Project 2: The Sweetness of Aspartame – Spectrophotometric Analysis and Receptor Binding

LABORATORY REPORT: Iterative Formal Writing Exercises

PRE-LAB ASSIGNMENT: DAY 1

- Read the entire laboratory project described in the following pages, as well as the *J. Chem. Educ.* source paper,[1] and section a1D in Skoog *et al.* (pp. 985-988).[2]
- Prepare, on a typed sheet of paper, the Project Objectives of this lab (please consult the appendix for an example); on the same sheet, complete the assignment below:

1) Use ChemDraw to draw the structure of aspartame at pH 7[3], and give its molecular weight. Read the MSDS for aspartame; record the HMIS Classification and health hazards.

2) An aspartame solution is made by dissolving 73.6 mg of standard pure aspartame in 25.00 mL of water. If this solution has an absorbance of 1.594 at 260. nm, what is the molar absorptivity of aspartame? Include correct units and significant figures.

3) An artificial sweetener solution is made by dissolving a 1.0 g packet of sweetener in 50.00 mL of water. If this solution has A_{260} = 0.397, and aspartame is the only component of the sweetener that absorbs at 260. nm, then use the molar absorptivity from the question above to calculate the mass of aspartame in the original sweetener packet.

4) The sweetener Equal is 3.67 % (mass/mass) aspartame, and you wish to make 10.00 mL of a solution that is 5.0 mM in aspartame. What mass of Equal will you need?

5) Starting with a 10.0 mM aspartame stock solution, calculate the volume of this stock that you would need to make 5.00 mL of 0.50 mM standard aspartame diluted solution.

---

[1] Stein, P. "The Sweetness of Aspartame" *J. Chem. Educ*. 1997, *74*, 1112-1113.
[2] Skoog, Holler, and Crouch, *Principles of Instrumental Analysis*, 6th ed., Thomson Brooks/Cole, Belmont, CA, 2007.
[3] Assume pK$_a$s of 3 and 8 for the carboxylate and amine groups. Draw the predominant form at pH 7.

6) The reported aspartame concentrations are 71.2 ± 2.7 and 73.8 ± 2.9 µM in samples 1 and 2, respectively.   Based on these results, can you state with confidence that the concentration in sample 1 is less than that in sample 2? Support your answer.

PRE-LAB ASSIGNMENT: DAY 2 (DUE IN CLASS ON THE FIRST FRIDAY)

As you have read in the introductory "Ligand Binding Theory" section, aspartame functions by binding to the sweet chemoreceptor. After agonist binding, the activated receptor initiates a biochemical signaling pathway that ends with an action potential and brain response (consult Scheme 1 in the laboratory Introduction). The aspartame-receptor binding equilibrium is given by

$$R + A \rightleftharpoons R{\bullet}A \tag{PL-1}$$

7) Write an expression for the equilibrium constant for the binding reaction (K_b), and also for the reverse reaction, dissociation (K_d).

8) What are the units of K_d (assuming that concentrations are not divided by 1 M)?

Receptor R is found in two forms in Equation PL-1, so the total concentration of R at any time (including at equilibrium) is given by the the *mass balance equation:*

$$[R]_{tot} = [R] + [R{\cdot}A] \tag{PL-2}$$

On the tongue, $[R]_{tot}$ is very low (nM) compared to typical tastant concentrations (µM to mM). Therefore the concentration of A bound to receptor at equilibrium is miniscule relative to the concentration of A initially present in the beverage, $[A]_0$, and thus

$$[A]_{eq} = [A]_0 - [R{\cdot}A]_{eq} \approx [A]_0 \tag{PL-3}$$

Because only the activated receptor, R·A, initiates sensory signaling, the perceived sweetness (S) depends on the equilibrium concentration of R·A; furthermore, the dependence is linear, so:

$$S = x\,[R{\cdot}A]_{eq} \tag{PL-4a}$$

When all receptors are activated ($[R \cdot A] \approx [R]_{tot}$), the receptors are saturated; further addition of A will cause no further increase in sweetness perception. At this point, the sweetness perception is maximal, S_{max}; the sweetness of solutions of A asymptotically approach S_{max} as $[A]$ increases. From Equation PL-4a,

$$S_{max} = x\,[R \cdot A]_{max} = x\,[R]_{tot} \qquad\qquad \text{(PL-4b)}$$

9) Use the equations above to derive: $[R \cdot A]_{eq} \cong \dfrac{([R]_{tot} - [R \cdot A]_{eq})[A]_0}{K_d}$

10) Use the equations above to derive: $S \cong \dfrac{(S_{max} - S)[A]_0}{K_d}$

11) Use the equation in (10) above to derive: $S \cong \dfrac{S_{max}}{1 + K_d /[A]_0}$

12) The equation in (11) above mathematically describes a hyperbolic saturation of S. Specify the value of S for the following three concentrations of A:

   $[A]_0 = 0$: $S \approx$ _____.

   $[A]_0 \approx \infty$ (infinite): $S \approx$ _____.

   $[A]_0 = K_d$: $S \approx$ _____.

13) A molecule with a relatively high K_d must be present in a relatively high or low concentration in order to reach half-maximal sweetness ($S = \frac{1}{2}\,S_{max}$).

INTRODUCTION

In the previous lab, we used a spectrophotometric calibration curve and focused on experimental uncertainties; we did *not* consider any uncertainty associated with the calibration curve itself. In this project, we will turn our attention to this additional source of error. Furthermore, you will gain experience using a second type of spectrophotometer, the Genesys UV10. The spectro-photometric calibration curve that you generate will be used to do both a quantitative analysis of the amount of aspartame in a packet of artificial sweetener, and a biochemical investigation of the sensory response to a particular activating ligand. In addition to spectrophotometry, you will gain experience in this lab with nonlinear curve-fitting, statistical analysis of error/uncertainty, ligand binding equilibrium, and the neurochemistry of sensory response.

We will apply Beer's Law ($A = \varepsilon bc = \varepsilon \ell c$) to the UV chromophore aspartame, an artificial sweetener. Aspartame, which is the methyl ester of the aspartate-phenylalanine dipeptide, is the active ingredient in Nutrasweet, Equal, and a number of generic store-brand artificial sweeteners. You will make standard solutions of known aspartame concentration, measure their absorbances, and from the slope of the calibration curve, determine the molar absorptivity of aspartame. You will then use this molar absorptivity to determine: (a) the amount of aspartame in a packet of artificial sweetener; and (b) the aspartame concentration in various Equal solutions for which you have both absorbance and biological taste measurements. You will use the absorbance and taste data to characterize the binding of aspartame to your sweet receptors.

