UCD 115 INSTRUMENTAL ANALYSIS

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UCD 115 Instrumental Analysis

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Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

Introductory Details

Preparation for the Laboratory

The laboratory part of this course is intended to give the student exposure to as many instrumental methods as possible during 60 hours of laboratory work. It is absolutely essential that the student be prepared for a given experiment by reading the laboratory outline before starting that laboratory work. Failure to do this will undoubtedly result in your failing to complete the laboratory, and, therefore, receive a poor grade. The laboratory outlines have been made as detailed as possible with respect to operation of the instruments, and, in theory, you should be able to proceed without the assistance of a TA if you are properly prepared.

Laboratory Work

Experiments have been set up for students working in small groups. Be sure to have a work plan that makes efficient use of your time. Lengthy sample preparation procedures should be started early in the six hours assigned to a given experiment. All students are expected to record laboratory observations and any numerical data in a hard-bound laboratory notebook. Each page should be titled, dated and signed. Results that are obtained from recorders, such as spectra, current-voltage curves, and chromatograms, do not have to be run twice for each student operating in a team. However, you may want to attach these results to your report, so photocopying may be necessary. If it is not too time-consuming, run the experiment twice so that you can check the reproducibility of your data. At the end of each lab, the TA must be asked to initial the pages of your lab book on which new data have been recorded.

The Laboratory Report

One of the most important skills you should be developing in this course is writing laboratory reports. As a student, the preparation of a well-written report requires careful consideration of the content of the report, hopefully leading to fuller understanding of the concepts involved in the experiment. In your future career, the ability to write clear, concise and well-organized reports will ensure success.

The reports submitted should be written using the standard format described below. Normally, they should be typewritten and double-spaced. The required format is:

- *Title page*: Title of experiment, course number, author, and date work was carried out, name of supervising TA, date report is submitted.
- *Introduction*: A statement of the purpose of the experiment; this requires 2 or 3 paragraphs at least.
- *Theory*: A brief paragraph describing the theoretical background for the analytical technique used; this section should contain a reference to the appropriate pages of the textbook, or other source material if you prefer.
- *Experimental*: A schematic or a box diagram is encouraged. The major pieces of equipment used should be listed. Also list standard solutions and reagents used to calibrate the instrument, and the nature and origin of any unknown samples analyzed. It is NOT necessary to copy experimental instructions from the laboratory manual, but you should refer to it here.
- *Data*: Summarize your data in numbered tables and graphs. Remember to label both axes on a graph, and to give it a title. Graphs should be referred to as Fig. 1, Fig. 2, etc., and tables as Table I, Table II, etc.
- *Results*: Present the results of your experiment based on the above data, showing all steps in calculations needed to reach a numerical result. Include an analysis of errors. The data should be analyzed to determine the amount of the unknown in the original sample.
- *Discussion*: Comment on the results. Explain any lack of success or limitations in the experiment. Discuss possible improvements if you see any.

References: List any references referred to in the report. This list should always include the textbook and the laboratory manual. In the text of the report, the reference is cited as a numbered footnote in parentheses: for example, "The drying procedure followed that outlined by Vogel (3)." 3. A.I. Vogel, Textbook of Quantitative Inorganic Analysis. Third Edition, Longmans, London (1961), p. 221.

Note: These report guidelines are not the same as those presented on the lab evaluation form.

Lab Evaluation Form

The lab evaluation form **must** be printed out and attached to your report between the Title page and the Introduction. This will be the criterion used for grading your lab report, although lab-specific questions will be introduced as necessary.

Here are a few more pointers

BE BRIEF AND CONCISE! This is one of the most difficult aspects of good writing to master. Read over your first draft and be ruthless in chopping out superfluous words, phrases, or sentences. Learn to be economical in expressing yourself. The total length of the lab report should be less than 15 pages. Short is sweet!

WATCH YOUR GRAMMAR! You may be a brilliant scientist in the lab, but your credibility will be diminished if you can't communicate your results. Science has two parts: doing novel and reproducible experiments, and being able to tell other people what you did!

WATCH YOUR SPELLING! You will not be marked down for the occasional typographical error, but a report full of spelling errors will be penalized. Take the time to proofread the draft of your text at least once.

Some particular comments on writing a scientific report:

- Avoid the use of personal pronouns, especially "I" and "we."
- Use the past passive tense in the Experimental and Results sections.
- All tables should be numbered and titled. The table headings should include the units.
- All figures should be numbered and titled. If the figure is a graph, the axes should be clearly labelled, including the units. If more than one set of data is included on the graph, use different symbols and a legend to identify them.
- Tables and figures should be presented on different pages, not included with the text. More than one table or figure can be placed on a page, however. Be sure to refer to any table or figure in the text, e.g. "The current and potential values from which E and n were calculated are reported in Table 2, and plotted in Figure 3."
- All equations presented in the text should be numbered and all symbols defined, as shown in the example below:

"The diffusion-limited current, i_d, is related to the bulk analyte concentration, c_a, by the following expression:

$$i_d = nFSD_a^{1/2}c_a/(\pi\Delta t)^{1/2} \tag{1}$$

where n is the number of electrons transferred, F is the Faraday constant, S is the electrode area, D_a is the diffusion coefficient of the analyte ion, and t is the time during which the current is sampled."

Remember, the all-important criteria in determining the quality of the report are that it be clear, concise, and provide enough information so that anyone else with your level of experience could repeat your work without consulting you. Students without experience in writing reports are often concerned about the length of the report and the time they take to write it. The first report may take longer, but with experience you will find that a good report can be written in a few hours. The most important aspect is that it presents the data and their analysis clearly and concisely. This can usually be done in 4 or 5 pages. If you have access to a word processor, you should use it to prepare reports. Editing is then very easy, and a neat copy is easily produced.

Finally, you are strongly advised to consult with the TA grading a given report before writing it. The TA can show you the important points to be emphasized in evaluating your data and will discuss his/her grading scheme. A list of TAs with their grading assignments and office hours is posted in the lab.

Your laboratory report is due one week after the lab is complete. The late hand-in penalty is **10 points (10%) per day**!

Lab Report Outline

(See pages 1-3 of Laboratory Manual)

I. Title page.

Include the title of experiment, course number, your name, and partners name(s), date of experiment performed, date lab report submitted and supervising TA.

II. Introduction.

Brief statement of purpose, which should indicate what was analyzed and the technique used. Limit to three to five sentences.

III. Theory

Describe the general theory used in the experiment. You may find that sections in your text and other textbooks could provide useful information in explaing the theory. Reference your sources of information.

IV. Experimental

Write down the major equipment used and list out the experimental conditions. DO NOT just reference the manual in terms of set up and procedure for this experiment. Plots will include copies of the chromatogram and mass spectrum of each standard and each unknown mixture.

V. Results & Discussion

Tables will include data (retention times and mass spectral information) obtained for standards used and labeled unknowns. From this information, you should be able to determine the compounds present in your unknowns.

Describe and explain your observations based on the experimental results. Also include possible structures of the standards and unknowns used in the experiment, based on your observations. Identify what your unknown mixtures contain. In your discussion, you should be able to determine the origin of the major peaks in the mass spectra. Use the references previously mentioned as an aid.

Explain possible sources of error and possible solutions to correct those problems.

VI. Conclusion

VII. References

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Chem 115 Lab Report Evaluation Form

Name:

Partner(s) Name(s)

Lab Report:

Experiment Number: #

Component	Received/Max	TA Comments
Title/ Author Proper Formatting of Title and Authors	/5	
Abstract Concise summary of purpose, results, conclusions	/10	
Introduction Importance clearly stated and referenced, Theory and goals clearly expressed, overall cohesion	/15	
Experimental Methods Equipment, methods and procedures clearly described	/10	
Results Data organized and presented clearly, error analysis, good selection of figures and tables, legends and titles. Evaluation of data **not mixed** with statement of results	/20	
Discussion Evaluation and interpretation of results. Are they significant? Do they support the hypothesis? What are the overall conclusions?	/10	
Conclusion A paragraph summarizing the main features of the report.	/5	
References Proper citing and use of references	/10	
Writing Quality Organization, coherence, conciseness, tense, third person, grammar, spelling, labels, units	/15	
Total	/100	

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Statistical Treatment of Data

Many times during the course of the Chemistry 115 laboratory you will be asked to report an average, relative deviation, and a standard deviation. You may also have to analyze multiple trials to decide whether or not a certain piece of data should be discarded. This section describes these procedures.

Average and Standard Deviation

The **average** or **mean** of the data set, $\bar{x}$, is defined by:

$$\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}$$

where x_i is the result of the ith measurement, i = 1,…,N. The standard deviation, σ, measures how closely values are clustered about the mean. The standard deviation for small samples is defined by:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N} (x_i - \bar{x})^2}{N}}$$

The smaller the value of σ, the more closely packed the data are about the mean, and we say that the measurements are **precise**. In contrast, a high **accuracy** of the measurements occurs if the mean is close to the real result (presuming we know that information). It is easy to tell if your measurements are precise, but it is often difficult to tell if they are accurate.

Relative Deviation

The relative average deviation, d, like the standard deviation, is useful to determine how data are clustered about a mean. The advantage of a relative deviation is that it incorporates the relative numerical magnitude of the average. The relative average deviation, d, is calculated in the following way.

1. Calculate the average, $\bar{x}$, with all data that are of high quality.
2. Calculate the deviation, $d=|x_i - \bar{x}|$, of each datum.
3. Calculate the average of these deviations.
4. Divide that average of the deviations by the mean of the data. This number is generally expressed as parts per thousand (ppt). You can do this by simply multiplying by 1000.

Report the relative average deviation (ppt) in addition to the standard deviation in all experiments.

Analysis of Poor Data

Sometimes a single piece of data is inconsistent with other data. You need a method to determine, or test, if the datum in question is so poor that it should be excluded from your calculations. Many tests have been developed for this purpose. One of the most common is what is known as the Q test (section 4-3). While it is very popular, it is not particularly useful for the small samples you will have (you will generally only do triplicate trials). Instead you will use what is commonly known as the 4d test. To use this test you need to follow the procedure outlined below.

1. Calculate the average, $\bar{x}$.
2. Calculate the deviation, d.
3. Calculate the average of these deviations.
4. Calculate the deviation of the "suspect" datum from the mean you calculated above, d_s. If this deviation is greater than 4 times the average deviation, then you should discard this datum.(If d_s >4d, then discard.)

Keep in mind that you also always have the right to discard a piece of data that you are sure is of low quality: that is, when you are aware of a poor collection. However, beware of discarding data that do not meet the 4d test. You may be discarding your most accurate determination!

Other important concepts and procedures

Associated topics you should be familiar with from your Chem 105 class:

- Normal error curve: Histogram of an infinitely large number of good measurements usually follow a Gaussian distribution

- Confidence limit (95%)
- Linear least squares fit
- Residual sum of squares
- Correlation coefficient

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Lab 1: Cyclic Voltammetry

GOALS

1. Students should be able to optimize the experimental condition to produce the best waveform.
2. Students should be able to extract key information from a CV waveform. (i.e. peak current and peak potential)
3. Students should be able to use the extracted information for practical application. (i.e. determining the unknown concentration of a compound)

Introduction

Cyclic voltammetry (CV) is a technique used to study reaction mechanisms that involve the transferring of electrons. The method involves linearly varying an electrode potential between two limits at a specific rate while monitoring the current that develops in an electrochemical cell. This experiment is performed under conditions where voltage is in excess of that predicted by the Nernst equation (Equation Lab 1.1).[1] Although CV is best at providing qualitative information about reaction mechanisms, several quantitative properties of the charge transfer reaction can also be determined.

$$E = E^{\circ} - \frac{RT}{nF} \ln Q \qquad \text{(Lab 1.1)}$$

Cyclic voltammetry involves applying a voltage to an electrode immersed in an electrolyte solution, and seeing how the system responds. In CV, a linear sweeping voltage is applied to an aqueous solution containing the compound of interest. A linear sweeping voltage is defined by the voltage (or potential) being varied linearly at the speed of the scan rate. The variation of the voltage can be seen in Figure 1.1. The voltage is initially given by Equation Lab 1.4 (see below). After the voltage reaches a certain maximum value, the potential is reversed and the sign of vt reverses and E_i becomes the maximum voltage, E_λ. The switch takes place at the peak which can be seen in Figure 1.1. The process can then be repeated in a periodic, or cyclic manner. The voltage after the potential sweep direction is switched is given by Equation Lab 1.5.

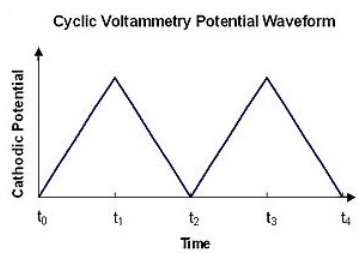


Figure 1.1: *Potential versus time program for cyclic voltammetry showing the forward and reversed linear potential ramp.*

As an important tool for studying mechanisms and rates of oxidation and reduction processes, CV provides the capability for generating a species during the forward scan and then probing its fate with the reverse scan or subsequent cycles. This process can occur within a few seconds. There is a unique aspect of cyclic voltammetry: three electrodes used. The electrodes are a working electrode, a reference electrode, and a counter electrode. The working electrode can be seen as a medium whose reductive or oxidative power can be externally adjusted by the magnitude of the applied potential. As the potential is increased or decreased linearly versus time, the working electrode becomes a stronger oxidant or reductant, respectively. Therefore, the working electrode, which typically consists of a chemically inert conductive material such as platinum, acts as a donor or acceptor of electrons. The general reaction written in Reaction Lab 1.2 refers to the addition of electrons to the oxidized electrode and the electrode transforming to its most reduced form.

$$O + ne^- \rightarrow R \qquad \text{(Lab 1.2)}$$

The reference electrode, typically AgCl or calomel, keeps the potential between itself and the working electrode constant. The potential is measured between the reference and working electrodes, and the current is measured between the working and counter electrodes. A counter electrode is employed to allow for accurate measurements to be made between the working and reference electrodes. The counter electrode's role is to ensure that the current does not run through the reference electrode since such a flow would change the reference electrodes potential. A voltage sweep from E_i (initial voltage) to E_f (final voltage) is produced using a

signal generator. The voltage is applied to the working electrode using a potentiostat. A potentiostat is an external power source. By sweeping the voltage slowly, information may be extracted from a graph of potential versus current going through the sample. Polarography utilizes this method of analysis where the limited current arising from a redox process in the solution during the sweep. This information is used to quantitatively determine the concentration of species that are electrochemically active in solution.

CV differs from polarography in two important ways. Firstly, the working electrode at which the reactions of interest occur has a constant area, not one which changes with time as in classical polarography. This electrode may be a solid such as graphite or platinum with a small surface area, or a stationary or hanging mercury drop. The latter type of electrode may have its surface renewed periodically. The second difference is that the potential of the working electrode is scanned rapidly over a wide potential range and then returned to its initial value using an applied potential signal which varies linearly with time between the initial value and the final value at the limit of the forward scan. Normally, this technique is applied so that currents due to reduction processes are observed during the forward scan and those due to oxidation on the reverse scan. The resulting cyclic voltammogram from a typical potential against time profile is shown in Figure 1.2.

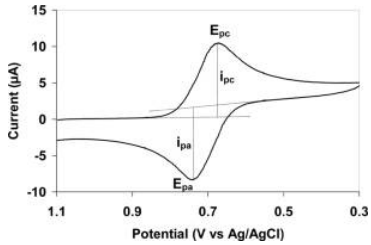


 Figure 1.2: *Cyclic Voltammogram of measured current versus applied potential. This diagram also shows the points where the y-values of peak anodic current (i_{pa}) and peak cathodic current (i_{pc}) as well as the x-values of peak anodic potential (E_{pa}) and peak cathodic potential (E_{pc}).*

Guided Example

Consider the response of the electron transfer reaction

$$A + ne^- \rightleftharpoons B^- \tag{Lab 1.3}$$

to the application of an electrode potential which is varying linearly with time. The electrode potential is given by the equation

$$E = E_i - vt \tag{Lab 1.4}$$

where E_i is the initial potential, v is the potential sweep rate (in volts s^{-1}) and t is time after the start of voltage sweep. After reaching some time, λ, the direction of the potential sweep is switched, and the equation describing the electrode potential becomes:

$$E = E_\lambda + v(t - \lambda) \tag{Lab 1.5}$$

where E_λ is the value of E at the switching point. Considering that the initial sweep is in the negative direction where reduction reactions are expected, it is clear that, if the sweep rate is sufficiently slow, the current against potential curve approaches that obtained by steady-state measurements. However, as v is increased, a peak develops on the i-E curve which becomes increasingly prominent (Figure 1.3). The peak is produced from the combined effects of high mass transfer rates in the non-steady state followed by the progressive depletion of the reactant in the diffusion layer.

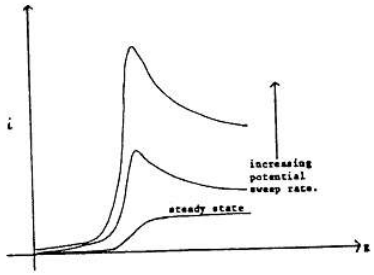


Figure 1.3. Effect of potential sweep rate on the PE curves in a linear potential sweep experiment adjacent to the electrode.

It should be noted that since E is a linear function of t, the potential axis is also a time axis.

In order to relate the observed current to the reactant concentration c_A, one must know how c_A varies with distance from the electrode, x, and with time, t. This variation is described by Fick's Law for mass transfer by diffusion:

$$\frac{\partial c_A}{\partial t} = D_A \frac{\partial^2 c_A}{\partial x^2} \tag{Lab 1.6}$$

where D_A is the diffusion coefficient of reactant A (units: cm^2/s). The solution of this second order partial differential equation requires specification of boundary and initial conditions and is described in textbooks on electroanalytical chemistry[1,2]. If the electron transfer reaction is sufficiently fast in Reaction Lab 1.3 to maintain a Nernstian equilibrium at the electrode surface (i.e., the reversible case), then the peak current, i_p for a negative sweep is given by Equation Lab 1.7. The variables are described in **Table 1.1**.

$$i_p = (2.69 \times 10^5)\, n^{3/2}\, S D_A^{1/2}\, v^{1/2}\, C_A, \tag{Lab 1.7}$$

***Table 1.1**: Variables of the modified Fick's Law, Equation* Lab 1.7

Variable	Description	Units
i_p	peak current	A
n	number of electrons transferred	-
S	Surface Area	cm^2
D_A	Diffusion coefficient	cm^2/s
v	scan rate	V/s
c_A	the concentration of compound A in the bulk solution	$mole/cm^3$

You may notice that the peak potential is *independent* of sweep rate and is related to the half-wave potential ($E_{1/2}$) by

$$E_p = E_{1/2} - \frac{0.0285}{n} \tag{Lab 1.8}$$

Furthermore, the shape of the peak is defined by the potential difference between the peak and the position when the current is **one half** of that at the peak:

$$E_p = E_{p/2} - \frac{0.0565}{n} \tag{Lab 1.9}$$

Thus, using the measured values of i_p, E_p, and $E_{p/2}$, one can determine n and D_A for a given electrode area and sweep rate. If the electrode reaction is slow so that the surface concentrations of A and B are no longer related by the Nernst equation at a given sweep rate, then the peak characteristics change such that

$$E_p - E_{p/2} > \frac{0.0565}{n} \tag{Lab 1.10}$$

and the peak potential now depends on the sweep rate. Using the appropriate boundary conditions to describe the rate of Reaction Lab 1.3 in the forward direction, Equation Lab 1.6 may be solved to obtain expressions for i_p and E_p which are now much more complicated.

After the direction of the potential sweep is reversed, a second current peak is observed corresponding to oxidation of the product B. When Reaction Lab 1.3 is reversible, implying that B is stable, the height of this peak is equal to that observed on reduction, but with the current flowing in the opposite direction. The method of estimating peak currents is illustrated in Figure 1.4, where i_{pc} is the peak potential in the cathodic sweep.

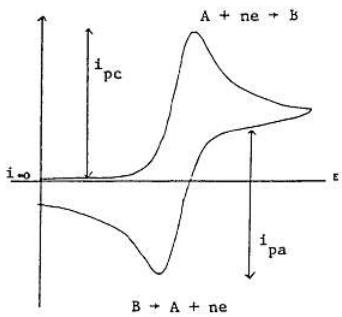


Figure 1.4: CV Waveform of Reaction Lab 1.3

The CV waveform in the above figure for the process Reaction \ (Lab 1.3\) and assumes that only A is initially present in the solution. ΔE_p is then defined as

$$\Delta E_p = |E_{pc} - E_{pa}|. \tag{Lab 1.11}$$

When the electrode process is **reversible** and it **independent** of sweep speed and is described by Equation Lab 1.12.

$$\Delta E_p = \frac{0.0565}{n} \tag{Lab 1.12}$$

As v is increased to the stage at which Nernstian equilibrium for Reaction Lab 1.3 cannot be maintained, ΔE_p increases with increasing sweep rate, and the shape and position of the peaks depend on both v and the kinetic parameters of the electrode reaction.

Reaction Mechanisms

One of the major uses of cyclic voltammetry is in the rapid qualitative identification of electrode reaction mechanisms. Organic molecules often undergo a rapid chemical reaction with the solvent or some other constituent of the solution after the electron transfer process. The resulting reaction scheme, referred to as the **ECE (electrode-chemical-electrode) mechanism**, can be written

$$A + ne^- \rightarrow B^{n-} \tag{Lab 1.13}$$

$$B + Z \rightarrow C \tag{Lab 1.14}$$

$$C + me^- \rightarrow D^{m-} \tag{Lab 1.15}$$

where Z is the solvent or some other species. The first and third reactions are labeled E since they involve the electrode, and the second step (or any other chemical step) is labeled C. Hence, the above three-step mechanism is referred to as the ECE reaction mechanism. It is possible to garner information about the (non-electrode-dependent) rate constant for step 2 (Reaction Lab 1.14) via cyclic voltammetry.

The standard potential for Reaction Lab 1.13 is generally different from that for Reaction Lab 1.15. A typical current-potential curve for such a system is shown in Figure 1.5. The current on the reverse sweep will depend on the sweep rate and the rate constant for Reaction \(Lab 1.14\, which is assumed to take place under pseudo first-order conditions ($c_Z \gg c_B$). For very fast sweep rates, very little B will react to form C, and the CV waveform will have the same appearance as the reversible case, with reduction and oxidation peaks at I and II, respectively. As v is decreased, peak II diminishes more rapidly and peak I less rapidly than the usual. The dependence $v^{1/2}$ would predict this because the chemical step removing species B becomes important and peak I has a contribution from Reaction Lab 1.15. In addition, a peak develops at III due to oxidation of D.

