BIOANALYTICAL CHEMISTRY LAB MANUAL
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LABORATORY NOTEBOOKS

In the "working world," a laboratory notebook is a legal document. It may be used as evidence in court for patent protection, for environmental litigation or as a document establishing or defending scientific misconduct. Therefore, the lab notebook must adhere to certain standards, such as those described below.

All recording in your lab book MUST BE IN NON-ERASABLE PEN. In your lab books enter the experiment in the table of contents at the front of your book, and the page number on which it starts. Title and date the experiment on that page (and list lab partners, if any), write a brief purpose (one or two sentences), a brief procedure (you may refer to the lab manual by saying "As directed in the CH 345 lab manual"), record the data in the data and observations section, and then staple in any LABELED hard copies you got from computers or graphs that you drew and any calculations you made. Alternatively, calculations may be stored as data files in programs like Excel with the filename noted in your notebook. Finally, write a conclusion paragraph, explaining your results and any deviations from the expected that you observed. For example, did the water weigh EXACTLY 1.0000g/mL? Should it have done so? This is the format we will expect for all of the labs.

Summary of lab book write-up:

1) Fill in the table of contents at the front of the notebook after each experiment. Write on only the right-hand pages in ball-point pen; you may use the left-hand pages to staple in neatly labeled and titled graphs, etc.

2) Title and date the experiment.

3) Give a short paragraph stating the purpose and main points of the experiment.

4) Procedure may be as directed in the lab manual, but add any modifications you make.

5) INCLUDE ALL DATA COLLECTED.

6) Include a sample calculation, so that you could repeat the work.

7) Include a BRIEF conclusion that states your result, the error and any explanation of unusually high error. Also include any changes that should be made in the future if you were to run this same lab again.

INTRODUCTION TO CHEMOMETRICS:
MICROPIPET CALIBRATION & HYPOTHESIS TESTING
(H. Drossman & N. Bower)

Objectives:
- Gain proficiency in the use of analytical micropipets, volumetric measurements, and analytical balances.
- Learn how to use a spreadsheet program.
- Use a practical example to learn statistical analysis.

Introduction:
Chemometrics has been defined by the International Chemometrics Society as "the chemical discipline that uses mathematical and statistical methods, (a) to design or select optimal measurement procedures and experiments; and (b) to provide maximum chemical information by analyzing chemical data." It includes such topics as statistics, optimization, signal processing, factor analysis and pattern recognition, resolution, calibration, modeling, library searching, structure-property relationships, and artificial intelligence. Every two years, Analytical Chemistry Fundamental Reviews (which has abstracted the chemometric literature since 1980) has between 500 and 1000 literature references to articles published on chemometrics, indicating the importance of this field as a separate discipline within analytical chemistry in the development of new methodology.

Analytical chemistry itself has been called "a science without a theory," but we would argue that it is composed of a body of theories dealing with widely ranging methods from the fields of spectroscopy, electro- and thermo-chemistry, titrimetry, gravimetry, and chromatography, to name only a few of the major ones. Integral to all of these is the discipline of chemometrics, which focuses on the theory of measurement and information optimization.

In this course we will try to introduce major techniques whose theory should be applicable to a wide variety of instruments, as the field of analytical chemistry is constantly in flux as new methods are developed. Among the major things we would like to get across will be a way of looking at information and how it is obtained. We would like to affect your attitude about the measurement process - to show that the "ends don't justify the means," but instead that the means describe the results! Therefore, we will be looking to improve your technique and how you process the information you obtain, trying to make you look at it critically, squeezing it for all of the information that you can, and not let you get away with using it merely to verify a preconceived theory.

Though the first two labs are fairly simple in thought, we would like you to approach them in a fresh way, exhausting the information they contain. Apply the principles of statistical analysis from the reading and lecture, and do not be afraid to go beyond what we ask at *any* point in the course. You will be doing experiments specifically designed to enable biochemists to become proficient in analytical chemistry. We hope you'll find it as interesting as we do.

Micropipet Calibration Procedure:
1) Calibrate the analytical balance by following the manufacturers suggested method.

2) When you are weighing an object, always close all of the doors on the balance and wait for the indicator before recording the weight. Record the weight right after the balance indication. The weight may continue to decrease due to evaporation of sample. In a more demanding experiment, you may have to keep the sample iced or covered to eliminate evaporation, but we will ignore this now.

3) Weigh the recommended amount (Table 1, end of lab) of distilled water added to a weighing boat from your micropipet. You may have to dry the weighing boat between weights with a Kimwipe® to avoid evaporative losses. Record each weight in your lab notebook. Repeat 10x. Repeat this procedure for each pipette at each interval listed in Table I (60 weights total).

4) Weigh the *empty* plastic weighing boat 10x. You should actually remove the boat and re-weigh each time. Though this may sound tedious, it will help you determine the differences in error between the scale and the pipette. (You wouldn't want to think that you can't pipette precisely, would you?; maybe you just can't weigh precisely?). If the weights you obtain for the boat are all identical, then assume that the scale precision is ±0.1 mg.

5) Pipette 200 μL of a viscous solution (50% glycerol) with the 20-200 μL pipet into a weighing boat. Repeat 10x. Record the density of the solution and the pipet that you used.

6) Record the barometric pressure and temperature in the laboratory (closest barometer is probably in the physical chemistry lab).

Data Analysis:
1) When you analyze the data to calibrate each pipette, use the Q-test to reject any suspect data. Note that the Q test can be applied more than once to a reasonably large data set, but if you find yourself trimming the data very much, you might question the whole data set and start over as the procedure was not "under control".

2) Correct the pipette weights for buoyancy effects and then convert the weights to volumes. (See Harris footnotes in Chapter 2).

3) Calculate the mean, standard deviation, precision (% RSD) and confidence interval for each pipette *volume*.

