data?

Left click on your data set and it will be highlighted in black (If you have more than one data set hold the shift key down so you can highlight multiple data sets)

Now click on the ‘Review Data’ button in this window that is a line or two above the actual data table

The data for the runs you want to review are now loaded into the ‘Review Data’ Window

On the left side of this window you will see a directory tree for each of the different runs, click the + or - boxes to expand the each run. As you click on the ‘injection’ for each run, the display on the right side of the window will display the data acquired during this run.

Play with this until you are comfortable with the directory tree and have located all of your runs. Now pick a single run and start the analysis

On the right hand window notice the 3 tabs along the bottom of the window 3D channels - 2D channels - Peaks

We don’t have any 3D data, so you can ignore that tab
2D channels is the important one for us. Click on this tab.
You should now see a data table with 2 lines
line 1 corresponds to the UV absorbance data
line 2 corresponds to the fluorescence data
Click on the ‘1’ or the ‘2’ on the left hand side of the data lines to toggle between the data sets
Let's start with the UV data set. Click on the ‘1’ and toggle into this set.

The UV data set is probably pretty well behaved, so the computer has already tried to integrate the areas under the peaks as best it can. You can see the chromatogram in black, with a single peak outlined in red. At the top of the peak is a number that identifies the peak by its elution time, and along the bottom you may see some lines that show how computer decided on the start, stop and baseline it used in the integration. If you want, you can right click anywhere on the chromatogram window and copy the chromatogram into a Word document at this point.

Now click on the ‘Peaks’ tab along the bottom of the window. There should now be a data table that displays the integrals of the peaks. Note how if you left click on an entry in the table that entry will get outlined in black and that peak will get outlined in red on the chromatogram. Using the left click and shift key or the cntrl key you can get one or all entries in the table outlined in black. Now right click on an entry that is outlined in black can copy it to the clipboard. You can now paste this into either a Word document for a write-up or Excel so you can do calculations.

Now go back to the 2D data tab and click on the ‘2’ and bring up the fluorescence data. Note that this data is very low intensity and noisy, so the computer has not tried to integrate it. That is now your job. Use the left button on the mouse to click on the left side of a peak in the fluorescence chromatogram, then hold down that button and move the mouse to the right side of a peak and then release the mouse button. A line should appear and the peak you are trying to integrate should be colored in red. Don’t like the points at that the computer grabbed for the start and finish of the peak? Grab them with a left click and move them to the place you want and release. Once you get the peak defined the way you want it, go to the ‘Peaks’ tab and grab your data and record it where you want it to go.

Those are the basics, identify the caffeine peaks in all chromatograms, get an integral for the peak in both absorbance and fluorescence data sets, and use the integral for the caffeine standard of known concentration to figure out the caffeine concentration in the sports drinks.

Now what does this tell you? The integrals are proportional to the amount of material in the peak. From your pure caffeine data and the water blank, you should be able to make a simple calibration curve for both fluorescence and absorption data. Use this calibration curve to calculate the amount of caffeine in the dilute energy drink sample, and in the original energy drink. If you hav any peaks other than caffeine, say something about them. One peak I was expected to see was Benzoic acid (It absorbs at 330 nm) How should we change the procedure if we wanted to quantitate this component?

Analysis (to be handed in)

Quantitative analysis of Caffeine

Make a data table that reports the UV and Fluorescence integrals for all the peaks run using the Caffeine2 method (Sample and standards). Plot two calibration curves (one for UV and one for fluorescence) for this data with Integral value on the Y-axis and total caffeine in sample on the X-Axis. Use this curve to determine the amount of caffeine in the 4 diluted sample. Use these values to determine the caffeine in the original, non diluted material.
Qualitative analysis of changing chromatographic conditions

Show the chromatograms of a single sample under the three different chromatographic conditions (Caffeine2, Caffeine3, and Caffeine4). Compare these chromatograms. Do the peaks come out in the same place? Do the peaks have the same resolution or bandwidth? Are any peaks poorly resolved in one method, but better resolved in the other? Calculate ‘Number of plates (N)’ and Plate height (H) for caffeine peak under the three different chromatographic conditions. The length of this column is 0.25 meters.

What I will be grading:

Hot and Spicy II
Write-up or spreadsheet was well organized and easy to read
(10 points) Calculation of N and H for all methods
(10 points) Comparison of 1 sample at low and high flow rates
R calculated for 2 peaks before and after
Comments? Any change in retention time, resolution, bandwidth?
(10 points) Comparison of 1 sample at low and high
R calculated for 2 peaks before and after
Comments? Any change in retention time, resolution, bandwidth?

Caffeine in Popular drinks II

Write-up or spreadsheet was well organized and easy to read
Quantitation of Caffeine
(10 points) Table of UV absorption integrals and caffeine concentrations
(10 points) Table of Fluorescence integrals and caffeine concentrations
(10 points) Plot of UV absorption integrals vs caffeine concentration for standards and line of best fit for this data
(10 points) Plot of Fluorescence integrals vs caffeine concentration for standards and line of best fit for this data.

(10 points) Final table of caffeine concentrations for samples.

Comparison of different chromatographic conditions
(10 points) Chromatograms under the three different conditions.
(10 points) Written analysis of the differences.
(10 points) Calculation of N and H under each different condition.