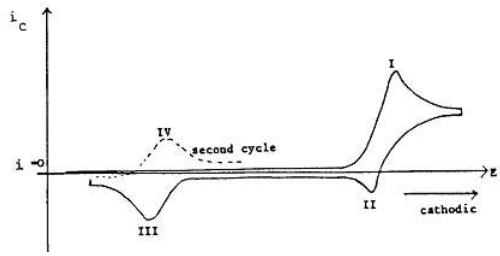


 ***Figure 1.5**: Cyclic voltammogram at an intermediate sweep rate for a system with an ECE mechanism.*

It should also be noted that the current-potential curves on the second and successive sweeps are not the same as that observed on the first. At very slow sweep rates, peak II disappears completely and peak I then corresponds to the process

$$A + (m+n)e^- \rightarrow D \qquad \text{(Lab 1.16)}$$

The variation of peak I with sweep rate is shown in Figure 1.6 for the case that $n = m = 2$.

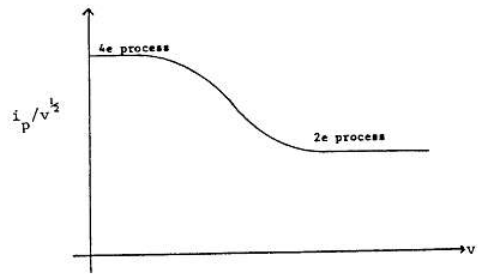


 ***Figure 1.6**: Variation of measured current for peak I from Figure 1.4 vs. potential scan rate, v.*

The rate constant k for the chemical Reaction Lab 1.16 can be obtained from an analysis of data obtained in the transition region of Figure 1.6 or from the ratio of peaks I and II at intermediate sweep rates in Figure 1.5.

Cyclic voltammetry can be applied to the analysis of many other reaction mechanisms including those with dimerization of the product of electron transfer, with preceding chemical steps, catalytic processes, etc.

The Instrument

The instrument used for this experiment is the BAS Epsilon potentiostat. It is controlled from a computer running Windows. A number of different electrochemical techniques are available in the Epsilon software, including cyclic voltammetry (current vs. potential for a linear potential sweep), chronoamperometry, time base, bulk electrolysis (current vs. time at a constant potential), and chronopotentiometry (potential vs. time at a fixed current).

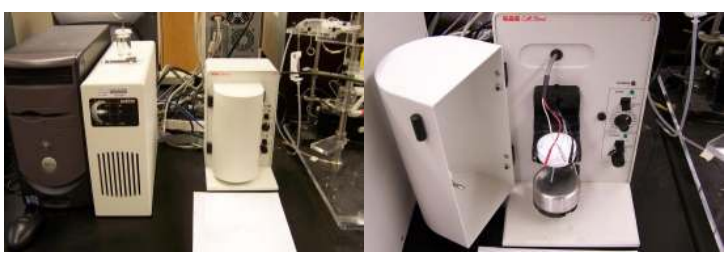


***Figure 1.7:** UCD cyclic voltammetry Instrument (including potentiostat)*

To begin an experiment, make sure that the Epsilon unit is turned on, and doubleclick the Epsilon icon on the desktop. Select **New** from the File menu or click the New icon. This will generate a menu that lists the available techniques. (This list can also be generated by selecting Select NEW Experiment from the Experiment menu or by using the F2 key.)

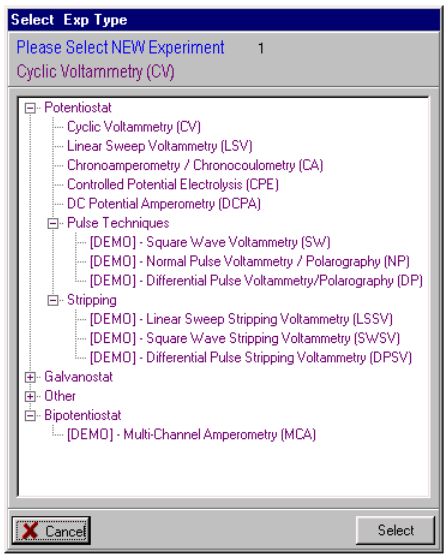


 Figure 1.8: *Computer Program*

Highlight Cyclic Voltammetry (CV), and click Select to confirm the selection. An experiment window containing an empty axis set is displayed (Figure 1.9), and the appropriate parameters are set in the various dialog boxes.

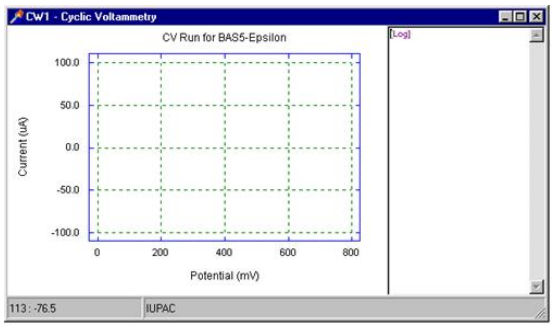


 Figure 1.9: *Computer Program*

The potential limits and the scan rate for CV are set using the Change Parameters dialog box (Figure 1.10) in either the Experiment menu or the pop-up menu (the pop-up menu is accessed with the right mouse button).

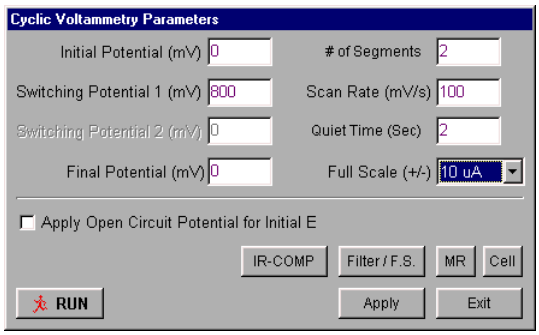


 Figure 1.10: *Change Parameters dialog box for cyclic voltammetry.*

1. Potential values are entered in mV, and the **Scan Rate** in mV/s.
2. If the **Apply Open Circuit Potential for Initial E** box is checked, then the open circuit potential will automatically be measured and used as the Initial Potential.
3. When the experiment is started, the cell is held at the **Initial Potential** for the number of seconds defined by the **Quiet Time**.
4. There are two gain stages for the current-to-voltage converter. The default values of these stages that are used for a given current **Full-Scale** value are determined by the software. However, they can be adjusted manually using the Filter / F.S. dialog

box. This dialog box is also used to change the analog **Noise Filter Value** settings from the default values set by the software. The **Full-Scale** value should be at the 10 mA/V setting at the start of the experiment, and then adjusted to a more convenient range depending on the maximum current observed in the experiment. Ask your TA about scale values to better observe your results.

5. The default condition of the cell is that the cell is **On** (i.e., the electronics are connected to the electrodes) during the experiment, and is **Off** between experiments. **THIS OPTION SHOULD NOT BE CHANGED SINCE CONNECTING OR DISCONNECTING THE ELECTRODES WHEN THE CELL IS ON CAN RESULT IN DAMAGE TO THE POTENTIOSTAT, THE CELL, AND/OR THE USER!**
6. Clicking the **IR-COMP** button activates the iR compensation option (compensates for the drop in voltage due to the resistance of the solution).
7. Clicking **Exit** will exit the dialog box without saving any changes made to the parameter values. Any changes can be saved by clicking **Apply** before exiting.
8. Range of allowed parameter values:
    - **Potential** = -3275 - +3275 mV
    - **Scan Rate** = 1 - 10,000 mV/s (also see below)
    - **Quiet Time** = 0 - 100 s
    - The **# of Segments** is limited by the total number of data points that can be stored (32,000) (note that in this initial version, the potential resolution of the current measurement is fixed at 1 mV).

10. Once the parameters have been set, the experiment can be started by clicking **Run** (either in this dialog box, in the **Experiment** menu, in the pop-up menu, on the Tool Bar, or using the **F5** key).

Experimental Procedure

Part 1: Reduction of the Ferricyanide Anion

In this portion of the experiment, you will be investigating the reduction of the ferricyanide anion. The purpose of this part of this experiment is to discover and develop an understanding of the properties of this reaction. This reaction can be important in the field of soil science because Iron is a comment element in soil. Iron can bind to cyanide ligands to make them less bioavailable in natural systems. The reduction of the Iron (III) complex can be represented in the following balanced half-reaction.

$$Fe(CN)_6^{-3} + e^- \rightarrow Fe(CN)_6^{-4} \tag{Lab 1.17}$$

Four conditions will be measured in this part in order to observe the effects of concentration and counter-ion selection on the voltammogram. To begin the experiment, take note of the solutions already prepared for you. These solutions can be found in the cabinet directly under the instrument. There should be solutions of 4 mM ferricyanide in 1 M KNO_3, 1 M KNO_3, and 4 mM ferricyanide in 1 M Na_2SO_4. If these solutions are empty or missing, notify your TA.

The four conditions in this part of the experiment will use the electrodes listed in **Table 1.2**. The electrodes can be found in a drawer. Do not attach the electrodes until your first solution is ready.

important

For the Platinum disc electrode, note the diameter of the electrode in your lab notebook; this value will be important for your post-lab calculations.

Table 1.2: Electrodes used for Part 1 of the Experiment.

Electrode Type	Electrode Material	Wire Color
Reference	Saturated Calomel Electrode (SCE)	White
Working	Platinum Disc	Black
Counter	Platinum Wire	Red

When using these electrodes, it is important that they are clean. If the electrodes are not clean, the CV waveform produced will not be accurate. Before beginning the experiment, have your TA demonstrate the cleaning procedure for the working electrode. **The cleaning procedure is as follows:** Put a few drops of the cleaning solvent on a cloth. Gently rub the working electrode on the damp cloth.

The initial settings will be the same for all four conditions. These settings are listed in **Table 1.3**. It is important to note that the "full scale" setting can be adjusted to view your peak more clearly. An important setting not listed in **Table 1.3** is the scan rate. The scan rates used will be given in the procedure sections of each condition.

Table 1.3: Initial Settings for Part 1.

Initial Potential	+700 mV
Switching Potential	-200 mV
Final Potential	+700 mV
# of Segments	2
Scan Rate	250 mV/s
Quiet Time	10 seconds
Full Scale	10 mA/V

Condition 1 – 4 mM Ferricyanide in 1 M KNO_3

1. Fill the cell with 4 mM Ferricyanide with 1 M KNO_3.
2. Deoxygenate the solution for 5-10 minutes using the gas tank provided. Your TA will set the pressure gauge as needed.
3. While the solution is deoxygenating, clean the electrodes, set the initial conditions in the computer software using the conditions from **Table 1.2** (The first scan rate will be **250 mV/s**), and prepare the dilution you will use in Condition 2 (the necessary dilution can be found in the Condition 2 section).
4. Once the solution has been deoxygenated, assemble the cell, place a small stir bar into the cell, and lower the electrodes into the cell.
5. Perform the scan using the procedure described in the Instrument Operation section.
6. After the scan (the scan will only take a few seconds), lift the electrodes out of the solution and stir the solution for 10 seconds or until the bubbles are removed. After stirring let the solution rest for 1 minute. **DO NOT stir the solution while electrodes are lowered or the scans are running, the stir bar can break the electrodes.**
7. Use the procedure in the Data Acquisition section to secure your data files.
8. Repeat steps 5-7 at scan rates of **160, 100, 50, and 20 mV/s**. Make sure to stir the solution between each scan.
9. After completing your scans, ask the TA if your results look reasonable. If they do not, the electrodes may need to be cleaned using the cleaning procedure.
10. Dispose of your solution in the specified waste container.

Condition 2 – 2 mM Ferricyanide in 1 M KNO_3

1. Dilute the 4 mM Ferricyanide with 1 M KNO_3 to 2 mM Ferricyanide with 1 M KNO_3. Perform the necessary dilution calculation in your laboratory notebook. This dilution needs to be done with the 1 M KNO_3 solution, otherwise, the KNO_3 concentration would be altered.
2. Deoxygenate the solution for 5-10 minutes using the gas tank provided. Deoxygenation can begin while the scans for Condition 1 are being performed.
3. Repeat steps 4-10 for this in the Condition 1 procedure for this condition running a scan with rates of **250, 160, 100, 50, and 20 mV/s.**

Condition 3 – 1 M KNO_3

1. Fill the cell with the 1 M KNO_3 solution.
2. Deoxygenate the solution for 5-10 minutes using the gas tank provided. Deoxygenation can begin while the scans for Condition 2 are being performed.
3. Assemble the cell with the 1 M KNO_3 solution, place a small stir bar into the cell, and lower the electrodes into the cell.
4. Perform the scan **(scan rate = 10 mV/s)** using the procedure described in the Instrument Operation section. Begin deoxygenating the solution in Condition 3.
5. Do not stir the solution, no other scans will be performed for Condition 3.
6. Use the procedure in the Data Acquisition section to secure your data files.
7. Look at your results and try to conclude if your scan looks reasonable. If you are having a difficult time determining this, ask your TA for help.

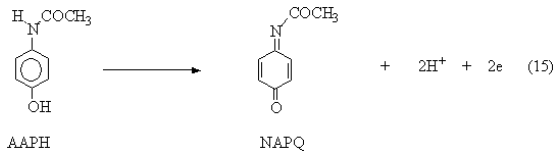
Condition 4 – 4 mM Ferricyanide in 1 M Na_2SO_4

1. Fill the cell with the 4 mM Ferricyanide in 1 M Na_2SO_4 solution.
2. Deoxygenate the solution for 5-10 minutes using the gas tank provided. Deoxygenation can begin as soon as the deoxygenation of Condition 3 is complete.
3. Repeat steps 4-10 in condition 1's procedure for scan rates of **250, 100, and 50 mV/s**.
4. After completing the last scan, disassemble the cell, rinse the electrodes with DI water and immerse them in a cell filled with DI water. Dry the electrodes and put them away into their respective cases.

When you are finished, rinse all three electrodes with deionized water and immerse them in the cell filled with deionized water.

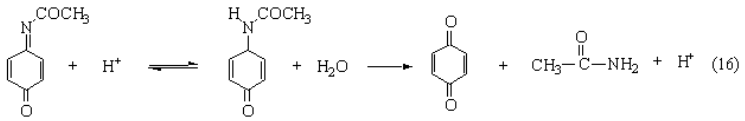
Part 2: The Oxidation of Acetaminophen

Tylenol is an over-the-counter pain relief medication that is commonly used in the United States. The reason that Tylenol work is because it contains the active ingredient Acetaminophen (AAPH). When someone takes Acetaminophen, also known as Paracetamol, the molecule undergoes a complete oxidation process in an aqueous solution. This oxidation reaction is the beginning of a reaction series that is demonstrated below. This is a pH-dependent process. The extent to which reaction Lab 1.20 occurs is dependent on the rate of reaction Lab 1.19.



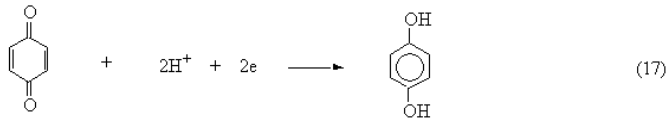
. (Lab 1.18)

The product can be protonated, and then undergo the elimination of acetamide to form benzoquinone (BQ):



. (Lab 1.19)

The rate of the first reaction is obviously pH-dependent. Finally, benzoquinone may be reduced to hydroquinone:



. **(Lab 1.20)**

In this portion of the experiment, the goal is to demonstrate this mechanism and determine the best pH conditions for this reaction. After you have determined the best conditions, you will determine the concentration of AAPH in a Tylenol tablet. The electrodes used in this part are listed in **Table 1.4** and the initial settings are listed in **Table 1.5**.

Table 1.4: Electrodes used for Part 2 of the Experiment.

Electrode Type	Electrode Material	Wire Color
Reference	Silver/Silver Chloride (Ag/AgCl)	White
Working	Glassy Carbon	Black
Counter	Platinum Wire	Red

Table 1.5: Initial Settings for Part 2.

Initial Potential	0 mV
Switching Potential	+1000 mV
Final Potential	-200 mV
# of Segments	2
Quiet Time	10 seconds
Full Scale	10 mA/V

Begin this part by preparing the solutions for conditions 1, 2, and 3 of pH 6, 2.2, and 1.8 respectively. Start by checking to see that there is an AAPH solution made for you. It may be in the refrigerator or in the same cabinet as the ferricyanide solution and counter-ion solutions. Once you have located the AAPH solution and three 100 mL volumetric flasks, begin by adding 5 mL of the AAPH solution to each of the volumetric flasks. The final concentration of AAPH should 3.5 mM. Fill one flask with pH 6 buffer to the line. Fill the next solution using pH 2.2 buffer and fill the last flask using 1.8 M Sulfuric Acid.

Condition 1 – pH 6

1. Deoxygenate the pH 6 buffer solution for 5-10 minutes. Your TA will reset the pressure gauge if this part is performed on the second day.
2. While the solution is deoxygenating, **clean the electrodes with the help of your TA** and set the initial conditions in the computer software using the conditions from **Table 1.5** (The first scan rate will be **250 mV/s**).
3. Assemble the cell with the electrodes from **Table 1.4** and the necessary solution. Place a small stir bar into the cell and lower the electrodes into the cell.

4. Perform the scan **(scan rate = 250 mV/s)** using the procedure described in the Instrument Operation section. Begin deoxygenating the solution in Condition 3.
5. After the scan (the scan will only take a few seconds), lift the electrodes out of the solution and stir the solution for 10 seconds or until the bubbles are removed. After stirring let the solution rest for 1 minute. **DO NOT stir the solution while electrodes are lowered or the scans are running, the stir bar can break the electrodes.**
6. Use the procedure in the Data Acquisition section to secure your data files.
7. Repeat steps 4-6 at scan rates of 100 and 40 mV/s. Make sure to stir the solution between each scan.
8. Look at your results and try to conclude if your scans look reasonable. If you are having a difficult time determining this, ask your TA for help. The electrode may need to be cleaned again.
9. Dispose of your solution in the specified waste container.

Condition 2 – pH 2.2

1. Deoxygenate the pH 2.2 buffer solution for 5-10 minutes. This process can begin as soon as the deoxygenation of Condition 1 is complete.
2. Repeat steps 3-9 in the Condition 1 section of Part 2.

Condition 3 – 1.8 M Sulfuric acid

1. Deoxygenate the 1.8 M Sulfuric Acid solution for 5-10 minutes. This process can begin as soon as the deoxygenation of Condition 2 is complete.
2. Repeat steps 3-9 in the Condition 1 section of Part 2.
3. Do not rinse all of the electrodes and put them away, they will be used in the determination of the AAPH in the Tylenol tablet.
4. As a group, determine the most appropriate condition pH condition for this reaction and the best scan rate. The best scan rate can be determined by seeing which scan gives you the clearest data. The best pH condition can be determined by picking the condition that gave the sharpest peaks.

Condition 4 – Tylenol Tablet in with the best conditions

1. Weigh the Tylenol Tablet.
2. Crush the tablet with a mortar and pestle into a fine powder. Transfer the fine powder into a 250 mL volumetric flask.
3. Fill the volumetric flask with the appropriate pH condition that you selected.
4. Deoxygenate the solution for 5-10 minutes.
5. Run the scan at your chosen scan rate using the initial settings listed in **Table 1.5**.
6. Perform the procedure in the Data Acquisition section.
7. Compare the peak height in this trial to the peak height of the scan performed with the same pH condition and scan rate. Use this information to determine the approximate concentration. Use this information to develop a concentration range that can be used to develop a calibration curve.
8. Dispose of your solution in the specified waste container.

Condition 5 – Calibration Curve (Solution #1)

1. Make the dilution using the standard AAPH solution used in conditions 1,2, and 3 in a 100 mL volumetric flask. Fill the flask to the line with your chosen pH condition.
2. Deoxygenate the solution for 5-10 minutes.
3. Run the scan at the chosen scan rate using the initial settings listed in **Table 1.5**.
4. Perform the procedure in the Data Acquisition section.

Condition 6 – Calibration Curve (Solution #2)

1. Repeat the procedure from Condition 5 for the second concentration used for the calibration curve.
2. After completing the last scan, disassemble the cell, rinse the electrodes with DI water and immerse them in a cell filled with DI water. Dry the electrodes and put them away into their respective cases.

Post-Lab Questions

Part 1: Reduction of the Ferricyanide Anion

****INCLUDE ALL CV WAVEFORMS****

Condition 1 – 4 mM Ferricyanide in 1 M KNO_3

1. Tabulate the measured values of i_{pc}, i_{pa}, E_{pc}, E_{pa}, and $E_{pc/2}$ for each scan rate.
2. Determine the Diffusion Coefficient (D_A) for each scan rate using Equation Lab 1.7.
3. Find the average, standard deviation, and relative deviation of D_A for the Condition 1 scans. Discuss potential errors that could account for this deviation.
4. Calculate $E_{1/2}$ for each scan rate using Equation Lab 1.8.
5. Test the reversibility of each scan rate using Equations Lab 1.11 and Lab 1.12. Is this reaction reversible?

Condition 2 – 2 mM Ferricyanide in 1 M KNO_3

1. Tabulate the measured values of i_{pc}, i_{pa}, E_{pc}, E_{pa}, and $E_{pc/2}$ for each scan rate.
2. Plot i_{pc} and i_{pa} versus $v^{1/2}$ for each scan rate. Make one plot i_{pc} versus $v^{1/2}$ that includes a series from the 2 mM data and a series from the 4 mM data. Make a second plot of i_{pa} versus $v^{1/2}$ that includes a series from the 2 mM data and a series from the 4 mM data.
3. Determine the Diffusion Coefficient (D_A) for each scan rate using Equation Lab 1.7.
4. Find the average, standard deviation, and relative deviation of D_A for the Condition 2 scans. Discuss protentional errors that could account for this deviation.
5. Calculate $E_{1/2}$ for each scan rate using Equation Lab 1.8.
6. Test the reversibility of each scan rate using Equations Lab 1.11 and Lab 1.12. Is this reaction reversible?

Condition 3 – 1 M KNO_3

1. Is there any current flowing through this cell? Explain.

Condition 4 – 1 M Na_2SO_4

1. Compare your voltammograms to the voltammograms that you acquired in Condition 1. Discuss the differences.
2. Does the charge of the supporting electrolyte affect the reaction?

Part 2: The Oxidation of Acetaminophen

****INCLUDE ALL CV WAVEFORMS****

1. Plot i_p versus $v^{1/2}$. On the plot, have three data series. The series one should be the data from Condition 1, the second series is the data from Condition 2, and the third series is the data from Condition 3.
2. Which system is the appropriate buffer system? Explain.

Determining the Unknown AAPH in Tylenol

1. Report the mass of the Tylenol Tablet.
2. Develop a calibration curve using your solutions from Condition 5 and 6. Use a third data point that uses the same pH condition and scan rate. This data point will come from the data gathered in either Condition 1, 2, or 3.
3. Use the calibration curve to determine the concentration of AAPH and report this value.
4. Calculate and report the mass of AAPH in the tablet.
5. Calculate and report the mass percent. Discuss any error in scans or in the determination for this value.