4) Considering your precision, determine if the accuracy is significantly (95% CI) different from the manufacturer's data? Assume the manufacturer's data is a known (true) value (the value you were trying to obtain). What statistical test is used here?

5) Convert the glycerol weights to volumes (use the recorded density) making sure to correct for buoyancy. Do an F-test and a t-test on the volumetric data to compare whether the micropipet

delivers different volumes with viscous solutions than with water. Remember that Excel's version of the F-test requires you to input the higher variance first.

6) Compare the variances for the pipette volumes between you and your partner (at each of the same volumes). Do the pipettes deliver a significantly different distribution of volumes from each other (F-test)? Do they deliver significantly different mean volumes (t-test)? How do you choose an appropriate t-test?

7) When drawing conclusions about your pipeting ability and your pipets, test the following: precision of pipet relative to manufacturer's precision, accuracy relative to the amount you attempted to deliver (what statistical test would you do for this) and accuracy compared with manufacturer's range (i.e. were you within their range considering your CI?). You should answer these questions as Y/N on the report form.

8) If you were not accurate and/or precise determine whether the likely cause is you or the pipet. Explain how you can tell the difference and what further tests might be needed to support your hypothesis.

APPENDICES
How to Use Excel

1) Turn on the power on the computers.

2) Double click the Excel icon.

3) Type in a label in column B and then the data using the return key (or down arrow is okay here, too. The enter key is really an **execute** key, so DO NOT use it here). You may move to new columns and rows with the arrows or with the mouse pointer. Enter the data for each pipette in separate columns.

4) You may correct errors while typing in with the delete (it acts as a backspace) key, or by highlighting the box with the error and retyping it. You may selectively edit one part of a box by using the scroll bar at the top of the sheet where the number is displayed. The scroll bar acts much the same way as a word processor.

5) Move the working box (either with the arrows or the mouse) to 6 boxes below your last entry in a column. Type in Q-test = and then return. Then in the box below perform a Q-test on any odd values by typing actual numbers.

$$Q = (\text{odd value - nearest neighbor}) / (\text{range})$$

NOTE: starting a box with the = sign means you want to do a formula and the result is to go in that box (or a set of boxes if you point the mouse to the starting box and then move to the ending box of a rectangle of boxes). For this calculation we did a Q-test and we can look in a

Q-table (see end of this lab for such a table) to see if this value is large enough to be rejected. Type in the value of Q_{crit} in box B14. (Put "Reject" in box B15 if it is, or "Accept" if not.). Repeat this procedure for any suspect values. You should do it for at least one value to show that you know how.

6) If you find a value that should be rejected, highlight the box with the value to be deleted and click on DELETE in the EDIT column. Then move pointer to okay box (or press RETURN) if you want to delete the value and have the boxes below move up to fill in the space. Then insert a column on the left of your data (for more labels) using the same EDIT function (plus insert) after making a block (by holding down the pointer) for as many labels as you may need.

7) We are now ready to calculate the averages (means) and standard deviations of each of the columns. You can type in a formula for this, or you can use one of the pre-packaged formulas available under f_x push button. Move the pointer below your last value for each pipette, then move the pointer to the f_x push button. You will get a menu of functions. Next, move the pointer to the down arrow on the right of the function box, or use the down arrow on the keyboard to select AVERAGE. Move pointer to Next, highlight the range of values and then push Finish (or press return). Label the appropriate adjacent box as MEAN=

8) The standard deviation is calculated in the same way. First put your label in the box below the mean label. (Labels are not required by Excel, but they make your spreadsheet easier to read afterward.) Move the working box to an appropriate place on the spreadsheet. Use f_x push button to select STDEV. (You can jump immediately to the S's by typing S on the keyboard, then using the down arrows or the mouse pointer placed on the down arrow.) Block out an appropriate section as before, then press return.

9) Repeat the steps necessary to calculate the mean and standard deviation for all additional sample columns. This can be done by dragging the formula across a group of rows. Excel is smart enough to know that it is to update the columns.

10) Label a row as "%RSD =" and calculate the % RSD's all across the columns. The RSD is the relative standard deviation or precision. %RSD = 100*(std dev/mean).

11) The newer versions of Excel have some useful statistical analysis tools. You can gain access to these tools under the Tools menu by highlighting Analysis Tools. The program will load the tools. Then you must again bring up the Tools menu and hit Data Analysis. You have a number of fascinating choices here:

• F-test: two sample for variances (use a separate worksheet ply)
• t-test: Paired two sample for means (use a separate worksheet ply)
• t-test: two sample assuming equal variances (homoscedastic)
• t-test: two sample assuming unequal variances (heteroscedastic)

12) In a new worksheet ply perform the appropriate t-tests and F-tests. Note that the newer versions of Excel do an appropriate t-test and return the probability of the two sets of data coming from the same population as well as a t-value, so you would not need to look up the t

value. If the probability is less than 0.05, there is 95% confidence there is a significant difference. Remember that the F-test requires the correct order for entering data.

13) Go to FILE, PRINT, and then PRINT again to get a printout of your worksheet.

14) Save your data to the CH345 class file as well as to your own network file. It would be good to have a CH345 file on your network drive.

Use the pointer to go to FILE and QUIT. Also use pointer to go to upper left corner little box to back out of programs, etc. When you get to SPECIAL on the control bar, select it and then SHUT DOWN.

