Determination of Red Food Coloring in Commercial Beverages

Introduction

Since a 1973 claim linking children's behavior and their consumption of food color additives, controversy over the safety of color additives in consumer products has brewed.[1-3] Federal oversight of these additives was established much earlier (1880s); however, not all coloring agents were found to be safe after further investigation. In fact, many additives were being used to mask inferior and defective food products. This prompted the Pure Food and Drug Act of 1906 whose enforcement was entrusted later to the Food and Drug Administration (FDA). Currently, FDA certification is required for all color additives in food, drugs, cosmetics and medical devices.[4]

There are currently nine color additives certified by the FDA for use in the United States. The most commonly used in consumer products is FD&C Red No. 40 (**Figure 1**). FD&C Red No. 40 additive was approved by the FDA in 1971, and an acceptable daily intake (ADI) calculated at 7 mg per kilogram bodyweight. For the average 60 kg (~132 lb) person, the ADI is 420 mg per day. Based on the amount of additive proposed for use in food manufacturing and those food products' consumption, the estimated daily intake (EDI) of Red 40 is 18 mg per day for the average 60 kg person.[4]
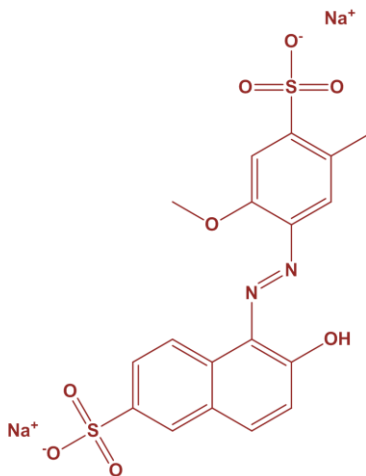

Figure 1 Chemical Structure of FD&C Red No. 40 color additive. Molecular Weight= 495.9987 g/mol.

In this laboratory experiment, you-the analyst-will measure the amount of FD&C Red No. 40 present in common commercial beverages and bakers' food dye provided in the lab. A spectrophotometer is an analytical instrument used to measure the light absorbed by samples. The simplest spectrophotometer is comprised of a light source (tungsten lamp generates the light), a monochromator (blocks all wavelengths of light except the one of interest), sample holder (stabilizes

the sample containing cuvette) and a detector (a phototube measures all the light remaining after the sample has absorbed light), **Figure 2**.
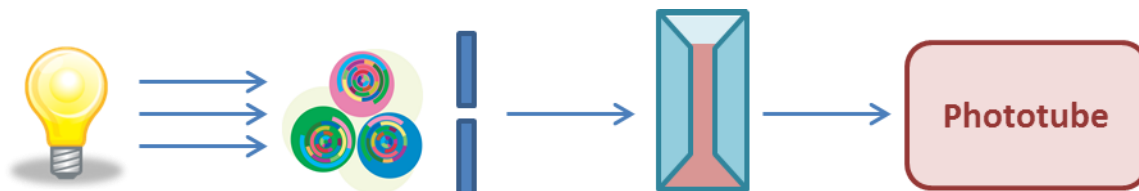

Figure 2 Spectrophotometer instrument diagram. The spectrophotometer's tungsten lamp releases white light that is filtered by a monochromator's diffraction gradient and sorted by an exit slit. The one wavelength of light released from the monochromator interacts with the sample in the cuvette, and any unabsorbed remaining light is measured by the phototube detector.

The spectrophotometer can report either a particular analyte's %Transmittance or Absorbance depending on the analyst's preference. These values can be calculated by the equations below:

$$\%\text{Transmittance (\%T)} = \frac{irradiance\ of\ light\ \textbf{exiting}\ the\ sample}{irradiance\ of\ light\ \textbf{entering}\ the\ sample} \times 100$$

and

$$\text{Absorbance (A)} = -\log(\frac{\%T}{100})$$

To determine the concentration of FD&C Red No. 40 in commercial products, we use a relationship called the Beer-Lambert Law or simply, Beer's Law.

Beer's Law: A = εbc

> where **A** is absorbance (unitless), **ε** is the molar absorptivity ($M^{-1}\ cm^{-1}$), **c** is the light-absorbing analyte's concentration (M) and **b** is the cuvette cell's pathlength (cm).

This relationship shows that an analyte's (i.e.- FD&C Red No. 40's) absorbance is directly proportional to its concentration in the sample. Therefore, the concentration of FD&C Red No. 40 can be determined in a number of commercial products like baker's food coloring, G2 Gatorade, Powerade, Publix strawberry soda and cherry 7 UP.

In order to determine the unknown concentration of FD&C Red No. 40 in a commercial product, the absorbance of a series of standard solutions with known FD&C Red No. 40 concentrations must first be measured. A serial dilution of these known concentrations should yield a linear range over which the spectrophotometer response will be proportional to the concentration. Remember that diluted solutions are prepared from more concentrated solutions by using the Dilution Formula.

Dilution Formula: $$M_1 \cdot V_1 = M_2 \cdot V_2$$

where **M** is the solution concentration and **V** is the solution volume. These variables may be any unit of analyte over the volume of the solution (i.e.- mol/L; g/mL; etc.) as long as the same units are used consistently on both sides.

If these concentrations and instrument responses are reported graphically, the data points should yield a visually straight line. The "best" straight line can be fitted to these data in Microsoft Excel by adding a *TRENDLINE* and displaying the trendline's equation and R^2 value on the graph. The resulting plot is known as a calibration curve.

Example Calibration Curve

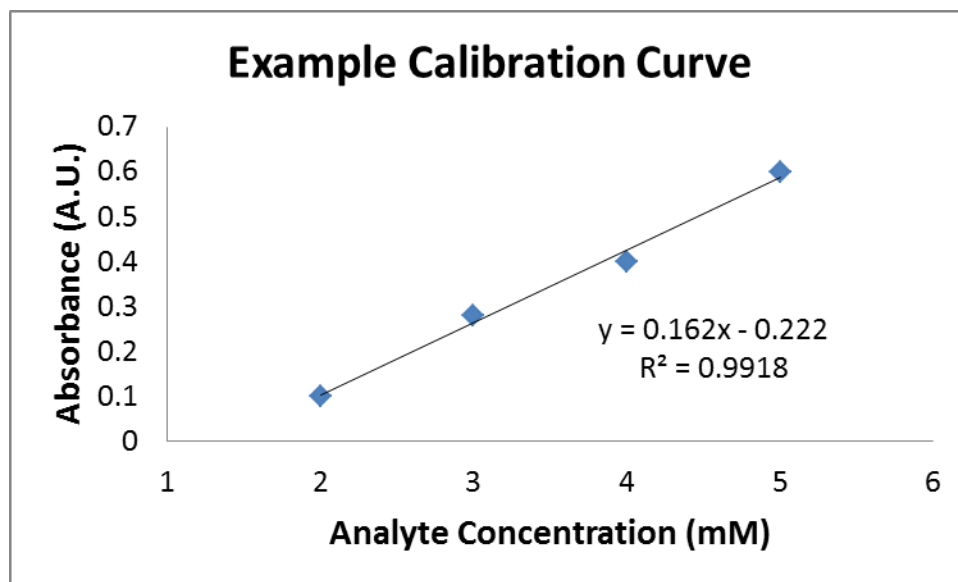$y = 0.162x - 0.222$
$R^2 = 0.9918$

Figure 3 Example calibration curve. Calibration curves are easily constructed in an Excel spreadsheet. [This calibration curve is only an example for instruction purposes and is not representative of experimental data.]