Outside Links

- http://www.chem.uoa.gr/applets/Apple...l_Diffus2.html
- http://pubs.acs.org/doi/abs/10.1021/j100661a017

References

1. Heineman, W. R.; Kissinger, P. T. Large-Amplitude Controled-Potential Techniques. In *Laboratory Techniques in Electroanalytical Chemistry*; Kissinger, P. T., Heineman, W. R., Eds.; Monographs in Electroanalytical Chemistry and

Electrochemistry; Marcel Dekker: New York, NY, 1984; pp 51-127.
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3. Compton, R. G.; Banks, C. E. *Understanding Voltammetry*; World Scientific: Hackensack, NJ, 2007.
4. Skoog, D., Holler, F. J., & Crouch, S. R. (2017). *Principles of Instrumental Analysis* (Seventh ed.). Boston, MA: Cengage Learning.
5. Elgrishi, N.; Rountree, K.J.; Brian D. McCarthy, B.D.; et al. A Practical Beginner's Guide to Cyclic Voltammetry. *J. Chem, Ed.* **2018** *95* (2), 197-206.

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Lab 2: High Performance Liquid Chromatography

Goals

1. Students should be able to develop an understanding of the principles of liquid-liquid partitioning.
2. Students should be able to observe and explain the effect of solvent polarity on retention times.
3. Students should be able to interpret a chromatogram and use the information to determine the components in a mixture as well as the concentration of those components.

Introduction

Samples collected from medical patients, industry products, and the environment are usually mixtures of many compounds. Often times, doctors, producers, and researchers are interested in specific components in these mixtures, so these mixtures need to be separated. High-performance liquid chromatography (HPLC) offers the ability to do just that. HPLC data can be used to complement gas chromatography (GC) or be an excellent alternative to GC when the components are nonvolatile or would thermodynamically decompose under high temperatures.

In order to separate mixture components, HPLC takes advantages of partitioning between a mobile and stationary phase under a uniform pressure that is typically between 500 to 5000 psi. High pressure is required to obtain a reasonable flow rate through the column. The process begins when a small amount of liquid sample is injected into the column that has a stream of liquid flowing through (which is known as the mobile phase). In partition chromatography, the column is packed with particles that are coated with the stationary phase. The polarity of the component and the type of HPLC being performed determines which phase the component is more attracted to. If the component is more attracted to the mobile phase, it will flow out of the column and have a shorter retention time. If the component is more attracted to the stationary phase, the component will be retained and will, therefore, have a longer retention time. Similar to Capillary Electrophoresis (CE) or Gas Chromatography (GC), these retention times can be used to determine components. Selecting the mobile phase (or solvent) is one of the most important steps when performing HPLC and is selected based on polarity. Solvent polarity relates to the ability of the components to partition into that phase. The polarity scale for different solvents can be found in Table 2.1. These solvents can be used exclusively or mixed to achieve the desired polarity.

***Table 2.1**: Relative polarities of the mobile phase solvent*

Solvent	Relative Polarity
Fluoroalkanes	<-2
Cyclohexane	.04
1-Chlorobutane	1.0
Carbon tetrachloride	1.6
Toluene	2.4
Tetrahydrofuran (THF)	4.0
Ethanol	4.3
Methanol	5.1
Acetonitrile	5.8
Ethylene glycol	6.9
Water	10.2

HPLC can be performed with fixed or variable solvent composition. When the solvent polarity is fixed, it is known as an isocratic run. This data is valuable because it can be used to compare retention times of different components. This method only works when the components have unique retention times under that condition. When the retention times of the components are very similar or

extremely different, not every component will be seen in the chromatogram. When solvent polarity is varied throughout the run, this is known as a gradient run. Gradient conditions can be optimized to improve the chance that all the components will be seen on the chromatogram. While all the retention times are not as comparable due to varying conditions, it is useful to see how many components are in the mixture and characterize the components.

During an HPLC analysis of a mixture, the components will separate based on their retention times. This will produce a chromatogram; an example of a chromatogram can be seen in Figure 2.1. Either the peak height or the peak area can be used to estimate the concentration. This can be done because these values are proportional to the concentration when the peaks are sharp, and the flow rate is carefully controlled. A calibration curve can be prepared by plotting either peak height or peak area as a function of concentration.

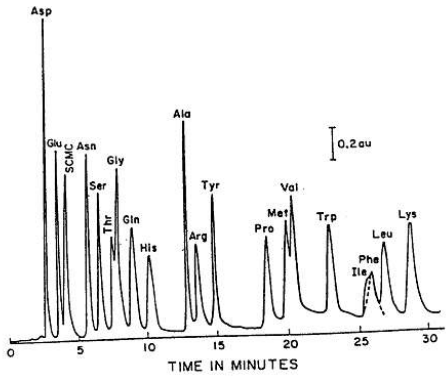

 ***Figure 2.1**: Reverse Phase (C18) Separation of Amino Acids.*

Normal Phase Chromatography

Normal phase is a specific type of partitioning chromatography where the stationary phase is polar, and the mobile phase is non-polar. While the compounds selected might not be particularly polar or non-polar, their relative polarities are the most important. As long as the stationary phase is more polar than the mobile phase, it is considered normal phase chromatography. The stationary phase is usually a column packed with silica particles that have R groups attached. Common R groups for normal phase are -C_2H_4CN or -$C_3H_6NH_2$.

When the mixture passes through the column, the polar components will be more attracted to the stationary phase and the non-polar components will be attracted to the mobile phase. This will lead the non-polar components to flow through (or elute) the column first. The more polar a compound is, the longer it will take to elute. By increasing the polarity of the mobile phase, the bound polar component will partition more into the mobile phase and elute from the column.

Reverse Phase Chromatography

Reversed phase chromatography is another type of partition chromatography but is opposite from normal phase. The mobile phase is more polar than the stationary phase. In this case, the most polar components will elute first. Figure 2.2 provides an image for how components are moving through a reversed phase column. Common R groups found on the silica particles for reverse phase are -n-octyl (C_8) or -n-octyldecyl (C_{18}).

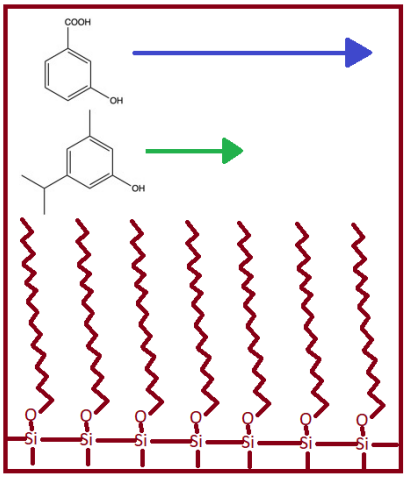

 Figure 2.2: *Reverse Phase Chromatography elution through a C-18 packed column.*

Adsorption Chromatography

The stationary phase is a solid of a polar nature such as particles of hydrated silica or alumina. The mobile phase and the solute (components in the sample) are in competition for active adsorption sites on the stationary phase particles. Thus, more strongly adsorbed components are retained longer than weakly adsorbed components. Because more polar compounds adsorb on a polar surface to a greater degree than do less polar compounds, retention in the column is related to sample polarity.

Ion Exchange Chromatography

Ion exchange HPLC is based on the partition of ions between a polar liquid phase and a stationary phase with ion exchange sites. The ion exchange sites are typically immobilized in small beads of resin that are formed by a cross-linked polymer. Bonded phase columns in which the ion exchanger is bonded to small particles of silica also are available. Cations are separated on cation exchange resins which contain negatively charged functional groups such as SO^{3-} and $–COO^{-}$. Anions are separated on anion exchange resins which contain positively charged functional groups such as CH_2N^{+} $(CH_3)_3$, a quaternary ammonium ion. Separation is based on ions partitioning into the ion exchange phase to varying degrees. The selectivity of a resin for an ion is determined primarily by the charge on the ion and its hydrated radius. Resin affinity increases with increasing charge density.

Instrumentation

The apparatus consists of a container of the mobile phase, a pump capable of pressures up to 4000 psi or greater, a valve for injecting the sample (usually 10 to 500 µL volumes), the column (sometimes thermostatted), a detector, electronics associated with the detector, and a recorder. A schematic of the HPLC instrument can be seen in Figure 2.3. This instrument in this lab used a C_{18} column.

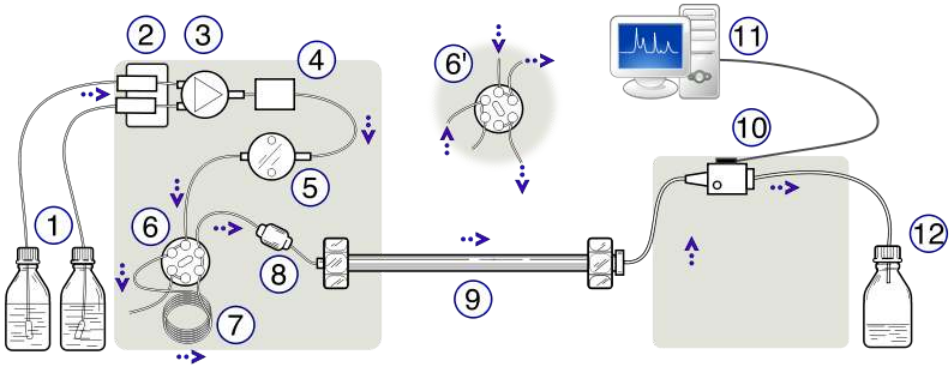

 Figure 2.3: *Schematic Diagram of a High-Performance Liquid Chromatograph. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position",*

(6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column or guard column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. Created by Yassne Mrabet.

UV-visible absorbance is the most commonly used mode of detection. Such detectors enable the component (or effluent) from the column to flow through an 8 to 10 µL spectrophotometric cell for detection of compounds at a particular wavelength (often in the ultraviolet, < 400nm, where many organic molecules absorb). Electrochemical and fluorescence detectors often are used to achieve lower detection limits. The other commonly used detector is based on a measurement of the differential refractive index.

Experimental Procedure

Part 1: Readying the HPLC for Operation

1. Ready the HPLC by making sure the solvent reservoirs are full and the waste bottles have at least 1 Liter of volume available to accommodate the waste solvent.

2. Launch Open Lab by clicking **HPLC1 (online)** (Figure 2.4)

 ***Figure 2.4**: HPLC1 (online) desktop icon.*

3. Use the Instrument Control Tab. Confirm that all components are green and indicate "ready." (Figure 2.5)

a. If this is not the case, press the "On" button.

b. The DAD will take a few minutes to warm up; if there is a lightning bolt through the purple lamp, wait until it has gone away, and the ready bar has turned green.

c. The fraction collector will not be used in this experiment.

 ***Figure 2.5**: "Instrument Control" tab indicted by top, green arrow. Bottom right, red arrow indicates the DAD window.*

Part 2: Separating a Paraben Mixture

Parabens are a class of chemicals widely used as preservatives in the cosmetic and pharmaceutical industries. Parabens are effective preservatives and are primarily used for their bactericidal and fungicidal properties. Parabens can be found in shampoos, shaving gels, personal lubricants, topical/parenteral pharmaceuticals, spray tanning lotion and toothpaste. Parabens can also be used as food additives. Recently, there has been an in-vivo and in-vitro studies have shown that parabens have weak estrogenic activity. Because parabens are endocrine disrupters, paraben concentrations have been mitigated and must not surpass the maximum permitted levels. Paraben mixtures are often used in order to increase the efficiency of the preservation. Take the time to look up general paraben structures to develop an understanding of their chemical structure.

Because parabens are often found in mixtures, HPLC can be used to separate the individual compounds. To determine the components in the mixture, **reverse phase HPLC** will be performed through a C_{18} column. In this portion of the experiment, gradient and isocratic elution data will be collected and compared. The paraben mixture has already been prepared for you and contains 4-hydroxy benzoic acid, methyl-4-hydroxy benzoate, ethyl-4-hydroxy benzoate, and propyl-4-hydroxy benzoate.

To begin, five isocratic experiments will be performed. The conditions for the five experiments are listed in Table 2.2.

***Table 2.2:** Isocratic Experiment Conditions*

Experimental Run	Mobile Phase Composition
1	100 % methanol
2	90 % methanol / 10 % water
3	80 % methanol / 20 % water
4	70 % methanol / 30 % water
5	60 % methanol / 40 % water

Preparing a Sequence for the Paraben mixture

1. Set up six methods. One for each of the 5 isocratic runs and one for the gradient run.****Your TA will be assisting you while you set up your sequence.****

2. Select "PARABENS.S" in the "sequence" menu bar.

3. Go to "sequence" and click "sequence template" and see the order of the samples. Each run will use the same sample which should be vial slot 1.

4. Each method should be different for each run. To check this, click the "method" menu bar, click "method" in the selection menu, and select "edit entire method."

5. Look through the method with your TA and check that all the parameters are correct. **DO NOT** change anything in the method unless you confirm with your TA that you are doing so. Save the method once you are done.

6. Confirm that you have enough of the paraben mixture in the sample vial and that the sample vial is in vial slot 1.

7. Click "run sequence."

8. You can print the reports after each run or after all runs are complete. You can find the data reports by clicking the "data analysis" tab in the bottom.

9. Before moving on, confirm that you have peaks for each of your runs. If you notice any issues with your data, talk with your TA.

The Paraben mixture runs will take approximately one hour to run, so the caffeine standards and the beverage samples can be prepared while they are running

Part 3: Analysis of Caffeine in Beverages

Caffeine is a common chemical that we interact with on a daily basis and people have access to it in many forms. They can drink it in many types of beverages, eat it in different types of food, and even take it in pill form. **Reverse phase HPLC** can be used to determine the amount of caffeine in these items. In this experiment, you will be determining the amount of caffeine in coffee, tea, and a soft drink. If the runs are performed with the same isocratic parameters, retention time can be used as a qualitative measure and peak area or peak height can be used as a quantitative measure of caffeine in a sample. In order to determine the concentration of caffeine in these samples, a calibration curve must be put together using a set of standard solutions comparing either the peak height or peak area to the known concentration of the solution.

Preparing the Standard Caffeine Samples

****DO NOT dispose of these samples; they will be used in Lab 6-Capillary Electrophoresis****

1. Accurately weigh out 10.0 mg of caffeine. The caffeine can be found on the shelf near the weigh station area.

2. Transfer the caffeine into a clean 100 mL volumetric flask.

3. Dilute to the mark with HPLC/CE grade water. The stock solution will have a final concentration of 0.1 g/L.

4. Carry out a series of dilutions to obtain standard solutions of 0.01 g/L, 0.025 g/L, 0.05 g/L, and 0.075 g/L. Make 10 mL of each solution and use HPLC/CE grade water to make the dilutions.

5. Shake each of the mixtures to ensure adequate mixing.

6. Filter the solutions using the provided filter. **Do not do this until you are ready to run your samples.**

a. The filter and syringe can be reused if the solutions are filtered from low concentration to high concentration.

b. The syringes can be found in the plastic drawers near the door of the lab.

c. Filter approximately 1 mL and dispose of it into a waste beaker to wash the filter.

d. Filter the solution into the appropriate vial. (Figure 2.6) DO NOT put tape on the vials; ask your TA for a sharpie to write directly on the vials.

 Figure 2.6: *Appearance of HPLC/CE vials. The HPLC can also use plastic vials that look quite similar. Image is taken from www.thermofisher.com*

Preparing the Beverage Samples

****DO NOT dispose of these samples; they will be used in Lab 6-Capillary Electrophoresis****

1. The beverages can be found in the refrigerator in the lab.

2. Prepare the Coffee Sample.

a. Pipette 5 mL of coffee into a clean and Dry 50 mL volumetric flask and dilute to the mark with HPLC/CE grade water.

b. Filter the sample using the provided filter.

c. Rinse the filter by filtering the first 1-2mL of the sample into the waste beaker.

d. Fill a vial with the appropriate volume and label the vial.

3. Prepare the Tea Sample.

a. Pipette 10 mL of tea into a clean and Dry 50 mL volumetric flask and dilute to the mark with HPLC/CE grade water.

b. Filter the sample using the provided filter.

c. Rinse the filter by filtering the first 1-2mL of the sample into the waste beaker.

d. Fill a vial with the appropriate volume and label the vial.

4. Prepare the Soft Drink Sample

a. If the soft drink you selected is carbonated, decarbonate the soft drink by pouring it back and forth between two beakers until the bubbles cease.

b. Pipette 25 mL of the soft drink into a clean and Dry 50 mL volumetric flask and dilute to the mark with HPLC/CE grade water.

c. Filter the sample using the provided filter.

d. Rinse the filter by filtering the first 1-2mL of the sample into the waste beaker.

e. Fill a vial with the appropriate volume and label the vial.

Preparing a Sequence for the Caffeine Standards and Samples

1. Set the method for this experiment. All of the samples will use the same method. ****Your TA will be assisting you while you set up your sequence.****

2. Put standard caffeine solutions in slots 1-5 with the least concentrated in slot 1 and the most concentrated in slot 5. Place the beverage samples in slots 6-8.

3. Select “CHE 115 CAFFEINE.M” in the “method” menu bar.

4. Click “method” in the selection menu and select “edit entire method.”

5. Confirm that the solvent mixture is 47% Methanol and 53% Water.

6. Go to “sequence” menu bar and select “CAFFEINE_LC.S.”

7. Click “sequence” followed by “sequence template” and check to see that your samples are in the correct sample order/slots. Click “run sequence.”

8. While the samples are running, make sure all of your bulk samples and caffeine standards are in containers that can be stored and are properly labeled if you are performing this lab before Lab 6 (CE experiment).

9. Confirm with your TA that your data looks appropriate before disposing of any solutions. You can dispose of the small vials for this experiment once you have collected all of your data. If you have already performed Lab 6, then dispose of your samples.

Treatment of Data

1. You can print the reports after each run or after all runs are complete. You can find the data reports by clicking the “data analysis” tab in the bottom.

2. Before completing the lab, confirm that you have peaks for each of your runs. If you notice any issues with your data, talk with your TA.

3. Create a calibration curve using the caffeine standard peak height or area versus the concentration. This can be done in *Chemstation* in the lab or at home in excel/google sheets.

a. To create a calibration curve in *Chemstation*, start by clicking the “data analysis” tab in the bottom left corner of the window. This will open and HPLC (offline) window.

b. Go to the CHE 115 file and find your data folder and select it.

c. Double click the first standard run in the sequence window.

d. Find the “Calibration” tab in the menu bar and select “New Calibration Table.”

e. A new window “Calibrate: HPLC1” will appear and select “Automatic setup.”

f. Set the “level” to 1 and put the concentration of the first run in the “Default Amount.” Press “OK.”

g. Double click the second run and go to the “Calibration” menu bar and click “Add Level.”

h. Set the “Level” to 2 and enter the second run’s concentration in the “Default Amount.” Press “OK.”

i. Repeat steps g and h for each of the remaining standard solutions.

j. The Calibration Table and the Calibration Curve can be viewed in the bottom of the window. Confirm that all the points are there.

Post-Lab Questions

Questions for Part 2: Separating a Paraben Mixture

****Include ALL Elution Data Reports****

1. How many compounds are observed in the paraben mixture? Identify each peak on the gradient chromatogram.

2. Compare the results from the isocratic runs to the results from the gradient run.

3. What advantages does the gradient elution offer?

4. What disadvantages does the gradient elution have compared to the isocratic elution?

5. Plot the results of the retention time of the last component (longest retention time in isocratic runs) versus percent Methanol for the series of isocratic runs. (plot log r.t. versus &MeOH). Do you obtain a linear plot? Explain.

Questions for Part 3: Analysis of Caffeine in Beverages

1. Using the calibration curve, determine the concentration of caffeine for each beverage in g/L.

a. Describe whether you used peak height, peak area, or both to estimate the concentration. Discuss the advantages to each method.

b. Make sure you take into account the dilutions you made when preparing the samples.

2. Discuss the errors potentially made during this experiment.

3. Discuss how retention times depends on methanol and the pH of the mobile phase. What factors contribute to the choice of mobile phase composition and pH in the present analysis.

4. If you have already performed Lab 6 – Capillary Electrophoresis, did you get the same concentrations as you calculated in the CE experiment? If the answers are different, discuss possible explanations.

References

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2. Larsson K, Ljung Björklund K, Palm B, et al. Exposure determinants of phthalates, parabens, bisphenol A and triclosan in Swedish mothers and their children. *Environ Int*. **2014**;73:323-33. pdf
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Lab 3: Fourier Transform Infrared Spectroscopy (FTIR)

Goals

1. Students should develop an understanding of molecular symmetry and vibrational modes.
2. Students should be able to use symmetry elements to predict which vibrational modes are IR active.
3. Student's should be able to use a solve the pollution mystery by determining the air pollutant and the source of the air pollutant.

Introduction

The procedure of this lab does not follow the normal format. In this lab, you have been hired by an environmental testing company to monitor air pollutants. By working through a series of exercises, you will gain important information to determine the air pollutant in an air sample and therefore determine the source of the pollution. The air sample was taken in a location 20 m away from an open farm field where strawberries were growing. In the field on one side, there were cows grazing; on the other side, there was a natural gas pumping station. Across the street, there was a gas station with an auto repair shop specializing in air conditioner repair and a dry cleaner. This information narrows the list of suspected chemicals to six. Test your air sample using an infrared spectrometer and determine the culprit.

The FTIR / Vibrational spectroscopy experiment is an adaptation of "Pollution Police" by Profs. Jodye Selco and Janet Beery at the University of Redlands, which was presented at the Division of Chemical Education Regional ACS meeting in Ontario, CA 1999. Many students currently in CHE 115 have already taken CHE 124A which focuses on symmetry, molecular vibrations, and point groups. For students that have taken CHE 124A, use this lab as an opportunity to use your knowledge and assist your lab partners that have not taken the class yet.

There are resources available to further an understanding of FTIR. The theory of FTIR spectrometer operation is discussed in SHN Chapters 16 and 17. The group theory and vibrational quantum mechanics are discussed in McQuarrie and Simon (Chem 110B text) Chapters 12 and 13. Principles of Infrared Spectrometry and its application can be found in Skoog, Holler, and Crouch (the textbook for CHE 115) in chapters 16 and 17.

The FTIR exercise follows the format of a detective story involving solving a series of problems rather than the normal lab format. The experimental portion of the exercises are problems #9 and #10. The computer in room 3475 will be used to complete problems #2, 7, and 8. The computers are set up to run HyperChem, Gaussian, and Spartan.

Setting the Scene

You have been hired by an environmental testing company to monitor air pollutants. Air pollutants can be released from many types of sources. A few examples of sources are: factories, cars, or cattle. Sometimes, volatile chemicals can evaporate from agricultural fields. Since most air pollutant chemicals can absorb infrared light, we are able to detect them with an infrared spectrometer. Some of the molecules commonly found in the atmosphere include those in Table 3.1. Water and small amounts of carbon dioxide, methane, and sulfur trioxide are found in “clean” air samples. Large amounts of any of the chemicals other than water usually indicate atmospheric pollution.

Table 3.1: List of compounds commonly found in the air.