Table 1: Experimental Parameters & Manufacturers Data:

Pipette	Aliquot Size (μL)	Accuracy Range* (μL)	Accuracy* (%)	Precision* (%)
1-10 μL	1	0.975-1.025	2.5	2.0
1-10 μL	10	9.9-10.1	1.0	0.5
2-20 μL	2	1.85-2.15	7.5	2.0
2-20 μL	20	19.8-20.2	1.0	0.30
10-100 μL	10	9.65-10.35	3.5	1.0
10-100 μL	100	99.2-100.8	0.8	0.15
50-200 μL	50	49.5-50.5	1.0	0.40
50-200 μL	200	198.4-201.6	0.8	0.15
20-200 μL	20	19.5-20.5	2.5	1.25
20-200 μL	200	198.0-202.0	1.0	0.50
100-1000 μL	100	97.0-103.0	3.0	0.60
100-1000 μL	1000	992-1008	0.8	0.13
200-2000 μL	200	194.0-206.0	3.0	0.60
200-2000 μL	2000	1985-2015	0.8	0.012

* From manufacturers specifications.[1]

Lab Report

Part 1: Complete the attached form and add your data to the CH 345 homework folder. Label your spreadsheet as Lab1initials where initials are your first and last initial.

Part II: Write a one-paragraph abstract that includes the overall conclusions about your pipeting skills (relative to your partner and manufacturer's suggested performance) and your pipets from the data you analyzed. Use the rubric for abstracts as a guide for good writing.

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[1]Rainin Instrument Company, Procedure for Evaluating Accuracy and Precision of Rainin Pipetman™ Pipets, Document AB-15, Revision 2, 1997, p. 14.

Chemistry 345
LAB #1: Pipet Calibration
REPORT FORM

Name: _____   **Partner:** _____

Pipet Set #: _____

Volume (Pipette)	Mean Adjusted Volume (μL)	Precision (% RSD)	Within Tolerance (p/at/am)?*		
2 μL (2-20)	_____	_____	Y / N	Y / N	Y / N
10 μL (2-20)	_____	_____	Y / N	Y / N	Y / N
100 μL (20-200)	_____	_____	Y / N	Y / N	Y / N
200 μL(20-200)	_____	_____	Y / N	Y / N	Y / N
200 μL (200-1000)	_____	_____	Y / N	Y / N	Y / N
1000 μL (200-1000)	_____	_____	Y / N	Y / N	Y / N

* Compared with manufacturer specs for **precision** (p), **accuracy using a t-test** (at), **accuracy compared with manufacturer's ranges** (am).

Analysis of variance:

1) Comparison between _____ μL (smallest volume pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

2) Comparison between _____ μL (smallest volume pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

3) Comparison between _____ μL (middle pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

4) Comparison between _____ μL (middle pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

5) Comparison between _____ μL (largest pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

6) Comparison between _____ μL (largest pipet) volumes of you and your partner:

F value: _____   critical F value: _____   hetero or homoscedastic

P-value: _____ t_{crit} value: _____   t value: _____

conclusion:

7) Comparison between _____ μL volumes of viscous solution and water:

F value: _____ critical F value: _____ hetero or homoscedastic

P-value: _____ t_{crit} value: _____ t value: _____

conclusion:

8) Assume the analytical balances have an error of ±0.20 mg and pipeting for a 2.0 μL pipet has a 5%RSD. What is the overall observed precision for the pipet? Show your work. How might you improve the procedure?

LAB #2: EXPERIMENTAL DESIGN
PROTEIN DETERMINATION IN SOILS

Learning and Skill Goals:
- learn how to correctly use a UV/Vis spectrophotometer
- learn how to correctly perform a protein assay
- familiarize yourself (again) with the use of a Spec 21
- understand linear and non-linear least squares
- learn how to use a spreadsheet for graphing, linear and non-linear regression
- learn how to propagate errors and display error bars in your graphs
- learn how to determine the limit of detection for an analytical procedure

Introduction:
Quantitative protein determinations are an important procedure for bioanalytical chemists and biochemists. A number of different quantitative methods exist for determining protein concentration; some of the most popular include the Kjeldahl, UV absorption, Biuret, Folin-Lowry, BCA and Bradford assays. The specifics of such assays are described in a number of biochemistry lab books. Each assay varies in a number of important ways including: sensitivity, specificity, amount of material required, time for analysis and instrumental technique. In this lab, you will be comparing three different soils and three different extraction methods for soils for the amount of protein as determined by the Bradford method. You will use the data generated to learn how to do a correct least squares analysis and associated error propagation. You will then analyze the variance to get more information about differences in samples, operators and methods by testing assumptions for and using analysis of variance (ANOVA).

The Bradford assay uses a dye (Coomassie Blue) whose negative charge binds with the positive charges on the protein. The dye has a red and blue form. The red form, dominant in solution, changes to a blue color when binding to a protein. The blue color is read and compared with standards at known protein concentrations. As you might expect, detergents like SDS, which are negatively charged, interfere by binding to the protein in preference to Coomassie Blue. Also, there can be an issue with soil compounds absorbing light or having soil constituents react with the Bradford reagent.

Experimental:

Soil Solution Preparation
1. Prepare the soil extracts by adding 4.00 g of fresh soil to a 50 mL plastic centrifuge vial. Record the exact soil mass added to the vial. To the vial, add 4.0 mL of Milli-Q water with a volumetric pipette. Check your work by weighing the vial on the analytical balance before and after the addition of dH_2O.
2. Vortex the soil and water until it forms a homogeneous slurry.
3. Extract the soils:
   a) Sonicate the slurry in an ultrasonic bath for 20 minutes (Extract Method 1).
   b) Heat the slurry in a 60C water bath for 20 minutes (Extract Method 2).

c) Shake the slurry on an orbital shaker for 20 minutes (Extract Method 3).
4. Centrifuge the soil slurry in a clinical centrifuge, making certain to balance the vial with an opposing vial of equal weight. This will centrifuge the soil at approximately 4,000-5,000 rpm, pelleting most of the solids to the bottom of the vial.
5. Pour the supernatant into micro-centrifuge vials, placing the same amount into each vial (roughly 1 mL). Centrifuge the soil extracts again at 14,000 rpm in the microcentrifuge. If you still see suspended sediment in the vial, filter the supernatant through a 0.45 micron syringe filter.