LIGAND BINDING THEORY

Ligand binding equilibria are at the heart of almost all biochemical processes, including enzyme activity, solute transport, hormone response, drug development, and sensory perception. Most sensory receptors bind their activating ligands (agonists) reversibly and non-covalently. For example, smell receptors bind "odorant" molecules, and taste receptors bind "tastant" molecules that may be sweet, sour, bitter, salty, or savory (umami). When an agonist binds to its receptor, it causes a conformational change that activates the receptor and leads to downstream signaling.[4]
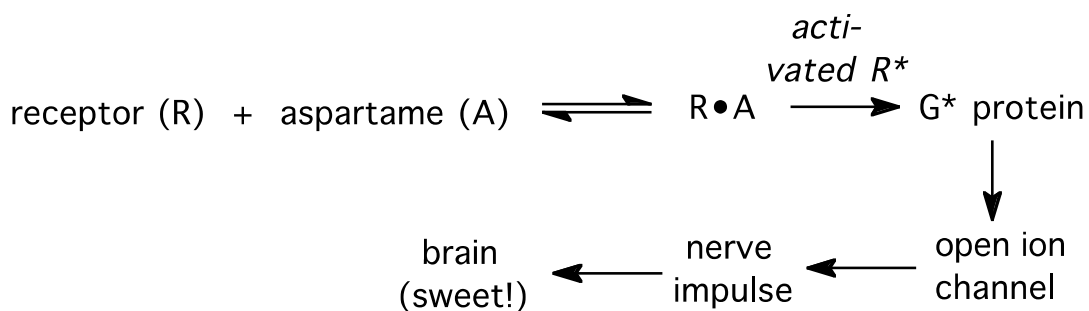
---

[4] Conversely, antagonists bind to a receptor without activating it. Because they block the agonist binding site on the receptor, antagonists serve as signaling inhibitors.

Sensory receptors convert signal reception, i.e. agonist binding, into a nerve signal which reaches the brain and is interpreted there (see Scheme 1 below).

The sweet receptors T1R2 and T1R3 are found mainly toward the front of the tongue; they are activated by natural sugar ligands such as glucose, fructose, and sucrose. Since the late 1800s, chemists have discovered a number of artificial sweeteners that serve as exquisitely strong agonists that bind to the receptors much more tightly than sugars. These include saccharin, which was first synthesized in 1879, aspartame (1965), stevia (1970 in Japan; centuries earlier in South America), and sucralose/Splenda (1976). Saccharin and sucralose are non-caloric, that is, they are mainly excreted without metabolic processing. Although stevia and aspartame are metabolized, they are so much sweeter than sucrose (per gram), that very little is needed to artificially sweeten food products. These four artificial sweeteners range from 200× (aspartame) to 600× (sucralose) sweeter than sucrose, per gram.

Agonists (A) like aspartame bind to their receptors (R) non-covalently to form the receptor-agonist complex (R·A, see Scheme 1 below). A subsequent conformational change activates the receptor, allowing it to activate subsequent downstream signaling proteins (e.g., G proteins, ion channels).

receptor (R) + aspartame (A) ⇌ R•A $\xrightarrow{\text{acti-vated R*}}$ G* protein

brain (sweet!) ⟵ nerve impulse ⟵ open ion channel

Scheme 1: The signal transduction pathway for the sweetness sensory response.

$$K_{\text{binding}}(\text{RA}) = K_b = \frac{[R \cdot A]}{[R][A]} \; ; \qquad K_{\text{dissociation}} = K_d = 1/K_b = \frac{[R][A]}{[R \cdot A]} \qquad (2)$$

In contrast to the formal definition of activities as used by physical chemists, biochemists typically do not divide the concentrations in equilibrium constant expressions by the standard

concentration of 1 M. Thus the dissociation equilibrium constant (K_d in equation (2) above) is not unitless; rather, it has units of mol/L = M.

There are two forms of the receptor, agonist-free (R) and agonist-bound (R·A); the *mass balance equation* gives the total concentration of receptor at any point, which is constant:

$$[R]_{total} = [R] + [R \cdot A] = R_{free} + R_{bound}$$
(3)

$[R \cdot A]_{max} = [R \cdot A]$ as [A] approaches infinity (saturating) = $[R]_{total}$ (i.e., 100% bound, 0% free)

You will derive the following equation for yourself: $[R \cdot A] = \dfrac{[R \cdot A]_{max}}{1 + K_d /[A]_0}$ (4)

Because your perception of sweetness intensity (S) is linearly proportional to [R·A] (equation PL-4a),

$$S = \frac{S_{max}}{1 + K_d /[A]_0} = \frac{S_{max} \cdot [A]_0}{K_d + [A]_0}$$
(5)

From equation (5), if $[A]_0 = K_d$, then $S = S_{max}/2$; therefore K_d, the dissociation equilibrium constant, is also the ligand concentration that yields $S = S_{max}/2$ (see Figure 1 below). Also, S_{max} is proportional to $[R]_{total}$, which differs for each person's tongue. "Supertasters" are people who are exquisitely sensitive to certain tastants, most likely due to a high $[R]_{total}$.

Equations 4 and 5 resemble the Michaelis-Menten equation typically applied to enzyme kinetics; however, recall that here, we are dealing only with the ligand binding equilibrium. In any case, equations (4) and (5) describe a curve that saturates hyperbolically: As concentration approaches infinity, the *y*-value approaches a finite asymptote, and the slope decreases monotonically to zero. Recall that:

A large K_d means more dissociation, looser binding, more sucrose needed to register a response.
A small K_d means less dissociation, tighter binding, less aspartame needed to register a response.

