

**INTRODUCTION TO CHEMOMETRICS:
MICROPIPET CALIBRATION & HYPOTHESIS TESTING
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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 50 μL of a viscous solution (50% glycerol) with the 100/200 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.

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

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. $\%RSD = 100 * (\text{std dev} / \text{mean})$.

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

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

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.

**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 (1-10)	_____	_____	Y / N	Y / N	Y / N
10 μL (1-10)	_____	_____	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

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

conclusion:

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

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

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

conclusion:

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

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

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

conclusion:

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

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

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

conclusion:

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

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

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

conclusion:

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

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

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

conclusion:

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

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

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

conclusion:

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