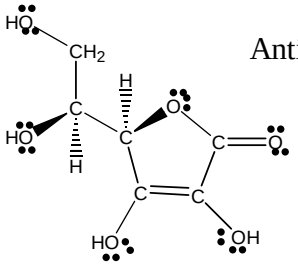
Name _____

People have known and believed for a long time that eating certain foods is good for their health. However, it is difficult to understand what about those foods is healthy. Is it just about vitamins and minerals? If that is the case, we could just take a vitamin/mineral pill each day and be healthy. In addition, we would like to know how we can measure the "healthiness" of food and beverages. We are now told that a dietary intake of food and beverages rich in antioxidants will prevent us from getting cancer, heart disease, and other disorders that might afflict us. A wide variety of foods, beverages, and dietary supplements are proclaimed to be high in antioxidants. Among these food items are: grape seed extract, cranberries, apples, green tea, chocolate, parsley, mojitos, ginger, etc…

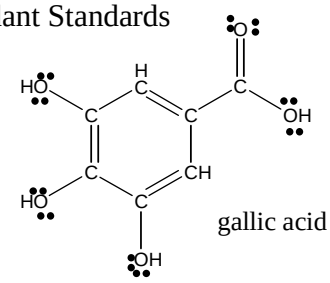
In the next two experiments we are going to explore the polyphenolic and antioxidant properties of popular tea beverages. We will be performing "*in vitro*" tests on beverages that claim to have "*in vivo*" antioxidant effects. That means that we are going to do chemical tests on substances that are purported to have beneficial properties in living organism such as ourselves. *In vitro* testing allows us to perform tests on biological properties of substances quickly and inexpensively. However, positive results from *in vitro* testing do not automatically mean that what we are testing is beneficial for humans. For example, the cytotoxicity of a compound may not be revealed by a simple benchtop test. That means that a compound may have a desirable biological activity in a cell-free solution but it turns out to be toxic to cells.

Many *in vitro* testing systems have been developed that can be monitored by UV-vis absorbance. Ultraviolet-visible absorbance is easy to measure and can be very sensitive. The sensitivity of a particular assay (test system) is maximized by measuring the UV-vis absorption of a compound at is maximum absorbance (lambda max). We will be using a best linear fit method of measuring the level of polyphenolics and antioxidants in beverages. In the best linear fit method a calibration study is first done with concentrations of a known standard. In this case, our known standard is also a pure compound.

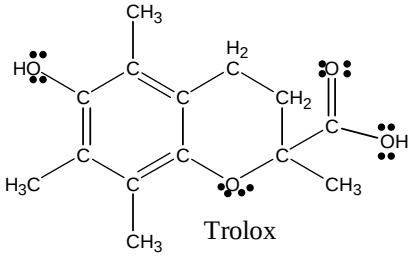
Antioxidant Standards



ascorbic acid (Vit C)