This is depicted in Figure 1 below, showing the dose response to these two sweeteners.
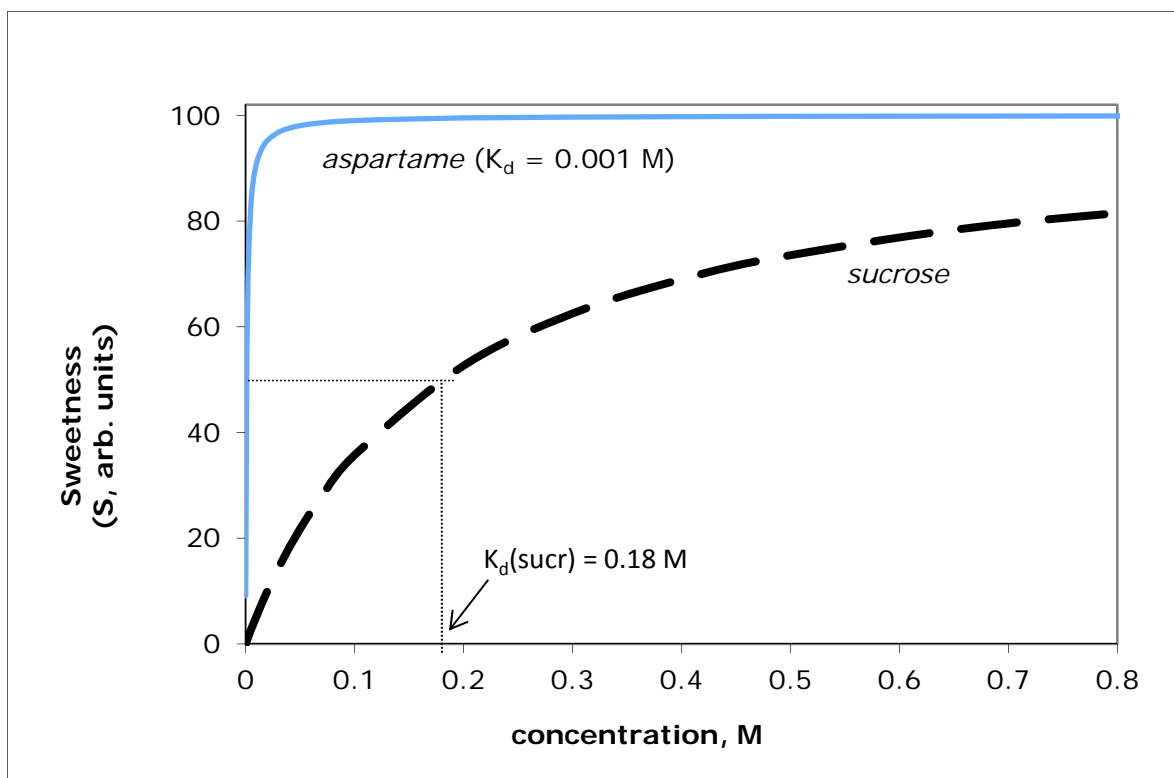

Figure 1: Sweetness intensity, *S*, saturates hyperbolically with agonist concentration. Aspartame (solid line) binds to the sweet receptor ≈ 200 times more tightly per gram than sucrose does (dashed line); this correlates to a K_d for sucrose (in units of M) which is ≈ 180 times higher than that of aspartame.

The common *Double-Reciprocal* form of the hyperbolic saturation equation (5) is:

$$1/S \; = \; 1/S_{max} \; + \; (K_d/S_{max})(1/[A]_0) \tag{6}$$

So a plot of $1/S$ (y-axis) vs. $1/[A]_0$ (x-axis) will be linear, with **y-intercept** = $1/S_{max}$, and **slope** = K_d/S_{max}. This type of plot is often used in older literature to determine S_{max} and K_d, but it is prone to error, especially for low values of $[A]_0$ where $1/[A]_0$ is very large. For each Equal solution made by the class, you will obtain average [aspartame] and *S*, and then plot these points and fit them to equations (5) and (6) to determine best-fit values for S_{max} and K_d.

EXPERIMENTAL: DAILY PLAN

Prior to beginning laboratory work, students should have in their notebooks a daily plan based on pre-lab discussions. For this lab, please see Appendix 1 at the end of this project for a list of all project activities. Before you begin your lab work each day, you must have the instructor check your daily plan. For Day 1 of the lab, each pair should prepare a plan that includes the information necessary to generate the calibration curve described below, e.g., mass of pure aspartame, volumes, and dilution factors. To plan for the analysis of Equal and store-brand sweetener packets, the sample size must be calculated: Stein reports that a 1 g packet of Equal contains 36.7 mg of aspartame (3.67%); a concentration of aspartame in the middle of the calibration curve (~5 mM) is desired, and 10 mL is a convenient volume. Using the molecular weight of aspartame and the given percentage of aspartame in Equal, calculate the mass of Equal powder needed to make the desired solution. This calculation, along with your entire daily plan, must be checked by the instructor.

For the second day in laboratory, the entire lab section will prepare and perform the taste test of aspartame solutions. Specifically, the plan should include the individual assignments of each student. Day 3 will be taken up with data analysis.

EXPERIMENTAL: CALIBRATE A SET OF PIPETTORS (SEE ALSO APPENDIX 2)

Each team should have a set of three adjustable micropipettors: large (100-1000 μL), medium (10-100 or 20-200 μL), and small (0.5-10 or 2-20 μL). In Project 1 you tested the accuracy and precision of the 1000 μL pipettor by dispensing water into a weigh boat on an enclosed analytical balance. Now you will do the same with a medium and small pipettor. Pipettors should deliver aliquots that are accurate and precise to within 3% of the selected volume.

Test your medium and small pipettors at both the minimum and maximum volumes, giving four tests in all. For each test, tare a weigh boat, select a pipettor volume, dispense an aliquot of water into the weigh boat, and record the mass in your lab notebook. Without taring, add at least four subsequent aliquots, recording the mass each time. Record as well the temperature of the lab room, and find the density of water at this temperature. Convert each of your mass readings to a dispensed volume of water.

1. *In your informal report, note the lab temperature and the density of water at this T. Cite your reference for the latter value.*

2. *Build a data table that includes, for each of the four tests:*
   a. *the pipettor used and the selected volume, V_{sel}*
   b. *average volume dispensed ± standard deviation = $V_{disp} \pm s_V$*
   c. *% error = $100 \times (V_{disp} - V_{sel})/V_{sel}$*
   d. *% relative error = $100 \times s_V/V_{disp}$*

All pipettors that perform within specification (% error and % relative error < 3%) should be labeled and color-coded for future use. Return any miscalibrated pipettors to the instructor with a note, and replace them with another pipettor (which must be calibrated).

EXPERIMENTAL (DAY 1): CALIBRATION CURVE

Prepare 50 mL of a 10 mM solution of aspartame in deionized water using an analytical balance, a small beaker for the initial solution, and a volumetric flask. (See Appendix 2, "Making a Standard Solution" for further details.) Calculate the exact concentration of the aspartame stock solution. Select 5-8 convenient dilutions to make standard aspartame concentrations that range from the 10.0 mM stock solution, to no lower than 0.4 mM. For each standard concentration, work out the volume of stock solution needed to make 5.00 mL of each standard solution (see Appendix 2, "Making Standard Dilutions" for further details). You will use micropipettors and 5 mL volumetric flasks for these dilutions. Please store these standard solutions in <u>labeled</u> clean, dry test tubes or vials in the refrigerator.