Standard Solution Preparation:

Prepare protein standards by dissolving bovine serum albumin (Sigma) in Milli-Q water. For your analysis, start with 50 mg of albumin in 100 mL dH_2O, and create four additional standards below this point (the highest standard will be equivalent to 500 mg/L protein).

Bradford method:
1. Add 5.0 mL of fresh Bradford Reagent to a glass vial. To each vial, add 500 uL of your albumin standards or soil extracts. Cap each glass vial.

2. Vortex the solution for 30-45 seconds.

3. Allow five minutes for the reaction to occur. The color will be maintained for one hour after the reaction, so don't allow your standards or samples to sit on the lab bench for very long.

4. Measure the absorption of each sample as well as an extract blank on the HP UV-Vis instrument to determine the λmax, and then use a Spec 21 to determine the transmittance of each sample and each standard. Be certain to measure the absorption of the Bradford Reagent to properly account for the blank.

Limit of detection:
You should estimate the detection limit using Equation 5-5 in Harris (p. 86)

Data Analysis:
Using Excel, prepare a calibration graph to test for the concentration of the soil protein. First, determine whether you should use linear or non-linear regression. Then, determine the regression statistics and from them the limit of detection in terms of weight of protein detectable.

Using Excel for Linear Regression:
1) Open a new worksheet under File and type in the concentration and transmittance data for the protein calibration data.

2) Write a formula to calculate absorbance from your transmittance data and copy the formula to all of the relevant cells.

3) Highlight a 2x5 set of cells starting with E1. Type the formula =LINEST. The format for this command is (y-values, x-values, 1,1). Use the mouse to box the y (dependent) data to be regressed. Then use the mouse to box the x (independent) data to be regressed. Follow the commands using TRUE (or 1) for both the y-intercept and stats questions. The last two commands will **not** force the y-intercept through zero and will provide extended statistical data. After this is done, press apple and return keys simultaneously (Command-Shift-Enter on a PC). Make sure the LINEST formula is ready to edit (blinking insert prompt). Then, press Command (the key with the apple on it) and Enter (not Return) together to execute this command. If all works well, ten numbers should appear as below. The values correspond to the slope and its standard deviation (m and s_m, respectively), y-intercept and its standard deviation (b and s_b, respectively), the correlation coefficient (r^2), the F-statistic, the degrees of freedom (df) and a few other mathematical terms that can be identified by reading Harris.

X	Y		LINEST RESULTS		
1	10	**m**	14.38	-2.92	**b**
2	23	**s_m**	1.63	4.24	**s_b**
2	29	**r^2**	0.96	3.71	**se_y**
3	44	**F**	78.29	3.00	**df**
4	52	**ss_{reg}**	1075.97	41.23	**ss_{resid}**

As it is important to be able to obtain all of the regression analyses in order to calculate detection limits in the rest of this course as well as in order to do multiple regressions in later courses, you should understand how to use this somewhat complicated command. It can also be used for multiple regressions (by putting the dependent variable in the first column, and the independent variables in a rectangle of columns where we have B2:B7 in our example). Alternatively, you can do the same analyses in this exercise in the Data Analysis Toolpack by using the regression function in which an ANOVA is run that gives the same ten values as above, Also, many statistical packages (Minitab, SPSS, etc.) also do the regression analysis.

4) Prepare a printout of the spreadsheet.

5) Get a plot of the data by highlighting the rows and columns with the labels and values (it is easiest if x values come first), then highlight INSERT, then New Sheet (unless you want the graph on the same sheet), and Okay. The menu will then ask a series of questions that are fairly intuitive (if you're confused, please ask).

6) Put error bars on your plot. First, generate the values of the error bars using a propagation of error or an estimate. For the x-error bars, calculate the errors in the x values and place them in columns labeled vol err. Assume that the absorbance error is 2% of the measured absorbance value.

7) Excel is also capable of doing a best-fit line, a regression equation (which you already have) and inserting x and y error bars. These are done by highlighting a point on the graph and going

to INSERT. To get a line, use the trendline feature and follow directions. To get error bars, highlight INSERT and the ERROR BARS and follow directions.

8) You may fool with the options to get your graph to look as nice as you can and then go to print preview under the FILE menu. Try to print the graph on a single page and fill the entire page.

9) Propagate the error for your soil protein determination by using the formula in Harris for s_x based on a regression line. First, do this the long way with a spreadsheet and then look at the values on the spreadsheet to see if you could this easier with either the regression or LINEST function.

LAB #2: REPORT FORM
PROTEIN DETERMINATION IN SOILS

Name: _____   Honor Code:

Add your data to the table below:

Standard Concentration (%w/w)	Standard Transmittance	Standard Absorbance

Provide a hard copy of your calibration graphs (with error bars) and the file name where you have your spreadsheet.

Provide the results for your data in the table below:

	Extract 1 (% w/w)	Extract 2 (%w/w)	Extract 3 (%w/w)
Soil 1			
Ave:			
Std Dev:			
Soil 2			
Ave:			
Std Dev:			
Soil 3			
Ave:			
Std Dev:			

Using the propagation formula from Harris or the non-linear regression method (whichever is applicable), calculate the standard deviation of your results for each brand by each method. Attach a spreadsheet with your calculations.

Questions:

1) Compare the LINEST results below with the values from the Analysis Tools Regression analysis (Summary Output) below. Label the values in LINEST that correspond to those in the regression data analysis tools.

