Phytochemical Antioxidants with Potential Health Benefits in Foods

A CASPiE Research Module

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Course Manual for UIC Chemistry 114 - 118

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Dr. Jay Burgess is an Associate Professor in the Department of Foods and Nutrition at Purdue University. His research focuses primarily on oxidative stress in mammals and the physiological results of such an accumulation of reactive oxygen species.

The Burgess lab has studied the role of reactive species in the pathophysiology of attention-deficit/hyperactivity disorder (ADHD) and has shown that some children with ADHD exhibit certain biochemical abnormalities indicative of cellular oxidative stress. They have also shown, in an animal model for ADHD, that supplementing with antioxidants can reverse the abnormalities and improve behavior.

Another project the Burgess lab has been working on involves flavonoid antioxidants. They have determined that such antioxidants can be helpful in reducing oxidative stress, but are not sufficient to compensate for a deficiency of essential antioxidant nutrients like vitamin E.

From this module, Dr. Burgess would like to gain a greater amount of knowledge of the antioxidant activity from different food products currently on the market. He is particularly interested at this time in the antioxidant characteristics of green tea and green tea mixtures with fruit juices, and what happens to these during digestion.

Recent Publications:


Introduction

We are constantly being told to eat fruits and vegetables, and that these types of plant foods contain chemicals that are good for us. Now the message is that we need to eat up to nine servings a day of fruits and vegetables because this will help prevent people from getting cancer, heart disease, and other disorders that might afflict us as we age. You may have many questions about these recommendations: Why do people have to eat so many fruits and vegetables to obtain these health benefits? What are the chemical components in fruits and vegetables that provide the proposed health benefits? It is common practice to use vitamin and mineral supplements to replace the need to consume so many fruits and vegetables. Are vitamins and minerals alone necessary to achieve the health benefits fruits and vegetables give us? Is this a misconception or has our understanding changed in recent years? You will explore the science that addresses some of these questions over the next six weeks.

The picture to the right illustrates an example of a vegetable source that is available in many of your local supermarkets. Note that under the name of the product contents is the description “with long lasting antioxidant activity.” Many would associate antioxidant activity with something good, but what does this really mean? As a consumer looking at this package you might wonder what would this so-called long lasting antioxidant activity do for you? Is it this characteristic of the sprouts that make them good for your health? Are these sprouts really better for you than the less expensive sprouts you can buy in the adjacent bin? The more detailed information on the back of the package identifies a specific compound, sulforaphane GS, as something in these sprouts that provides “long lasting antioxidant and cellular function.” The sulforaphane GS is not an essential nutrient such as vitamin C which is also found in these sprouts. This chemical substance is one of a multitude of substances in plants (called phytochemicals) that are thought to explain an observed association between high fruit and vegetable consumption and lower incidence of killer diseases such as cancer and heart disease. Antioxidant activity is hypothesized to be one mechanism by which these chemical substances might exert this protective effect. This characteristic is cited because many of these chemical substances show potent antioxidant activity in the test tube. Despite these observations many questions remain concerning which chemical antioxidants in fruits and vegetables really contribute to a lower risk for chronic diseases and how the substances from multiple food sources interact with one another in a mixed food diet to provide such benefit.
This introductory section describes the background information on food, nutrients, and antioxidants and their relationship to health. A second section will describe phytochemicals and indicate the chemical characteristics that make them good antioxidants. The final section will provide an overview of techniques you will use in this module and how these assessments are used to evaluate the potential health benefits of phytochemicals as antioxidants.