In this lab, record the linear equation (y=mx+b) of best fit and the goodness of fit of the data to the line (R^2). Using the line formula, y = mx +b, the concentration of an unknown substance can be determined. The closer the data's R^2 value is to 1 (i.e.- R^2 ≥0.995), the better the linear fit. Thus, the R^2 value is often used as an experimental diagnostic. If R^2 decreases after a method is validated, something has changed from the validated procedure.[5]

Quality controls (QC) are samples containing a known or statistically established concentration of the method target (ex. FD&C Red No. 40) and are used as a check of the overall work process. These samples are selected or designed to fall within a calibration linear range and typically are representative of the low, mid, and high concentration region of a calibration curve. Multiple QC samples are routinely prepared and analyzed along with every calibration curve and set of unknown

samples. If the observed QC concentration values are within the established acceptable range, the work process is considered to be stable. When the observed QC concentration values exceed the expected variation, it indicates a source of error has been introduced into the work process and must be fixed. Until corrective action is taken, the results from the method are untrustworthy.

References:

[1] C.K. Conners, Psychopharmacology Bulletin, 9 (1973) 24-85.
[2] C.K. Conners, Food additives and hyperactive children, Springer, New York, 1980.
[3] S. Kobylewski, M.F. Jacobson, in, Center for Science in the Public Interest, http://cspinet.org/new/pdf/food-dyes-rainbow-of-risks.pdf, 2010.
[4] FDA/CFSAN, in: F.A. Committee (Ed.), 2011.
[5] D.C. Harris, Quantitative chemical analysis, W.H. Freeman and Company, New York, 7th ed. 2007.

A few notes BEFORE you begin:

1. The cuvettes are expensive. Please be careful with them.
2. For your entire experiment, you should use the same cuvette. Each cuvette has microscopic imperfections that are accounted for when you "blank" the solution on the instrument.
3. Do not put liquid in (or take liquid out) of the cuvette while it is in the instrument.
4. Shut the door of the instrument carefully between measurements.

Experimental Procedure

1. Make all the dilutions at your bench and then take your samples to the spectrophotometer to measure the absorbance.

2. In a 50 mL beaker, pour 20 mL of 50.0 µM red food coloring.

3. Using a 10.0 mL volumetric flask and a 5.0 mL or 10.0 mL Mohr pipette, dilute the 50.0 µM solution of red food coloring to make the following concentrations:

2.5 µM
5.0 µM
10.0 µM
15.0 µM
20.0 µM
25.0 µM

These samples will be used to construct the calibration curve.

Using the dilution procedure described, make a 1:10 and a 1:5 dilution of the G2 fruit punch Gatorade.   These samples will be used as the QC low (1:10 dilution) and the QC high (1:5 dilution).

Additionally, select two unknowns. Prepare the appropriate dilution needed via the table in step #12 to determine the concentration of the unknowns using Beer's Law.

Note: Our Mohr pipettes are not calibrated to include the liquid below the 5.0 mL mark. For example, if you wish to add 1.0 mL, you would fill the pipette to 4.0 mL and empty until the meniscus is sitting on the 5.0 mL denotation.

4. Once your dilutions are made, measure the absorbance of each concentration using the spectrophotometer.

5. Before measuring the absorbance, make sure the wavelength is set at 505 nm.

6.   Hold the cuvette on the opaque side of the glass. Wipe the clear side of the glass with a chemwipe to remove any fingerprints or dust that may interfere with the measurement.

7. Fill the cuvette with distilled water to the level of the yellow square.

8. Align the clear side of the cuvette with the holes for the light in the instrument.

9. Place the cuvette in the spectrophotometer.

10. Close the door and push the "0 Abs 100% T" button. This is the blank for the measurement.

11. Remove the water from the cuvette using a disposable transfer pipette.

12. You are now ready to measure the two concentrations of G2 Gatorade (QC low and high) and **two** beverage unknowns from the list below. For the most accurate results, measure the least concentrated to most concentrated.

Powerade Zero	(1:10 dilution)
Cherry 7 Up	(No dilution)
Sparkling Ice Black Raspberry	(1:20 dilution)
Publix Strawberry Soda	(1:10 dilution)
Diet Oceanspray Cranberry Juice	(1:10 dilution)

Note: To accurately determine the concentration of red food coloring in G2 Gatorade and the two unknowns, the absorbance must fall within the range of the known concentrations.

Lab Report

1. Show the calculations for the dilutions neatly below. Remember significant figures and units. Show all your work. If there is no work, there will be no credit. (**1 point**)

2. Record your data in the table below. <u>Write the name of your unknown in the space provided under the "Product" column.</u> (**1 point**)

Product	Concentration	Absorbance
FD&C Red No. 40	2.5 μM	
	5.0 μM	
	10.0 μM	
	15.0 μM	
	20.0 μM	
	25.0 μM	
G2 Gatorade – QC Low	1:10 dilution	
G2 Gatorade – QC High	1:5 dilution	
Unknown 1: _____	_____:_____ dilution	
Unknown 2: _____	_____:_____ dilution	

3. Attach a graph of concentration (x axis) versus absorbance (y axis) of the red food coloring. Fit the line using a linear trendline, display the equation and R^2 on the chart. Email your excel spreadsheet to your professor. (**1 point**)

4. Using the equation from your calibration curve, what is the <u>undiluted</u> concentration of each of the two G2 Gatorade QCs and the two unknowns? Show your work. If work is not shown, no credit will be given. (**2 points**)

5. The absorbance of a solution of red food coloring is 0.204 at 500 nm. The pathlength is 1.00 cm and the concentration of the solution is 3.175 mM. What is the molar absorptivity? (**1 point**)

6. Evan obtained the following absorbance data:

Concentration (M)	Absorbance
1.05	0.231
2.10	0.402
3.20	0.691
4.40	0.837

A graph of this data revealed the line equation was y = 0.1885x + 0.0366 with an R^2 = 0.98064.

a. Evan measures a solution with an absorbance of 0.548. What is the concentration of the analyte in solution? (**0.5 point**)

b. Evan has a solution that has an analyte concentration of 1.5 M. What would the expected absorbance of the solution be according his calibration curve? (**0.5 point**)

c. What does the R^2 value mean? What does your R^2 value from Question #3 mean? (**0.5 point**)

7. Using the undiluted G2 Gatorade Fruit Punch concentration determined in Question #4, calculate the amount of red food coloring present in a normal 32 oz. (946 mL) container of G2 Gatorade Fruit Punch. Report your calculation in milligrams. Show all work. (**0.5 point**)

b. Given an ADI of 420 mg/day for a 60 kg (132 lbs) person, what is the percent contribution of one G2 Gatorade Fruit Punch to his/her ADI? (**0.5 point**)

c. Calculate the red food coloring ADI for a 160 lb adult assuming a safe daily value of 7 mg/kg bodyweight. (**0.5 point**)

8. You work as a scientist for the International Olympic Committee and are responsible for testing athletes for anabolic steroids as part of the anti-doping program for the Olympic Games. Your calibration curve has a R^2 of 0.943. Your QC low sample is within the expected range but the QC high sample is well above the acceptable range. Of the 10 urine samples tested, you observed 8 positive results for anabolic steroids. What could you report back to the International Olympic Committee? (**1 point**)

Red Food Coloring Instructor Guide

Learning Outcomes

1. The students will demonstrate the ability to make a serial dilution of red food coloring.
2. The students will demonstrate the ability to obtain, record and analyze spectroscopy data of a known sample.
3. The student will demonstrate the ability to determine the concentration of an unknown sample using Beer's Law.