Chemical Name	Chemical Formula	Description
Water	H_2O	Ubiquitous in the atmosphere, even on dry days.
Carbon Dioxide	CO_2	Produced by the combustion of fuels (including the food we eat) and is a major source of greenhouse warming in the atmosphere.
Methyl halides	CH_3X	Used as pesticides that are sprayed on agricultural fields. They currently are under strict EPA controls and are partially responsible for the ozone hole.
Dichlorodifluoromethane	CCl_2F_2	refrigerant that is partially responsible for the ozone hole.
Ethylene	C_2H_4	A component of natural gas. Is used to ripen bananas.
Acetylene	C_2H_2	Fuel for high-temperature torches.
Sulfur trioxide	SO_3	Originates from the burning of sulfur-containing fuels such as coal. Reacts with water to form acid rain.
Benzene	C_6H_6	A component of gasoline; is carcinogenic.
Dichloroethylenes (DCE)	$C_2H_2Cl_2$	Used in the dry-cleaning and polymer industries.
Percholoethylene (PCE)	C_2Cl_4	Used in the dry-cleaning and polymer industries.
Hydrogen Peroxide	HOOH	A component of smog.

When testing, a test sample is first obtained by taking an evacuated cell to the target location, opening a valve, and allowing the ambient air to fill the cell. An FTIR – Fourier Transform Infrared Spectrometer will be used to perform Infrared Spectrometry. The final output from the spectrometer called an infrared spectrum (Figure 3.1), is a plot of the intensity of light reaching the detector divided by the initial intensity of light, as a function of frequency (%Transmittance= I/I_o vs. frequency). The goal of this project is to gain a better understanding of group theory and to identify atmospheric pollutants from their infrared spectra.

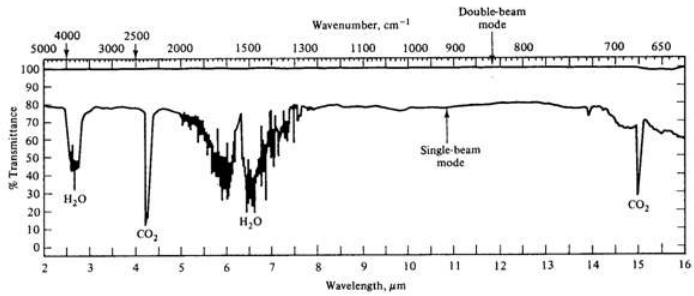

 ***Figure 3.1**: An infrared spectrum of air. The lower, single-beam trace clearly shows the absorption of atmospheric gases. The top, double-beam trace shows that the reference beam compensates nearly perfectly for the air absorption, producing a stable 100% T.*

For the molecules listen in Table 4.1, examine the three-dimensional ball-and-stick models for these molecules and compare them with the two-dimensional representations of the molecules in Figure 3.2. In the drawings in Figure 3.2, straight lines represent

bonds that lie in the plane of the paper; two lines between a pair of atoms represent a double bond; a filled arrowhead indicates a single bond that is angled out of the plane of the paper toward you; and the dashed arrowhead indicates a bond angled into the plane of the paper away from you. Carbon dioxide is an example of a linear molecule; water, ethylene, sulfur trioxide, and benzene are planar molecules.

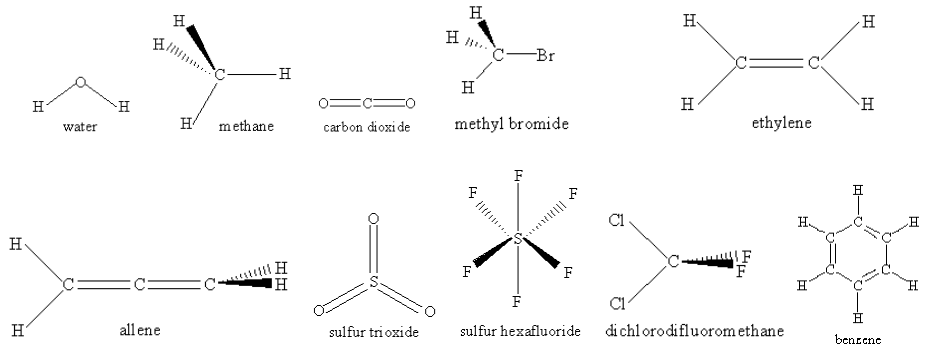

Figure 3.2: *Molecules of Study originally listed in Table 4.1.*

Problem #1: Center-of-mass and Coordinate Axes

In order to accomplish our goal of identifying the chemical pollutant you are investigating from its infrared spectrum, we must know which infrared frequencies, if any, the molecule absorbs. To decide which molecules absorb infrared radiation and at which frequencies, we will need to examine various properties of the molecules themselves, including their centers of mass, their Cartesian coordinate axes, their vibrations, and their symmetries. Specifically, we will compare the actions of the symmetry operations of a molecule on its Cartesian coordinate axes with the actions of the symmetry operations on its vibrations.

When a molecule absorbs infrared radiation of a given frequency, this energy causes the molecule to vibrate in a specific way; the atoms bounce against each other much like balls connected by a spring. The vibrational motions of a molecule that absorb infrared radiation are the ones that exhibit the same behavior as do the Cartesian coordinate axes of the molecule when the atoms of the molecule are permuted in certain ways. This is a result of the orthogonal interaction between the electromagnetic field of the light and the electric field of the molecule itself. Therefore, you will begin by drawing in a three-dimensional coordinate system for each of the molecules in the set assigned (see Table 3.2). The convention for molecules is that the origin of the axis system is placed at the center of mass of the molecule. (This is the weighted average of the positions of the atoms.) First, determine approximately where this should be. (You might want to reexamine the ball-and-stick models.) Remember, the masses of the different atoms are different. To find out how much each atom weighs; consult a periodic table of the elements. The mass number for each type of atom appears at the bottom of the square in which the atomic symbol appears.

By convention, **the z-axis is the unique axis,** if there is one. This axis is also called the molecular axis, or axis of highest symmetry. In a linear molecule, it corresponds to the line formed by the molecule; this is true for carbon dioxide. For benzene, the z-axis is the out-of-plane axis since it is the unique axis. If there doesn't seem to be a unique axis, then place the heaviest atoms in the molecule along the z-axis (often, there is more than one way to do this). Once the z-axis is assigned, the in-plane axis usually is the y-axis and the out-of-plane axis is the x-axis. In addition, axes should be placed along the molecular bonds whenever possible.

Example 3.1

The y- and z-axes for water and carbon dioxide are shown in Figure 3.3. The x-axis is out-of-plane from the origin (just below the O atom and pointing straight out at you for water, but centered in the C atom and pointing directly away from you for carbon dioxide).

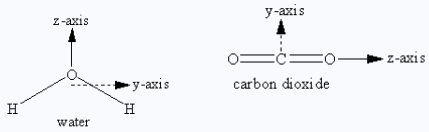

Figure 3.3: *Cartesian coordinate axes for water and carbon dioxide*

Assignment

Draw in the three Cartesian coordinate axes in a picture of each molecule assigned to you in Table 3.2 as outlined in the steps below. (If you do not assign them correctly now, you will have a chance later to re-label them.)

a. Estimate, by eye, the center of mass for each molecule, keeping in mind the atomic masses for each type of atom.
b. Draw in the z-axis using the rules above.
c. Draw in the two remaining axes using the rules above.

Table 3.2: Assigned molecules for each group

Group Letter	Assigned Molecules
A and E	dichlorodifluoromethane, hydrogen peroxide, nitrous oxide, sulfur trioxide
B and F	*trans*-1,2-dichloroethylene, ethylene, nitrous oxide, methyl bromide
C and G	*gem*-1,1-dichlorethylene, dinitrogen tetroxide, nitrous oxide, ammonia
D and H	*cis*-1,2-dichloroethylene, perchloroethylene, nitrous oxide, methyl iodide

Problem #2: Molecular Vibrations

The types of molecular vibrations a molecule has determine whether or not it absorbs infrared light. Hence, you need to determine the types of vibrations your molecules make. To ensure that all of them have been identified, we need to know how many are possible. Consider a molecule that is a collection of N atoms connected together in a specific way by chemical bonds. In order to describe the motions of the molecule, we need to consider the motions of each individual atom. This means that we need 3 degrees of freedom for every atom within the molecule for a total of 3N degrees of freedom. However, the atoms within the molecule have a specific geometric relationship to the other atoms in the molecule; this results in a redistribution of the number of independent degrees of freedom. The motion through space of the molecule uses three degrees of freedom, reducing the 3N degrees of freedom to 3N - 3. Since the molecule also can rotate (like a spinning baton or Frisbee), we require two degrees of freedom to describe the coordinates about which a linear molecule can spin and three degrees of freedom for a non-linear molecule (for a linear molecule there is no concerted rotation about the molecular axis (z-axis). This leaves 3N - 5 degrees of freedom for the linear molecule and 3N - 6 for the non-linear molecule still unaccounted for; each of the remaining degrees of freedom describes a distinct coordinated internal motion, or vibration, of the atoms within the molecule.

For example, when there are only two atoms in the molecule (e.g. O_2, N_2, or CO), there is only one vibrational motion: 3(2) - 5 = 1. In the case of benzene (C_6H_6) there are 12 atoms and 3(12) - 6 = 30 vibrational motions possible! As it turns out, not all of these vibrations are capable of absorbing infrared radiation. For the simplest molecules, such as water, it is easy to draw pictures representing the vibrational motions.

Example 3.2

Water has 3(3)-6=3 vibrations and carbon dioxide has 3(3)-5=4 vibrations as shown in Figures 3.4 and 3.5.

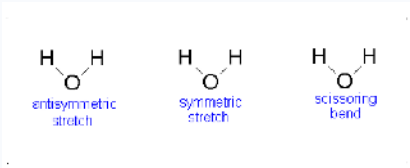

Figure 3.4: *Vibrations of water*

Consider the water molecule as it undergoes the asymmetric stretch; as it reaches its most extreme position, it has one “arm” extended and the other “arm” contracted. The bend is a change largely in angle and not inter-atom distances.

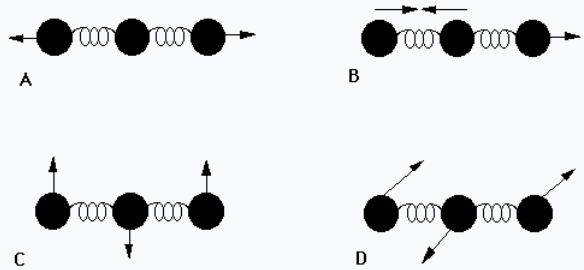

 ***Figure 3.5**: Vibrations of carbon dioxide*

The four vibrations for CO_2 consist of two stretches (one symmetric and one asymmetric) and two bends, that are degenerate (with the same energy) but involve perpendicular motions.

Assignment

For each molecule, calculate the number of vibrational motions using the formula given above. Then, use a computer program to view the vibrational motions for water, carbon dioxide and the other molecules assigned. Record the motions of the atoms in the molecule using the symbols from the examples above, and record the frequency calculated for each vibration. Note that some of these motions are out-of-plane motions. Be sure to rotate the molecules on the computer screen so that you examine the motions from many different angles. (Many of the programs calculate the frequencies in cm^{-1}; this is actually the frequency, in s^{-1}, divided by the speed of light (3.00×10^{10} cm/s). In this exercise, make note of the frequencies in cm^{-1} given by the HyperChem program.) Again, you are to complete the following steps for the molecules assigned to you in Table 3.2. This assignment will require a lot of space in your lab notebook due to all of the vibrations you will be drawing.

a. Determine the number of vibrational motions, 3N – 6 or 3N – 5, to make sure that you know how many vibrational motions to record.
b. Record the vibrational motions for your molecules, following the notation used in the examples above.
c. Record the frequency for each of these vibrations.

Spartan '16 Operation

1. Double-click “PC Spartan '16” on desktop. The Spartan computer is located in the TA area of 3475 and has a bright orange name tag.
2. Click the new page icon in the top left corner. Atoms with various bond choices will appear. If you prefer a more advanced setting, click the Expert tab.
3. Draw your molecule by clicking on the atom with the correct number of bonds needed. To join two atoms, repeat this process by touching the mouse arrow to the open bond.
4. Once you are done drawing your molecule, click the Glasses ("View") icon located just below the Geometry scroll-down. This will finalize your drawing.
5. Next, go to the Setup scroll-down and select Calculations. The following will be your entries:
   i. Calculate: **Equilibrium Geometry** with **Hartree-Fock 3-21G(*)**
   ii. Compute: **IR**
   iii. Print: **Vibrational Modes**
6. Go to Setup and scroll down to Submit. Make a new folder with your group's letter in the Chem 115 folder. Label within your folder as you see fit.
7. After you save the file you will be prompted twice that Spartan has started and completed. Press OK both times.
8. Go to Display and scroll to Spectra. Here you will find the frequencies associated with the different vibrations of your molecule. Click on any of the checkboxes to view the animation for the vibration associated with that particular frequency.

Problem #3: Symmetry Elements and Symmetry Operations

Because the molecules we are examining are very small, the rules of quantum mechanics govern the processes in which we are interested in. According to quantum mechanics, not all light absorption processes are allowed; many are "forbidden" by symmetry. If we want to determine which molecular vibrations absorb infrared light, we need to examine the actions of the symmetries of the molecules on the coordinate axes and on the molecular vibrations. We begin by determining the symmetry elements that each molecule possesses.

A symmetry operation on a molecule is an action that moves the molecule into a position that is indistinguishable from the starting position. A symmetry element of a molecule is a geometric feature of the molecule about which a symmetry operation is performed. Symmetry elements include planes and axes; symmetry operations include reflections across planes and rotations about axes. In the case of water, 180° rotation about the z-axis is a symmetry operation, denoted C_2; while the z-axis itself is a symmetry element, a C_2 axis. The symbol C_3 indicates a three-fold axis of symmetry, a symmetry element; while C_3 indicates a 120°rotation about a C_3 axis, a symmetry operation. Rotation by 240° about a C_3 axis is denoted C_3^2. Rotation by 360° about a C_3 axis is equivalent to doing nothing; that is, $C_3^3=\hat{E}$, where $\hat{E}$ is the identity operation.

***Table 3.3**: A list of all symmetry elements and operations*

Symmetry Element		Symmetry Operation	
Symbol	Description	Symbol	Description
E	Identity	$\hat{E}$	No change
C_n	n-fold axis of symmetry	$\hat{C}_n$	Rotation about the axis by 360°/n
σ	Plane of symmetry	$\hat{\sigma}$	Reflection through the plane
i	Center of Inversion	$\hat{i}$	Reflection through the center
S_n	n-fold rotation-reflection axis of symmetry, also called improper rotation axis	$\hat{S}_n$	Rotation about the axis by 360°/n followed by a reflection through a plane perpendicular to that axis

The plane of symmetry, σ, is also referred to as a reflection plane or mirror plane. The symbol σ_v is used to denote a "vertical" plane of symmetry that is parallel to an axis of highest symmetry (z-axis, or C_n with largest n), while the symbol σ_h is used to denote a "horizontal" plane of symmetry that is perpendicular to the axis of highest symmetry (taken as z-axis). The symbol σ_d denotes a "dihedral" plane of symmetry that bisects an angle between atoms.

Example 3.3

Figures 3.6 and 3.7 illustrate several of the symmetry elements listed in Table 3.3.

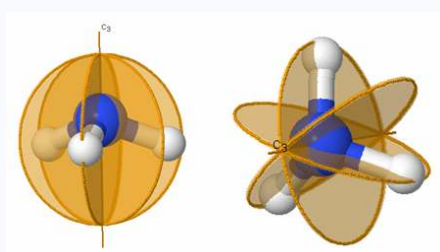

***Figure 3.6**: Symmetry elements for two views of ammonia (NH_3). The C_3 axis is labeled and the three mirror planes, σ_v, σ_v', σ_v'' are labeled as gold circles.*

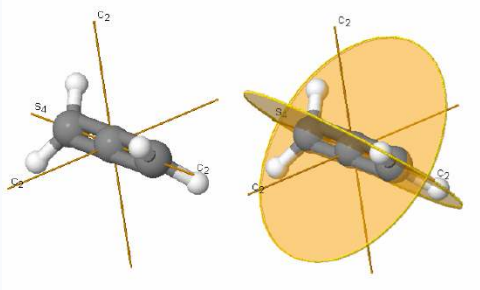

 Figure 3.7: *Symmetry elements for allene (C_3H_2). The C_2 and S_4 axis is labeled and the two mirror planes, σ_d, $\sigma_{d'}$ are labeled as gold circles.*

Example 3.4

Figures 3.8 and 3.9 show the symmetry elements and operations of water and carbon dioxide, respectively.

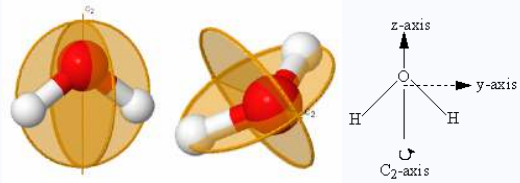

 Figure 3.8: *Symmetry elements of water viewed from differing perspectives*

In the case of water the symmetry elements are E, C_2 (shown), σ_v (xz-plane, perpendicular to the plane of the molecule), and $\sigma_{v'}$ (yz-plane, the plane of the molecule). Note that it does not matter whether σ_v represents the xz- or yz-plane. The corresponding symmetry operations for water are $\hat{E}$, C_2, σ_v, and $\sigma_{v'}$.

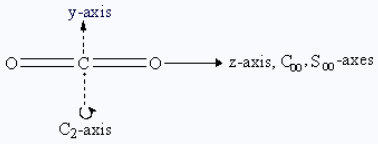

 Figure 3.9: *Symmetry elements of carbon dioxide*

The symmetry elements not shown above are E, σ_v (yz-plane, the plane of the paper), σ_h (xy-plane, perpendicular to the C_2 axis), and i (center of inversion at the coordinate origin). The symmetry operations for carbon dioxide are $\hat{E}$, infinitely many C_2, C_∞, infinitely many σv, S_∞, σ_h, and i. A subscript of ∞ means that rotation through any angle about that axis results in a valid symmetry operation. Note that σ_h is identical to S_0 and is often omitted.

Assignment

Reexamine the ball-and-stick models for molecules assigned to you in Table 3.2. Determine all of the symmetry elements and corresponding symmetry operations for each molecule. (Hint: At least one molecule on the list contains the symmetry element σ_h and one contains an S_3 symmetry element.)

There are a few things to keep in mind while trying to determine the symmetry elements for chemical compounds. The first is that molecules are three-dimensional objects. This means that we can tell the difference between the “front” and “back” or the “top” and “bottom” of planar molecules. For instance, the $\sigma_{v'}$ reflection of the water molecule across the yz-plane is not the same as the identity operation $\hat{E}$. Second, since atoms of the same kind (or color) are indistinguishable, you may want to number the atoms in the models in order to keep track of the results of the symmetry operations. Finally, when molecules have hexagonal rings with alternating double bonds, all of these bonds---both single and double---are equivalent (e.g. benzene and toluene). It is only the orientation of the atoms themselves that can be “seen” spectroscopically and hence needs to be considered here.

After you have determined the symmetries of the molecules, you can double-check your axis assignments. The z-axis should be the axis of highest C_n symmetry. In H_2O, there is only one C_n axis, C_2, so it is the z-axis. In CO_2, there is a C_2 axis and a C_∞ axis, so

the C_∞ axis is the z-axis. Check the other molecules to make sure that the z-axis you assigned is the one of highest symmetry. Remember that the axis of highest symmetry may not be unique.

Problem #4: Orders of Symmetry Operations

The *order* of a symmetry operation is the number of times the operation must be applied to obtain the identity operation, $\hat{E}$. More specifically, the order of a symmetry operation $\hat{A}$ is n, if n is the smallest positive integer such that $\hat{A}^n = \hat{E}$. For instance, for H_2O, the non-identity symmetry operations each have order 2. Note that an inversion always has order 2. The symmetry operation S_4 has order 4 because it must be applied four times in succession to return the molecule to its original orientation when the outside atoms are labeled. (Try it for methane!) Therefore, S_4 generates 4 symmetry operations: S_4, $S_4^2 = C_2$, S_4^3, and $S_4^4 = \hat{E}$ of orders 4, 2, 4, and 1, respectively.

Assignment

For each of the molecules assigned to you in Table 3.2, find the order of each symmetry operation. Use the description of order to aid you.

Problem #5: Symmetry Groups

You may have noticed that the set of symmetry operations forms a group under composition of operations, called the symmetry group of the molecule. The symmetry elements of a molecule can be used to determine the group to which the molecule belongs. Using Figure 3.10 and the molecule's symmetry elements, the group can be identified.

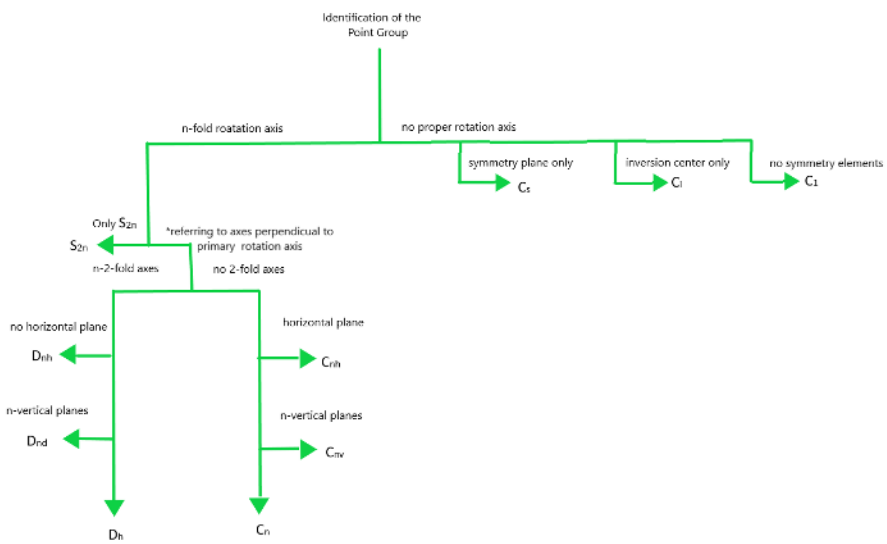

Figure 3.10: *Flow Map for identifying the symmetry group to which any molecule belongs.*

Assignment

Identify the symmetry group for each of the molecules assigned to you in Table 3.2. The order of a symmetry group is the number of operations which comprise the group. What is the order of each group? Verify by determining the group for your molecules from HyperChem and by examining the character tables in Chapter 12 of McQuarrie and Simon (Chem 110B text) or **Chemical Applications of Group Theory**, by F. A. Cotton. There are also tutorials available on to help identify the group and practice finding groups of other molecules.

Problem #6: Action of Symmetries on Coordinate Axes

Your overall goal is to identify the molecular origins of the infrared peaks observed in a spectrum of contaminated air. Since a molecule's infrared light absorption depends on how the Cartesian axes transform under the symmetry operations, the next step is to determine what happens to each of the Cartesian axes as the different symmetry operations are performed upon the molecule.

Example 3.5

In Figure 3.11 and Table 3.4, we illustrate this process for water. Note that under the identity operation, Ê, none of the axes are inverted or reversed. When the molecule is rotated about the C_2 axis, the orientation of the z-axis remains the same but the x- and y-axes are oriented in the opposite direction; each point (x,y,z) is moved to the point (-x,-y,z). In this case, the rotation is equivalent to multiplying the x and y values by -1. For any operation, -1 indicates that there is a reversal in the orientation of the axis relative to the original orientation, whereas +1 indicates that the orientation remains the same.