**Essential Nutrients in Food**

Food provides chemical substances that are required by heterotrophs\(^1\) to allow for growth, reproduction, and the overall maintenance of health. By the first part of the twentieth century the chemical constituents in food that supported these outcomes for humans were identified and classified into groups: water, carbohydrates, lipids, proteins, vitamins and minerals. It was observed during this time that if one did not consume sufficient amounts of these essential nutrients that specific deficiency disorders would develop. A good example is the disease scurvy, which occurs as a result of insufficient consumption of vitamin C. This scientific discovery process was carried out by chemists, biochemists, physiologists, and nutritionists and eventually led to government-supported recommendations for the minimum amount of these nutrients that healthy people should consume to prevent the development of deficiency diseases. Today most disorders resulting from nutritional deficiencies are uncommon in developed countries, but still often occur in the developing world.

You probably recognize the acronym RDA, which stands for Recommended Dietary Allowance. The RDAs define specific amounts for each nutrient which must be consumed to prevent deficiency in healthy people. The U.S. Dietary Guidelines\(^2\) and the Food Guide Pyramid\(^3\) are more practical tools that consumers can use to select combinations of foods to create diets that provide the RDA for all of the nutrients. In recent years it has become apparent to many scientists who study human health that in addition to providing nutrients that prevent deficiency diseases, consumption of certain foods appears to be associated with a lower occurrence of diseases that occur more frequently as we age. These chronic diseases, which generally require decades to develop, include heart disease, cancer, diabetes, hypertension, and Alzheimer’s disease, and are among the leading causes of death in the U.S. population. Fruits and vegetables are the types of foods that are most often cited as helping to protect people from developing chronic disease. However, the essential nutrient content of fruits and vegetables, although abundant, does not appear to account for all of these health benefits. Thus, over the past decade a great deal of research has been conducted to identify how other chemical constituents in fruits and vegetables might help prevent the development of chronic disease in people.

**Chronic Disease and Oxidative Damage**

Understanding the processes by which chronic diseases develop has led to the identification of key mechanisms underlying the development of a disease state. Some of

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1 Heterotrophs feed partially or exclusively off of other forms of life.
2 http://www.health.gov/dietaryguidelines/
3 http://www.nal.usda.gov/fnic/Fpyr/pyramid.html
these processes include carcinogenesis, the inflammatory process, over stimulation of programmed cell death, and oxidative stress. Oxidative stress is defined as the accumulation of reactive oxygen species (ROS) in living systems to a sufficient degree to cause measurable damage to cells and tissues. Reactive oxygen species are defined as partially reduced forms of oxygen that are either radical species themselves or can easily form radical species. As aerobic organisms that undergo respiration, humans use oxygen during the process of respiration to obtain energy from fuel sources. Molecular oxygen is reduced by four electrons to produce water in the mitochondria of cells (the proton gradient resulting from this electron transfer process drives the formation of ATP). Neither the starting material, oxygen, nor the final product, water, that results from this reduction process is very reactive with large macromolecules that make up the structure of the cells. But univalent (one-electron) reduction of oxygen can form species that are much more prone to react with cellular macromolecules.

As illustrated in the first equation below, one-electron reduction of oxygen leads to the formation of superoxide.

\[
\text{O}_2 + e^- \rightarrow \text{[O}_2^-] \quad (1)
\]

\[
2\text{[O}_2^-] + 2H^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (2)
\]

\[
\text{H}_2\text{O}_2 + 2e^- + 2H^+ \rightarrow 2\text{H}_2\text{O} \quad (3)
\]

\[
\text{O}_2 + 4e^- + 4H^+ \rightarrow 2\text{H}_2\text{O} \quad \text{(overall)}
\]

Eq. 1

Addition of a second electron along with a source of protons can lead to the formation of hydrogen peroxide (H$_2$O$_2$). Two-electron reduction of H$_2$O$_2$ will lead to the formation of the final product, water. Alternatively, addition of only one electron to H$_2$O$_2$ leads to the formation of hydroxyl radical and hydroxyl anion.

\[
\text{H}_2\text{O}_2 + e^- \rightarrow \cdot\text{OH} + \text{OH}^- \quad \text{Eq. 2}
\]

Superoxide, hydrogen peroxide and hydroxyl radical are considered ROS because they will either directly or indirectly react with biological macromolecules in living cells such as proteins, lipids and DNA. This interaction will often alter the structure of the macromolecule and destroy its function.