EXPERIMENTAL (DAY 1): ABSORBANCE MEASUREMENTS WITH THE GENESYS-10

The instructor will show pairs of students how to use the Genesys-10; detailed instructions are given in Appendix 3. The Genesys-10 is a single-beam instrument, so a reference spectrum of water must be collected before scanning a sample. Scan the water in a UV-transparent plastic cuvet in slot B from 240-300 nm. You only need to do this once, unless you change scan wavelength parameters. Next, scan the 10 mM stock solution of aspartame. Use the instrument software to find λ_{max} and A_{max}. If the measured A_{max} is > 2, scan a more dilute solution until A_{max} is ≤ 2. Print your scan and record λ_{max} and A_{max} for this stock aspartame solution in your lab note-

book. Next, with your water reference in the Blank (B) slot of the spectrophotometer, use either the "Advanced A-%T-C" or the "Abs_5samples" test to measure the absorbance at λ_{max} of each of your standard solutions in disposable UV-transparent cuvets in slots 1-5. Take replicate (4-7) absorbance measurements for each standard. Plot your standard curve and check its linearity.

EXPERIMENTAL (DAY 1 OR 2): ANALYSIS OF SWEETENER PACKETS

The stockroom will supply retail size 1.0 g packets of Equal and store-brand sweeteners. Student pairs should confirm the mass of product in each type of sweetener packet by combining the contents of five packets of each and weighing on an analytical balance.

> *3.   Record the average mass per packet, and the % error in this value.*

The two sweetener types weighed above can now be used to make replicate sample solutions for spectrophotometric analysis. Prepare three to five replicate sample solutions of each type of sweetener in 10 mL volumetric flasks using the mass calculated in your "daily plan" section above. Record the exact mass of each replicate as measured on an analytical balance. Finally, measure the absorbance of each replicate on the Genesys-10 spectrophotometer; you may choose to make multiple measurements of each sample. Record all of the values in your notebook.

EXPERIMENTAL (DAY 2): EQUAL TASTE TEST

Students in each lab section will divide the work to make 50 mL of each of the following Equal solutions (all concentations in mg Equal/mL[5]): 3, 4, 6, 10, 15, 20, 30, and 40. You will also have available a pre-prepared 50 mg/mL Equal solution to compare as *S*(sweetness) = 5.0.

You will <u>taste</u> these solutions, so the following solution-making process will be performed at a tasting table outside of the lab room. Add your massed portion of Equal to a Dixie cup, and fill with bottled drinking water to the 50 mL mark on a previously prepared wooden dipstick. Stir with a *clean* wooden splint. For the higher concentrations of Equal, you may have to rest your Dixie cup in a beaker of hot water while stirring to speed up dissolving. Label the cup containing your solution with your letter code. Pour 20 mL of your Equal solution into a small clean, dry

---

[5] For example, 50 mL of a 2 mg/mL solution requires 100 mg of Equal.

beaker; you will bring this portion into the lab room for absorbance measurements (below). Insert a sterile plastic dropper in the remaining 30 mL of Equal solution, and leave the labeled Dixie cup on the tasting table outside of the lab.

Back in the laboratory room, add portions of your Equal solution to a UV disposable cuvet to measure replicate absorbance values at the aspartame λ_{max}. (Do not scan, just measure absorbance at the single λ_{max} wavelength.) Empty and refill the sample cuvet, and measure a total of 4-5 times. If any replicate absorbances are off by more than 10%, repeat measurements until you get improved agreement. Finally, take one measurement of the absorbance of the pre-prepared high-concentration 50 mg/mL Equal solution. Be sure to record all of these absorbance values both in your lab notebook and also in the lab spreadsheet. Do NOT leave the lab until you have ascertained that your absorbance values make sense and are duly recorded.

At the tasting table outside of the lab room, taste all of the Equal solutions made by your classmates. Use the plastic dropper in each Equal solution to dispense a few drops onto your own plastic spoon, which you will rinse and dry between each solution tasting. Start by placing a few drops of the stockroom-prepared high concentration Equal solution (50 mg/mL) on your own plastic spoon and taste it. Keep this taste in mind as a "5" on your 0-5 scale. Clear your palate by tasting a few drops of bottled water; this is "0". Note the identifying letter code on each cup, and taste all of the Equal solutions made by your classmates by placing a few drops on your spoon. Clear your palate with water after each tasting. Rank each solution in sweetness intensity (*S*) on a 0-5 scale; you may use fractional values if you wish. Record your *S* values both in your lab notebook and also in the lab spreadsheet.

When you are finished, record in the class Google doc spreadsheet your name and:
  (a) the letter code identifying YOUR Equal solution;
  (b) your sweetness ratings (*S* values, 0-5 scale) for ALL of the section's Equal solutions
  (c) your 4-5 replicate absorbance readings for YOUR Equal solution, and
  (d) your single absorbance reading for the stockroom-prepared 50 mg/mL Equal solution

Before you leave lab for the day, you MUST record the data above (a-d) in the class's Google doc spreadsheet.

DATA ANALYSIS: CALIBRATION CURVE

Use Excel to make an aspartame calibration curve. (Consult instructions from the previous lab, if necessary.) The slope of your straight line is related to the molar absorptivity of aspartame at λ_{max}. Use a statistical package to determine the standard error in your molar absorptivity, as well as the standard error of the linear regression.

4. *Include in your informal report a high-quality calibration curve with absorbance error bars, fit equation, and R^2.*

5. *Include a copy of the regression statistical analysis in your informal report.*

6. *Report the molar absorptivity of aspartame (ε), with proper units, uncertainty, and significant figures. Note that the units may differ from those of the slope.*

DATA ANALYSIS: ASPARTAME IN EQUAL AND STORE-BRAND SWEETENER PACKETS

For each of the 3-5 replicate Equal sample solutions, calculate* an aspartame concentration from the measured absorbance (or the average *A* if multiple readings were taken). Use the calibration curve error equation (Equation CCE, Appendix 4A) to determine* the uncertainty in aspartame concentration stemming from the calibration curve. This uncertainty then determines the correct number of significant figures in the reported aspartame concentrations in each Equal sample solution. Now determine* the mass of aspartame in each sample solution, from the reported aspartame concentration and volume of each solution. From this aspartame mass per sample, calculate* the milligrams of aspartame per gram of Equal. Finally, for the 3-5 Equal samples, calculate the mean, standard deviation, and 95% confidence intervals for mg aspartame per gram of Equal. Now perform the same set of calculations for your 3-5 store-brand sample solutions.

7. *Prepare a data table with columns for mass of sample, average absorbance, aspartame concentration, and mg aspartame per gram of each sweetener. For the latter value, mg aspartame per gram, give a statistical summary including mean,*

> *standard deviation, and 95% confidence interval. *Provide a reference to the exact*
> *pages in your laboratory notebook where sample calculations* can be found.*

Taking 36.7 mg aspartame per gram as an accepted value for the composition of Equal, determine if there is any bias in your analysis of aspartame in Equal.