Laboratory Preparation

Using McCormick's Red Food Coloring, dilute 1.0 mL of the food coloring to 1000 mL in a volumetric flask using distilled water. This is considered a 50 μM stock solution. The QC was made from G2 Fruit Punch Gatorade. The Powerade Zero unknown is the Fruit Punch flavor. *Note: If you are using the same glassware for the aspartame analysis, make sure you thoroughly rinse the glassware. The copper biuret will quench the red food coloring.*

Topics to Cover in Pre-lab Lecture

1. **Beer's Law Overview**
2. **Example of solving for concentration using y=mx +b**
3. **How to properly make a dilution using volumetric glassware (DEMO)**
4. **How to use a Mohr pipette (DEMO)**

Lab Report – Key/Scoring Rubric

Please tally all points earned for the lab report <u>first</u> (10 points total). Feel free to adjust this to your class' grading scale later, but for the purposes of reporting in this project, please keep your submitted tallies to a 10 point scale.

From the total points earned, consider the following possible general deductions:

-0.2 incorrect use of significant figures when reporting calculations
-0.2 units not reported or reported incorrectly with numerical calculations

Please mark any lab reports turned in Incomplete with an "I" next to the numeric score. (Incomplete = not all questions were attempted)

1. Show the calculations for the dilutions neatly below. Remember significant figures and units. Show all your work. If there is no work, there will be no credit. (**1 point**)

Using $M_1V_1 = M_2V_2$, the students should be able to derive the amount of concentrated solution to use.

For example, 50 µM (X) = (2.5 µM)(10 mL)
X = 0.5 mL

Likewise,

For the 5 µM concentration, 1 mL of the 50 µM solution should be diluted to 10 mL.
For the 10 µM concentration, 2 mL of the 50 µM solution should be diluted to 10 mL.
For the 15 µM concentration, 3 mL of the 50 µM solution should be diluted to 10 mL.
For the 20 µM concentration, 4 mL of the 50 µM solution should be diluted to 10 mL.
For the 25 µM concentration, 5 mL of the 50 µM solution should be diluted to 10 mL.

+ 0.4 use of $M_1V_1=M_2V_2$
+ 0.6 dilution calculated correctly (+0.1 EACH)

2. Record your data in the table below. <u>Write the name of your unknown in the space provided under the "Product" column.</u> (**1 point**)
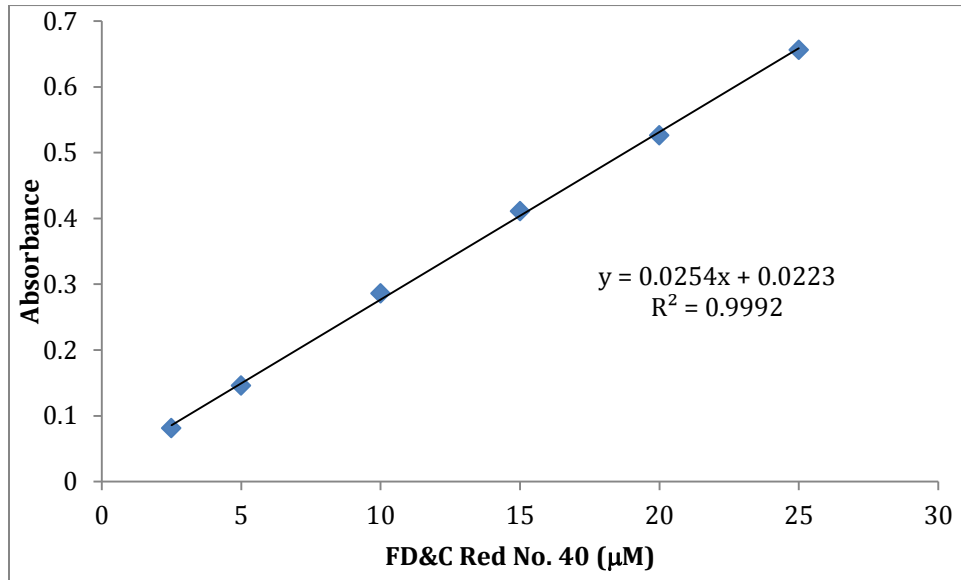
Product	Concentration	Absorbance
FD&C Red No. 40	2.5 µM	*0.081*
	5.0 µM	*0.146*
	10.0 µM	*0.286*
	15.0 µM	*0.411*
	20.0 µM	*0.526*
	25.0 µM	*0.656*
G2 Gatorade – QC Low	1:10 dilution	*0.242*
G2 Gatorade – QC High	1:5 dilution	*0.466*
Powerade Zero	(1:10 dilution)	*0.332*
Cherry 7 Up	(No dilution)	*0.204*
Sparkling Ice Black Raspberry	(1:20 dilution)	*0.454*
Publix Strawberry Soda	(1:10 dilution)	*0.221*
Diet Oceanspray Cranberry Juice	(1:10 dilution)	*0.189*

+ 0.3 recording Abs to three significant figures (as appropriate to lab spectrophotometers used)
+ 0.3 recording all calibration absorbance data
+ 0.4 recording QC and unknown data (+0.1 per line)

3. Attach a graph of concentration (x axis) versus absorbance (y axis) of the red food coloring. Fit the line using a linear trendline, display the equation and R^2 on the chart. Email your excel spreadsheet to your professor. (**1 point**)

Chart: Absorbance (y-axis) versus FD&C Red No. 40 (μM) (x-axis).

$$y = 0.0254x + 0.0223$$
$$R^2 = 0.9992$$

+ 0.3 Linear trendline graphed
+ 0.2 R^2 value present
+ 0.2 correct axes labels (+0.1 EACH)
+ 0.3 line equation present

4. Using the equation from your calibration curve, what is the <u>undiluted</u> concentration of each of the two G2 Gatorade QCs and the two unknowns? Show your work. If work is not shown, no credit will be given. (**2 points**)

Each student should have 4 calculations (2 QCs & 2 unknowns) worth 0.5 points each.

When the above data is graphed, the line equation is $y = 0.0254x + 0.0223$ ($R^2 = .99917$). This equation was used to solve for the red food coloring in the unknowns.