How Antioxidants Prevent Oxidative Damage

In living systems the potential sources of ROS include mitochondrial respiration, enzymes such as NADPH oxidase\(^4\) and other types of oxidases, and exposure to environmental chemicals/toxins such as alcohol, cigarette smoke and surface ozone. Transition metals such as iron and copper that donate and accept electrons easily also contribute to ROS production. To prevent damage due to ROS, living systems have a multitude of defense systems. These systems include primary antioxidants that are

\(^4\)NADPH oxidase is nicotinamide adenine dinucleotide phosphate-oxidase.
substances that directly inactivate ROS. Primary antioxidants donate a neutral hydrogen atom (with one proton and one electron) to a free radical ROS, thereby terminating the radical (allowing for the formation of an electron spin-pair in the outer orbital of the ROS) and preventing the molecule from causing damage. Hydrogen-donating antioxidants form more stable, less reactive radical species than the ROS. **Secondary antioxidants** prevent damage due to ROS by a variety of other mechanisms including metabolism of non-radical ROS, chelation\(^5\) of transition metals and repairing the damage that ROS cause to lipids, protein and DNA.

A good example of an interaction between an ROS, cellular macromolecules and an antioxidant is observed in foods and biological systems that contain polyunsaturated fatty acids (PUFA). In living systems, PUFA are important constituents of cell membranes and serve as precursors to hormones. The PUFA chemical structure is characterized by a long hydrocarbon chain that contains multiple double bonds separated by methylene groups. This arrangement makes the molecule susceptible to hydrogen atom removal and oxygen incorporation. Interaction with an ROS can initiate oxidation of PUFA, which can then lead to continual formation of more ROS as illustrated generally in Equation 3 below.

\[
\begin{align*}
  \text{LH} + \text{X}^\cdot & \rightarrow \text{L}^\cdot + \text{XH} \\
  \text{L}^\cdot + \text{O}_2 & \rightarrow \text{LOO}^\cdot \\
  \text{LOO}^\cdot + \text{LH} & \rightarrow \text{L}^\cdot + \text{LOOH}
\end{align*}
\]

Eq. 3

A more specific example is given in Equation 4, where oleic acid forms a peroxyl radical by reaction with hydroxyl radical (step one) and then oxygen (step two). The peroxyl radical can continue to damage other molecules of oleic acid.

\[
\begin{align*}
  \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \cdot\text{OH} & \rightarrow \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \text{H}_2\text{O} \quad (1) \\
  \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \cdot\text{OH} & \rightarrow \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \text{H}_2\text{O} \quad (2) \\
  \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \cdot\text{OH} & \rightarrow \text{HCH(\text{CH}_2\text{CH}_2\text{CH}_2)}_2\text{C}==\text{CH}\text{CH}(_2\text{CH}_2\text{CH}_2\text{CH}_3)\text{OH} + \text{H}_2\text{O} \quad (3)
\end{align*}
\]

Eq. 4

\(^5\) Chelation is the process of reversible binding of a ligand to a metal ion, forming a complex.
Unless an antioxidant is present to stop this continual chain reaction, all the PUFA present will be modified via incorporation of oxygen which will significantly change their characteristic from being very hydrophobic to more hydrophilic. If this happens in a cell membrane, it will lead to cell death.