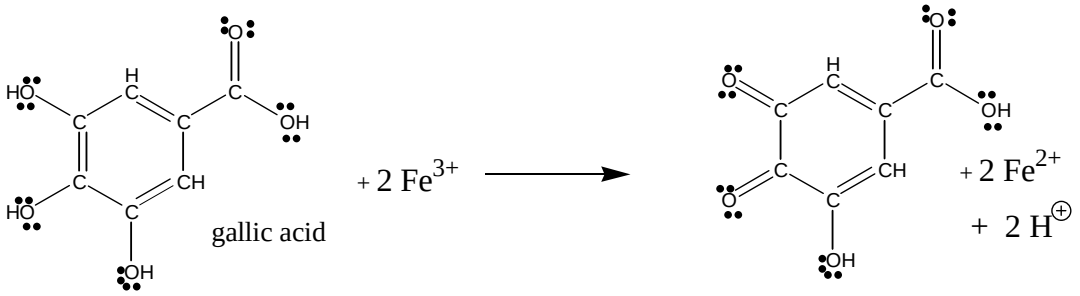
gallic acid



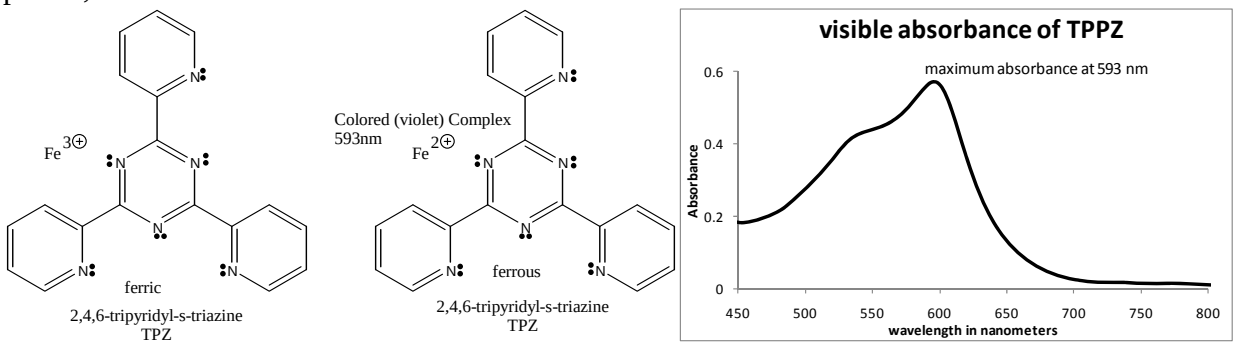
Trolox

The sample, which is a mixture, is compared to the known compound. This allows us to test a wide variety of beverages with different concentrations of diverse constituents. Since every sample is compared to a standard compound, the variations in the samples are all measured on a common scale. A best linear fit can be easily created by measuring the absorbance of a series of concentrations created by diluting a stock solution of known concentration.

We will be using the FRAP (ferric reducing antioxidant power) assay to determine antioxidant strength. This assay works on the principle that ferric ion is reduced to ferrous ion in the presence of an antioxidant. An antioxidant is defined in this experiment as a molecule that can donate one or more electrons to an electron acceptor such as ferric ion.



A synthetic dye measures the presence of ferrous ion in the solution. The more antioxidant power, the more ferric ion is reduced.



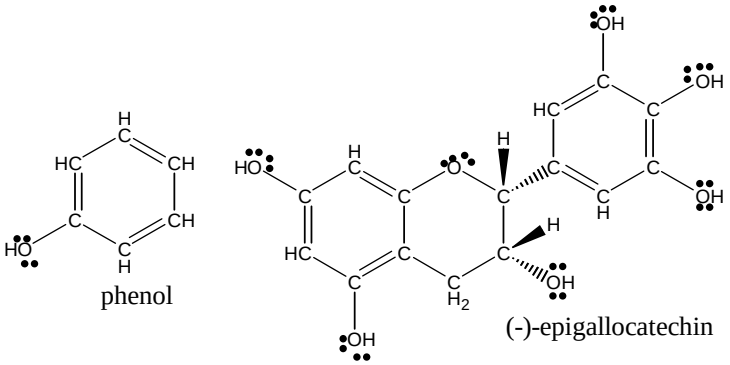
The visible absorbance spectrum of 2,4,6-tripyridyl-s-triazine shows that the maximum absorbance is close to 593 nanometers.

Let's say that we test different concentrations of ascorbic acid with the FRAP assay. These are the results we obtain:

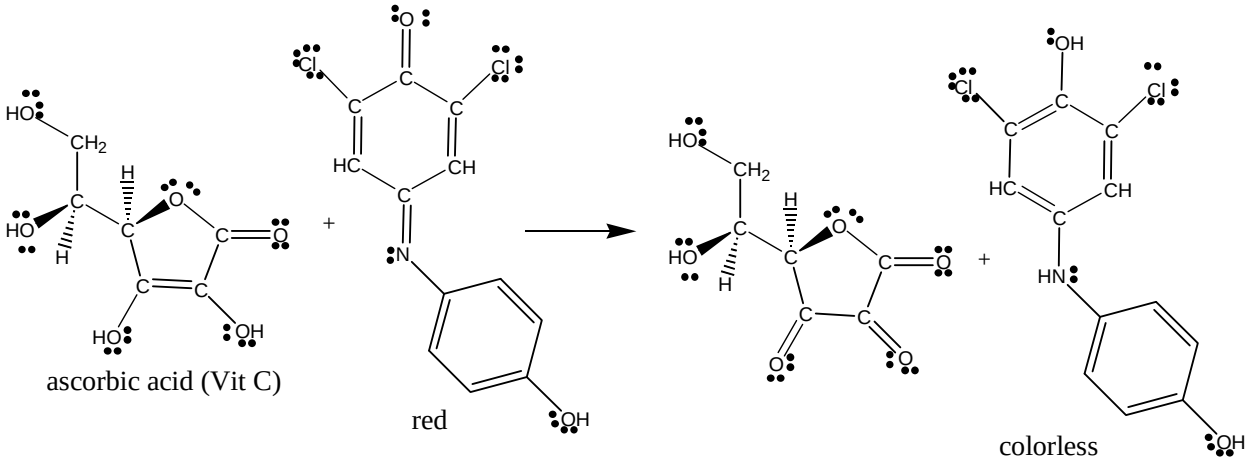
concentration in mM	60	30	15	7.5	3.75	unknown
Absorbance at 593 nm	1.47	0.562	0.349	0.245	0.198	0.405