X is concentration. Y is absorbance. Solving for x in the equation:

$$x = (y - .0223)/.0254$$

G2 Gatorade 1:10 dilution

$x = (.242 - .0223)/ .0254$ **+ 0.1 line equation**

$x = 8.64\ \mu M$ **+ 0.1 correct variable substitution**

Since 1.0 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1 = M_2V_2$**

$(8.64\ \mu M)\ (10.0\ mL) = (1.00\ mL)\ (M_2)$ **+ 0.2 correct variable substitution**

$M_2 = 86.4\ \mu M$

G2 Gatorade 1:5 dilution

$x = (.466 - .0223)/ .0254$ **+ 0.1 line equation**

$x = 17.46\ \mu M$ **+ 0.1 correct variable substitution**

Since 2.0 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1 = M_2V_2$**

$(17.46\ \mu M)\ (10.0\ mL) = (2.00\ mL)\ (M_2)$ **+ 0.2 correct variable substitution**

$M_2 = 87.34\ \mu M$

Powerade Zero

$x = (.332 - .0223)/ .0254$ **+ 0.1 line equation**

$x = 12.2 \ \mu M$ **+ 0.1 correct variable substitution**

Since 1.0 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1=M_2V_2$**

$(12.2 \ \mu M) \ (10.0 \ mL) = (1.0 \ mL) \ (M_2)$ **+ 0.2 correct variable substitution**

$M_2 = 122 \ \mu M$

Cherry 7 UP

$x = (.204 - .0223)/ .0254$ **+ 0.1 line equation**

$x = 7.15 \ \mu M$ **+ 0.1 correct variable substitution**

No dilution, so the concentration of red food coloring in the soda is 7.15 μM.
         + 0.3 no dilution

Sparkling Ice Black Raspberry

$x = (.454 - .0223)/ .0254$ **+ 0.1 line equation**

$x = 17.0 \ \mu M$ **+ 0.1 correct variable substitution**

Since .50 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1=M_2V_2$**

$(17.0 \ \mu M) \ (10.0 \ mL) = (0.50 \ mL) \ (M_2)$ **+ 0.2 correct variable substitution**

$M_2 = 340. \ \mu M$

Diet Oceanspray Cranberry Juice

x = (.189 - .0223)/.0254   **+ 0.1 line equation**

x = 6.56 μM   **+ 0.1 correct variable substitution**

Since 1.0 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1=M_2V_2$**

(6.56 μM) (10.0 mL) = (1.0 mL) (M_2)   **+ 0.2 correct variable substitution**

M_2 = 65.6 μM

Publix Strawberry Soda

x = (.221 - .0223)/ .0254   **+ 0.1 line equation**

x = 7.82 μM   **+ 0.1 correct variable substitution**

Since 1.0 mL of the soda was diluted to 10 mL of the measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of red food coloring in the undiluted soda.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1=M_2V_2$**

(7.82 μM) (10.0 mL) = (1.00 mL) (M_2)   **+ 0.2 correct variable substitution**

M_2 = 78.2 μM

5. The absorbance of a solution of red food coloring is 0.204 at 500 nm. The pathlength is 1.00 cm and the concentration of the solution is 3.175 mM. What is the molar absorptivity? (**1 point**)

Convert mM to M. c = 0.003175   **+ 0.3 conversion to M**

A = εbc   **+ 0.4 Beer's equation**

ε = A/bc

ε = 0.204/(1.00 cm ∗ 0.003175)   **+ 0.2 substitution of variables**

ε = 64.3 cm^{-1} M^{-1} **+ 0.1 correct answer**

6. Evan obtained the following absorbance data:

Concentration (M)	Absorbance
1.05	0.231
2.10	0.402
3.20	0.691
4.40	0.837

A graph of this data revealed the line equation was $y = 0.1885x + 0.0366$ with an R^2 = 0.98064.

a. Evan measures a solution with an absorbance of 0.548. What is the concentration of the analyte in solution? (**0.5 point**)

$x = (y-0.0366)/0.1885$ **+ 0.1 the equation**

$x = (0.548 – 0.0366)/0.1885$ **+ 0.3 substituting the correct variable**

$x = 2.71$ M   **+ 0.1 correct answer**

b. Evan has a solution that has an analyte concentration of 1.5 M. What would the expected absorbance of the solution be according his calibration curve? (**0.5 point**)

$y = 0.1885x + 0.0366$ **+ 0.1 the equation**

$y = 0.1885 (1.5) + 0.0366$ **+ 0.3 substituting the correct variable**

$y = 0.319$ **+ 0.1 correct answer**

c. What does the R^2 value mean? What does your R^2 value from <u>Question #3</u> mean? (**0.5 point**)

The R^2 value describes the "goodness of fit" of the data to the linear trendline. The closer the R^2 value to 1, the better the fit. My R^2 =0.9992 (from question #3); this implies my data is a "good fit" to the trendline because the $R^2 \geq 0.995$.

+ 0.3 variations of- R^2 is the description of a trendline's fit to the data
+ 0.1 desired $R^2 \geq 0.995$ or approaching 1
+ 0.1 if <u>their</u> $R^2 \geq 0.995$

7. Using the undiluted G2 Gatorade Fruit Punch concentration determined in Question #4, calculate the amount of red food coloring present in a normal 32 oz. (946 mL) container of G2 Gatorade Fruit Punch. Report your calculation in milligrams. Show all work. (**0.5 point**)

0.946 L x 0.00008734 M = 0.0000826 moles   **+ 0.2 conversion to mol**

0.0000826 moles x 495.7 g/mol = 0.0410 grams   **+ 0.2 conversion to g**

0.0410 g x 1000 = 41.0 mg   **+ 0.1 conversion to mg**

There is 41.0 mg red food coloring in a 32 oz. bottle of G2 Gatorade Fruit Punch.

b. Given an ADI of 420 mg/day for a 60 kg (132 lbs) person, what is the percent contribution of one G2 Gatorade Fruit Punch to his/her ADI? (**0.5 point**)

(40.95 mg/ 420 mg) x 100 = 9.8 % of his/her ADI

+ 0.2 numerator from 7a
+ 0.2 correct denominator
+ 0.1 multiply by 100 to get percent

c. Calculate the red food coloring ADI for a 160 lb adult assuming a safe daily value of 7 mg/kg bodyweight. (**0.5 point**)

160 lbs x 0.454 kg/lb = 73 kg   **+ 0.2 conversion to kg**

73 kg x 7 mg/kg = 511 mg = 510 mg   **+ 0.2 conversion to mg**

The adult's red food coloring ADI is ~510 mg/day.   **+ 0.1 correct answer**

8. You work as a scientist for the International Olympic Committee and are responsible for testing athletes for anabolic steroids as part of the anti-doping program for the Olympic Games. Your calibration curve has a R^2 of 0.943. Your QC low sample is within the expected range but the QC high sample is well above the acceptable range. Of the 10 urine samples tested, you observed 8 positive results for anabolic steroids. What could you report back to the International Olympic Committee and <u>why</u>? (**1 point**)

Since the QC is above the acceptable range, the results for the samples potentially containing anabolic steroids cannot be trusted. The R^2 value also implies that the data's TRENDLINE is not a good linear fit to the data acquired. The 8 positive results cannot be trusted therefore athlete samples will need to be re-evaluated after corrective action has been achieved.