A good antioxidant that typically protects PUFA in foods and living systems is vitamin E, which is a group of structurally similar lipophilic molecules collectively known as tocopherols. Each form of tocopherol is indicated by a different Greek letter. The vitamin possesses a phenolic ring structure attached to a hydrocarbon chain. It can readily donate a hydrogen atom to a lipid peroxyl radical (such as the product of step 2 in Eq. 4) terminating the radical and preventing the further propagation of what is referred to as the lipid peroxidation process.

\[
\text{LOO}^- + \text{EH} \rightarrow \text{LOOH} + \text{E}^+ \quad (1)
\]

\[
\text{E}^- + \text{appropriate reducing agent} \rightarrow \text{EH} \quad \text{Eq. 5}
\]

In biological systems chemical antioxidants such as vitamin E and vitamin C (L-ascorbic acid or just “ascorbate”) are constantly reused because other supporting systems help to keep them in a reduced state. These systems are mostly enzymatic and don’t function in foods. Thus, antioxidants are often added to foods that contain PUFA to prevent lipid peroxidation and to preserve the concentration of essential antioxidant nutrients. In fact ascorbate can reduce vitamin E and is sometimes added to foods as a preservative.

**Phytochemicals as antioxidants**

There is a multitude of chemical substances in foods that possess antioxidant properties. These fall into numerous antioxidant classes, as shown in Figure 3.

---

6 The opposite of oxidation is reduction. A “reducing agent” will counteract oxidation.
The various chemicals that behave as antioxidants have different properties, for example some are soluble in fats/non-polar solvents and some are soluble in water/polar solvents. In this module we will focus on a class of substances referred to as flavonoids. Flavonoids all possess a basic 3-ring structure as illustrated in Figure 4.

![Figure 4. Basic Flavonoid Structure.](image)

Different subclasses of flavonoids vary in the structure of the C-ring: it may include a double bond between carbons 2 and 3, a carbonyl group at carbon 4, or a hydroxyl group at carbon 3. Within each subclass the specific species vary based on the substitution of hydroxyl or O-methyl groups at positions 5, 7, 3’, 4’, and 5’. Because these are polyphenolic compounds they all possess some degree of antioxidant activity when evaluated in a test tube. Generally, those chemical species with a greater number of hydroxyl substituents on the ring possess greater activity in the test tube. The flavonoid called quercetin possesses adjacent hydroxyl substituents on the B-ring at positions 3’ and 4’. A proposed scheme for how quercetin acts as an antioxidant is illustrated in Figure 5 in which two lipid radicals are reduced sequentially. The single dot next to some of the O atoms indicates an unpaired electron, which indicates a radical chemical species.

![Figure 5. Flavonoid Antioxidant Mechanism.](image)

Flavonoids are abundant in common fruits and vegetables. Table 1 summarizes which types of flavonoids are found in fruits and vegetables.

---

7 A polyphenolic antioxidant is a compound having multiple phenols, or benzene rings with –OH (hydroxyl) substituents.
Table 1.  
**Flavonoid subclass** | **Major Food Sources**
--- | ---
Flavonols (e.g. quercetin) | Onions, kale, broccoli, apples, cherries, berries, tea, red wine
Flavones | Parsley, thyme
Flavanones | Citrus
Catechins (e.g. epicatechin) | Apples, tea
Anthocyanidins | Cherries, grapes
Isoflavones | Soya beans, legumes

**Measurement of Antioxidant Activity**

Several methods have been developed to measure the total amount of antioxidant activity that a pure chemical, complex mixture, food or biological sample possesses. All of the methods involve the generation of ROS and the termination of the ROS by the antioxidant being tested. One common method for assessing total antioxidant activity in a food or biological sample, like serum, is the Trolox equivalent antioxidant capacity (TEAC) assay. This is a method that you will use during this module. This method measures the ability of a test antioxidant to reduce a radical species. The radical species exhibits a characteristic absorption spectrum. Therefore, reduction is associated with loss of color at a specific wavelength. Potential antioxidant preparations are tested at several different concentrations and compared to Trolox, which is also tested at several concentrations. Good antioxidants will reduce the absorbance of the radical species at lower concentrations than poor antioxidants. Figure 6 illustrates the overall antioxidant activity of common fruits and vegetables observed when measuring their TEAC activities. Larger values indicate greater antioxidant activity. Notice the value for strawberries is more than two times greater than the value for onions.