> 8. *In your Discussion section, show the calculation of bias for Equal brand sweetener, and report the result.*

Compare the results for Equal and store-brand sweeteners. Do a *t*-test to determine if the two brands contain the same mass of aspartame per gram of sweetener.

> 9. *In your Discussion section, show the calculation and report the results of the t-test. Use Excel to perform the t-test (Two-Sample, Equal Variances) and report the P-value for a two-tail comparison. Do both the calculated t-test result and the Excel-returned P-value lead to the same conclusion?*

DATA ANALYSIS: EQUAL TASTE TEST

For each Equal solution in the taste test, calculate the average absorbance and the standard deviation: $A_{avg} \pm s_A$. Use the molar absorptivity to convert* each A_{avg} to [aspartame]. Determine* the "uncertainty in *x*-value derived from calibration curve" using the calibration curve error (CCE) equation as described in Appendix 4A. Report, for each Equal solution, average aspartame concentration ± uncertainty, with correct units and significant figures. The uncertainty will be plotted as *x*-error bars in your final *S* vs. [aspartame] scatter plot.

Determine the **Average Sweetness Intensity,** *S*, for all Equal solutions: Use the class tasting results in the spreadsheet to calculate an average *S* value for each Equal solution, and also the standard deviation for each average value. These standard deviations will be plotted as *y*-error bars in your final *S* vs. aspartame concentration scatter plot.

10. *Prepare a data table for the aspartame concentration and S data. .   *Provide a reference to the exact pages in your laboratory notebook where sample calculations* can be found.*

DATA ANALYSIS: HYPERBOLIC SATURATION

The *S* vs. aspartame concentration scatter plot will be fit to a hyperbolic saturation curve by the graphing program Kaleidagraph using non-linear regression. First, use Kaleidagraph to fit the practice data in Table 1 in Appendix 4B to the equation for hyperbolic saturation.

> *11. Include a printout of the plot of the sample data with the best-fit line and fitting*
> *parameters.*

Once you are familiar with Kaleidagraph, plot *S* vs. [aspartame], and add both *x* and *y* error bars to the data points. Fit the data to the equation for hyperbolic saturation.

> *12. Include a printout of your plot with the best-fit line and fitting parameters.*

Prior to the easy availability of software that performed nonlinear regression, nonlinear data had to be linearized and then fit using linear regression. Data that saturate hyperbolically can be linearized using the double reciprocal equation (6), after calculating $1/y$ and $1/x$ for each point.

> *13. Include a printout of your double reciprocal plot with the best-fit line and fitting*
> *parameters.*

> *14. Report fitted values for K_d and S_{max} with their associated uncertainties, derived from*
> *both nonlinear regression (hyperbolic saturation) and linear regression (double*
> *reciprocal plot).*

> *15. Are K_d and S_{max} more reliable as derived from the nonlinear hyperbolic saturation*
> *plot, or the linear double reciprocal plot? Support your conclusion using statistical*
> *arguments such as relative uncertainties, the P-value of the double-reciprocal y-*
> *intercept, and the mathematics of reciprocals of error-prone small values.*

> *16. Discuss the significance of K_d and S_{max}. Compare to literature values[6], if possible.*

---

[6] Some literature values are available in the P. Stein *J. Chem. Educ.* source paper[1] for this project, and also in papers listed in "Further Reading" below. You can also Google "aspartame sweet receptor (or T1R2) dissociation constant (or binding affinity)."

FURTHER READING

"The Sweetness of Aspartame" Stein, P.J. (1997) *J. Chem. Educ*. **74**, 1112-1113.

"Sweet Taste Receptor Gene Variation and Aspartame Taste in Primates and Other Species"
        Li, X. et al, (2011) *Chem. Senses*, **36**, 453-475.

"Characterization of the Modes of Binding between Human Sweet Taste Receptor and Low-Molecular-Weight Sweet Compounds"
        Masuda, K. et al (2012) *PLoS ONE* **7**(4), e35380

"Different functional roles of T1R subunits in the heteromeric taste receptors"
        Xu, H. et al (2004) *Proc. Natl. Acad. Sci. USA* **101**(39), 14258-14263.

APPENDIX 1: ASPARTAME LAB ACTIVITIES

DAY 1:

1. Calibrate a set of three pipettors (student pair)

2. Make a 10 mM standard aspartame solution (student pair)

3. Make 5-8 standard aspartame dilutions (student pair)

4. Obtain a UV scan of standard 10 mM aspartame (student pair)

5. Take absorbance measurements of the 5-8 standard aspartame dilutions (student pair)

DAY 2:

6. Make 3-5 replicate Equal and store-brand sweetener solutions (student pair)

7. Make a selected Equal solution and measure its A_{max} (each student)

8. Taste and rate ALL Equal solutions (each student) made in #7 above.

9. Each student will record on a class spreadsheet

    a. Replicate A_{max} values for YOUR Equal solution

    b. One A_{max} value for the pre-prepared Equal solution (50 mg/mL)

    c. Your own taste ratings for ALL Equal solutions

Because activities 1, 2, and 6 above all require use of the analytical balance, you may have to use a balance outside of your lab room. Balances are available in the EB and EC wet labs, the Introductory Chemistry and Organic Chemistry labs on the fourth floor, and student research labs on the third floor. Aim to complete activities 1-5 on day 1, and the rest on day 2. Day 3 will be reserved for data analysis.

APPENDIX 2: LABORATORY TECHNIQUES

Use of Adjustable Pipettors: You have five adjustable micropipettors to choose from.

Volume range	Scale maximum	Type of tips
100-1000 µL:	'100' = 1000 µL (so '10' = 100 µL)	large blue tips
20-200 µL:	200 = 200 µL	small yellow tips
10-100 µL:	100 = 100 µL	small yellow tips
2-20 µL:	20 = 20 µL	small yellow tips
0.5-10 µL (P10):	'100' = 10 µL (so '10' = 1 µL)	tiny white tips

Pipettors have less relative error at the upper end of their range, so choose accordingly: To deliver 20 µL, choose the 2-20 µL pipettor rather than the 10-100 µL one. When you use a pipettor, fill the tip by pressing down on the plunger to the first stop point and releasing slowly. To deliver the aliquot, rest the tip against the side of the vessel, and slowly press the plunger all the way, past the first stop point.