The graph of this data gives a best linear fit of y = 0.0226x + 0.0402. The value for "y" is absorbance (A). The value for "x" is concentration of ascorbic acid in mM. A solution of unknown composition had an absorbance of 0.405 A. To calculate the antioxidant power in ascorbic acid equivalents we need to solve the linear equation for "x." In this case x = (y - 0.0402)/0.0226. For the sample of unknown concentration y = 0.405 putting that values of "y" in the equation gives x = 16 mM of ascorbic acid. The solution of unknown composition, therefore, has an antioxidant power of 16 mM ascorbic acid.

We will be using a second assay to determine the polyphenolic compounds in our samples. The reagent we are using is called the Folin-Ciocalteau method of measuring polyphenolics. The main constituents of the Folin-Ciocalteau reagent are phosphotungstic acid, phosphomolybdic acid, and phenol. The solution is originally yellow, and when it is reduced by the phenolic groups it form a bright blue complex. The final product absorbs light at a wavelength of 590 nanometers. Epigallocatechin is a common polyphenolic compounds found in teas.



A third assay we are doing in this experiment is the determination of ascorbic acid (Vitamin C) using an indicator dye. Ascorbic acid ($C_6H_8O_6$) is a water-soluble vitamin. Vitamin C is easily oxidized, and the majority of its functions in vivo rely on this property. It plays a key role in the body's synthesis of collagen and norepinephrine by keeping the enzymes responsible for these processes in their active reduced form. Vitamin C is found to some extent in almost every fruit and vegetable. It is used as a beverage additive since it improves flavor at the same time it stabilizes the beverage. The amount of vitamin C in a sample will be determined by a reduction/oxidation titration using the reaction between ascorbic acid and 2,6-dichloroindophenol (DCIP). DCIP is used as the titrant because it should 1) only oxidize ascorbic acid and not other substances that might be present, and 2) act as a self-indicator in the titration. DCIP colorless in the presence of ascorbic acid and red when ascorbic has all reacted.



In acidic solutions DCIP is red, but if ascorbic acid is present, it will be reduced to a colorless substance. The solution will remain colorless as more DCIP is added until all of the ascorbic acid has reacted. As soon as the next drop of DCIP solution is added the solution will be light red, due to the excess DCIP and the end point of the titration has been reached.

Please work in pairs for this experiment.

Make sure the spectrophotometer is turned on and the wavelength is set at 593 nm.

Part I. Making serial dilutions of a stock solution.

Set up 5 test tubes in a test tube rack

Obtain a stock solution of either gallic acid, ascorbic acid, or trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Name of Antioxidant Standard _____

With a plastic pipette, remove 3 ml of solution from the stock solution and add it to the first test tube. Add 3 ml of distilled water to the solution in the first test tube. Mix the contents of the first test tube by shaking and/or swirling it.

With a new plastic pipette, remove 3 ml of solution from the first test tube and add it to the second test tube. Add 3 ml of distilled water to the solution in the second test tube. Mix the contents of the second test tube by shaking and/or swirling it.

With a new plastic pipette, remove 3 ml of solution from the second test tube and add it to the third test tube. Add 3 ml of distilled water to the solution in the third test tube. Mix the contents of the third test tube by shaking and/or swirling it.

With a new plastic pipette, remove 3 ml of solution from the third test tube and add it to the fourth test tube. Add 3 ml of distilled water to the solution in the fourth test tube. Mix the contents of the fourth test tube by shaking and/or swirling it.

Add water to the final test tube. This is your water blank.

Obtain a test tube or vial with a solution of unknown concentration.

Part II. Antioxidant measurement using the FRAP method.

Obtain 7 square plastic spectrophotometer cuvettes.
Add the amounts on each reagent specified on the table.
Use a new small plastic pipette each time.

cuvette	
1	4 drops stock solution
2	4 drops test tube 1
3	4 drops test tube 2
4	4 drops test tube 3
5	4 drops test tube 4
6	4 drops water
7	4 drops unknown

Add 3 ml (measured with a plastic pipette) of FRAP solution to each cuvette.
Measure the absorbance of each cuvette at 593 nm.
Zero the spectrophotometer with cuvette 6 before each reading.

Complete the following table with you values
Do a second Absorbance reading on each cuvette to determine if the Absorbance has stabilized.

Tube	Absorbance	Concentration of antioxidant _____
1		
2		
3		
4		
5		
6	0 mmol	0
	0 mmol	
7 (unknown)		

Add data to a spreadsheet before you leave the lab.