![Figure 6. TEAC values of various foods.](image)
Another method for measuring antioxidant activity focuses on a different class of antioxidants: polyphenolics. This measurement uses a reagent, the Folin-Ciocalteau reagent, that reacts with polyphenolic antioxidants to form compounds that absorb light in the visible region. As the concentration of polyphenolics increases, so does the amount of light absorbed by the solution. As with the TEAC assay, there is a standard that is known as epicatechin. Epicatechin is reacted with the Folin-Ciocalteau reagent at different concentrations to form a standard curve. Then potential antioxidants are also reacted with the reagent at multiple concentrations. The absorbance of the potential antioxidants is used in conjunction with the standard curve of epicatechin to find the concentration of polyphenolics in the sample.

The final analysis method that you will use is L-ascorbic acid (Vitamin C) quantification via high-performance liquid chromatography (HPLC). In this analysis method, you will make a standard curve of L-ascorbic acid as well as multiple concentrations of your food samples. The HPLC will separate the components of your solutions, both standard and sample, and quantify the amount of L-ascorbic acid. You can use this to determine the concentration of L-ascorbic acid in your sample solutions and calculate the amount in your dry samples.

**What we don’t know and what you are going to do in this module**

Although a number of studies have been conducted with pure phytochemicals and food sources of phytochemicals, much is still unknown concerning the contribution of these non-nutrient compounds to health. Many questions remain unanswered about how these phytochemicals interact in a complex diet both before and after we consume them. Some of the questions are listed below with example hypotheses statements. When these hypotheses are tested, they may help answer each question.

- **Is the antioxidant activity of spices affected by cooking?**
  - Hypothesis: The antioxidant activity of curry powder, a spice known to contain antioxidants, will not be changed by heating the spice to simulate cooking.

- **Are dried fruits effective for use in making antioxidant-rich foods as a substitute for fresh fruits?**
  - Hypothesis: Raisins, the dried analogue of grapes, which are known to be high in polyphenolics, are not high in antioxidant activity.

- **Does the digestive process significantly alter antioxidant activity of common foods?**
  - Hypothesis: Conditions that simulate digestion such as changing pH, mixing, and exposure to digestive enzymes will significantly reduce the antioxidant activity of a dried fruit or spice.

In this module you will apply several of the fundamental properties of chemistry to address a research question about antioxidant properties of food substances that may be beneficial to health. Specifically, you will be examining the effect of the digestion process on green teas and juice/green tea mixtures. Key chemistry topics that will be used in this module include the properties of mixtures, organic compounds (the atomic properties of carbon), and chemical equilibrium. During the first three lab periods you will learn how to measure total antioxidant activity (via the TEAC method), ascorbate
concentration and total phenolic concentration in standard and food samples. During lab four you will begin to plan your own research project. As a means of learning how to best design your own research project, you will review a very recent paper testing the effect of processing on antioxidant activity of a food substance. During this lab period you will also identify the experimental question that your group would like to address and design the experiment that you will conduct during the subsequent laboratories. The following table illustrates this proposed schedule.

**Module Calendar**

The three main measurements for the upcoming labs are total antioxidant activity as TEAC, ascorbate concentration (using HPLC), and total polyphenolics in standard and test samples. The TEAC measurement estimates total antioxidant activity contributed by both known (ascorbate and polyphenolics) and unknown chemical components for a given sample. Measurement of total polyphenolics concentration is used in this module to reflect the amount of flavonoid compounds present in a test sample.

You will use a few standard substrates in the TEAC assay: epicatechin, quercetin, and Trolox. Epicatechin is a common flavonoid that is found in apples and tea and it is also a component of compounds that are found in cherries and grapes. Quercetin is one of the more abundant flavonoids in our food supply; one of the richest sources of quercetin is fried onions. Trolox is a more polar form of vitamin E, lacking the long hydrocarbon side chain in α-tocopherol. Ascorbate is a known essential nutrient antioxidant that functions as such in the body.