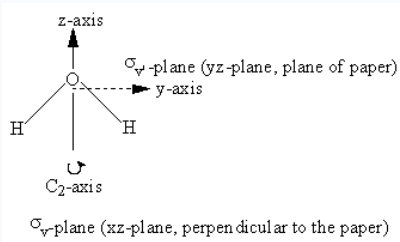

Figure 3.11: The coordinate axes and symmetry elements for water

Table 3.4: *How the axes of water transform under the symmetry operations*

H_2O	Ê	C_2	σ_v (xz)	σ_v' (yz)
X	1	-1	1	-1
Y	1	-1	-1	1
Z	1	1	1	1

Assignment

Construct similar tables for the **FIRST THREE** molecules assigned to you in Table 3.2. First, check with your TA to make sure you have drawn the Cartesian coordinate axes in the standard way for each of these molecules.

Problem #7: Action of Symmetries on Vibrational Motions

The vibrational motions of the molecule that absorb infrared radiation are the ones that transform under the symmetry operations of the molecule in the same way as do the Cartesian coordinate axes of the molecule. Therefore, we need to determine how the molecular vibrations behave under each of the symmetry operations, so that we can compare them to the transformations of the Cartesian coordinate axes.

Example 3.6

Let us examine the vibrations of water again (Figure 3.12).

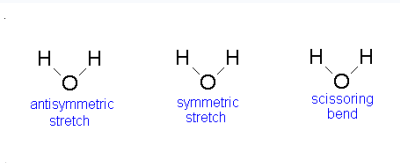

Figure 3.12: Vibrations of water

If we ask how each of the vibrations of water behaves under each of the symmetry operations, we can add more entries to Table 3.5. When we examine what happens to the vibrating molecules as the symmetry operations are performed, we are interested only in whether or not the geometrical orientation of the molecule has changed. For instance, consider the water molecule as it undergoes the asymmetric stretch. Imagine the molecule (or stop the computer program) when it reaches its most extreme position, with one “arm” extended and the other “arm” contracted. Now perform the C_2 operation (rotation by 180°) on this “distorted” molecule. Its orientation after the C_2 operation is different from its orientation before. Note that the configuration has been reversed. The fact that its new position is distinguishable from its original position is represented in Table 3 by -1.

Now let's examine the water molecule as it undergoes the bend or symmetric stretch. If we perform the C_2 operation (rotation by 180°) on this vibrating molecule, its geometric orientation is unchanged. Its new position is indistinguishable from its original position. The fact that it appears unchanged is represented in Table 3.5 by +1.

Table 3.5: *How the axes and vibrations of water transform under the symmetry operations*

H_2O	$\hat{E}$	C_2	σ_v (xz)	$\sigma_{v'}$ (yz)
X	1	-1	1	-1
Y	1	-1	-1	1
Z	1	1	1	1
bend	1	1	1	1
symmetric stretch	1	1	1	1
asymmetric stretch	1	-1	-1	1

Assignment

Construct tables as in example 3.6 for the **FIRST THREE** molecules assigned to you in Table 3.2. Examine the motions of the atoms for each different vibration. If you were to imagine the molecule (or stop the computer program) when it reaches its most extreme position, consider how that "version" of the molecular shape would behave under each of the different symmetry operations. That the molecule is indistinguishable after the symmetry operation is indicated by a 1, while a distinguishable molecule is represented by a -1. Fill in a line in your table for each vibrational motion. Note this procedure works for most, but not all simple molecules. For example, this procedure will not work for your third molecule.

Problem #8: Comparing Symmetry Operations on Axes and Vibrations

When a symmetry operation and an axis transform in the same way, the frequency associated with that symmetry operation will absorb light. By comparing the tables you generated in

Example 3.7

In the case of water, as illustrated in Table 3.5, the bending vibration transforms under the symmetry operations in the same way as does the z-axis. This is also true for the symmetric stretching motion. On the other hand, the asymmetric stretch transforms in the same way as does the y-axis. In this case, all three of the vibrational motions of water would absorb infrared light since each one of them transforms under the symmetry operations as does one of the Cartesian coordinate axes. All of the vibrations also have frequencies that are in the appropriate frequency range. We would expect the infrared spectrum of water to have three peaks corresponding to frequencies of 1840, 3587, and 3652 cm^{-1}.

Assignment

For the **FIRST THREE** molecules assigned to you in Table 3.2, use the tables you constructed in Problem #7 to determine how the vibrational motions transform under the symmetry operations. List the frequency calculated of the ones that transform as do the x-, y-, or z-axes. (You listed the frequencies you need for this problem in Problem #2.) Given that infrared spectrometers operate in the range of about 600 cm^{-1} to 4000 cm^{-1} (wavenumbers), which vibrational frequencies should you observe in the infrared spectra for your molecules? Make a rough sketch of the infrared spectrum you would expect to see for each of your molecules, labeling peaks with their frequencies. Assume that if peaks are separated by less than 25 cm^{-1}, they will not be resolved and will appear as a single peak.

Problem #9: Obtaining Infrared Spectra

You have now reached the experimental portion of this exercise. Before you can measure your contaminated air sample. You will need to measure some common air pollutants. The IR spectra that you measure for these molecules will help you decide which compound is in your air sample.

Assignment

Breathing into the sample compartment of the infrared spectrometer can generate the spectrum of water and carbon dioxide. You will then take spectra of **methyl iodide, ethylene, acetylene, trans-DCE,** and a sample of **contaminated air**, which have already been prepared in infrared cells. Do not be overly concerned if the windows on the cells appear to be hazy; they will work fine. Do you observe the predicted absorptions? If not, try multiplying all of your vibrational frequencies by 0.89 (a factor theoretical chemists recommend to compensate for over calculations). Do they come closer to what you observe now?

Operating the Bruker FTIR Spectrometer (room 3475)

1. Start the **OPUS 6.0 software** on the computer next to the instrument.
2. In the **Measure Menu,** go to the **Advance tab** to set the parameters for the experiment
   - Scans: 16 for sample and 16 for background
   - Resolution: 2.0
   - Signal Gain: 1
   - IR Data Type: Transmittance
   - Go to **Path:** and select the Chem115 directory for data storage
     - Name your sample in the **Filename:** field
3. Close the sample compartment and wait 2-3 minutes for the sample compartment to be purged of air.
4. In the **Measure Menu**, go to the **Basic tab**. Click the “Background Single Channel” button, the instrument will start scanning the background. The progress status will be shown at the bottom of the screen.
5. Open the chamber, and load your sample in. Close the chamber.
6. To acquire your sample scan. Give your sample a description under the **Basic tab**. Run the sample spectrum by clicking on the **“Sample Single Channel”** button. The progress of the scan can be seen at the bottom of the screen.

Operating the FTIR Spectrometer (room 3480)

1. Start the **OPUS 7.2 software** on the computer next to the instrument.
2. In the **Toolbar Menu,** find the **Advance Measurement tab** to set the parameters for the experiment

   - Scans: 16 for sample and 16 for background
   - Resolution: 2.0
   - Signal Gain: auto
   - IR Data Type: Transmittance
   - Go to **Path:** and select the Chem115 directory for data storage
     - Name your sample in the **Filename:** field
3. Close the sample compartment and wait 2-3 minutes for the sample compartment to be purged of air.
4. In the **Measure Menu**, go to the **Basic tab**. Click the “Background Single Channel” button, the instrument will start scanning the background. The progress status will be shown at the bottom of the screen.
5. Open the chamber, and load your sample in. Close the chamber.
6. To acquire your sample scan. Give your sample a description under the **Basic tab**. Run the sample spectrum by clicking on the **“Sample Single Channel”** button. The progress of the scan can be seen at the bottom of the screen.

2017-An Agilent 630 FTIR might be available, ask Paul

Collect a spectrum of water and carbon dioxide:

1. Open the sample compartment.
2. Exhale into the sample compartment.

3. Collect the spectrum by clicking on the "**Sample Single Channel**" button on the **Basic Tab**.

Collect spectra of the four reference samples and your unknown:

1. Open the sample compartment.
2. Put the cell holder with the cell into the sample compartment. Align the holes in the bottom of the holder with the holes in the plate in the bottom of the compartment.
3. Wait 2-3 minutes for the compartment to purge.
4. Name the file.
5. Acquire the spectrum by pressing **Sample Single Channel** button.

To pick peaks: (This procedure may not work depending on the instrument you are using.)

Click on the peak picking icon, or select **Peak Picking** from the **Evaluate** menu.

- The peak picking screen will show up. Choose the **Interactive mode**. Sliding the Threshold square up or down so that all the peaks are below it.
- Each peak above the threshold will be labeled with its frequency and the peak list will be written to a report file that you must save by selecting Save Report from the File pulldown menu.
- You can annotate the plot by selecting the annotator options from the Tools pulldown menu.
- The file path and name are the default title for your plot. You can change this by selecting Title under the Display pulldown menu.
- Once you have picked your peaks and annotated your plot, save the window as before by selecting Save Sample from the File pulldown menu.
- Select Plot from the File pulldown menu. In the Plot menu select the window you wish to print. Check that the size of the plot is correct and then click on the "plot". This will take 1-2 minutes. Be patient. You can then select another window to print or click on the "done" button.

Problem #10: Identifying the Pollutant

As a reminder: The sample was taken in a location 20 m away from an open farm field where strawberries were growing. In the field on one side, there were cows grazing; on the other side, there was a natural gas pumping station. Across the street, there was a gas station with an auto repair shop specializing in air conditioner repair and a dry cleaner. This information narrows the list of suspected chemicals to six. Test your air sample and determine the culprit. The spectrometer automatically purges the cell cavity with N_2, which is transparent in infrared. This removes water and carbon dioxide, which exhibit strong IR absorptions, so you should be able to see the pollutant's spectrum relatively easily.

Assignment

Examine the spectrum from your sample of the unknown, contaminated air. Note that it is a very different spectrum from that of carbon dioxide and water, which is shown in Figure 3.1. Which of the chemicals that you studied is responsible for this spectrum? To which potential polluter described above would you attribute this pollution?

Post-Lab Questions

1. Include all of the information recorded during the assignments.

2. Include all of the FTIR spectra

3. Identify the pollutant and predict the source of the pollutant.

4. Explain the process used to identify the pollutant.

Outside Links

- http://symmetry.otterbein.edu/index.html
- Point group symmetry character tables

References

1. McQuarrie, D. A.; Simon, J. D. *Physical Chemistry: A Molecular Approach*; University Science Books: Sausalito, CA, 1997.
2. Cotton, F. A. *Chemical Applications of Group Theory*; Wiley: New York, NY, 1990.
3. Drago, R. S. *Physical Methods in Chemistry*; W. B. Saunders: Philadelphia, PA, 1977.
4. Skoog, D. A.; Holler, F. J.; Crouch, S. R. Principles of Instrumental Analysis, Seventh Edition; Cengage Learning: Boston, MA, 2016.

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Lab 4: Molecular Fluorescence

goals

1. Students should have an in-depth understanding of fluorescence after the completion of this lab exercise.
2. Students should be able to optimize the scan parameters to achieve the best emission and excitation spectra.
3. Students should be able to extract key information from the emission and excitation spectra and determine the unknown concentration of a compound.

Introduction

When a molecule absorbs a photon in the ultraviolet or visible (UV VIS) region (180 - 780 nm), an electronic transition occurs within the molecule. This transition involves moving an electron from the singlet ground state to a singlet excited state. After excitation, the molecule will undergo de-excitation to regain its ground state electronic configuration. De-excitation of the molecule can occur in three distinct ways: by collisional deactivation (external conversion), fluorescence, or phosphorescence (Figure 4.1).

The first relaxation mechanism, **collisional deactivation**, occurs when the excited molecule transfers its excess energy to molecules with which it collides without photon emission. The second mechanism, **fluorescence**, involves the electron returning from the excited singlet state to the ground singlet state accompanied by the emission of a photon of lower energy (longer wavelength) than the absorbed photon; the energy loss is due to vibrational relaxation while in the excited state. When fluorescence is favored, it occurs within about 10^{-8} seconds after absorption. For reference, a picosecond is 10^{-9} seconds. **Phosphorescence**, the third route for de-excitation, occurs when the excited electron enters the lowest triplet state from the excited singlet state by intersystem crossing and subsequently emits a photon in returning from the lowest triplet state to the singlet ground state. These excited singlet-triplet and triplet- ground singlet transitions involve electron reversal (Figure 4.2), which is a low probability occurrence. Thus, the time between absorption and phosphorescence can be from 10^{-2} seconds to several minutes.

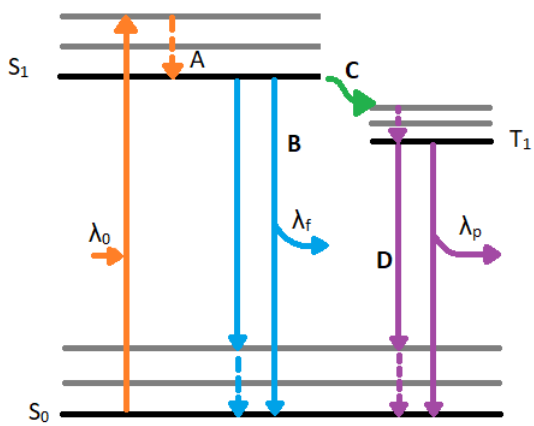

 Figure 4.1: *Jablonski diagram displaying excitation and de-excitation pathways of a molecule. λ_0 indicates the incident wavelength that is absorbed by the molecule. Once the molecule reached the excited singlet state (S1) from the ground state singlet (S_0), is can experience vibrational relaxation as seen in A. After relaxing to the lowest energy excited state, a molecule can de-excited by fluorescence as seen in path B. The fluorescence path emits a wavelength (λf) which is longer than the absorbed wavelength (λ0). The alternative, unfavored path moves along path C which involves intersystem crossing. Intersystem crossing involves a conversion from an excited singlet state(S1) to an excited triplet state (T1). After reaching the triplet state and experiencing vibrational relaxing, the molecule will phosphoresce by emitting an even shorter wavelength (λp) to reach the ground state singlet (S_0).*

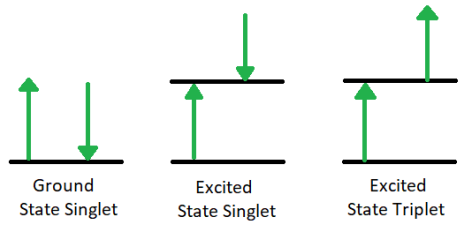

 Figure 4.2: *Transition from Ground Singlet (A) to Excited Singlet (B) to Excited Triplet (C).*

Since our interest involves fluorescence, a typical experimental arrangement is shown in Figure 4.3. This is used in either of two distinct ways. The first, which holds the excitation monochromator M1 fixed, and varies the emission monochromator M2, yields an emission spectrum (the wavelength distribution of light emitted by the excited singlet state). When generating an emission section, the intensity of the transmitted light (I) is measured and compared to the transmitted light's measured wavelength (λ_f). Alternately, M2 can be fixed and M1 varied; this procedure produces an excitation spectrum (a plot of fluorescence intensity as a function of excitation wavelength). The excitation spectrum can be produced by measuring the intensity of the transmitted light (I) and comparing it to the measured wavelength of the incident light (λ_0). Often, the excitation spectrum of a pure compound has exactly the same profile as the absorption spectrum (Figure 4.1). The principles described by Figure 4.1 can be observed in the excitation and emission spectra. An example of these spectra can be seen in Figure 4.4.

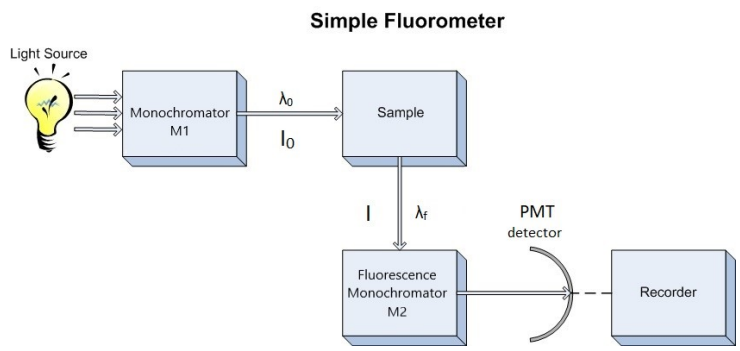

 Figure 4.3: *Schematic Diagram of Fluorometer. M1 = excitation monochromator, M2 emission monochromator, Photo Multiplier Tube detector.*

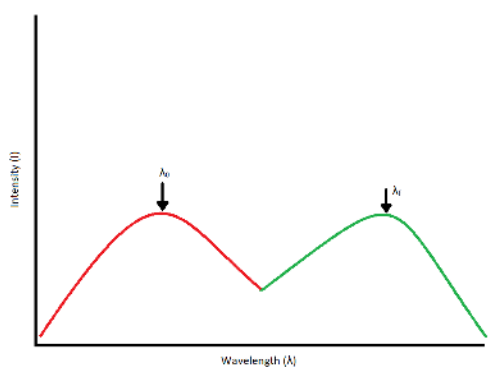

 Figure 4.4*: Excitation/Absorbance Spectrum (Red) coupled with an emission spectrum (Green). Each spectrum peaks at its respective wavelength. Excitation and emission spectra should overlap and be mirror images of each other.*

Coupling the above techniques with a relationship between fluorescence intensity and concentration would be exceptionally useful. Such a relationship can be derived from Beer's Law, which states that the fraction of light intensity transmitted by a sample is

$$\frac{I}{I_o} = 10^{-\epsilon bc} \tag{Lab 4.1}$$

where,

- I is the transmitted light intensity,
- I_0 is the incident intensity,
- ε is the molar absorptivity at a given wavelength in units of $L*mol^{-1}*cm^{-1}$,
- b is the cell path in centimeters, and
- c is the concentration in moles per liter.

The fraction of absorbed light is:

$$1 - \frac{I}{I_o} = 1 - 10^{-\epsilon bc} \tag{Lab 4.2}$$

from which it follows that the absolute amount of absorbed light is equal to:

$$I_o - I = I_o - I_o\left(10^{-\epsilon bc}\right) \tag{Lab 4.3}$$

The fluorescence intensity, F, is proportional to the amount of light absorbed and fluorescence quantum yield, Φ. Thus,

$$F = kI_o\phi\left[1 - \left(10^{-\epsilon bc}\right)\right] \tag{Lab 4.4}$$

where k is a proportionality constant. **If dilute solutions are used**, so that less than 2% of the excitation energy is absorbed, the exponential term in Equation Lab 4.4 can be approximated by the first two terms in the corresponding Taylor series expansion

$$e^x \approx 1 + x \tag{Lab 4.5}$$

Then,

$$F = kI_o\phi\epsilon bc \tag{Lab 4.6}$$

hence fluorescence intensity is **proportional to concentration, but only at low concentration:**

$$F \propto c \tag{Lab 4.7}$$

Instrumentation

In this experiment, two instruments will be used. First, the Agilent HP8453 UV-Vis spectrometer to measure optimal excitation wavelength. You used this instrument in CHE 105, so you may remember how to use it. For the second portion of this experiment, the Varian Eclipse Fluorescence Spectrometer (Figure 4.5) will be used. This instrument is computer-controlled through software running on Windows XP. Detailed operating instructions for the Eclipse will be in the procedure section.

Figure 4.5: UCD's Varian Eclipse Fluorescence Spectrometer

Experimental Procedure

This experiment involves the analysis of vitamin B_2, riboflavin, by measuring its native fluorescence. Riboflavin (Figure 4.6) is a common water-soluble vitamin found in eggs, milk, and other foods, that strongly fluoresces and is very sensitive to light. Its two predominant irradiation decomposition products are lumichrome and lumiflavin, which are themselves highly fluorescent.

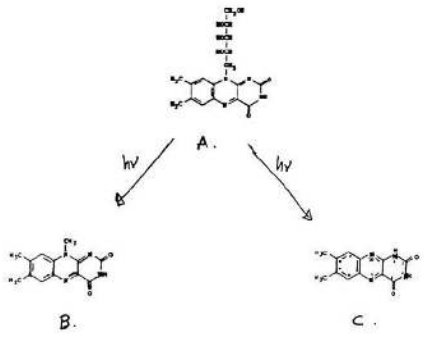

Figure 4.6: Riboflavin (A) and the two most predominant irradiation decomposition products, lumiflavin (B) and lumichrome (C).

The analysis method requires working with riboflavin samples at concentrations well below the part per million (ppm) level. Therefore, it is extremely important that the utmost care is exercised in cleaning and handling all of the glassware and solutions during this experiment.

In the first lab period, the standard and unknown solutions mentioned below should be prepared. Later during this period, you should consult the T.A. about operating the UV VIS spectrophotometer as well as the fluorescence spectrometer. You should also obtain the absorption spectra of riboflavin and perform a practice run on the fluorescence spectrometer.

The second lab period should be used to complete the experiment and Part II of the unknown analysis.

Stock Solutions

1. Obtain the 100 ppm stock standard solution of Riboflavin from the refrigerator or from the T.A.
2. Allow the solution to sit on the counter for a few minutes to equilibrate to room temperature. This is to ensure consistent volume measurements.
3. Deliver 1 mL of the stock solution to a 10 mL volumetric flask and dilute using deionized water. This is your 10 ppm solution.
4. Using a volumetric pipet, deliver 1.00 mL of the 10 ppm solution to a 100 mL volumetric flask, and dilute using deionized water. This is your 100 part per billion (ppb) stock solution.
5. Fill in Table 4.1 with the correct amounts of 100 ppb riboflavin to make the remainder of your standards. Check the table with your TA before you begin the experiment.
6. Make the solutions 2-6 from the table and dilute with deionized water. Make certain that your glassware is clean and rinsed with deionized water.

***Table 4.1**: Standard Riboflavin Solutions*

Solution #	mL of 100 ppb sol.	Dilute to (mL)	Rib. Conc (ppb)
2		50	2
3		50	10
4		50	20
5		50	40
6		50	50

Unknown Solution

The dry milk sample should be reconstituted as indicated on the jar. Record your work (this is a quantitative experiment). The milk samples require special treatment in order to remove interfering fats and proteins, which would otherwise make analysis difficult or impossible. The procedure to accomplish this is outlined below.

1. Find the dry milk sample on the shelves above the weigh station.
2. Reconstitute the dry milk sample by following the directions listed on the sample jar.
3. Take 25 mL of milk sample and add exactly 75 mL of a solution consisting of equal parts 3M HAc and 3M NaCl. Do not use a volumetric flask for this. It is too difficult to stir the solution. Use your 25 mL pipet and a 250 or 400 mL beaker.
4. Stir for 20 minutes.
5. Filter the solution using a Buchner funnel and the vacuum line. Make sure you have a trap between the filtering flask and the house vacuum line.
6. Take a 5 mL aliquot of the filtrate and dilute to 100 mL with deionized water in a volumetric flask. This solution will be used for your analytical measurements.