LINEST Results

5.565576324	-0.004839564
0.147588033	0.018135921
0.997894826	0.028966384
1422.060249	3
1.193181646	0.002517154

Regression Analysis
from Data Tool Pack

Regression Statistics	
Multiple R	0.998946858
R Square	0.997894826
Adjusted R Square	0.997193101
Standard Error	0.028966384
Observations	5

ANOVA

	df	SS	MS	F	Significance F
Regression	1	1.193181646	1.193181646	1422.060249	4.102E-05
Residual	3	0.002517154	0.000839051		
Total	4	1.1956988			

	Coefficients	Standard Error	t Stat	P-value
Intercept	-0.004839564	0.018135921	-0.266849631	0.806876711
Std Conc	5.565576324	0.147588033	37.71021413	4.102E-05

2) Compare the s_x equation in Harris using the matrix method of calculation with the results from LINEST and regression. Write a short summary of how the values in LINEST and regression relate to each other.

3) Which variable in this experiment is the dependent variable and which is the independent variable? What is the significance of the difference between these in a linear regression? Explain whether this was a good choice and why.

4) If it was done, use the data provided to you in class to calculate the limit of detection for the Bradford method using the procedure outlined in Harris.

5) How good is the Bradford method with respect to each parameter below. Be quantitative.

Linearity:

Sensitivity:

Detection Limit:

Reproducibility:

A1: ANOVA Activity

Name: _____   Honor Code: _____

The following questions should be answered and handed in for full credit. Not all require the use of ANOVA. Hand in a copy of the appropriate statistical tests either as a paper copy or electronically. If electronically, give the name of the file and folder.

File: _____   Folder: _____

1. Write the complete design for the experiment analyzed.

Source	df	EMS	F

2. Is there a significant difference among the extraction methods for determining the protein concentration in soil? Append the results or note the file that allows you to make your conclusion. Explain your conclusion based on comparison of p and F values.

3. Is there a significant difference in the results among the soils. Append the results or note the file that allows you to make a conclusion. Explain. If the methods are different, give a reason why they might be different.

4. How did you insure there was no bias by student in your method?

5. Write an abstract for the laboratory including both protein determination and ANOVA results.

Activity #2: Alpha Fractions: A Useful Way to Solve Acid/Base Problems

Alpha fractions are defined as the fractional amount of acid that exists in a certain form. The total amount of an acid added to a solution is called either the analytical concentration (C_A) or the formal concentration (F_A) of the acid. Thus, if 0.100 M acetic acid is added to a solution, $C_A = F_A = 0.100$ M, no matter what the ratio of the acid and its conjugate base. The actual concentrations of the dissociated and undissociated forms are denoted as the equilibrium concentrations and written with the usual symbols ($[A^-]$ and $[HA]$).

Let's look at how the alpha fraction works for a simple monoprotic weak acid. We define α_{HA} as the fraction of C_A that exists as HA and α_A as the fraction that exists as A^-. The fractions are written as follows:

$$\alpha_{A^-} = \frac{[A^-]}{[HA] + [A^-]} \qquad\qquad \alpha_{HA} = \frac{[HA]}{[HA] + [A^-]}$$

$$\alpha_A = \frac{[A^-]}{C_A} \qquad\qquad\qquad \alpha_{HA} = \frac{[HA]}{C_A}$$

1) Write a reaction for the dissociation of the generic acid, HA, in water.

2) Write an expression for the acid dissociation constant, K_a, for HA in terms of equilibrium molar concentrations.

3) Solve for $[A^-]$ in terms of $[HA]$, $[H_3O^+]$ and K_a.

4) Using this derived expression for $[A^-]$, solve for α_A in terms of $[HA]$, $[H_3O^+]$ and K_a only.

5) Now, eliminate [HA] from the previous expression and solve for the alpha fraction for A⁻ in terms of K_a and $[H_3O^+]$ only.

6) If you've done this correctly, you should be able to simplify your expression to the following:

$$\alpha_{A^-} = \frac{K_a}{K_a + [H_3O^+]}$$

Because the fractions of HA + A⁻ must add up to equal 1:

$$\alpha_{HA} + \alpha_{A^-} = 1$$

7) Using this knowledge, derive an expression for α_{HA} in terms of K_a and $[H_3O^+]$ only (there should be no "1" in the answer when you have simplified the expression).

These are general expressions for any monoprotic acid.

8) When $[H_3O^+] = K_a$ what are the values of α_{A^-} and α_{HA}? Does your answer make sense?

9) Complete the following by adding the missing term:

$$[A^-] = \alpha_{A^-} * \underline{\quad}$$

$$[HA] = \alpha_{HA} * \underline{\quad}$$

Graphing Alpha Fractions vs. pH

It is easiest to display data in a log format. This is usually done by graphing $\log \alpha$ (y-axis) vs. pH (x-axis).

10) Solve for $\log \alpha_{HA}$ in terms of pH, K_a and $[H_3O^+]$.

11) Solve for $\log \alpha_A$ in terms of pK_a, K_a and $[H_3O^+]$.

12) Solve for $\log \alpha_{A^-}$ and $\log \alpha_{HA}$ in terms of pK_a and pH when $K_a \gg [H_3O^+]$.

13) Solve for $\log \alpha_{A^-}$ and $\log \alpha_{HA}$ in terms of pK_a and pH when $K_a \ll [H_3O^+]$.

14) What are the slope and y-intercept of a plot of $\log \alpha_{A^-}$ vs. pH and $\log \alpha_{HA}$ vs. pH in the two regions ($K_a \ll [H_3O^+]$ and $K_a \gg [H_3O^+]$) of the graph?

15) On a 1 mm ruled graph paper, plot both of the dependent variables, $\log \alpha_{A^-}$ and $\log \alpha_{HA}$ vs. pH (independent variable). Use a y-scale of 0 to -14 and an x scale from 0-14.

16) On the same graph, plot the $\log [H_3O^+]$ vs pH and $\log [OH^-]$ vs. pH using the expression $K_W = [H_3O^+] [OH^-]$ or from your knowledge of the relation between pH and $\log [H_3O^+]$, and between pOH and $\log [OH^-]$.