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<td><strong>Lab 2:</strong> TEAC Measurement – Conduct the TEAC measurement with Trolox and the flavonoids epicatechin and quercetin, and explore the equilibrium of the reaction.</td>
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<tr>
<td><strong>Lab 3:</strong> Ascorbate and Total Phenolic Measurement - Introduction to HPLC and the use of a standard curve to determine ascorbate and total phenolics concentrations in foods.</td>
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<tr>
<td><strong>Labs 4-7:</strong> Independent Research Project - Conduct experiments to test the chosen hypothesis and complete analysis - repeat tests as necessary.</td>
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Laboratory 1: Making Solutions and Spectral Scanning of the Trolox Equivalent Antioxidant Capacity (TEAC) Substrate.

Overview of this Laboratory Activity
During this laboratory period you will make a variety of stock solutions that you will be using throughout the remaining weeks of this module. You will also determine the exact concentration of a solution, which has been previously prepared for you, by using the spectrophotometer.

Introduction to Making Solutions
Understanding how to make solutions and how to measure their properties is fundamental to many kinds of research, simply because so many substances that are important to life are either solutions or inhomogeneous mixtures. The liquids and solids that make up living systems and foods are mixtures of two or more substances physically mixed together but not chemically combined. Mixtures possess two important defining characteristics: variable composition and retention of individual component properties. Solutions and colloids are two common types of mixtures. A solution is a homogeneous mixture with each component dispersed evenly throughout the space or phase. Salt dissolved in water is an example of a solution. Heterogeneous mixtures exist in separate phases. A colloid is an example of a heterogeneous mixture in which one component is dispersed evenly as very small particles in the other. Milk is an example of a colloid; to the unaided eye it appears to be a homogeneous mixture. Applying centrifugal force to a milk sample will separate the phases and reveal the colloidal nature of the food. Solutions and colloids differ because the particles in solutions are individual atoms, ions or molecules whereas in colloids the particles are large macromolecules or aggregates of smaller molecules that are still small enough to remain dispersed. In this module you will encounter both solutions and colloids. The chemical reagents that you will use to carry out the experiments in each lab are solutions, whereas the foods that you will analyze may be either solutions or colloids.

Solutions are usually defined as one substance (solute) dissolved in another (solvent) that is more abundant. The solubility of a solute is the maximum amount of the chemical that will dissolve in a particular volume of solvent, usually 1 liter. (Some substances will mix together in any proportion and are said to be miscible.) A major factor which influences the solubility of a solute in a solvent is the relative strength of the intermolecular forces within and between solute and solvent. These forces and their effects are discussed in detail in most Chemistry textbooks. Here, however, we will discuss issues relating to making solutions of various desired concentrations.

Common conventions for expressing concentration include molarity, molality, percent mass and percent volume.
- Molarity (M) = moles of solute / volume of solution (L)
- Molality (m) = moles of solute / mass of solvent (kg)
- Percent mass = mass of solute / total mass of solution x 100
- Percent volume (v/v) = volume of solute / total volume of solution x 100
Percent volume (w/v) = mass of solute / total volume of solution x 100

In this module, we will primarily be using molarity to express concentration units, but you will also see some substances described with the other units so it is important to be familiar with them, and the differences between them. When making a solution of a desired concentration, say 25 mM for example, you need to determine two things:

- Total volume of solution that you need
- Mass of solute that you must add to get the desired number of moles.