Observations and comments:

Part III. Total phenolics measurement using the Folin-Ciocalteau reagent.
Set the spectrophotometer to 750 nm.
Set up 7 square plastic cuvettes.
To each cuvette add 5 drops of the solution from the test tubes using a 3 ml plastic pipette following the table below. Use a new small plastic pipette each time.

cuvette	
1	5 drops stock solution
2	5 drops from test tube 1
3	5 drops from test tube 2
4	5 drops from test tube 3
5	5 drops from test tube 4
6	5 drops water
7	5 drops of unknown

Then add 1.5 ml of Folin-Ciocalteau reagent to each cuvette.
Mix the solution in each cuvette by shaking and/or swirling.
Let them set for 7 minutes at room temperature.
Add 1.5 ml of $NaCO_3$ solution to each cuvette.
Mix the solution in each cuvette by shaking and/or swirling.
Allow the cuvettes to sit for 30 minutes.
Read of absorbance of cuvettes 1 – 5 & 7 at 750 nm.
Zero the spectrophotometer with cuvette 6 before each reading.

Tube	Absorbance	Concentration of antioxidant _____
1		
2		
3		
4		
5		
6	0 mmol	0
7 (unknown)		

Add data to a spreadsheet before you leave the lab.
Observations and comments:

Part IV. Standardization of 2,6-dichlorophenol reagent with ascorbic acid.

Obtain a sample of ascorbic acid of known concentration.
Record the concentration of the ascorbic acid sample _____
Place 5 ml (measure with a 10 ml graduated cylinder) of this solution in a 125 ml Erlenmeyer flask.
Add 5 ml (measure with a 10 ml graduated cylinder) of acetic acid buffer to the Erlenmeyer.
Fill a 50 ml burette with DCIP solution.
Run a little DCIP solution through the burette to get air bubbles out of the burette tip.
Record the starting point (the level of DCIP solution in the burette) of your titration.
_____ ml
Add the DCIP solution to the erlenmeyer drop by drop.
Swirl the flask to mix the solutions.
When you near the end point of the titration the pink color will persist a few seconds before disappearing.
The end point in reached when the pink DCIP does not disappear after mixing.
Record the end point of your titration.
_____ ml

The molarity (concentration in mM) of the DCIP solution is determined by the relationship
$M_1V_1 = M_2V_2$.
M_1 is the concentration of the ascorbic acid sample.
V_1 = 5.0 ml.
V_2 is the volume of DCIP solution added to the ascorbic acid (ending ml – beginning ml).
M_2 is the concentration of the DCIP solution.

Reading at the end of titration.

Add data to a spreadsheet before you leave the lab.

Observations and comments:

Lab Report.
Spreadsheet Data for the class will be available on the MyDU website.

1. Using the FRAP best linear fit for your antioxidant standard (gallic acid, ascorbic acid, or trolox) calculate the antioxidant power of your unknown.

2. Using the Folin-Ciocalteau best linear fit for your polyphenolic standard (gallic acid, ascorbic acid, or trolox), calculate the polyphenolic content of your unknown.

3. Compare the best linear fit graphs for the 3 different antioxidant standards for the FRAP assay. What are your conclusions?

4. Compare the best linear fit graphs for the 3 different antioxidant standards for the Folin-Ciocalteau assay. What are your conclusions?

5. Consider the best linear fit graph for the DCIP ascorbic acid titration results. What are your conclusions?

Prelab: Hand in at the beginning of the lab period.
For Part I., The stock solution in the first test tube has a concentration of 50.0 mM trolox.
Fill in the table below with the concentrations of trolox in tubes 1, 2, 3, and 4
$M_1V_1 = M_2V_2$.
M_1 = concentration of the trolox solution added to the test tube.
V_1 = volume of solution added to the test tube.
V_2 = total volume of solution in the test tube after addition of water.
M_2 = final concentration of trolox in the test tube.

cuvette	Absorbance	Concentration of trolox
1	0.927	50.0 mM
2 (tube 1)	0.588	
3 (tube 2)	0.363	
4 (tube 3)	0.277	
5 (tube 4)	0.240	
6	0.000	00.0 mM
7 (unknown)	0.405	

Part II.
Use Excel or a graphing calculator to determine the best linear fit of x = concentration, y = absorbance plot of the data.

Equation of the line _____

What is the concentration of trolox in the unknown?

Part IV.
The titration data is as follows.
Concentration of ascorbic acid 100.0 mM
Beginning burette reading 1.5 ml
Ending burette reading 35.2
What is the concentration (ascorbic acid equivalents) of DCIP solution in mM?