+ 0.4 QC is out therefore sample results cannot be reported with confidence

+ 0.4 R^2 value is below desired 0.995 so the TRENDLINE does not fit the data

+ 0.2 Sample results cannot be reported at this time

Determination of Aspartame in Commercial Sweeteners

Introduction

Aspartame is a non-nutritive sweetener found in over 6,000 consumer products.[1] It is marketed as a table top sweetener by the brand name Equal and is an ingredient in NutraSweet, Canderel, TriSweet and many other food products. The most common use of aspartame in the United States is its use in sweetening low-energy commercial beverages like zero-calorie, diet soft drinks and juices.

Aspartame is a synthetic methyl ester of a dipeptide containing the amino acids phenylalanine and aspartic acid (**Figure 1**). Under dry conditions, aspartame is stable, but when in solution, it degrades. The rate of this degradation is influenced by changes in pH and temperature.[1] While aspartame has an equal caloric intake as sugar at 4 kcal/g, it can be used at much lower levels than sugar to reach the same degree of sweetness.[2] Therefore, a lower caloric intake can be achieved since less of it is needed.
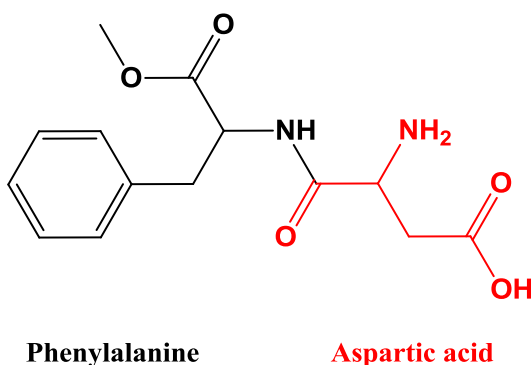

Phenylalanine   **Aspartic acid**

Figure 1 The chemical structure of aspartame. Aspartame is a dipeptide composed of phenylalanine methyl ester (**Black**) and aspartic acid (**Red**). It's molecular weight is 294.1216 g/mol.

Aspartame was approved for use in manufactured food products in the early 1980s by the FDA, but controversy continues to follow this product's use. As of current, aspartame doses up to 4000 mg/kg bodyweight per day have shown no adverse effects in acute, subacute and chronic toxicity experiments in mice, rats, hamsters and dogs.[1] The FDA estimates an acceptable daily intake (ADI) of 50 mg aspartame/kg bodyweight per day in humans.[1] For the average 60 kg (~132 lbs) person, the ADI would be 3000 mg per day.

In this experiment, you will use the Biuret Reaction by Gornall, *et. al.* of 1949 to perform a quantitative analysis of aspartame in Equal sweetener packets.[3] A compound with peptide bonds (like aspartame) treated with biuret reagent under alkaline conditions (pH>7.4) will form a dark blue/purple complex with the copper salts of the reagent (**Figure 2**). A deep blue color arises because transition metals like copper will absorb energy from a wavelength in the visible light spectrum, and their electrons will become excited causing them to leave the lower energy d-

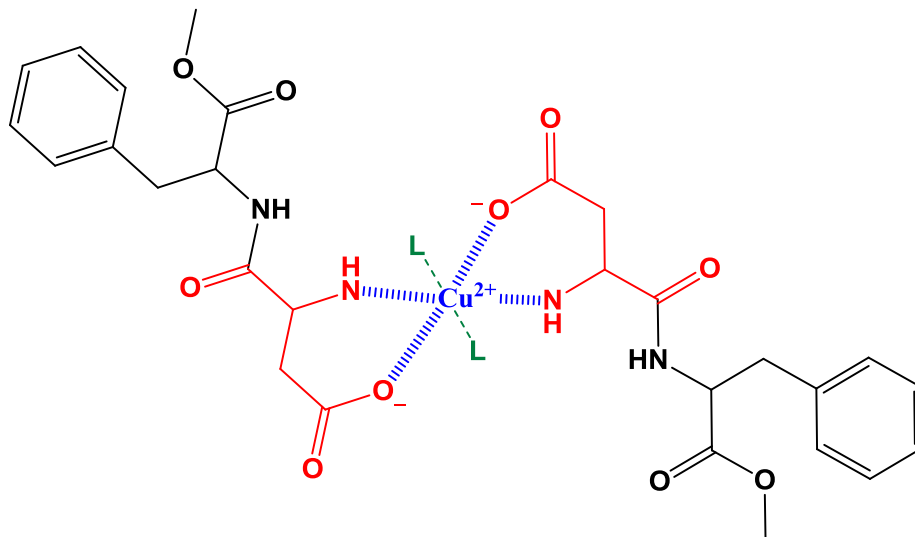orbitals where they normally reside. This resultant color can be measured by colorimetric analysis.[4]

Figure 2 Copper (II)-Aspartame Complex. Aspartame is comprised of aspartic acid (**Red**) and phenylalanine (**Black**). This compound will form a copper (II) (Cu^{2+}) complex (**Blue**) that absorbs light and can be measured at a wavelength of 629 nm. Water molecules and other ligands that do not have a high affinity for the cupric metal center will also bind (**Green L**).

You'll remember from your earlier determination of FD&C Red No. 40 in commercial beverages that Beer's Law allowed you to calculate the concentration of an analyte in a beverage by measuring the light it absorbed. The light absorbed by an analyte is proportional to its concentration as shown in the equation below:

Beer's Law: $A = \varepsilon bc$

> where **A** is absorbance (unitless), **ε** is the molar absorptivity (M^{-1} cm^{-1}), **c** is the light-absorbing analyte's concentration (M) and **b** is the cuvette cell's pathlength (cm).

You will again create serial dilutions of the analyte of interest (aspartame), measure the absorbance of the dilutions and graph the concentration of the dilutions versus the corresponding absorbance. Remember that diluted solutions are prepared from more concentrated solutions by using the Dilution Formula.

Dilution Formula: $M_1 \cdot V_1 = M_2 \cdot V_2$

> where **M** is the solution concentration and **V** is the solution volume. These variables may be any unit of analyte over the volume of the solution (i.e.- mol/L; g/mL; etc.) as long as the same units are used consistently on both sides.

When graphed (Absorbance y-axis versus Concentration x-axis), the measurements from a serial dilution will be the calibration curve you use for the determination of aspartame concentrations in this experiment.

A few notes BEFORE you begin:

1. The cuvettes are expensive. Please be careful with them.
2. For your entire experiment, you should use the same cuvette. Each cuvette has microscopic imperfections that are accounted for when you "blank" the solution on the instrument.
3. Do not put liquid in (or take liquid out) of the cuvette while it is in the instrument.
4. Shut the door of the instrument carefully between measurements.
5. Use the same spectrophotometer for all your measurements.

Experimental Procedure

1. Calculate and make all the dilutions at your bench and then take your samples to the spectrophotometer to measure the absorbance.

2. In a 50 mL beaker, pour 30 mL of 5.00 mg/mL aspartame solution. In a separate beaker, pour 30 mL of biuret reagent.

3. Using a 10.0 mL volumetric flask and a 5.0 mL or 10.0 mL Mohr pipette, add 2.0 mL of biuret reagent to the 10.0 mL volumetric flask. Dilute to volume with water. This is your blank.