An important point to remember about solutions is that the concentration of the solution will be the same throughout the whole solution. For example, if you have a liter of solution with a concentration of 50 mM and you pour half of that into another container, then the amount you have poured out also has a concentration of 50 mM (as well as the amount you left in the original container.) This may seem like such an obvious point that it shouldn’t even deserve mention here. However, this simple point has a very practical purpose when you are making solutions in the laboratory. If you need a very small amount of solution (for example, 5 mL) and it will be a very dilute solution, then it may be difficult or even impossible for you to weigh the proper amount of solute to make the proper concentration of solution at only 5 mL. But you could instead make 100 mL of the solution at the desired concentration and simply use 5 mL of it, if 100 mL is a more practical volume for weighing your solute. Alternatively, you could make a more concentrated solution and use what is known as a serial dilution technique to arrive at the more dilute concentration that you desire. We will talk about each of these approaches. In either case, your own common sense is important in deciding how you will arrive at your final desired volumes and concentrations. (For example, if you are going to make a larger volume than you need, don’t pick such a large volume that you would be wasting large amounts of solute – especially if it is an expensive solute.)

Proper Technique for Weighing Solids

You will be using analytical balances for weighing solids in the lab. You should keep in mind that these balances are not reliable below about 10 mg (0.010g). So, if your calculations show that you need to weigh out less than this amount, you should consider making a more concentrated solution and diluting to the required concentration. Once you’ve determined an amount larger than 10 mg to weigh out, you still need to be careful about using proper technique so that you don’t waste materials or weigh your sample incorrectly. (Remember, many of the substances that you will be working with in this module are very expensive – as is often true with chemicals used in research studies.)

When you use the balances, you should first place your weighing paper or weighing boat onto the balance and “tare” the balance. You will then place both hands into the balance, one from each side of the balance. In one hand you will hold the container for the solid sample you are weighing, and in the other you will hold the tool (such as a spatula) with which you are scooping it onto your weighing paper or boat, as shown in the image below.
When you have the desired amount in your weighing paper or boat, leave your sample on the scale but remove both your hands and close the doors. The mass displayed with the doors closed is the correct mass for the amount of sample you have weighed out. Be sure that you have not spilled any solid sample on the weighing pan outside of your weighing boat, since this will give you an incorrect reading.

**Using Dilution to Reach a Desired Concentration**

You can make a more concentrated solution than you need, and then dilute it volumetrically to reach the concentration you desire. When you are trying to achieve very specific concentrations, you should always be sure to use volumetric flasks to make your solutions. In particular, it is very important to never fill past the line on the neck. You cannot fill past the line and then remove some liquid, because that will change your concentration.

When diluting a solution, you need to keep track of the number of moles (i.e. amount of solute) that you are moving from one place to another. Conveniently, the number of moles can be calculated by multiplying concentration and volume:

\[(\text{conc}) \, \text{mol/L} \times (\text{volume}) \, \text{L} = \text{mol}\]

Let’s take an example where we will start out with a “stock” solution that has a concentration of 25.0 mM (that is 2.50 x 10^{-2} M). A “stock” solution is the solution that you start with, and you usually will have a relatively large quantity of it so that you will use it repeatedly to make your other solutions. For this reason, it is very important that you make your stock solutions carefully and make sure they do not become contaminated.
(such as by putting a dirty pipette into them.) Let’s assume that you want to make 25 mL of a 0.50 mM solution starting with this stock solution. This means you will transfer some small amount of your stock solution into a 25 mL volumetric flask, and then carefully dilute it with the proper solvent up to the line on the next of the flask. The question is – how much should you transfer?

Remember to **always think about the moles**! A 50.0 mL solution of 0.500 mM concentration has \( (5.00 \times 10^{-2} \text{ L}) \times (5.00 \times 10^{-4} \text{ mol/L}) = 2.50 \times 10^{-5} \text{ mol} \) of solute in it. So, you need an amount of your stock solution that will put this many moles into your new flask. You can find this by dividing this number of moles by the concentration of your stock solution:

\[
\frac{2.50 \times 10^{-5} \text{ mol}}{2.50 \times 10^{-2} \text{ mol/L}} = 1.00 \times 10^{-3} \text{ L} = 1.00 \text{ mL}
\]