Here are some reminders regarding the correct usage of pipettors:

    a. First, select the best pipettor to use (middle to upper end of volume range)

    b. Be sure to dial in the correct volume; <u>predict</u> the fill level you expect to see in the tip.

    c. To fill, depress to first stop, then suck up.

    d. Check tip for air bubbles, leakage, and appropriate filling. When in doubt, start over!

    e. To deliver, rest tip against vessel above liquid surface, and depress past the first stop.

    f. When finished, store the pipettor upright in a rack; never lay it down on the lab bench

Making a Standard Solution: If you know that the standard solute is soluble, and you only need to add solvent and dissolve, then you may add the weighed solute directly to the appropriate volumetric flask using a funnel. It is very often advisable, however, to first add the weighed solute to a beaker; this allows you to heat the solution, stir it, or adjust its pH before bringing to volume. Add to the beaker a volume of solvent that is less than the final solution volume and make any necessary adjustments (heat, stir, pH, etc.). Finally, quantitatively transfer the solution in the beaker to the appropriate volumetric flask, including several rinse portions in the beaker.

Making Standard Dilutions: To make standard dilutions, you must use the equation $V_{dil}M_{dil} = V_{conc}M_{conc}$. For example, to make 5.00 mL of a diluted 80.0 μM standard, starting with a 10.0 mM stock solution, you may calculate that 40.0 μL of the stock are necessary. Thus, you must pipet 40.0 μL of stock solution into a 5 mL volumetric flask, add water to the mark, cover with Parafilm and mix thoroughly. In biochemistry, dispensing small volumes such as 40 μL is often necessary, thus requiring the use of adjustable pipettors, as opposed to the volumetric pipets preferred by analytical chemists.

Aspartame Calibration Curve Dilutions: In order to prepare a calibration curve for aspartame, you will need a series of 5-8 known dilutions of the stock 10.0 mM aspartame solution so that your absorbances span the range from about 0.04 up to about 2. If the stock 10.0 mM aspartame solution has an absorbance of 1.5, then this solution can serve as the highest concentration standard solution for your calibration curve. To get an absorbance of 0.05, you will need a solution that has a 1.5 ÷ 0.05 = 30× lower concentration, or 10.0 mM ÷ 30 = 0.33 mM. Therefore, your 5-8 known dilutions should range from 0.4-10 mM.

Every dilution you make involves pipetting errors, and these errors magnify when you make serial dilutions (using a diluted sample to make a further dilution). Therefore, it is best to use the ORIGINAL stock solution, as long as you work within the limits of the smallest pipettor available to you. Thus, try to use as few "stock" solutions as possible when preparing your dilutions.

For example, given a stock 10.0 mM aspartame solution, to make two dilutions of final concentrations 5.0 and 0.4 mM, all with final volume = 5.00 mL, the best way is:

[asptm], mM:	5.0 mM	0.40 mM
Dilution:	2× (1:1)	25× (1:24)
Vol. of 10 mM stock:	2.5 mL	200 μL
Vol. of water: (fill to mark on 5.00 mL volumetric flask)	≈ 2.5 mL	≈ 4.8 mL

All standard dilutions can be made from a single stock solution, using only the 200 and 1000 μL pipettors. Recall that you have access to five pipettors, covering the entire range of 1-1000 μL.

APPENDIX 3: USING THE GENESYS-10

Collect a Baseline Absorbance Scan: Use water in a *UV disposable cuvet*[7] as a blank reference cell, placing it in slot B of the Genesys-10 spectrophotometer sample compartment. The cuvet's *transparent windows must face the light beam*. Close the compartment lid. Press the green 'Test' button (bottom center of keypad), select the "Scanning" test (press 'enter'). Set the start and stop wavelengths (low and high λ, respectively) to cover 240-300 nm. Press the 'Run Test' button, then when a plot shows on the screen, press the 'Collect Baseline' button. This will take awhile. You only need to do this once, unless you change scan wavelength parameters.

Collect an Absorbance Spectrum of Stock Aspartame Solution: <u>Student pairs</u> place 3-4 mL of the stock 10.0 mM aspartame solution in a UV disposable cuvet, place the cuvet in sample slot #1 and press "Measure Sample" to collect an absorbance scan. Estimate the wavelength of maximum absorbance (λ_{max}) of asparatame, and the absorbance at this wavelength (A_{max}). If the measured A_{max} is > 2.0, dilute appropriately and scan again until A_{max} is ≤ 2.0. When you have a usable scan, press "Edit Graph", then "Math", then "Peaks and Valleys". Select only "Label Peaks", then "escape". Finally, print your scan and record λ_{max}, A_{max}, and the concentration of this stock aspartame solution in your lab notebook. BE ADVISED that this is the <u>only</u> scan that you need to do in this lab; for all further measurements, you will simply read the absorbance at λ_{max}. (Recall that λ_{max} is the wavelength of maximum absorbance, i.e., the absorbance peak; it is NOT the longest wavelength in your scan.)

With water in slot B of the Genesys-10 spectrophotometer, load your standard solution UV disposable cuvets into slots 1-5. Select "Test" = "Advanced A-%T-C" or "ABS_5samples". Set the wavelength to aspartame's λ_{max}. Check that "Number of Samples" is set to how many UV disposable sample cuvets you have to read. Press "Run Test", and once your reference (water) is in slot B and your samples are loaded in slots 1-5, press "enter". Record absorbances in your lab notebook. For any subsequent measurements, just press "Measure Samples", load your cuvets, and press "enter".

---

[7] For mid-to-low UV absorbances, we use UV disposable semi-micro cuvets; these have a narrowed interior. They hold a maximum of 3 mL, and can be reliably read with only 1.0 mL samples.

APPENDIX 4A: DATA ANALYSIS – UNCERTAINTY DERIVED FROM A CALIBRATION CURVE

To calculate the uncertainty (u_{unk}) in the value of x_{unk} determined from the calibration curve we will use a slightly modified version of the "calibration curve error" equation from Skoog et al[2]:

$$u_{unk} = \frac{s_r'}{m}\sqrt{\frac{1}{N_{std}}+\frac{1}{N_{unk}}+\frac{(\bar{y}_{unk}-\bar{y}_{std})^2}{m^2\sum_{i=1}^{N_{std}}(d_i)^2}} \qquad \text{where}$$

m = slope of the calibration curve straight line

N_{std} = number of standard points on the calibration curve straight line

N_{unk} = number of replicate absorbance measurements made on the unknown sample

$\bar{y}_{unk}$ = average of replicate absorbance measurements made on the unknown sample

$\bar{y}_{std}$ = average absorbance for all n_{std} points on the calibration curve

d_i = difference between the concentration (x) the ith standard point in the calibration curve and the average concentration for all standard points in the curve: $x_{std}(i) - \bar{x}_{std}$

The definitions above are all identical to those found in Skoog and in the appendix at the end of this lab manual. The difference for our calculation is that s_r' will reflect both the standard error of the regression (s_r), as well as the standard deviation of the replicate absorbance measurements made on your chosen Equal solution (s_A from p. 23). Specifically, $s_r' = \sqrt{s_r^2 + s_A^2}$.