Instrumental

Part 1: Approximate Excitation Wavelength

The instrument you'll be using in this portion of the experiment is an Agilent HP8453 UV-Vis spectrometer.

The first step in conducting the analysis is to determine the optimum excitation wavelength for riboflavin. When no prior information is available, as in this case, the absorption spectrum can be used to approximate the best excitation wavelength.

Look near the Agilent HP8453 UV-Vis spectrometer for a pack of cuvettes. If you cannot find a pack, ask your T.A. for a new pack. Use the 10 ppm solution to obtain an absorption spectrum of riboflavin from 280 to 600 nm. Use distilled water as your reference solution. If you do not recall how to acquire an absorbance spectrum, check with your TA for instructions. The wavelength of maximum absorbance should be used as an initial excitation wavelength. There may be more than one absorbance peak.

Print the absorbance spectrum

Part 2: Actual Excitation and Emission Maxima

In this part of the experiment, you will determine the optimal instrument operation parameters. Using the approximate excitation wavelengths determined above from the UV-Vis absorbance spectrum, obtain two emission spectra. The emission maximum observed in the two emission spectra will then be used to collect an excitation spectrum to "refine" the choice of excitation wavelength. **You will see that the fluorescence excitation spectrum closely follows the absorbance spectrum.**

You should collect the following spectra:

1. Two emission spectra of the 50 ppb standard solution will be taken. Collect the first spectrum from the first excitation wavelength (~370 to 700 nm). Collect the second spectrum from the second excitation wavelength (~440 to 700 nm). The two approximate excitation wavelengths determined in part 1 should be used to determine the wavelength ranges.
2. Two excitation spectra. One spectrum of the 50 ppb standard and one spectrum of the milk sample you prepared. (280 to ~500nm) Select the scan maximum by taking the larger of the two emission wavelength and subtracting 30 nm as described in step 5 of the "Using the Varian Eclipse Fluorimeter."
3. Print all of the spectra from this section.

Using the Varian Eclipse Fluorimeter

- Ask your TA for the necessary Cuvette for the instrument.

Emission Spectrum

1. Power up the Eclipse with the switch on the front panel, if necessary. The dim light on the front will become green when ready.
2. If the computer is not logged in, log into "Fluorimeter" by clicking on its icon.
3. Double click on the **Scan** icon on the desktop THEN Click on the **Setup** button.
4. Set the excitation wavelength to either of the absorption maxima in the UV-Vis spectrum
5. Set the scan limits. The starting wavelength for the **emission spectrum** should be 30 nm above the excitation wavelength to avoid Rayleigh scattering. The upper wavelength should be 700nm.
6. The scan speed should be set to **Slow** (120 nm/minute) and both slits should be set to 10nm.
7. Click on the **Reports** tab. Set the **Threshold** value to 5. Click the OK button.
8. Click on the green traffic light icon to start the scan.

9. A window will appear where you can name your sample. Put in your desired name and click OK.
10. When the scan is complete, print the spectrum by clicking on the **Print** button.

Excitation Spectrum

1. Obtain **excitation spectrum** by repeating the above steps, start by clicking the **Setup** button. Click on the box that says excitation.
2. Set the emission wavelength to the value you determined above.
3. Record the excitation spectrum from 280nm to about 30nm below the emission wavelength.
4. Collect excitation spectra under the identical conditions for the milk sample you prepared.

Part 3: Standard Curve

At two excitation wavelengths (both ~370 and ~440 nm) you determine the exact wavelengths to use.

1. Double click the **Simple Reads** icon on the desktop.
2. Click on the **Setup** button, and set the emission wavelength and excitation wavelength in the window.
3. Set the excitation and emission slits at 10 nm, and the Average Time at 5 seconds.
4. Click on **Read** to begin data acquisition.
5. Change the excitation wavelength and repeat the procedure.
6. Do this for all your standards, and repeat three times for the unknown sample.
7. Print the data files when you are done.
8. When you are done with the fluorimeter, exit the programs, switch out the instrument, and remember to sign the logbook.
9. Create a standard curve in Excel or Google sheets to determine the concentration of Riboflavin in your unknown solution.

Post-Lab Questions

1. Include all spectra generated during this lab.
2. Include the calibration curve you created in excel.
3. Indicate the calculated concentration of Riboflavin in the milk sample.
4. Describe the relationship between the emission and excitation spectra that you obtained. Are they mirror images?

References

1. Skoog, D. A.; Holler, F. J.; Crouch, S. R. Principles of Instrumental Analysis, Seventh Edition; Cengage Learning: Boston, MA, 2016.

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Lab 5: Gas Chromatography/Mass Spectrometry (GC/MS)

Goals

1. Students should develop an understanding of gas-liquid partitioning.
2. Students should gain the skill of using the mass spectra data library to determine the components in a mixture.

Introduction

Similar to the CHE 105, you will be responsible for separating the components of a mixture by comparing the results to standard solutions. This lab has an added challenge of first identifying what the contents of those standards are before being drawing conclusions about the mixtures. The analysis of different unknown mixtures will be performed via high resolution capillary gas chromatography (GC) coupled with an ion trap detector (ITD). The ITD is a variation of a quadrupole mass spectrometer and is designed to function specifically as a GC detector. Due to the design variances of the ITD compared to a true quadrupole mass spectrometer, the ITD mass spectrum of an organic compound may not be identical (but should be very similar) to its classical mass spectrum measured by an electron impact (EI) detector. These classical spectra are used in the National Bureau of Standards library of mass spectra for comparison differences. In this lab, the ITD spectrum you measure of the unknown will be compared to the EI spectra of several different classes of compounds found in the library. Thus, the characteristic features of a mass spectrum for a given class may be recognized, and the chemical structure determined. The NIST 2005 library, which is a software feature of the GC/MS data acquisition system, will be utilized to confirm the identification of the components of the unknown.

Gas chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one being a stationary bed of large surface area, and the other a gas that percolates through the stationary bed. When the stationary phase is a solid, the separation process is more precisely called gas-solid chromatography. This technique is generally used to separate volatile compounds in a gaseous solution. The more common technique (which will be used in this experiment) is gas-liquid chromatography (GLC) in which the stationary phase is a porous solid covered with an absorbing liquid. GLC is used to separate a wide variety of organic compounds. The basic requirements for GLC are that the sample be volatile and that it does not decompose in the vaporization process. Since the vaporization occurs in an inert atmosphere, decomposition of the sample is generally not a problem.

Separation of a mixture into its components depends on the solubility differences of the sample vapor in a liquid (the stationary phase). The stationary phase is coated in a thin layer on solid particles (solid support) of large surface area and then packed uniformly into a column. A constant flow of the carrier gas passes through the column and transports solute molecules in the gas phase. The column is wound to fit inside an oven for precise temperature control.

A sample of the analyte is introduced by syringe injection into the heated injector tube, where it is vaporized and mixed with a carrier gas. As the sample vapor is carried through the column by the carrier gas, the analyte partitions between the gas and liquid phases according to the analyte components' solubility in the liquid at the column operating temperature. This equilibrium partitioning continues as the sample is moved through the column by the carrier gas. The rate at which the sample travels through the column is determined by the sample solubility in the stationary phase, the carrier gas flow rate, and the temperature. Each component travels at a characteristic rate, and if the column has sufficient length and resolving power, the sample will be completely separated by the time it reaches the detector.

The detector located at the column exit is the ITD mass spectrometer. It records the total number of ions entering the mass analyzer from the column. The chromatogram produced is called the total ion chromatogram. Each point in the chromatogram is a mass spectrum.

Each component is identified by comparing its "retention time", the length of time that it remains in the column, to that of a standard. The retention time of a vapor depends on the column temperature limits and ramp rate, the column length, type of stationary phase, and carrier gas velocity. If these variables are kept constant, the retention time of a component may be tentatively identified by comparison to the retention time of a known standard run under identical operating conditions.

If the response of the detector is linear, the area under a peak accurately represents the quantity of the component present. If it is not, calibration for detector response to the types of components expected to be in the analyte yields a set of response factors which convert the reported area percentages to quantitative weight percentages.

For a given gas chromatography column, the van Deemter theory is useful for determining the flow rate, which gives optimum efficiency at a given column temperature for a particular compound. The van Deemter equation is:

$$HETP = A + \frac{B}{v} + Cv \tag{5.1}$$

where A, B, and C are constants and v is the carrier gas flow rate. HETP is the "height equivalent to a theoretical plate," and results from the treatment of gas chromatographic separations in terms of repeated equilibrations between a moving and a stationary phase. HETP is calculated in equation 5.2 for a particular gas flow rate. It is calculated from the total number of theoretical plates (N) and column length (L).

$$HETP = \frac{L}{N} \tag{5.2}$$

Where,

$$N = 16\left[\frac{t_R}{w}\right]^2 \tag{5.3}$$

The retention of the component is t_R and w is the width of the elution peak at its base. The first term in the van Deemter equation accounts for eddy diffusion, the second term accounts for molecular diffusion, and the third term accounts for non-equilibrium effects due to flow of the mobile phase. For a particular column at a constant temperature, the optimum carrier gas flow rate is that for which the HETP is a minimum. By measuring the HETP at several linear gas velocities (flow rates), the parameters A, B, and C in eq. (1) can be determined and the optimum velocity defined.

If the sample contains materials with a wide range of boiling points, separation of all components isothermally is not practical. When the column is operated at low temperatures, the more volatile components will be distributed between the gas and liquid phases and will pass rapidly through the column, giving sharp, well-resolved peaks. The high-boiling components, however, will remain dissolved in the stationary phase and will be eluted very slowly, if at all. Since the vapor pressure of the latter solutes is low, partitioning will occur over broad bands of stationary phase, resulting in broad, poorly resolved peaks.

If the column is operated at a temperature which gives well-defined peaks for the less volatile components, the low boiling fraction will pass through the column with very little partitioning into the liquid phase. As a result, it will appear as one or two sharp, poorly resolved peaks, often with retention volumes approaching the dead space of the column.

By utilizing temperature programming, all the compounds can be eluted at temperatures approximating the ideal temperature for separation from adjacent solutes. By employing a low initial temperature, the low boiling components will be distributed between both phases in the column and will appear at the detector as sharp, well-resolved bands. The higher boiling fractions will remain 'frozen' at the injection point. As the column temperature is raised, the vapor pressure of the less volatile components increases and they distribute themselves between the two phases. As a result, they move down as well-defined bands, eluting at characteristic temperatures. By careful choice of the temperature ramp rate and carrier gas flow rate, each component can be eluted at a temperature approximating the optimum for separation from adjacent solutes. Although the resolution of closely spaced peaks cannot be improved over that at a single optimum temperature, the resolution of widely spaced peaks can be improved considerably.

Gas Chromatography / Mass Spectrometry

The experiment concerns the actual identification of an unknown using GC/MS. The system you will be using is menu driven. Your TA will show you how to set up a file and acquire data.

The power of the GC/MS technique comes from the fact that not only are components of a mixture separated and detected quantitatively, but the detector (the ITD) also provides information concerning the structure of each of the components. Therefore, compounds can be identified not only by comparing the retention time to a standard, as in conventional GC but also by its mass spectrum. An unknown can also be identified in most cases based solely on its mass spectrum, eliminating the need to run standards for retention time data. Therefore, it is not necessary to know what you are looking for, as in the case of GC.

The chromatography for GC and GC/MS is identical in theory. However, the column used in the GC/MS experiment is a capillary column as opposed to the packed column used in the GC experiment done in Chemistry 105. A capillary column is simply a long

tube made of glass with a small internal diameter. For this experiment, a 30 cm column with an internal diameter of 0.25 mm is used. The stationary phase is actually bonded to the interior of the glass capillary, eliminating the need for packing a solid support in the column. Different columns may have bonded phases of different characteristics depending on the type of separation to be carried out.

After the components of a mixture are separated in the column, they reach the ion trap detector as pure compounds (if the separation was successful). The compounds are ionized by electron impact (EI) by passing the stream of gas over a beam of electrons accelerated to an energy of 70 eV. This energy is used to form ions by stripping away an electron and may break some of the bonds of the compound. Differing populations of the ions will have differing amounts of internal energy. Some of the molecules will become ionized but will not fragment, forming a "parent ion". A parent ion, or molecular ion, has the same mass in atomic mass units as the neutral molecule (it differs by only the mass of an electron). It is the highest mass peak in the spectrum. Many of the ions formed may have sufficient internal energy to fragment, forming a smaller mass ion and a neutral. (The neutrals formed are not detected. Only ions are detected). By using the same energy electrons to ionize the compounds, the resulting mass spectra are highly reproducible, not only on a given instrument but on other instruments using 70 eV electron impact ionization. In this way, libraries of mass spectral data have been generated, so that an unknown can be identified by searching through and matching the mass spectra.

Different classes of compounds have some fragmentation characteristics that can be used to help identify unknown compounds. For example, compounds with many strong bonds, such as aromatic compounds, may be less likely to fragment. These compounds are characterized by mass spectra which are dominated by a single peak, the molecular ion. Straight chain hydrocarbons, however, fragment much more easily and may show little or no abundance of the molecular ion in their mass spectra. Attached to this manual is a reference describing characteristic fragmentations of various classes of compounds. An excellent reference that describes the fragmentation of classes of compounds is "Interpretation of Mass Spectra" by Fred McLafferty. Another is "Spectrometric Identification of Organic Compounds", by Silverstein, Bassler and Morrill. You should use these references, along with your text, to help explain the mass spectra of your unknown compound(s).

Instrumentation

A basic chromatography instrument consists of the following:

1. A sample port or injector for introduction and vaporization of the sample;
2. A separating column, consisting of metal tubing packed with a solid material coated with a stationary absorbing liquid;
3. A carrier gas, usually N_2 or He, to sweep the sample through the column;
4. Flow control equipment to maintain a constant flow of carrier gas through the column;
5. The detector for measuring the quantity of a separated component;
6. Ovens and heaters for temperature control of the column, detector, and injector;
7. An integrator or integrator/strip chart recorder combination to provide a permanent record of the analysis.

Figure 5.1*: Retired Ion Trap GC/MS instrument*

The instrument used in this experiment is an Agilent 6890N GC/MS (low-resolution mass spectrometer) pictured in Figure 5.2. The mass spectrometer and GC are controlled by *Chemstation* software on the computer. This instrument can be found in room 3475.

Figure 5.2: GC/MS Instrument used in this experiment.

Experimental Procedure

No sample preparation is required for this experiment. You will be provided with 6 standards.

- Standard 1: straight alkane
- Standard 2: aromatic halide
- Standard 3: alkyl halide
- Standard 4: aromatic ether
- Standard 5: aromatic halide
- Standard 6: alkane

You will also be provided with **three** unknowns that are mixtures of two or more of the above compounds. You will be required to identify the unknowns provided based on both the mass spectra and retention times of the peaks.

6890N GCMS Operation Procedures

1. Enter the console and locate the GC/MSD icon located in the upper left corner of the desktop. Open the GC/MSD by clicking on the icon (*Figure 5.3*) if *Chemstation* is not already running.
2. Allow a few minutes for the software to initialize. The green bar along the bottom of the *Chemstation* window indicates action completion. When prompted, Click OK to confirm firmware configuration. Once the software initialization is complete, the green action bar will read “Welcome to the Enhanced Agilent MSD *Chemstation*”. The next step is to load the desired method if it is not already loaded by accessing the method tab. The methods are found following the tree from D: drive > MSDCHEM > 1 >Methods. Load CHE_115_ALS_Default_Split100_9.5MIN.M. The currently loaded method is displayed in the blue bar across the top of the *Chemstation* window. For example, the method “CHE_115_ALS_Default_Split100_9.5MIN.M” is loaded as seen in Figure 5.4. Choose your method and load, loading takes a few minutes. The green action bar will confirm when the method loading is complete. Have your TA check the method to make sure students did not save changes to it accidentally.
3. Make sure the ALS tower is in place. Check the liquid level in your vial standards, they should contain at least half full.
4. Now it is time to set up the analysis by clicking on the green arrow. A new window will open up (Figure 5.5). First, set the user (or the group) name. Next, set your data pathway by following the tree from D: drive > MSDCHEM > 1 > DATA > 2014 > CHE 115 > the appropriate folder (Figure 5.6). Name your file making sure to retain the “.D” suffix. The recommended file naming format is using the date and underscores such as “2014.01.02_standard1”. Add the other information: user, sample name, info. It is imperative that you enter the correct Vial #. Standard 1 is in position 1, 2 in 2... Unknown (UK) 1 is in position 10, UK2 in 15, UK3 in 20.
5. Click “OK and Run Method” and the GC/MS will begin the automation.

 Figure 5.3: *Icon on the desktop to open the software.*

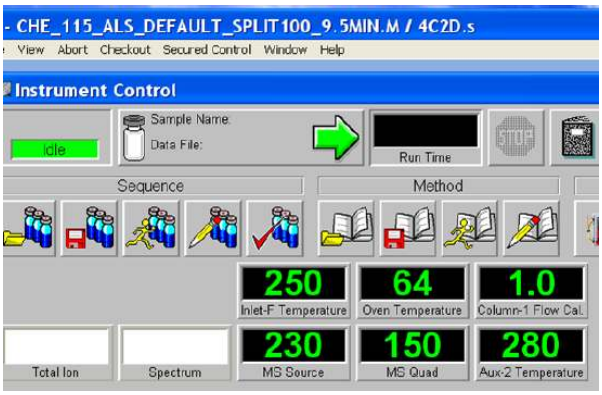

 ***Figure 5.4**: Main operating screen on* Chemstation*; note the method name on the top of the window.*

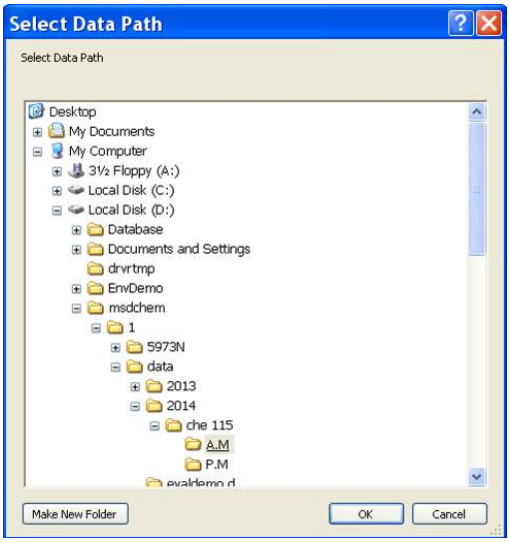

 ***Figure 5.5:** File tree search window.*

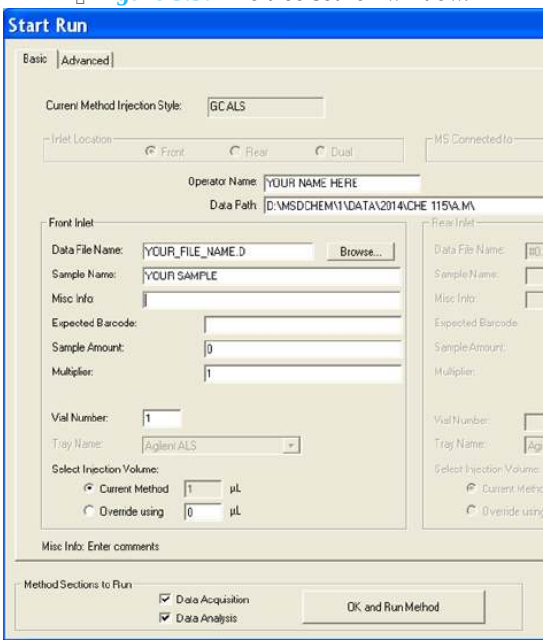

 ***Figure 5.6:** File naming window.*

6. Once the run begins, you will be prompted to override the solvent delay. **NEVER OVERRIDE THE SOLVENT DELAY**, doing so will drastically shorten the lifetime of the filaments inside the Mass Spec, potentially render the instrument inoperable, and likely result in increased chemistry laboratory fees.

7. As your run progresses and the approximate 3-minute solvent delay has passed, you should see peaks appear in the smaller subwindows. It is possible to begin data analysis by taking a snapshot in the data analysis program. If not already open, launch the Enhanced Data Analysis program by clicking on the bottom icon seen in Figure 5.3. Under the file tab of the Enhanced Data Analysis window, choose 'take a snapshot'. The snapshot of the current run is then displayed. You can zoom in on a peak or region by clicking and dragging the cursor over the 'square' area desired to be blown up. Within the chromatogram subwindow, the cursor becomes a vertical line. Double right-clicking on the peak of interest brings up the mass spectrum in another subwindow below the chromatogram (Figure 5.5). Different regions of the peak can give varying information; therefore, it is important to click in several places within the same peak to obtain the best data. It is possible to take multiple snapshots during a run; however, once the last minute of the run has begun, the snapshot option becomes unavailable.
8. There is useful data that can be obtained from these sub-windows. Notice the peak's retention time is displayed at the top of the mass spectrum sub window. The abundance is observed as the X-axis of both the chromatogram and mass spectrum. These abundances should be in the $1x10^5$- $1x10^6$ range. Abundances of $1x10^7$ and greater are too large and indicate a possible over-injection, too concentrated of a sample, and/or wrong method. Extremely large abundances will shorten the filament lifetime. Conversely, abundances less than $1x10^5$ are also not desired and indicate injector problems, diluted sample, and/or the wrong method. Report abundances that are too high or too low to your TA immediately.
9. In the mass spectrum sub-window, a library comparison search can be performed by double right-clicking inside the lower sub-window. The mass spectrum of the top library hit is displayed under your sample's mass spectrum for ease of comparison along with a small table of compound identity, mass values, and other data. It is important to visually compare the two, often similar spectra with analogous peak passes are comparable but close attention to detail will discriminate them apart. There is also a confidence measure that is listed under the 'Qual' tab, the closer to 100 the value is the greater the confidence.
10. Make sure to analyze the entire spectrum to ensure your snapshot included every peak. Snapshot data are typically stored in a 'snapshot' folder located on underneath the data file located on the same data path level. The standards should be single components, while the unknowns are mixtures. Standards will become contaminated as the course progresses; you should consult with your TA and focus on the largest peak. If several peaks are observed of the relative same abundance, especially in the unknowns, then fresh samples may be required. Again, consult with your TA.
11. Once your run has completed, you can begin the next sample. The instrument will require a few minutes to return the GC oven temperature back to the 40 °C starting point. During this time, you can work up your data and print your spectra. Return to #4 and begin the next analysis.

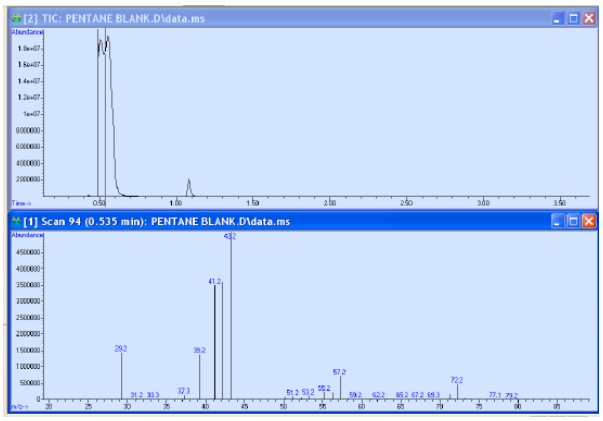

 Figure 5.7: *Example of an appropriate result.*

Post-Lab Questions

1. Include the following data Reports:

I. Standards

1. A good chromatogram of each standard sample.
2. A mass spectrum of each standard, that is, 6 total (with labeled peaks).