4. Using a clean 10.0 mL volumetric flask and a 5.0 mL or 10.0 mL Mohr pipette, add 2.0 mL of biuret reagent to the volumetric flask. Add the calculated volume of aspartame from the 5.00 mg/mL stock solution to the volumetric flask so that a final concentration of 0.50 mg/mL will be obtained. Dilute to volume with water. Allow the reaction to react for 15 minutes. Once mixed, you can transfer this to a test tube or beaker and continue to make your dilutions.

Using the same method, determine the dilutions needed to make the following concentrations:

> 1.0 mg/mL
> 1.5 mg/mL
> 2.0 mg/mL
> 2.5 mg/mL
> 3.0 mg/mL

Using the same method, you will determine the amount of aspartame in two Quality Control (QC) samples of Equal. For the QC Low, add 3.0 mL of the provided QC Equal solution to a 10.0 mL volumetric flask, 2 mL of buiret reagent and dilute to volume. In a separate 10.0 mL volumetric flask, prepare the QC High sample by adding 6.0 mL of the provided QC Equal solution, 2 mL of buiret reagent and dilute to volume.

You will also determine the amount of aspartame in an unknown packet of Equal. You will prepare this unknown by recording the weight of 1 package of Equal,

transferring it to a 10.0 mL volumetric flask and diluting to volume. Add 5 mL of this solution to a clean 10.0 mL volumetric flask, add 2 mL of the biuret reagent and dilute to volume. Let the solution stand 15 minutes and measure its absorbance with the spectrophotometer.

Note: Our Mohr pipettes are not calibrated to include the liquid below the 5.0 mL mark. For example, if you wish to add 1.0 mL, you would fill the pipette to 4.0 mL and empty until the meniscus is sitting on the 5.0 mL denotation.

4. Once your dilutions are made, measure the absorbance of each concentration using the spectrophotometer.

5. Before measuring the absorbance, make sure the wavelength is set at 629 nm.

6.   Hold the cuvette on the opaque side of the glass. Wipe the clear side of the glass with a chemwipe to remove any fingerprints or dust that may interfere with the measurement.

7. Fill the cuvette with the blank to the level of the yellow square.

8. Align the clear side of the cuvette with the holes for the light in the instrument.

9. Place the cuvette in the spectrophotometer.

10. Close the door and push the "0 Abs 100% T" button. This is the blank for the measurement.

11. Remove the blank from the cuvette using a disposable transfer pipette. Collect this as waste in a beaker. Once you are done with all your measurements, dispose the waste liquid in a container on the cart.

12. You are now ready to measure your Quality Controls and Unknown solutions of Equal.

Note: To accurately determine the concentration of given solutions, the absorbance must fall within the range of the known calibration curve standards.

References:
[1] B.A. Magnuson, G.A. Burdock, J. Doull, R.M. Kroes, G.M. Marsh, M.W. Pariza, P.S. Spencer, W.J. Waddell, R. Walker, G.M. Williams, Critical Reviews in Toxicology, 37 (2007) 629-727.
[2] Journal of the Academy of Nutrition and Dietetics, 112 (2012) 739-758.
[3] A.G. Gornall, C.J. Bardawill, M.M. David, The Journal of Biological Chemistry, 177 (1949) 751-766.
[4] N. Kaufman, C.J. Fenk, Journal of Chemical Education, 84 (2007) 1676-1678.
[5] B.A. Magnuson, in, The Coca-Cola Company Beverage Institute for Health and Wellness, http://www.beverageinstitute.org/en_us/pages/expert-straight-facts-on-aspartame-and-health.html, 2012.

Lab Report

1. Show the calculations for the calibration curve sample dilutions neatly below. Remember significant figures and units. Show all your work. If there is no work, there will be no credit. (**1 point** total)

2. Record your data in the table below. (**1 point** total)

Product	Concentration	Absorbance
Aspartame Solution	0.50 mg/mL	
	1.0 mg/mL	
	1.5 mg/mL	
	2.0 mg/mL	
	2.5 mg/mL	
	3.0 mg/mL	
*Equal – QC Low	3.0 mL	
*Equal – QC High	6.0 mL	
Unknown _____ g	_____ mg/mL	Not measured
Unknown Dilution	5.0 mL	

3. Attach a graph of concentration (x axis) versus absorbance (y axis) of the aspartame analysis. Fit the line using a linear trendline, display the equation and R^2 on the chart. Email your excel spreadsheet to your professor. (**1 point** total)

4. Using the equation from your calibration curve, what is the <u>undiluted</u> concentration of the aspartame in both QC and unknown samples? Report your calculations in **mg aspartame/ g Equal**. (*Note:* The initial concentration of QC solution was 100 mg/mL.) (**1.5 points** total)

QC Low Determination:

QC High Determination:

Unknown 1 Determination:

5. The absorbance of a solution of aspartame is 0.372 at 629 nm. The pathlength is 10.0 mm and the molar absorptivity is 0.543 cm^{-1} M^{-1}. What is the concentration of the solution? (**1 point** total)

6. Evan obtained the following absorbance data:

Concentration (mM)	Absorbance
13.0	0.095
16.2	0.143
19.4	0.205
22.6	0.241
25.8	0.312

A graph of this data revealed the line equation is y = 0.0166x – 0.1223 with an R^2 = 0.99234.

a. Evan measures an unknown solution with an absorbance of 0.448. What is the concentration of the solution? (**0.5 point** total)

b. Evan takes 1.0 mL of a solution and dilutes it to 50.0 mL. The absorbance is 0.192. What is the concentration of the original solution? (**0.5 point** total)

7. Mary is taking absorbance measurements of aspartame. She is consistently getting higher absorbance readings than the known values. What are two <u>experimental</u> mistakes Mary could be making? (**1 point** total)

8. Your colleagues at another university report the aspartame concentration in a packet of Equal as 38.0 mg/g.[4] Assuming their reported value is the theoretical value, use your Unknown <u>undiluted</u> calculation of aspartame from Question # 4 to calculate the percent error of your measurement. <u>Show all work</u>. (**1 point** total)

9.   James, a 185 lbs male, drinks three 20 oz. bottles of Diet Coke within 24 hours. Assuming this is James' only source of aspartame for that day, did he exceed his aspartame ADI?   (**Helpful Notes:**   The FDA estimates an ADI of 50 mg/kg bodyweight per day.[1] The Coca Cola Company estimates 125 mg aspartame in an 8 oz. can of diet coke.[5] There is 0.4538 kg per 1 lb.) Show all work. (**1.5 points** total)

Aspartame Determination Instructor Guide

Learning Outcomes

1. The students will demonstrate the ability to measure an analyte using a biuret reagent.
2. The students will demonstrate the ability to obtain, record and analyze spectroscopy data of a known sample.
3. The student will demonstrate the ability to determine the concentration on an unknown sample using Beer's Law.

Laboratory Preparation

This lab is modified from "A New Colorimetric Assay of Tabletop Sweeteners Using a Modified Biuret Reagent" by Fenk, Kaufman and Gerbig, J. Chem. Ed., Vol. 84, No. 10, 1676 – 1678.

The reagent preparation from the supporting information from the above article:

Reagent Preparation

Biuret Reagent (250 mL): Copper (II) sulfate pentahydrate (3.75 g, 15.0 mmol) was added to a 250 mL volumetric flask. Water was added (40 mL) and the copper (II) sulfate pentahydrate was completely dissolved. A solution of potassium sodium tartrate tetrahydrate (18.82 g, 52.5 mmol) in water (60 mL) was prepared in a separate container and combined with the copper (II) sulfate solution. Finally, dilute the mixture to volume using 1 M sodium carbonate solution.