17) There are three principal intersections on the graph. Circle the intersections and solve for pH at each intercept. The intersections represent the initial, midpoint and equivalence points in a titration of your weak acid by a strong base. Identify each titration point.

Polyprotic Acids

We can derive the alpha fractions for a diprotic acid in a similar manner. The only difference is that we now have three terms to consider ($[H_2A]$, $[HA^-]$ and $[A^{2-}]$). The denominator will still be the analytical concentration, but this time we must derive each concentration in terms of two K_a's and the $[H_3O^+]$. The expressions for the three alpha fractions are:

$$\alpha_{H_2A} = \frac{[H_3O^+]^2}{[H_3O^+]^2 + K_1[H_3O^+] + K_1K_2}$$

$$\alpha_{HA} = \frac{K_1[H_3O^+]}{[H_3O^+]^2 + K_1[H_3O^+] + K_1K_2}$$

$$\alpha_A = \frac{K_1K_2}{[H_3O^+]^2 + K_1[H_3O^+] + K_1K_2}$$

Problem: Using your powers of induction from the monoprotic and diprotic acids, derive the alpha fraction expressions for a triprotic acid in terms of the equilibrium constants and $[H_3O^+]$ only. You will need these for the spreadsheet exercise.

Activity #3
Spreadsheet Calculations: Amino Acid pH Distribution

In this activity, you will be modeling the acid/base chemistry of amino acids. To do this, you will need to set up a spreadsheet in Excel. Across the sixth row, label the columns as [H$^+$], [OH$^-$], denom, pH, α_{H3A}, α_{H2A}, α_{HA}, α_A, pH, log [H$^+$], log [OH$^-$], log [H$_3$A], log [H$_2$A], log [HA], and log [A] (pH is done twice for ease of graphing; denom will hold the denominator for the alpha fractions so you do not enter it multiple times). Under the pH column add numbers from 0 to 14 in increments of 0.25. You can do this easily by typing in the values 0 and 0.25 to cells D8 and D9. Highlight these two cells and drag to box D65 (until pH 14). The numbers will all fill in sequence. In boxes A1-A5 write the names K_{a1}..K_{a3} and C_a. In cells C1-C3 write the names pK_1..pK_3. Add the pK_a values that you are assigned to cells D1..D3. In cells B1-B3, place the formulas to determine K_a from pK_a. Highlight box A1, go to the **Insert** window and highlight **Name** and then **Define.** Excel assigns the name K_{a1} automatically, so press **OK**. Enter a value of 0.10 next to the C_a cell (B4) and define it. By defining these values, you insure they are constants rather than variables (you can also use the label of B1 in your mathematical formulas to show that the value in box B1 is a constant rather than a variable, but as you will see, the define name command is easier here).

In the appropriate (A) cell, write a formula to determine the concentration of the H$^+$ based on the pH. After writing the formula, drag it down to fill in the entire column to a pH from 0-14. Next do the same for OH$^-$ and fill in the B column. In the appropriate box, write a formula that represents the denominator for the alpha fractions. Fill in the appropriate C column cells with the formula. Now write formulae for the **alpha fractions** of H$_3$A, H$_2$A, HA, and A based on the [H$^+$], the appropriate equilibrium constants (that you defined earlier) and the denominator that you calculated (in column C) of the amino acid. Next, fill in the rows for the log [H$_3$A], log [H$_2$A], log [HA], and log [A] (using the analytical concentration and the alpha values already calculated). When you are finished drag down the formula for all the appropriate rows. Highlight all the cells that should have a scientific number format, pull down the **Format** command and highlight **Cells** and then **Number**. Click on **Scientific** and then on **OK**. Next click on the cell in the extreme upper left (above the number 1 and next to A). This cell highlights the entire spreadsheet. Pull down the **Format** command and highlight **Column** and **Auto Fit Selection**.

Plotting: Highlight the boxes pH, log [H$^+$], log[OH$^-$], log [H$_3$A], log [H$_2$A], log [HA], and log [A] (including the labels). Choose the **Chart button** and insert the graph as a new worksheet. Follow the directions (**X-Y Scatter**, etc.). You now have a plot of log [X] as a function of pH. To make it look better, double click on any axis that you want to change. A dialog box will come up. Click on **Scale** to change the scale and position of the axes (make sure you undo the x-marked auto boxes). The suggested scale is log [X] from 0 to -14 and pH from 0 to 14. Remove the grey background by clicking on the grey color and highlighting none for background (this leaves a white color). When you are satisfied with the graph (confirm with professor), then you can print a copy. To print a copy, choose the **File** command, then **Print Preview**. Check with your professor and then instruct the program to **Print**.

Different Amino Acids and Analytical Concentration: Save the spreadsheet to temporary storage (in the CH 345 folder) now. Vary the analytical concentration and note what happens to your graph. Next, return the analytical concentration to 1 M and instead of plotting $\log \alpha$ vs pH, plot α vs. pH for all the species. Watch the change in the graph as you change the values of pK_a. After a few tries, predict what will happen to the graph as you raise and lower the values of the pK_a's.

Questions:

1) On your log [A] vs. pH graph (at $C_a = 0.10$ M), identify the predominant species of the amino acid in each pH range by writing its chemical structure. Make sure you correctly write the formal charges on each atom, where appropriate.

2) Calculate the isoelectric pH of your amino acid? Explain your calculation briefly.

3) Circle and identify the significance of the most important titration intersections (initial point, midpoints and equivalence points) on your graph. For **each** intersection:

    a) Write an equation to solve for pH by setting the appropriate equilibrium concentrations equal.

    b) Substitute alpha values for concentrations for any species other than [H⁺].

    c) Make any appropriate approximation.

    d) Solve for pH at each intersection using your approximation.

    e) If species are more than 2 **log** orders different, you should note the nature of the approximation and indicate a more exact equation (but you need not solve that one).