3. A library print out of each known, that is 6 total.

II. Unknowns (mixture containing 2 or more of the known standards)

I. Chromatographs and mass spectra of all 3 unknown mixtures.

II. Identify each unknown and report.

2. Identify the retention time, compound category, the compound matched in the library, and boiling points of the compounds.

3. Discuss the differences between the observed retention time values and the known boiling points. How can molecular structure affect these characteristics?

4. Identify the retention time(s) observed when performing the experiment on the unknown mixtures. Identify which compounds are present in each unknown.

5. Discuss any differences you observed in the mass spectra of the compounds.

References

1. McLafferty, F. *Interpretation of Mass Spectra*; 3rd ed.; University Science Books: Mill Valley, CA, 1980.
2. Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Interpretation of Organic Compounds*; 4th ed.; Wiley: New York, NY, 1981.
3. Skoog, D. A.; Holler, F. J.; Nieman, T. A. *Principles of Instrumental Analysis*, Fifth Edition; Harcourt Brace: Philadelphia, 1998; 591-621.

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Lab 6: Capillary Electrophoresis

Definition

1. Students should develop an understanding of the similarities between electrophoretic methods.
2. Students should develop an understanding of how Electroosmotic Flow and Electrophoretic Mobility effect retention time.
3. Students should use CE data to complement data for gas or liquid chromatography.
4. Students should know how the addition of a component affects partitioning.

Introduction

Electrophoresis is a class of separation techniques in which we separate analytes by their ability to move through a conductive medium in response to an applied electric field. This conductive medium is usually an aqueous buffer. Generally, when the electric field is applied, cations migrate toward the electric field's negatively charged cathode. Anions migrate toward the positively charged anode and neutral species do not experience the electrical field and remain stationary. Ions with larger charge-to-size ratios —which favors ions of larger charge and of smaller size—migrate at a faster rate than ions with smaller charge-to-size ratios. High-Performance Liquid Chromatography (HPLC) or Gas Chromatography (GC) use liquid and gas respectively to force the sample through a column, but electrophoresis utilizes an electric field.

In capillary electrophoresis (CE), the conducting buffer is retained within a capillary tube whose inner diameter is typically 25–75 µm. Samples are introduced into one end of the capillary tube. As the sample migrates through the capillary its components separate and elute from the column at different times. When a component in the sample migrates at a lower rate, the component will be retained and will have a longer retention time. When a sample migrates at a faster rate, the retention time is shorter. Similar to HPLC and GC, the retention times can be used to identify components and determine the amount of component that was in the original sample. In capillary electrophoresis, the movement of ions through the capillary is defined by the ion's electrophoretic mobility. A basic schematic of the instrument can be seen in Figure 6.1.

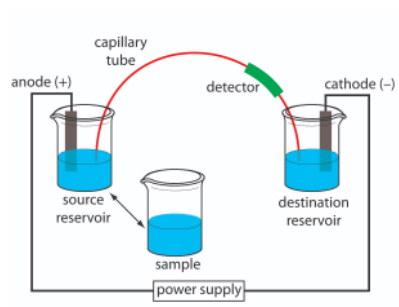

***Figure 6.1**: Schematic diagram of the basic instrumentation for capillary electrophoresis. The sample and the source reservoir are switched when making injections.*

Electrophoretic Mobility

The electrophoretic mobility (μ_e) describes the ability of a component to migrate. The electrophoretic mobility (equation 6.1) of an object in an applied electric field is determined by the charge on the molecule divided by the frictional coefficient (f). Stokes' Law (Equation 6.2) gives the frictional coefficient of the molecule (f). This value is dependent on size and shape of the molecule as well as the viscosity of the solvent(η).

$$\mu_e = \frac{q}{6\pi\eta r} \tag{Lab 6.1}$$

$$f = 6\pi\eta r \tag{Lab 6.2}$$

The velocity of the particle in an applied field is described by equation 6.3. The variable E is the applied electric field. The velocity describes the rate at which the components are moving through the capillary.

$$\nu = \mu_e E \tag{Lab 6.3}$$

Gel Electrophoresis

One of the most common forms of electrophoresis that students use is slab gel electrophoresis. In a slab gel, he conducting buffer is retained within a porous gel of agarose or polyacrylamide. Slabs are formed by pouring the gel between two glass plates separated by spacers. Typical thicknesses are 0.25–1 mm. Gel electrophoresis is an important technique in biochemistry where it is frequently used for separating DNA fragments and proteins. Although it is a powerful tool for the qualitative analysis of complex mixtures, it is less useful for quantitative work. Gel electrophoresis is commonly used in biochemistry to separate macromolecules, nucleic acids and proteins. Proteins and nucleic acid fragments are separated by differences in mobility through a sieving gel under the force of an applied electric field. An Example of a gel can be seen in Figure 6.2.

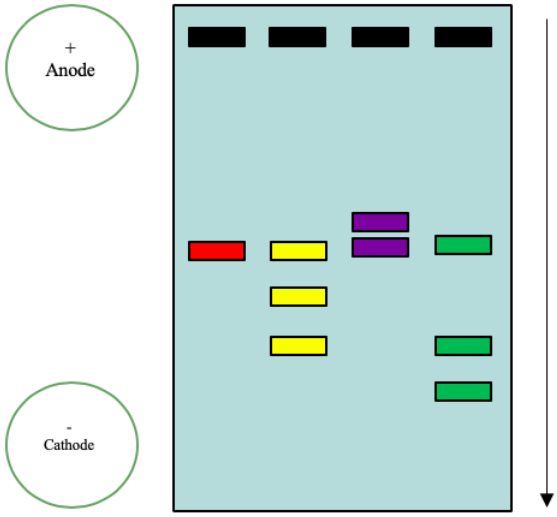

***Figure 6.2**: Schematic of a standard gel electrophoresis set-up. The travels in the direction of the arrow*

Capillary Zone Electrophoresis

The simplest CE method is capillary zone electrophoresis (CZE), a method by which molecules, ions, or particles are separated **solely** by their electrophoretic mobility. This technique is based on the idea that the velocity is proportional to the charge to mass ratio. The capillaries are usually made of silica. In uncoated capillaries at pH greater than 3 the SiOH groups are ionized to SiO^- (Figure 6.3). The deprotonation creates a negative charge along the capillary wall. This leads to a phenomenon called electroosmotic flow (EOF). The EOF is the overall movement of the sample from the anode to the cathode.

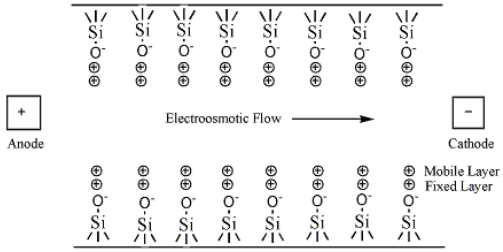

***Figure 6.3**: SiOH groups being ionized and creating the electroosmotic flow inside of the capillary.*

The negative charge on the capillary wall leads to the formation of a double layer of cations along the wall. The inner layer of cations is tightly bound to the capillary wall (cations directly bound to the oxygens of the SiO^- group) and the outer layer is a diffuse layer of cations. A **zeta potential** forms at the boundary between the inner and outer layers of cations. When an electric field is applied, the cations in the diffuse layer move towards the cathode. The cations are more solvated than the anions and pull the bulk solvent towards the cathode. This movement defines the EOF. (Figure 6.4). The relative mobilities of the particles are the same, but now neutral molecules and negative particles are pulled toward the cathode by the EOF.

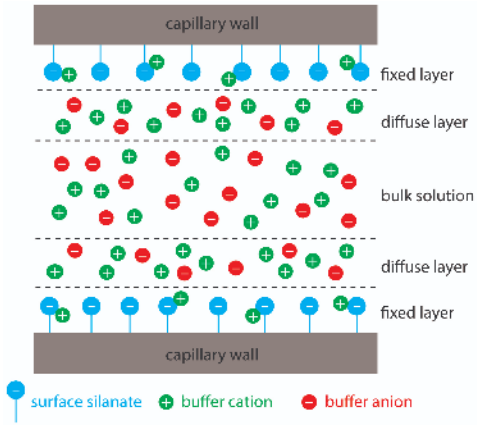

Figure 6.4: Schematic diagram showing the origin of the double layer within a capillary tube. Although the net charge within the capillary is zero, the distribution of charge is not. The walls of the capillary have an excess of negative charge, which decreases across the fixed layer and the diffuse layer, reaching a value of zero in bulk solution.

While the EOF is affecting all of the components in the sample, the cations have a faster velocity because they are attracted to the cathode, anions are unattracted to the cathode, and neutral molecules are neither attracted or unattracted. Neutral molecules will not be separated from one another. Negatively charged particles will be separated because the electrophoretic mobility counters the EOF. A solute's total velocity (v_{tot}) as it moves through the capillary is the sum of its electrophoretic velocity and the electroosmotic flow velocity. This concept is demonstrated in Figure 6.5. Cations elute first in an order corresponding to their electrophoretic mobilities, with small, highly charged cations eluting before larger cations of lower charge. Neutral species elute as a single band with an elution rate equal to the electroosmotic flow velocity. Finally, anions are the last components to elute, with smaller, highly charged anions having the longest elution time.

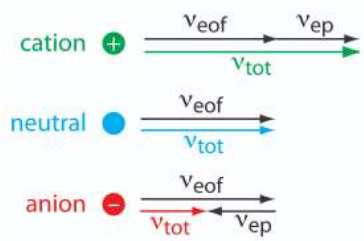

 Figure 6.5: Electroosmotic and Electrophoretic flow. A visual explanation for the general elution order in capillary electrophoresis. Each species has the same electroosmotic flow. Cations elute first because they have a positive electrophoretic velocity, v_e. Anions elute last because their negative electrophoretic velocity partially offsets the electroosmotic flow velocity. Neutrals elute with a velocity equal to the electroosmotic flow.

 Figure 6.5 can also be described using the following equations:

$$\nu_{tot} = \nu_{ep} + \nu_{eof} \tag{Lab 6.4}$$

$$(\nu_{tot})_{cations} > \nu_{eof} \tag{Lab 6.5}$$

$$(\nu_{tot})_{neutrals} = \nu_{eof} \tag{Lab 6.6}$$

$$(\nu_{tot})_{anions} < \nu_{eof} \tag{Lab 6.7}$$

The EOF is extremely useful for separating molecules with both positive and negative charges, but the EOF is not necessary. The EOF can be abolished by changing the buffer conditions. Using running buffer at very low pH will abolish the EOF. If low pH is a problem for the stability of the samples, the inside of the capillary can be coated with an uncharged layer. Low concentrations of ionic detergent, below the critical micelle concentration, will also diminish the EOF. There is no separation of molecules with similar charge to mass ratios. It is frequently desirable to improve or alter the separation. Molecules with similar electrophoretic mobilities can be separated by the addition of carrier compounds to the running buffer.

Micellar Electrokinetic Chromatography

The addition of a new species to the running buffer will separate the molecules in the initial sample. This separation can occur based on the molecules' affinities for the added species. For example, uncharged, nonpolar molecules can attract to neutral species, while charged species can attract to added ionic species. There are several different ways to perform this experiment based on the properties of the added species. The addition of cyclodextrins (or micelles) to the running buffer allows the separation of chiral species. This is described as MEKC, or Micellar Electrokinetic Chromatography. This technique can separate compounds (which had similar mobilities in CZE experiments) due to the difference in affinities of the sample molecules for detergent micelles.

Neutral species will partition between the running buffer and the hydrophobic interior of the micelles. The micelles, which are negatively charged, have a retention time greater than the EOF. Thus, as molecules enter the micelles they are slowed down. The stronger an affinity the neutral species has for the micelle, the longer its retention time. The more nonpolar neutral species have the highest affinity for the micelles. Charged particles that have hydrophobic groups will also be retained by interaction with the hydrophobic core of the micelle. Highly positively charged particles will interact with the surface of the micelle and also be retained. These interactions can be seen in Figure 6.6. You can see that the separation of the species in the mixture will be changed by the addition of detergent to the running buffer. You can tailor your separation to exactly suit your needs by experimenting with different additions to the running buffer.

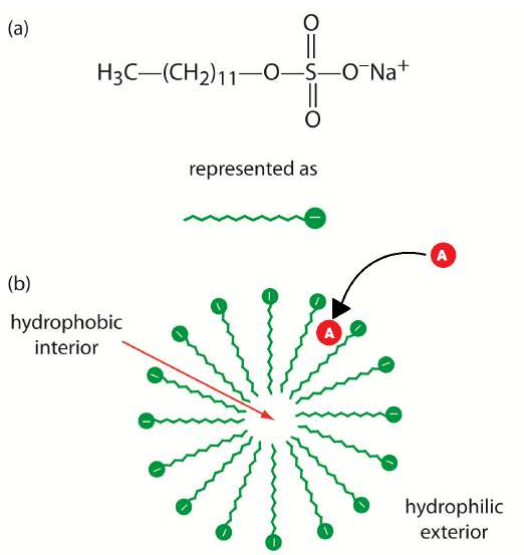

 Figure 6.6: Micellular Interactions within the Capillary. (a) Structure of sodium dodecylsulfate and its representation, and (b) cross-section through a micelle showing its hydrophobic interior and its hydrophilic exterior. The cross-section shows the migration of neutral compound "A" into the hydrophobic interior. The v_{ep} of "A" can be retarded due to the partitioning into the micelle.

Instrumentation

In this experiment, you will repeat the analysis you did (or will do) in the HPLC experiment. You will compare two different modes, Capillary Zone Electrophoresis (CZE) and Micellar Electrokinetic Capillary Chromatography (MEKC), to achieve the same separation. Differences in resolution and retention times will be observed and explained. Differences, if any, between the results obtained by HPLC and CE will also be addressed. The Instrument used can be seen in Figure 6.7

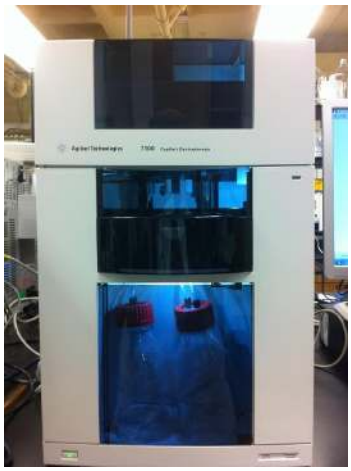

 Figure 6.7: Agilent Technologies 7100 Capillary Electrophoresis, UCD Capillary Electrophoresis instrument. Note that multiple vials can be probed sequentially if required. This instrument is located in room 3475.

Experimental Procedure

Part 1: Preparing the Required Solutions

1. The solutions from Lab 2: High-Pressure Liquid Chromatography (HPLC) will be used. If you have already performed that lab, gather your samples. If your group has not performed Lab 2, refer to Part 3: Analysis of Caffeine Beverages in the Procedure section of Lab 2: HPLC.

2. Check the counter with the buffer solutions and identify two buffers that have already been prepared. The buffers are:

- **a.** Solution A: 0.05 M borate buffer, pH=9.0.
- **b.** Solution B: 0.05M Sodium dodecyl sulfate (SDS), 0.05 M borate buffer, pH=9.0.

3. Filter the stock caffeine solutions using the provided filter and syringe. Add approximately 10 mL into a labeled CE vial. **DO NOT fill the vials more than 75%.**

- **a.** The filter and syringe can be reused if the solutions are filtered from low concentration to high concentration.
- **b.** The syringes can be found in the plastic drawers near the door of the lab.
- **c.** Filter approximately 1 mL and dispose of it into a waste beaker to wash the filter.
- **d.** Filter the solution into the appropriate vial. (Figure 6.8) **DO NOT** put tape on the vials; ask your TA for a sharpie to write directly on the vials.

4. Filter the beverage samples into labeled vials using the same technique described in step 3.

5. Fill a labeled CE vial with filtered Solution A buffer and another labeled vial with filtered solution B buffer each about 75% full.

Figure 6.8: Appearance of HPLC/CE vials. The HPLC can also use plastic vials that look quite similar. Image is taken from www.thermofisher.com

DO NOT dispose of these samples if Lab 2: HPLC has NOT been performed; after completing Lab 2 AND Lab 6, the solutions can be disposed of.

Part 2: Running a Capillary Zone Electrophoresis Experiment

1. Turn on the instrument (Agilent Technologies 7100 Capillary Electrophoresis Instrument), if it is not already on, by pressing the power button at the bottom left of the front face of the CE. The light in the middle of the button should be green when it's on.

2. Open the *Chemstation* software if it's not already on by double-clicking the "Instrument 2 Online" icon with an image of the CE. You will start in "Instrument View."

3. The instrument must first be initialized.

a. Click the "On" button next to the question mark button on the bottom right of the "CE" window.

b. Click on the power button icon in the top left corner of the "DAD" (diode array detector) window to "make device ready." Wait until the system goes to "Ready." This may take some time if the temperature-controlled zones need to stabilize.

4. Wait until the software goes to "Ready."

a. In the "CE" window, right-click "Inlet" and unload by selecting "Unload Inlet Lifter."

b. Do the same for the "Outlet." You should then hear the instrument lowering the vials in positions 1 and 2.

c. Right-click the sample wheel and select "Get Vial."

d. "Vial" should be set to "1" - click "Get."

e. The tray will rotate vial 1 to the front of the instrument.

f. Open the tray door and remove vials 1 and 2, if they are present.

5. Place the prepared 75% full Solution A vial in space number 1.

6. Fill a second vial 50 % full of Solution A and label this "Buffer A outlet." Place this Vial is space number 2 in the sample wheel.

7. Close the tray door. The tray will rotate back to the operating position.

8. Make sure to use the features on the screen to insert Vial 1 as the inlet and Vial 2 as the outlet.

9. Right click on the regulator pressure icon in the "CE" window and click "Flush" on the menu. Enter a time of "600" seconds and click "OK". The pressure should increase to about 930 mBar and slowly decrease as liquid is pushed from one vial to the other during the flush time. This will wash out any residues from the previous runs and equilibrate the capillary with the running buffer.

10. Make sure that the method is "CAFFEINE.M" and that the sequence is "CAFFEINE.S."

a. The method and sequence names can be found in the drop-down boxes in the main menu bar.

b. Check and make sure the CZE method is using 20 kV by selecting "Method" then "Edit Entire Method". Check with your TA what the run time is for the current capillary.

c. Press "OK" in the first two windows that appear until you get to the "Setup Method" window.

d. Select the "CE" tab to check the voltage and run time under "Stop Time."

e. Check that under "Injection," on the right side of the "CE" tab window, there is one row that under "Function" has "Apply Pressure" and under "Parameter" it says "50mbar for 5s (Inlet: Injection Vial Outlet: Outlet Home Vial)." Click "OK".

f. "OK" through or cancel through the remaining windows.

11. Right click on the tray icon and select "Get Vial."

a. Set the "Vial" number to 7 and click "Get."

b. After the tray rotates to the correct position. open the tray door and put all your samples in.

c. Start at position 4 (leaving position 3 empty) with the 0.01 g/L standard followed by the rest of the standards in increasing concentration, then the three beverage samples. Make sure you keep track of the order in which the samples are run.

d. Close the tray door.

12. Review the sequence parameters.

a. On the menu bar, click "Sequence" and select "Sequence Parameters".

b. Enter an operator name, make sure "Prefix/Counter" is checked, and enter a prefix for your datafile names.

c. Click “OK.”

13. Run the sequence by clicking the “Sequence” Play button above the “DAD Window.”

14. After the sequence has started, watch the "Online Plot." You should see a peak for your first standard about 2-3 minutes into the run. The electrical current, which can be monitored in the "CE" window should be 15-16 microamperes.

15. You can print the reports after each sample is run.

a. When the entire sequence has completed, click "Data Analysis" at the bottom left of the window.

b. Click on "Chem_115" in the data tree and select your sequence.

c. Select the data you want and print.

d. Repeat for all your other data files.

e. A prompt to save each trial as a PDF will appear after each trial.

f. Create a file and save the trials to the file.

g. Print the PDFs.

16. When the sequence is complete, remove the inlet and outlet.

a. In the "CE" window, right-click "Inlet" and unload by selecting "Unload Inlet Lifter."

b. Do the same for the "Outlet." You should then hear the instrument lowering the vials in positions 1 and 2.

IMPORTANT

Make sure that you get a peak for each standard. You can always abort the run and restart it if there is a problem.

Part 3: Running a Micellar Electrokinetic Capillary Chromatography Experiment

1. Wait until the software goes to "Ready."

a. In the "CE" window, right-click "Inlet" and unload by selecting "Unload Inlet Lifter."

b. Do the same for the "Outlet." You should then hear the instrument lowering the vials in positions 1 and 2.

c. Right-click the sample wheel and select "Get Vial."

d. "Vial" should be set to "1" - click "Get."

e. The tray will rotate vial 1 to the front of the instrument.

f. Open the tray door and remove vials 1 and 2 from Part 2 of the experiment.

g. Place the prepared 75% full Solution B vial in space number 1.

h. Fill a second vial 50 % full of Solution B and label this “Buffer B outlet.” Place this Vial is the space number 2 in the sample wheel.

i. Close the tray door. The tray will rotate back to the operating position.

2. Make sure to use the features on the screen to insert Vial 1 as the inlet and Vial 2 as the outlet.

3. Right click on the regulator pressure icon in the "CE" window and click "Flush" on the menu. Enter a time of "300" seconds and click "OK."

4. Make sure that the method is "CAFFEINE_MEKC.M" and that the sequence is "CAFFEINE_MEKC.S."

a. The method and sequence names can be found in the drop-down boxes in the main menu bar.

b. Check and make sure the MEKC method is using 15 kV by selecting "Method" then "Edit Entire Method". Check with your TA what the run time is for the current capillary.

c. Press "OK" in the first two windows that appear until you get to the "Setup Method" window.

d. Select the "CE" tab to check the voltage and run time under "Stop Time."

5. The samples should already be in the correct position after the CZE experiment.

6. Run the sequence by clicking the “Sequence” Play button above the “DAD Window.”

7. You can print the reports after each sample is run.

a. When the entire sequence has completed, click "Data Analysis" at the bottom left of the window.

b. Click on "Chem_115" in the data tree and select your sequence.

c. Select the data you want and print.

d. Repeat for all your other data files.

e. A prompt to save each trial as a PDF will appear after each trial.

f. Create a file and save the trials to the file.

g. Print the PDFs.