Sodium Carbonate (1 M): Sodium carbonate (105.99 g, 1 mol) was added to a 1 L volumetric flask and dissolved into 750 mL water. The solution was then diluted to volume with water.

Aspartame Stock Solution: Prepare an aspartame stock solution by dissolving 250 mg aspartame into 50.00 ml water (5.00 mg/ml).

The QCs are made from Equal. Buy individual packets. 1 packet is approximately 1 gram Equal.

Topics to Cover in Pre-lab Lecture

1. **Beer's Law Overview**
2. **Example of solving for concentration using y=mx +b**
3. **How to properly make a dilution using volumetric glassware (DEMO)**
4. **How to use a Mohr pipette (DEMO)**

Lab Report

Please tally all points earned for the lab report <u>first</u> (10 points total). Feel free to adjust this to your class' grading scale later, but for the purposes of reporting in this project, please keep your submitted tallies to a 10 point scale.

From the total points earned, consider the following possible general deductions:

-0.2 incorrect use of significant figures when reporting calculations
-0.2 units not reported or reported incorrectly with numerical calculations

Please mark any lab reports turned in Incomplete with an "I" next to the numeric score. (Incomplete = not all questions were attempted)

1. Show the calculations for the calibration curve sample dilutions neatly below. Remember significant figures and units. Show all your work. If there is no work, there will be no credit. (**1 point** total)

Using $M_1V_1 = M_2V_2$, the students should be able to derive the amount of concentrated solution to use.

For example, 5.00 mg/mL (X) = (.5 mg/mL)(10 mL)
                   X = **<u>1.0 mL</u>**

Likewise,

For the 1.0 mg/mL concentration, **<u>2 mL</u>** of the 5.00 mg/mL solution should be diluted to 10 mL.
For the 1.5 mg/mL concentration, **<u>3 mL</u>** of the 5.00 mg/mL solution should be diluted to 10 mL.
For the 2.0 mg/mL concentration, **<u>4 mL</u>** of the 5.00 mg/mL solution should be diluted to 10 mL.
For the 2.5 mg/mL concentration, **<u>5 mL</u>** of the 5.00 mg/mL solution should be diluted to 10 mL.
For the 3.0 mg/mL concentration, **<u>6 mL</u>** of the 5.00 mg/mL solution should be diluted to 10 mL.

+ 0.4 use of $M_1V_1=M_2V_2$

+ 0.6 dilution calculated correctly (+0.1 EACH)
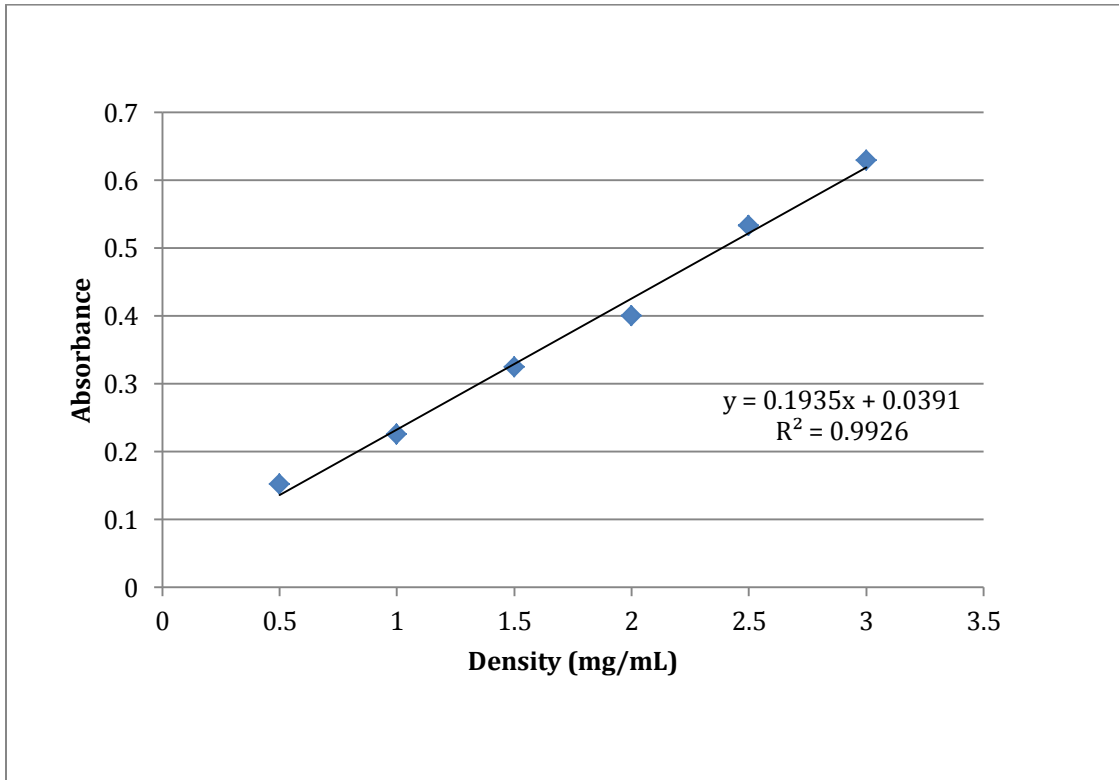
2. Record your data in the table below. (**1 point** total)

Product	Concentration	Absorbance
Aspartame Solution	0.50 mg/mL	0.152
	1.0 mg/mL	0.226
	1.5 mg/mL	0.325
	2.0 mg/mL	0.400
	2.5 mg/mL	0.533
	3.0 mg/mL	0.630
*Equal – QC Low	3.0 mL	0.238
*Equal – QC High	6.0 mL	0.442
Unknown __1.039____ g	_104_____ mg/mL	Not measured
Unknown Dilution	5.0 mL	0.478

+ 0.3 recording Abs to three significant figures (as appropriate to lab spectrophotometers used)
+ 0.3 recording all calibration absorbance data
+ 0.4 recording QC and unknown data (+0.1 per line)

3. Attach a graph of concentration (x axis) versus absorbance (y axis) of the aspartame analysis. Fit the line using a linear trendline, display the equation and R^2 on the chart. Email your excel spreadsheet to your professor (professor@school.edu). (**1 point** total)

Chart showing Density (mg/mL) on x-axis and Absorbance on y-axis with equation $y = 0.1935x + 0.0391$ and $R^2 = 0.9926$

+ 0.3 Linear trendline graphed
+ 0.2 R^2 value present
+ 0.2 correct axes labels (+0.1 EACH)
+ 0.3 line equation present

4. Using the equation from your calibration curve, what is the <u>undiluted</u> concentration of the aspartame in both QC and unknown samples? Report your calculations in **mg aspartame/ g Equal**. (*Note:* The initial concentration of QC solution was 100 mg/mL.) (**1.5 points** total)

<u>Each student should have 3 calculations (2 QCs & 1 unknown) worth 0.5 points each.</u>

When the above data is graphed, the line equation is y = 0.1935x + 0.0391 (R^2 = 0.99263). This equation was used to solve for the aspartame in the QCs and unknown.