4) Write a proton balance equation for adding 0.10 M NaH_2A and 0.005 M Na_2HA and use alpha methods to solve for the pH of the solution.

Activity #4
Enzyme Purification Computer Laboratory

The purpose of this lab is to learn how the different techniques are used to separate proteins. Start the program by double clicking on the protein purification program icon. It can be found at the following URL:

http://home.btconnect.com/agbooth/archive/swingPP/ProtLab.html

To learn how to use the program, complete *all* the exercises on the web page and answer the associated questions.

For the activity, you may wish to practice on proteins 15, 17, 18 and 19. When you feel competent, see how well you can do on proteins 4 and 5. You may try each one as often as you like, but only your last effort will count for each. The object is to design a reasonable strategy and explain it, not necessarily to get the fewest number of steps (though too many costly steps will be frowned upon).

A grading rubric will be developed based on the following criteria:

        30% for turning in the activity
        20% for completing the exercises as demonstrated by answering the questions
        20% for a reasonable strategy
        30% for an explanation of why you chose each strategy, what you hoped would happen at each step and why what happened did happen for proteins 4 and 5.

You can copy your results and paste them in a word processing program for display. Below is one of my results for getting the total mixture to a pure protein (#20).

Example: Purification results for protein 20

Step	Procedure	Protein (mg)	Enzyme Yield (%)	Enzyme (units)	Enrichment (ph/100 u)	Cost
1	Initial	511.0	100	5400	1.0	
2	Proc. 1	60.4	100	5400	8.5	0.11
3	Proc. 2	17.6	97.5	5266	28.4	0.34

Activity #5
Spectrophotometry Practicum

The purpose of the spectrophotometry practicum is to assess your understanding of how different spectrophotometers work and provide feedback to improve your understanding. In order to study for the practicum, you should read Chapter 21 of your text, as suggested below. The focus of the practicum will be on identifying the most important parts of several instruments, understanding the role of each component and why different instruments work in different ways. Your grade will be based on your preparation as well as your ability to teach about the functioning of the instruments.

Practicum Reading
Chapter 21: deals with instrumentation for spectrophotometry. As part of a practical "quiz" you will be asked to identify the components in one or two instruments based on your reading. There is a lot of detail in this chapter and a lot of formulas describing optical behavior. You need not concern yourself with the formulas, but you should qualitatively understand how the components of the instruments function and how they fit together to give an absorption measurement.

Project Lab #1
Clinical Analysis of Standard Human Blood Serum

Analytes:

        Cations: Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}
        Anions: Cl^-, PO_4^{3-}, SO_4^{2-}
        Glucose
        Creatine and Uric Acid
        Cholesterol

Techniques: chromatography (reverse phase, CE, IC); electrochem (ion selective electrodes); spectroscopic (UV-Vis, fluorescence, optodes, Raman)

Preliminary proposal (due first Wed of block from manager)

References: literature reference(s) for the paper you will be basing your method.

Method: a brief (1 paragraph) outline of how the method works. Describe how the instrumental technique is suited to the analysis and what calibration methods (internal standard, standard addition, etc.) will be used for analysis.

Background: What are the normal ranges for the analyte in serum?

Materials: what chemicals and equipment (including vol flasks, etc.) will be needed. Include costs of chemicals if we may have to purchase them.

Standards & samples preparation: what concentrations you will use for samples and standards? How much sample will be required?

Calculations: how will you perform the calculations?

Grading:
You will present the data from your analysis as a written scientific paper. The assignment will be graded based on a detailed rubric that follows the following criteria:

Written report (submitted by manager in *Analytical Chemistry* format):

        Title, author, location (5 points)

        Abstract (15 points)**:**
        Brief summary of experiment and results
        see Abstract rubric for details

Introduction (15 points):
Why is analysis important in a medical context?
What are typical values and the meaning of atypical values?
What was the main purpose of this experiment?
How does the experimental technique work?

Methods (10 points):
Can someone reproduce the work from what you have given and get the same results because you have provided all the details necessary?

Results (40 pts.):
The reader (professor) must have all the information available to calculate the final results from the data given? (if not, paper cannot be graded and many points will be deducted!)
Are appropriate graphs (with legends, labels and error bars) providede"
Is primary data available in appropriate tables?
Based on the data presented, are the calculations done correctly? (if not, a poor grade will result!)
Is the data accurate compared to known results? Is the data precise?
Did you compare your data with literature values?

Conclusions (15 points):
How good are the results?
If the results are not good, how could they be improved?
How does the standard addition/internal standard method compare with the calibration method?

Project Lab #2
Qualification (and Optimization) of a Bioanalytical Procedure

Learning Goals:
- learn to use a literature procedure to guide your experimental protocols
- become more proficient in troubleshooting
- learn how to write a journal style manuscript
- improve your skills in oral presentation
- gain familiarity with a new technique (electrochemistry, spectroscopy, chromatography)

Introduction:
You are to choose a laboratory procedure that has at least three variables that can be optimized. You may choose to adapt experiments appropriate to bioanalytical chemistry from journals such as *Biochemical Education*, *Journal of Chemical Education* or a current research journal like *Analytical Chemistry* or *Analytical Biochemistry*. Some ideas for how to optimize a method are included in the pages following. Briefly, the optimization should consist of two stages: a factorial design and a simplex optimization. In some cases (of hard luck), your optimization may just consist of getting the experiment to work.

The report for this experiment will be in standard *Analytical Chemistry* format. It should contain a title, introduction, detailed procedure, data analysis and conclusion as in the first report.