8. When the sequence is complete, remove the inlet and outlet.

a. In the "CE" window, right-click "Inlet" and unload by selecting "Unload Inlet Lifter."

b. Do the same for the "Outlet." You should then hear the instrument lowering the vials in positions 1 and 2.

c. Right-click the sample wheel and select "Get Vial."

d. "Vial" should be set to approximate “4” - click "Get." (This will ensure that you can remove all of the vials from the CE)

e. The tray will rotate vial 4 to the front of the instrument.

f. Open the tray door and remove all of your vials.

g. Close the tray door.

Treatment of Data

1. Before completing the lab, confirm that you have peaks for each of your runs. If you notice any issues with your data, talk with your TA.

2. Create a calibration curve using the caffeine standard peak height or area versus the concentration. This can be done in *Chemstation* in the lab or at home in excel/google sheets.

a. To create a calibration curve in *Chemstation*, start by clicking the “data analysis” tab in the bottom left corner of the window. This will open and offline window.

b. Go to the CHE 115 file and find your data folder and select it.

c. Double click the first standard run in the sequence window.

d. Find the “Calibration” tab in the menu bar and select “New Calibration Table.”

e. A new window “Calibrate: HPLC1” will appear and select “Automatic setup.”

f. Set the “level” to 1 and put the concentration of the first run in the “Default Amount.” Press “OK.”

g. Double click the second run and go to the “Calibration” menu bar and click “Add Level.”

h. Set the “Level” to 2 and enter the second run’s concentration in the “Default Amount.” Press “OK.”

i. Repeat steps g and h for each of the remaining standard solutions.

j. The Calibration Table and the Calibration Curve can be viewed in the bottom of the window. Confirm that all the points are there.

3. If you cannot make a calibration curve in *Chemstation* or the curve did not use all of the points, use the data to develop a calibration curve in Excel or Google Sheets

Post-Lab Questions

****Include ALL Data Reports and Calibration Tables****

1. Explain the difference in retention time for the two different experiments.
2. What would you expect to happen to the retention time of the caffeine peak if you decreased the run voltage for the first experiment to 10 kV?
3. Did you get the same answer for the two different CE experiments? Explain.
4. Did you get the same answer as you did for the HPLC experiment? Is this surprising? If the answers are different, suggest some possible explanations. If you have not completed HPLC, you do not need to answer this question.

References

1. Copper, C. L. Capillary Electrophoresis Part I. Theoretical and Experimental Background. *J. Chem. Ed.* **1998**, *75*, 343-347. pdf
2. Copper, C. L.; Whitaker, K. W. Capillary Electrophoresis Part II. Applications. *J. Chem Ed.* **1998**, *75*, 347-351. pdf
3. McDevitt, V. L.; Rodriguez, A.; Williams, K. R. Analysis of Soft Drinks: UV Spectrophotometry, Liquid Chromatography, and Capillary Electrophoresis. *J. Chem. Ed.* **1998**, *75*, 625-629. pdf
4. Skoog, D. A.; Holler, F. J.; Nieman, T. A. *Principles of Instrumental Analysis*, Fifth Edition; Harcourt Brace: Philadelphia, 1998; 591-621.

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Lab 7: Electrospray Mass Spectrometry

Goals

1. Students should gain familiarity with the operation of the instrument.
2. Students should observe the multiple ionization states of a complex molecule.
3. Students should be able to determine the optimal conditions to produce the best mass spectrum.
4. The students should understand how to pick peaks and determine the charge and molecular weight of a complex compound.

In this experiment, you will learn the mechanics of electrospray mass spectrometry and use this technique to study the effect of different solvents on the three-dimensional structure of proteins.

Introduction

Every mass spectrometer has three essential parts, an ionization source, a mass analyzer, and a detector. There are many ionization techniques. In this class, you will encounter two different ionization methods. In the GC/MS experiment, the molecules of interest were ionized by electron impact. Not only were the molecules ionized they were fragmented into smaller pieces. The pattern of ionized fragments produced is characteristic of the compound and allows one to identify compounds by the mass spectrum produced. This is called a hard ionization technique; in contrast, electrospray ionization is a soft ionization technique. The solution containing the compound of interest is sprayed through a nebulizer to create a fine mist of droplets. The droplets are charged by the large potential difference between the end of the nebulizer needle and the entrance of the capillary (Figure 1). The droplets are dried by the drying gas inside the capillary. As the droplets shrink, the electrostatic repulsion between the charged particles causes some of them to enter the gas phase. The gas-phase ions are drawn through the capillary by the high vacuum in the analyzer. The molecules in solution are ionized but not fragmented. Each molecule in the solution can have a different number of charges attached. This creates a mass spectrum of differently charged particles.

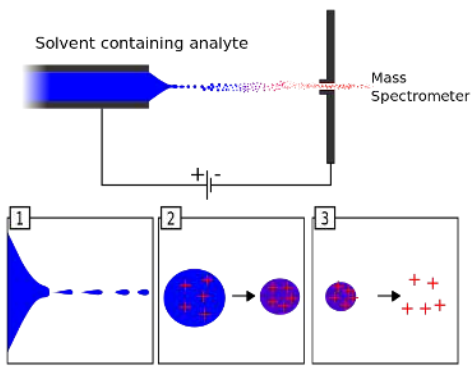


Figure 7.1: *Diagram of Electrospray Ionization. (1) Under high voltage, the "Taylor Cone" emits a jet of liquid drops (2) The solvent from the droplets progressively evaporates, leaving them more and more charged (3) When the charge exceeds the Rayleigh limit the droplet explosively dissociates, leaving a stream of charged ions. Image used with permission (CC-SA-BY 3.0;* Evan Mason)

In this instrument, the mass analyzer is a modified quadrupole. The quadrupole detector comprises four rods, two positively charged and two negatively charged, maintained by a variable DC potential difference (Figure 2). A variable AC potential also exists between the two rods. The mass of ions that pass through the quadrupole is determined by the strengths of the applied fields. During a scan, the voltages are ramped up but the ratio of the ac and dc fields is held constant. See section 11B-2 in Skoog for a detailed description. Scans are very quick, on the order of 100 ms. Because of the speed of data acquisition, the quadrupole detector is ideal for the analysis of HPLC output. Once the ions make it through the quadrupole they must be counted by a detector. The detector in your system is a HED (high energy dynode) detector, which is a modified electron multiplier that is more sensitive at higher masses.

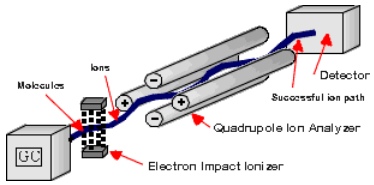


Figure 7.2. Quadrupole Ion selector

The instrument is extremely flexible and can be run in either positive or negative ion selection mode. It can also be run in scan mode where a wide range of masses is detected or it can be used in selected ion mode where only a single ion is detected.

The technique is used primarily for determining the molecular weights of proteins and components of multi-protein complexes. For proteins larger than 3000 daltons, no molecular ion can be observed in this mass spectrometer. A series of ions is produced from each protein molecule. The pattern of peaks is called the charge state envelope. The molecular weight of the protein can be determined based on the distribution of ions in the envelope. The first step is to determine which ions are related. Usually this involves selecting the largest peaks in the mass spectrum. Remember that you are detecting protein molecules to which additional H^+ atoms are attached. The m/z of a particular peak is:

$$\frac{m}{Z} = \frac{M + nH^+}{n} \tag{Lab 7.1}$$

where M is the mass of the uncharged protein and n is the number of additional protons attached and H^+ is the mass of a single proton. Using several peaks in the charge state envelope you can determine the molecular weight of the protein.

Electrospray ionization is also used to study protein denaturation. When a protein is folded into a compact globular structure many of the ionizable side chains are buried in the core of the protein and are not accessible to solvent. So, they will not be ionized. As the protein unfolds the buried acidic or basic side chains are exposed to solvent and protonated or deprotonated. Thus, the more peaks at high m/z the more unfolded the protein.

Example

When determining the molar mass of a compound, it requires using two adjacent peaks seen in the mass spectrum. Usually, the tallest peak and its neighbor are the best peaks to select. When performing the calculation, the following two equations represent the two adjacent peaks:

$$X = \frac{M + z}{z} \tag{Lab 7.2}$$

$$Y = \frac{M + z + 1}{z + 1} \tag{Lab 7.3}$$

The X equation represents the peak with the larger m/z value. This peak is not as ionized as the Y equation which is the peak that has the smaller m/z value. Equations 7.2 and 7.3 can be combined to find the z value or the charge of the molecule (Equation 7.4).

$$z = \frac{Y - 1}{X - Y} \tag{Lab 7.4}$$

This z value can then be used to solve for the molecule weight. By performing a simple rearrangement of equation 7.2, the molecular weight can be solved for. The rearranged equation can be seen as equation 7.5.

$$M = z(X - 1) \tag{Lab 7.5}$$

Solution

In the following example, the charge and the molecular weight will be calculated for the following molecule.

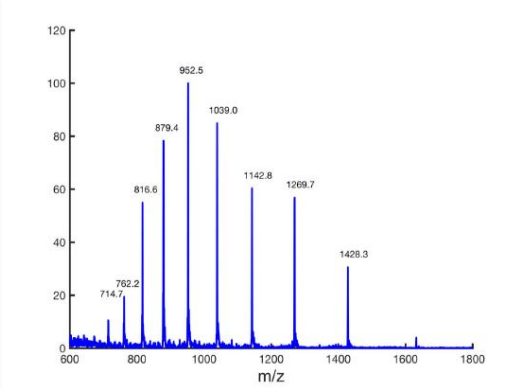


Figure 7.3: Example of a Mass Spectrum.

The first task will be to start by picking two adjacent peaks. The tallest peak is 952.5 m/z, so this will be the Y peak. The X peak will be the adjacent peak with the larger m/z value. In this example, the 1039 m/z peak will be used. These values will be plugged into equation Lab 7.4)to determine the charge.

$$z = \frac{952.5 - 1}{1039 - 952.5} \tag{Lab 7.6}$$

After plugging in these values, this equation results in a z value of +11. This z value can be used to determine the molecular weight by using equation Lab 7.5).

$$M = 11(1039 - 1) \tag{Lab 7.7}$$

After completing this calculation, the molecular weight is calculated to be 11,418 amu. This value is an approximation, but after finding out that the molecule is putidaredoxin which has an actual theoretical molecular weight of 11,419 amu, it shows that this method could approximate the mass very closely.

Principles of protein structure

Proteins are polymers of 20 amino acids. The amino acids can be divided into groups based on their chemical properties. The hydrophobic or non-polar amino acids are leucine, isoleucine, valine, alanine, phenylalanine, methionine, proline and tryptophan; the polar, uncharged residues are glycine, serine, threonine, tyrosine, glutamine, asparagine and cysteine. The most important for mass spectrometry are the charged amino acids, glutamate and aspartate are the acidic side chains, lysine, arginine, and histidine are the basic side chains. The structures for the 20 side chains are shown in Figure 3. Note that the figure contains a few errors: under the positively charged side groups heading, the Asparagine amino acid listed should actually be Arginine (Arg, R) and under the polar, uncharged side groups heading, the Aspartate amino acid listed should actually be Asparagine (Asn, N). Also, Proline should be listed in the nonpolar, aliphatic side groups section as it is hydrophobic.

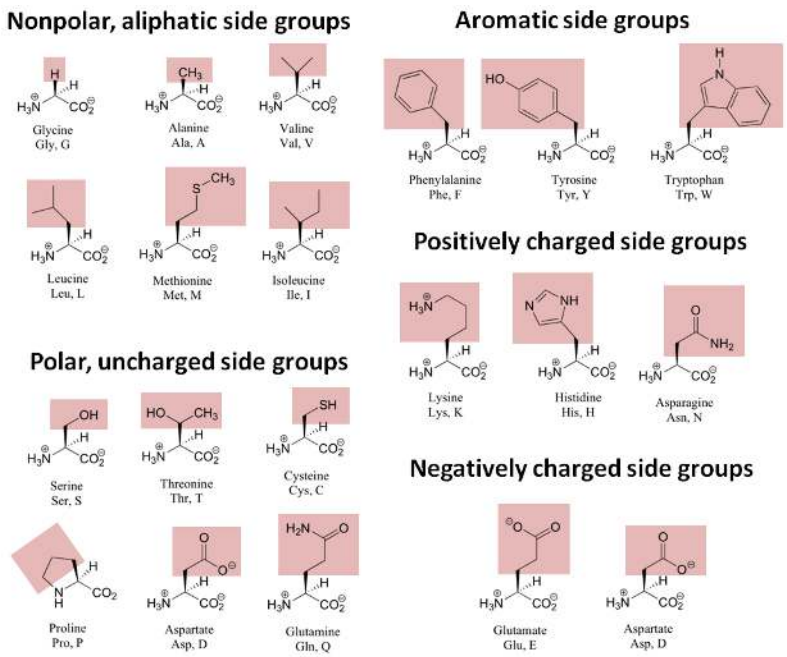


Figure 7.4: The structures of the 20 naturally occurring amino acids.

At pHs above the pK_a for the acidic side chains, those side chains will carry a negative charge. The basic side chains are positively charged at pH less than the pK_a. The pH of the solution in which your protein is prepared will determine how the proteins are studied by mass spectrometry. At low p,H the proteins will carry a net positive charge and at higher pHs, the charge will go through neutral and become positive as the pH increases. The pK_a's of the amino acids are shown in table 1.

Table 1: *pK_a Values for the 20 naturally occurring Amino Acids*

Amino Acid	α-carboxylic acid	α-amino	Side chain
Alanine	2.35	9.87	
Arginine	2.01	9.04	12.48
Asparagine	2.02	8.80	
Aspartic Acid	2.10	9.82	3.86
Cysteine	2.05	10.25	8.00
Glutamic Acid	2.10	9.47	4.07
Glutamine	2.17	9.13	
Glycine	2.35	9.78	
Histidine	1.77	9.18	6.10
Isoleucine	2.32	9.76	
Leucine	2.33	9.74	
Lysine	2.18	8.95	10.53
Methionine	2.28	9.21	
Phenylalanine	2.58	9.24	
Proline	2.00	10.60	
Serine	2.21	9.15	
Threonine	2.09	9.10	
Tryptophan	2.38	9.39	
Tyrosine	2.20	9.11	10.07
Valine	2.29	9.72	

The forces that stabilize the three dimensional or tertiary structures of proteins are hydrophobic interactions between the non-polar side chains, hydrogen bonds between carbonyl oxygens and amide nitrogens, electrostatic interactions, usually on the surface of the protein, between charged side chains. Denaturation occurs when the tertiary structure of the protein unfolds (imagine scrunching up a rubber band and then letting go of it.) As the tertiary interactions unfold, so do the secondary structure elements, the alpha helices, and the beta sheets. The pH of the solvent will affect not only the charge of the ionizable side chains it will also destabilize the three-dimensional structure of the protein by disrupting charge-charge and electrostatic interaction that stabilize the structure. Organic solvents also destabilize the structure by interfering with the hydrophobic interactions between nonpolar side chains. Hydrophobic interaction is a term used to describe the phenomenon that nonpolar groups prefer to be in a nonpolar environment. In aqueous solution, the nonpolar side chains will "stick together" to decrease the surface area in contact with the solvent.

Denaturants that are frequently used include, heat, urea, salt, pH and organic solvents. The effect of these denaturants on protein stability give insight into the type of interactions that are more important in stabilizing the tertiary structure.

Experimental Procedure

In this experiment, you will acquire mass spectra of the protein ubiquitin under several different solvent conditions.

- First, you will investigate the effect of pH on protein stability by looking at the mass spectrum at different pH values.
- Make 100 mLs each of the following 5 solutions: Water and acetic acid at pH=7, 2.75, 2.5, 2.1, and 1.8.

- You will be given a 10^{-3} M solution of ubiquitin in water. Prepare 1mL dilutions of 10^{-6}M ubiquitin by diluting the stock ubiquitin in each of the solutions.

HINT: Glacial acetic acid is 100% and the molarity is 17.4. The K_a for acetic acid is 1.76×10^{-5}. Also, remember the ICE table approach. Use this information to calculate what percentage of acetic acid solutions you need to make. Do this before you come to the lab.

You will also investigate the effect of organic solvents on the stability of ubiquitin.

Make 10 mLs each of the following 4 solutions:

- 20% acetonitrile + 80% pH=2.75 acetic acid and water
- 5% acetonitrile + 95% pH=2.75 acetic acid and water
- 20% methanol + 80% pH=2.75 acetic acid and water
- 40% methanol + 60% pH=2.75 acetic acid and water.

Use the same stock solution of ubiquitin to prepare 1 mL of 10^{-6} M ubiquitin by diluting the ubiquitin in each of the solutions.

Instrumentation

(For a 2013 update go to http://chemwiki.ucdavis.edu/User:labgod4)

The instrument you are using is a Varian 325-MS with a Primeline LC Pump (instrument picture below is not the MS-325-TQ).

Figure 7.5: Mass Spectrometer in Chem 115. The ESI needle in the middle image.

First, make sure that the solvent reservoirs are sufficiently filled with HPLC grade solvent. Open **System Control,** if it is not already open, by clicking on the top and most left icon (*Figure 7.6*) on the toolbar at the top of the monitor. This opens up the **Quad View** (*Figure 7.7*). Make sure you are using view **III** (*Figure 7.8*) so that the proper quadrants are viewable. Make sure the correct method is loaded. This software should look familiar because it is similar to that used for the HPLC experiment. The desired method for running the ubiquitin samples is '115 50-50 posneg 4 min'. It is a 4-minute method. Changing methods is done by activating the desired method under the file tab of **System Control**.

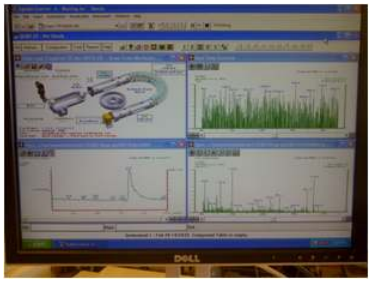


Figure 7.6 Figure 7.7

Figure 7.8 Figure 7.9

Click **Turn Spray On** in the Quad view (*Figure 7.10*) to turn on the gases and heaters. Once the temperature reaches 75% of the set value (275-300ºC) activate the pumps under the Windows tab under **System Control,** click on 212.24, then "Activate." The spray icon now has a red stream coming from it (*Figure 7.11*), and the pump lights have turned green.

Figure 7.10 Figure 7.11

A few minutes is required for the temperature to stabilize, flush out the sample loop during this time. Add ~10 µL of water into the sample loop in the INJECT position, twice. When you are ready to load your sample, make sure the valve is in the LOAD position. Dilute the first sample (pH 7) 10x to check concentration, and inject around 10. Then, under the Windows tab in **System Control**, click on the Sample List, click Data Files, and change the sample name. Once the API and 325 indicate they are ready (*Figure 7.12*), it is time to turn on the analyzer. The button turns green when it has been activated (*Figure 13*).

Now toggle back to the 212.24 LC pump window and click **Start Run** on the pumps. This starts the run and the green analysis timer begins in the area to the right of '325 MS: ready to collect' seen in *Figure 7.10*. Push the valve button to INJECT your first sample into the LCMS after the run has gone for 1 min. After each run, make sure the instrument is in the "Ready" and "Ready to collect" mode.

To print your chromatograms and mass spectra, click on your file name above **System Control** at the top of the monitor (*Figure 7.14*) which opens the window that appears in *Figure 7.15* and select: View/edit chromatogram. Click on the desired peak and the mass spec data pops up below (*Figure 7.16*). Use the arrow keys to locate the highest RIC for the peak of interest. Right click on the mouse to label the peak as desired by choosing **New Label for Plot 1** (*Figure 7.17*). Hover the mouse of the label; when the arrow turns to a 'hand' the label and its indicator can be dragged to their desired locations. Print, using the printer icon, choose active chromatogram and spectrum. Then click on the short stack printer icon to send the data to the printer. Each click prints one copy.

Repeat this for all samples, starting with the flushing of the sample loop.

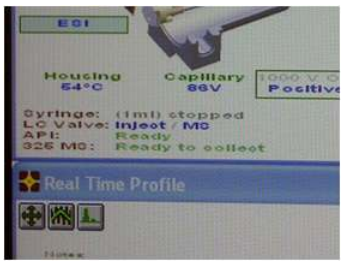


Figure 7.12 Figure 7.13

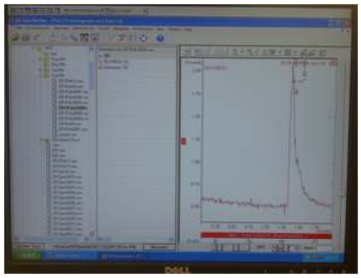
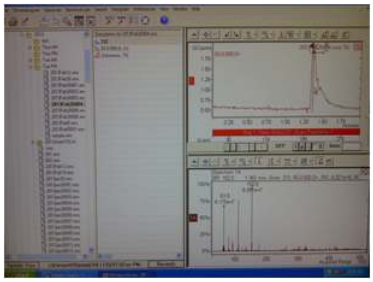


Figure 7.14 Figure 7.15



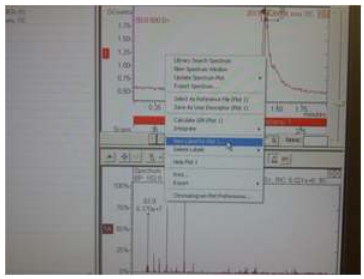


Figure 7.16 Figure 7.17

<u>Shutdown Protocol:</u> Once the analysis is complete, **turn off the spray** by clicking the icon in *Figure 7.11*. The icon now reverts back to how it appears in *Figure 7.10*. Verify that the spray has become a constant drip in the API window. Allow the drip to continue for a few minutes. Toggle back to the **Quad View** to verify that the **Spray is off** and appears as it does in *Figure 7.10* (no red coming out). If it has not been dripping, allow the drip to continue to flush out the needle and chamber for a few minutes. Using the Windows tab in **System Control**, toggle to the 212.24 window and click **Stop Pumps**. It is now safe to close out **System Control** and exit as normal. Make sure to log off to restrict the access to the instrument.

Post-Lab Questions

1. Print the chromatogram and the mass spectra for each "peak" in the chromatogram.
2. Using the pattern of peaks in your mass spectrum of the pH=2.75 sample calculate the molecular weight of ubiquitin. Show all your work.

Include the answers to the following questions in your report:

1. Why is the peak for the pH=7 sample so much smaller than the peaks for all the other conditions?
2. Discuss the differences in the distribution of ions for all the different conditions.
3. What happens to the 3-dimensional structure of the protein as the pH is decreased?
4. What happens as the concentration of the organic solvent is increased? Explain the differences you see with methanol and acetonitrile.

References

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2. Loo, J. A.; Ogorzalek-Loo, R. R.; Udseth, H. R.; Edmonds, C. G.; Smith, R. D. Solvent Induced Conformational Changes of Polypeptides Probed by Electrospray Ionization Mass Spectrometry. *Rapid Comm. in Mass Spec.* **1991**, *5*, 101.
3. Hofstadter, S. A.; Smith, R. D. Electrospray Ionization Mass Spectrometry. *J. Chem. Ed.* **1996**, *73*, A82.
4. For a 2013 Update go to http://chemwiki.ucdavis.edu/User:labgod4

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