X is concentration. Y is absorbance. Solving for x in the equation:

$$x = (y - 0.0391)/0.1935$$

<u>QC Low, 3 mL dilution</u>

x = (.238 - .0391)/.1935   **+ 0.1 line equation**

x = 1.03 mg/mL Aspartame   **+ 0.1 correct variable substitution**

Since 3.0 mL of the initial solution was diluted to 10 mL for measurement, use M_1V_1 = M_2V_2 to determine the concentration of Equal in the undiluted QC solution.

M_1V_1 = M_2V_2 **+ 0.1 M_1V_1=M_2V_2**

(M_1) (10.0 mL) = (3.00 mL) (100 mg/mL) **+ 0.1 correct variable substitution**

M_1 = 30 mg/mL Equal

To calculate the mg aspartame/g Equal, convert the calculated concentration of Equal to g/mL units and divide:

1.03 mg/~~mL~~ Aspartame   /   0.030 g/~~mL~~

=   34 mg aspartame/g Equal   **+ 0.1 correct result conversion**

<u>QC High, 6.0 mL dilution</u>

x = (0.442 - 0.0391)/0.1935   **+ 0.1 line equation**

x = 2.08 mg/mL   **+ 0.1 correct variable substitution**

Since 6.0 mL of the initial solution was diluted to 10 mL for measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of Equal in the undiluted QC solution.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1 = M_2V_2$**

$(M_1)(10.0 \text{ mL}) = (6.00 \text{ mL})(100 \text{ mg/mL})$ **+ 0.1 correct variable substitution**

$M_1 = 60$ mg/mL Equal

To calculate the mg aspartame/g Equal, convert the calculated concentration of Equal to g/mL units and divide:

2.08 mg/~~mL~~ Aspartame / 0.060 g/~~mL~~

=   35 mg aspartame/g Equal   **+ 0.1 correct result conversion**

<u>Unknown, Equal Packet</u> *(take note with this grading, slightly different)*

x = (0.478 - 0.0391)/0.1935   **+ 0.1 correct line equation substitution**

x = 2.27 mg/mL

Calculate the initial concentration of the Equal solution and convert to mg/mL:

1.039 g / 10 mL = 0.104 g/mL x 1000

= 104 mg/mL   **+ 0.1 unknown initial concentration**

Since 5.0 mL of the initial solution was diluted to 10 mL for measurement, use $M_1V_1 = M_2V_2$ to determine the concentration of Equal in the undiluted QC solution.

$M_1V_1 = M_2V_2$ **+ 0.1 $M_1V_1 = M_2V_2$**

$(M_1)(10.0 \text{ mL}) = (5.00 \text{ mL})(104 \text{ mg/mL})$ **+ 0.1 correct variable substitution**

$M_1 = 52$ mg/mL Equal

To calculate the mg aspartame/g Equal, convert the calculated concentration of Equal to g/mL units and divide:

2.27 mg/~~mL~~ Aspartame / 0.052 g/~~mL~~

=   44 mg aspartame/g Equal   **+ 0.1 correct result conversion**

5. The absorbance of a solution of aspartame is 0.372 at 629 nm. The pathlength is 10.0 mm and the molar absorptivity is 0.543 cm^{-1} M^{-1}. What is the concentration of the solution? (**1 point** total)

Convert mm to cm. b = 1.0 cm   **+ 0.3 conversion to cm**

A = εbc   **+ 0.4 Beer's equation**

c = A/bε

c = 0.372/(1.00 cm ∗ 0.543 cm^{-1} M^{-1})   **+ 0.2 substitution of variables**

c = 0.685 M   **+ 0.1 correct answer**

6. Evan obtained the following absorbance data:

Concentration (mM)	Absorbance
13.0	0.095
16.2	0.143
19.4	0.205
22.6	0.241
25.8	0.312

A graph of this data revealed the line equation is y = 0.0166x – 0.1223 with an R^2 = 0.99234.

a. Evan measures an unknown solution with an absorbance of 0.448. What is the concentration of the solution? (**0.5 point** total)

The absorbance is outside the range of absorbance measurements of the known concentration. The concentration cannot be determined.   **+0.5 point**

b. Evan takes 1.0 mL of a solution and dilutes it to 50.0 mL. The absorbance is 0.192. What is the concentration of the original solution? (**0.5 point** total)

y = 0.0166x – 0.1223   **+ 0.1 the equation**

x = (y+0.1223)/0.0166

x = (0.192+0.1223)/0.0166   **+ 0.1 substituting the correct variable**

x = 18.9 mM

$M_1V_1 = M_2V_2$ **+ 0.1 M_1V_1=M_2V_2**

(x)(1.0 mL) = (50 mL) (18.9 mM)   **+ 0.1 substituting the correct variable**

x = 945 mM   **+ 0.1 correct answer**

7. Mary is taking absorbance measurements of aspartame. She is consistently getting higher absorbance readings than the known values. What are <u>two</u> <u>experimental</u> mistakes Mary could be making? (**1 point** total)

There could be several. Here are a few: **+ 1.0 correct answer (0.5 each)**
1. Fingerprints on the cuvette
2. A more concentrated solution is being used (did not fill the volumetric flask to the line properly)
3. Placing the cuvette in the holder so that the frosted side is in the light beam

8. Your colleagues at another university report the aspartame concentration in a packet of Equal as 38.0 mg/g.[4] Assuming their reported value is the theoretical value, use your Unknown <u>undiluted</u> calculation of aspartame from Question # 4 to calculate the percent error of your measurement. <u>Show all work</u>. (**1 point** total)

My Coke Zero value from Question #4 is 44 mg aspartame/g Equal

Percent Error = $\frac{|Actual - Theoretical|}{Theoretical} \times 100$ **+ 0.2 the equation**

$\qquad = \frac{|44 - 38|}{38} \times 100$ **+ 0.3 variable substitution**

$\qquad = \frac{|6|}{38} \times 100$

$\qquad = 0.16 \times 100$ **+ 0.2 correct answer**

$\qquad = 16\%$ error **+ 0.3 if < 25% error**

9. James, a 185 lbs male, drinks three 20 oz. bottles of Diet Coke within 24 hours. Assuming this is James' only source of aspartame for that day, did he exceed his aspartame ADI? (**Helpful Notes:** The FDA estimates an ADI of 50 mg/kg bodyweight per day.[1] The Coca Cola Company estimates 125 mg aspartame in an 8 oz. can of diet coke.[5] There is 0.4538 kg per 1 lb.) <u>Show all work</u>. (**1.5 points** total)

185 lbs x 0.4538 kg/lb = 83.92 kg James   **+ 0.2 conversion**

83.92 kg x 50 mg/kg = 4196 mg aspartame allowed in James' ADI
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ **+ 0.3 calculation**

3 bottles x 20 oz. x (125 mg aspartame/8 oz.) = 937.5 mg aspartame consumed by James via the 3 bottles of Diet Coke.   **+ 0.5 calculation**

James has consumed approximately a quarter of his aspartame ADI so he has not exceeded his ADI.   **+ 0.5 correct answer**