Timelines:
1) Due to restrictions of the block, it is necessary that I receive a proposal for what you intend to do for your special project no later than 5 PM on the second Monday of the block. Any requests later than this that result in delays of the experiment are YOUR problem. This proposal will include a copy of the lab or paper from the literature that you will be attempting to optimize. A short description of how you intend to optimize the lab procedure (unless it is suitably challenging as is) should be included in one or two well-written, typed paragraphs. A list of all the chemicals that are required with a supplier, cost, stock number and amount must be included. You may want to check with the stock room or with me to see if we have the required chemicals and equipment.

I urge the managers to discuss the proposed lab with me as soon as possible.

2) We will order the chemicals no later than Wed of Week 2, so that they should be here by the following Monday. You will have four afternoons to complete your project in Week 3.

3) You will deliver an oral summary to the class on Friday morning of Week 3. This is a chance to have your work questioned and commented upon before you submit your final written report.

4) Your final written report is due no later than 9 AM on the final Monday of the block.

Project Lab #3
Experimental Design

The purpose of this lab project is to use your knowledge of ANOVA and experimental design to design an experiment with at least two sources of fixed variance and one source of analytical variance (the last one is easy-it's always there). Since you only have two days to complete the lab, you should choose a procedure that is robust and not too time consuming. Some examples of experiments that have worked well in the past (with associated variance terms) include:

- Chloride in three different brands of pretzels, potato chips or other snacks by ISE (analytical, sampling and error)

- Caffeine in three different coffee types by three different extraction procedures by HPLC (coffee type, extraction method and error)

- Morphine in urine after poppy seed ingestion by radioimmunoassay (fluid intake, sugar intake, error).

- Riboflavin (fluorescence), glucose (colorimetry), or organic acids (IC) in Kombuchka broth (tea type, tea concentration, error).

- Ergosterol in dried fungi by HPLC (species, fungal part, error)

- Loss of ergosterol by HPLC upon soil incubation (soil source, time of incubation, soil moisture, error)

Oral summaries and written reports will be due by 9 AM on the last day of class.

Writing Scientific Papers
(L. Murata & H. Drossman)

Your lab report will follow the format of papers published in scientific journals. All lab reports should be typed and double-spaced. You may work together on interpreting and graphing results and formatting papers, but all written work must be original. Figures and tables should be numbered and given a descriptive title. They are referred to by number in the body of the text (such as "see Figure 2 and Table 2"). Figures and tables may be placed within the text or attached at the end of the report. For examples of scientific papers, refer to journals such as *Analytical Chemistry or Biochemistry*. These papers are organized into discrete sections, each of which describes a specific aspect of the report. For your report, use the following format, and label each section as such ("Abstract", etc.) in the paper. You may submit a preliminary report to me at least two days in advance of the due date for review.

I. Abstract

This is a short section (less than 200 words) that **briefly** describes the purpose of the experiment, the methods used, the important results, and conclusions drawn. Abstracts serve to **summarize** the report so that a potential reader need only read the abstract to understand the general purpose, conclusions, and implications of the experiment. Generally, only a researcher in the field (or a professor) would then go on to read the rest of the report, which contains the details of the experiment. An example is given below:

ABSTRACT

Many chocoholics feel that world production of chocolate is insufficient to meet their needs. Here, we examined the potential of newly discovered plant, *Cocolatus emmandemmus,* as a new source for chocolate candies. By varying the temperature, light intensity, and nutrient sources of *C. emmandemmus* plants, we determined growth conditions for optimal production of chocolate candies. Production was maximized at 37 °C when plants were fed a nutrient mixture of sugar dissolved in 2% (butterfat) milk. Mixing a small amount of peanut butter (5% w/v) into the nutrient mixture promoted the development of peanut-containing candies in addition to the normally-present plain candies. The color of the candy shell varied with the wavelength of light present during growth. *C. emmandemmus* has little potential as a major source of chocolate, however, since even under optimal growth conditions each plant produces only one ounce of candy per year and consumes twenty gallons of milk.

II. Introduction

This section contains sufficient **background** for the reader to understand the relevance of the experiment, its purpose, and the rationale for the experimental design. In the example above, the introduction might include brief descriptions of other chocolate-producing plants and comparisons of their productivity to that of *C. emmandemmus*, statistics on world chocolate production, a brief account of the discovery of *C. emmandemmus*, and a summary of the results of previous productivity experiments on *C. emmandemmus*. The introduction would also include a brief description of the experimental approach and reasons why this approach is more suitable or will yield more information than approaches used in previous experiments.

III. Materials and Methods
This section describes the **methods** and **materials** used in the experiments in sufficient **detail** for another researcher to exactly duplicate the experiment. Sources and names of reagents should be given. Times, temperatures, volumes, procedures, etc. should be explicitly stated. If the experiment follows a previously published procedure, then it is sufficient to give the reference for the procedure along with exact descriptions of any changes. In the example above, this section might include the source of the *C. emmandemmus* plants, their size at the start of the experiment, methods for controlling ambient temperature, volumes and frequencies of nutrient application, and numbers of plants in experimental and control groups.

IV. Results and Discussion
This section **describes** and **interprets** the **results** of the experiment and any relevant **observations**. In the example above, this section might include a chart showing the average growth rate (including standard deviations) of each group of plants, numbers and colors of candies produced, and number of plants that died during the course of the experiments. The dependence of productivity on growth conditions would be described explicitly and possible reasons for variation would be given. Suggestions for changes in future experiments might be given.

V. Conclusions
This section, which may be presented as a list of numbered items, summarizes the overall conclusions of the experiments.

VI. Acknowledgments
This section acknowledges and thanks any outside sources of help received, including the provider of the plants, the mother-in-law who suggested the experimental design, and the guy on the bus who provided the breakthrough insight.

VI. References
Any references cited or consulted should be listed here.