For the given chromophore absorbance data set in the spreadsheet below, the uncertainty in the concentration value due to both the calibration curve *and* the absorbance standard deviation, calculated from the equation above, turns out to be 18.15 µM. This calculation is described briefly here, using data from the spreadsheet below: (1) The standard deviation in the average aspartame absorbance from replicate measurements on the unknown solution $= s_A = 0.01058$.
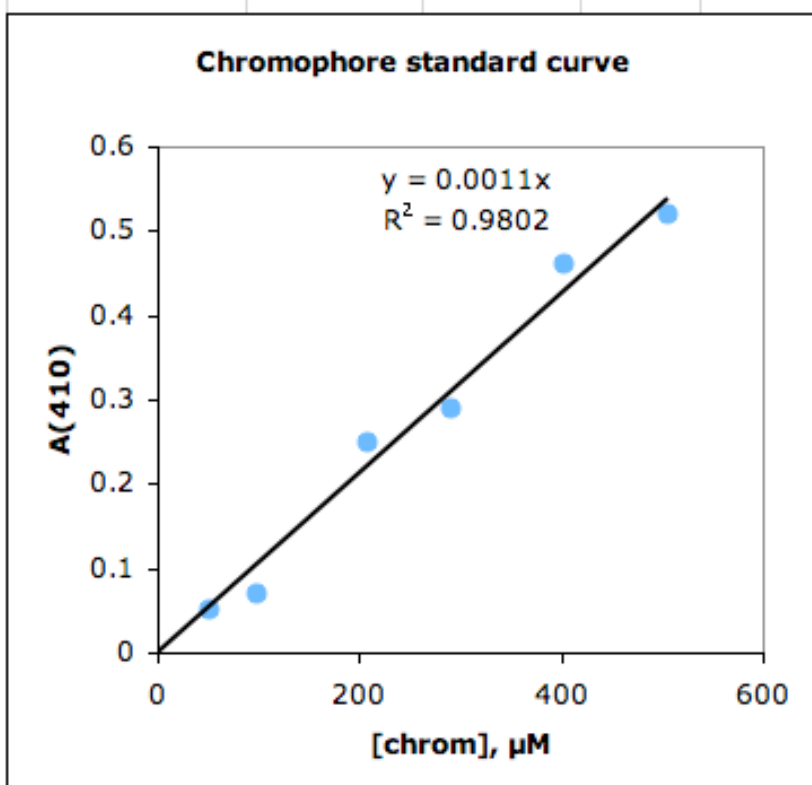
(2) The standard error of the regression $= s_r = 0.02725$; so $s_r' = \sqrt{0.02725^2 + 0.01058^2} = 0.0292$.

(3) The slope of the calibration curve $= m = (1.06 \pm 0.04) \times 10^{-3}$ µM^{-1}cm^{-1}, so the molar absorptivity $= \varepsilon_{410} = 1060 \pm 40$ M^{-1}cm^{-1}.

standard curve:			unknown sample		x(i) - x(avg	d(i)^2
[chromphor], µM	A(410)		A(410)			
51	0.05		0.33		-208.5	43472.25
98	0.07		0.318		-161.5	26082.25
208	0.25		0.342		-51.5	2652.25
291	0.29		0.338		31.5	992.25
403	0.46		0.332	A(avg)	143.5	20592.25
506	0.52		0.0106	A(s.d.)	246.5	60762.25
259.5	0.27333	avg			sum(d(i)²)=	154553.5

SUMMARY OUTPUT			312.05 [chrom], µM
Regression Statistics			9.9472 c(s.d.)
Multiple R	0.99707689		
R Square	0.99416232		molar absorptivity =
Adjusted R Square	0.79416232		$(1.06 \pm 0.04) \times 10^{-3}$ µM⁻¹cm⁻¹
Standard Error	0.02724981		1060 ± 40 M⁻¹cm⁻¹
Observations	6		

	Coefficients	Standard Error	t Stat	P-value	Lower 95%
Intercept	0	#N/A	#N/A	#N/A	#N/A
X Variable 1	0.00106392	3.646E-05	29.181	8.86E-07	0.0009702

Chromophore standard curve

$y = 0.0011x$
$R^2 = 0.9802$

(plot: A(410) vs [chrom], µM, data points with fitted line)

m (slope)	0.001064
n(std)=	6
n(unk)=	4
y(avg,unk)	0.332
y(avg,std)	0.273333
sum[d(i)^2	154553.5
S(r)=	0.02725
A(s.d.)=	0.010583
S(r)'=	0.029233
S(r)'/m=	27.47645
fraction =	0.019674
uncerty=	18.14986

final [chrom] =
312 ± 18 µM

(4) The average A_{410} of the unknown sample is 0.332, so [chromophore] = 312.054 µM.

(5) Furthermore, n_{std} = 6; n_{unk} = 4; $\bar{y}_{unk}$ = 0.332; $\bar{y}_{std}$= 0.273; and the sum of all d_i^2 = 154,553.

(6) From these values we can calculate, using the uncertainty equation above, u_{unk} = 18.15 µM. Hence, the final reported value for chromophore concentration is: **312 ± 18 µM.**

APPENDIX 4B: DATA ANALYSIS – CURVE-FITTING AND REGRESSION

Regression uses a mathematical algorithm to find the equation for a curve that best-fits experimental data.   Regression is usually carried out by graphing applications like Excel, Kaleidagraph, PeakFit, SigmaPlot, etc. Regression results return values for parameters in an equation for a curve that matches experimental data points as closely as possible.

Regression works by minimizing, for each data point, the difference between the experimental *y*-value and the *y*-value calculated from the equation; this difference is known as the *residual*. Goodness of fit is judged by high R^2 values ($R^2_{max} = 1$). Best-fit parameters determined by regression should always be reported along with their associated uncertainties, to the correct number of significant figures. For linear regression, fitted parameters are slope and *y*-intercept. For hyperbolic saturation ($y = \dfrac{y_{max}}{1 + K/x}$), fitted parameters are y_{max} and K.

NON-LINEAR CURVE-FITTING

Nonlinear curve-fitting is done best with plotting software like Kaleidagraph, Excel "Solver", PeakFit, or SigmaPlot.   The following are general instructions for Kaleidagraph, using the practice data set in Table 1 below:

Table 1:

[chrom], M:	0.01	0.02	0.05	0.10	0.25	0.50
Absorbance:	0.104	0.238	0.507	0.839	1.222	1.425
± s_A:	0.007	0.011	0.028	0.015	0.022	0.019

Plot these sample data, absorbance vs. [chromophore], in a simple *xy* scatter plot: Input the data into a Kaleidagraph spreadsheet,[8] being sure to label each data column by double-clicking on the column number. Next, select from the menu "Gallery/Linear/Scatter", and choose your *x* and *y* columns (concentration = *x*, absorbance = *y*). Set the minimum value on each axis to zero by

---

[8] Numbers in Kaleidagraph spreadsheets are always right-justified. If your data are left-justified, then you must convert the column from "text" to "number" format by selecting, under the "Data" toolbar menu, "Column Format", then under "data type" on the top right, select "float" instead of "text". In this box you can also fix number format (decimal vs. scientific notaton) and # of digits shown.

double-clicking on each axis and specifying "min". Label this plot "Sample nonlinear data set: Absorbance vs. concentration" by double-clicking on the plot title.   Add the absorbance uncertainty ($\pm s_A$) as *y*-error bars: Under "Plot" at the top, select "Error Bars", "Y err"; select "Link Error Bars", and then next to the "+" bar, select "Data Columns" and select the column that contains your error bar values.

Note that the data points show hyperbolic saturation: At first absorbance rises roughly linearly with [chromophore], but then it starts to level off, asymptotically approaching a maximum value (A_{max}). The mathematical equation describing this type of plot is

$$A = \frac{A_{max}}{1 + K/[chrom]} = \frac{A_{max} \cdot [chrom]_0}{K + [chrom]}$$

Fit your data set to this hyperbolic saturation equation, using the asymptote A_{max} and the equilibrium constant *K* as fittable parameters. Select your plot, and select from the menu "Curve Fit/General". If you see "Michaelis-Menten" as a choice, choose it, and check the box by your *y*-value column. If not, then choose "Edit General", and click the "Add" button. Select the "New Fit" at the top of the left column, and in the dialog box below, rename it "hyperbolic satn". Click "Edit" and in the dialog box write:

m1*m0/(m0 + m2); m1=1; m2=1;

Here m0 stands for your *x*-value ([chromophore]), m1 is your first fittable parameter (A_{max}), and m2 is your second fittable parameter (*K*). The last half of the line sets your fittable parameters to their initial values of, in this case, one.

Click "OK", and your new "hyperbolic satn" fit should be selected in the left column. Click "OK" again. Go back in the menu to "Curve Fit/General", select "hyperbolic satn", and check the box by your *y*-value column. The fit (red line) should run close to your data points, and the red fitted parameter data box should appear on your plot. (If the red parameter box disappears from

your plot, in the top menu choose "Plot/Display Equation". If you make any changes in your data spreadsheet, you can incorporate these changes into your plot by selecting "Plot/Update Plot".)

For the purpose of graph tidying, the axis maximum values should be close to your data maxima. Double click the *x*-axis and set "max" to 0.52. In the menu, select "Format/Curve Fit Options" and check the boxes for "R^2 instead of *R*", "extrapolate to axis limits", and "force fit through zero". We choose R^2 because it is the goodness of fit parameter that is most useful; in this case the fit is forced through zero because a correctly zeroed spectrophotometer will give a reading of zero if the chromophore concentration is zero. Keep in mind however that not all curve fits should be forced through zero.

In the red fitted parameter box on your plot, m1 = the fitted value for A_{max}, the *y*-axis asymptote. The error is the uncertainty in the fitted parameter. Uncertainty is reported to two significant figures if its first digit is one or two, but to only one significant figure if its first digit is three or higher.   Since your A_{max} error is 0.04963, report only one significant figure, i.e., 0.05. Furthermore, the number of reported significant figures in the reported value is controlled by the final decimal place in the reported uncertainty, so you report your value of A_{max} only out to the hundredths place. Your fitted value of A_{max} should therefore be reported as A_{max} **= 1.79 ± 0.05** (unitless). Likewise, your fitted value of K **= 0.121 ± 0.009 M**. Note that *K* (m2) has units of M, the same units as [chrom] (m1). Since absorbance (*y*) is unitless, the *y*-asymptote A_{max} must also be unitless. Your R^2 value should be 0.997(93).

Recall that for hyperbolic saturation, $K = C_{50}$, the [chrom] that gives $A = A_{max}/2$. On your plot, you can see that the [chrom] for which $A = 0.9$ has a value of about 0.12 M, which matches the fitted value of $K = 0.121$ M. In general, a small value of *K* denotes saturation at low [chrom], i.e., an avid reaction that requires only low substrate concentration to saturate the reaction. Conversely, a large value of *K* denotes saturation at high [chrom], i.e., a weak reaction. So *K* is inversely related to reaction "strength".

Finally, when you finish with Kaleidagraph, save your files to your H: drive. Data (spreadsheet) files are saved as filename.qda, whereas plots are saved separately as filename.qpc. When you're

all done, after choosing 'quit', be sure to check the boxes "data, plots, layout, macros, and style" in the "Save Changes" dialog box.

Aspartame Sweetness Plot: Use Kaleidagraph to plot an "Aspartame Binding/Sweetness Curve", average *S* vs. average [asptm]. Each of the class's Equal tasting solutions will be one point on this curve. For each point include error bars in the y direction (standard deviation in average S) and in the x direction (net uncertainty in average [asptm]). To do this, include the error values in separate columns (one for y error, one for x error) in the Kaleidagraph spreadsheet. After plotting the points, select "Plot/Error Bars", and select the box below "x err". In the "% of value" box, select "Data Column" and then choose the data column with your x error values. If the "Link Error Bars" box is selected (it should be already), you'll get the same bars on either side of the point. Do the same procedure for your y error bars.

This curve should exhibit hyperbolic saturation, so you should get a good fit by fitting to the hyperbolic saturation (or Michaelis-Menten) equation:

$$S = \; S_{max} \cdot [asptm]/([asptm] + K_d \,) \;\; = \; m1*m0/(m0 + m2)$$

where $m0 = x = [asptm];$ $m1 = S_{max};$ $m2 = K_d;$

As long as your chosen initial values for the fittable parameters are close enough to the final best-fit values, Kaleidagraph will return best-fit values *and* their associated uncertainties for the equilibrium dissociation constant, K_d, and the asymptotic maximum sweetness, S_{max}.   If Kaleidagraph returns a "matrix error," then you must adjust the initial values for m1 and m2 so that they are closer to the final best-fit values. You can estimate this by a visual examination of your plotted points: m1 should be the y-asymptote (S_{max}), and m2 the concentration that gives S = $S_{max}/2$ (i.e., C_{50}). Remember to save your files to your H: drive: name.qda for data; name.qpc for plots.