Therefore, you need to put 1.00 mL (measured in a volumetric pipette) from your stock solution into your 25 mL volumetric flask, and dilute to the line. Another way to arrive at this calculation is to remember the equation \( V_1 C_1 = V_2 C_2 \). In this equation, \( V \) stands for volume and \( C \) stands for concentration. If you take the volume and concentration of the solution you want as \( V_2 \) and \( C_2 \), and the concentration of your stock as \( C_1 \), then you can rearrange the equation to find out the amount of solution that you need to transfer from the stock to the new flask:

\[
V_1 = \frac{V_2 C_2}{C_1} = \frac{(5.00 \times 10^{-2} \text{ L})(5.00 \times 10^{-4} \text{ mol/L})}{2.50 \times 10^{-2} \text{ mol/L}} = 1.00 \times 10^{-3} \text{ L} = 1.00 \text{ mL}
\]

Notice that this yields the same answer and is, in fact, the same calculation, all in one step. You must not forget, however, that you are actually achieving this by making the number of moles equal on the two sides of the equation.

**Introduction to TEAC Assay: Spectrophotometric Measurement of ABTS⁺ (the ABTS Radical Cation)**

One of the solutions that you will make during this lab period provides the radical substrate for the Trolox equivalent antioxidant capacity (TEAC) assay. (An assay is an analysis to determine the presence, absence or quantity of some component in a mixture or substance.) This assay compares the total antioxidant activity in a sample to a standard, Trolox. Because this assay aims to quantify the total amount of antioxidant activity in a complicated test mixture of interest, the substrate of choice is a radical chemical species that exhibits characteristics that can be exploited for quantification purposes. The radical chemical species of the monocation \( 2,2' \)-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), abbreviated ABTS⁺, is ideal for this purpose. It is relatively stable, is readily reduced by important antioxidant compounds and exhibits a characteristic absorption spectrum in the visible range of light. This last property is one that can be used for quantification purposes. The ABTS⁺ is synthesized prior to its use by first making separate solutions of ABTS and potassium persulfate in water. Equal volumes of these two solutions are combined and the reaction (outlined in Eq. 6 below) is...
allowed to continue until it reaches equilibrium after about 12 hours. One electron is withdrawn from each of 2 molecules of ABTS by potassium persulfate leading to the formation of potassium bisulfate and ABTS$^{+}$. The product formed has characteristic absorbance at several wavelengths. This type of oxidation reaction with ABTS is utilized in many common medical analyses, such as enzyme-linked immunosorbent assays (ELISA) to measure the concentration of important biomolecules in blood or urine.

Colored solutions absorb light in the visible range (400-700 nm) of the electromagnetic spectrum. A compound that would absorb all wavelengths of light in this range would appear black. Others which absorb only at distinct regions within this range appear as different colors. The ABTS$^{+}$ actually absorbs at several distinct regions in the visible light spectrum. We will use this property to quantify the amount of the ABTS$^{+}$ in solution using a spectrophotometer and exploiting the **Beer-Lambert Law**, or “Beer’s Law”. This law states that the concentration (C) of a chemical compound is proportional to its absorbance (A) divided by a constant (molar extinction coefficient or absorptivity [$\varepsilon$]) and path length (l), as follows:

$$C = \frac{A}{\varepsilon l}$$

These websites explain this in more detail:  
http://www.shu.ac.uk/schools/sci/chem/tutorials/molspec/beers1.htm  
http://dl.clackamas.cc.or.us/ch105-04/beer's.htm

\[ \varepsilon = 1.5 \times 10^4 \text{ mol}^{-1}\text{Lcm}^{-1} \]

You will use this constant to calculate the concentration of ABTS\(^{+}\) in substrate solutions.

**Pre-Lab Requirements**

Write an introduction and experimental section for this laboratory period. Your experimental section should include a description of what you plan to do in lab, in your own words, such that you could follow the instructions direction out of your own lab notebook. In addition to writing introduction and experimental sections for this laboratory, you will also need to calculate the masses and volumes needed to make all solutions and dilutions for this procedure before coming to lab. You should also research proper volumetric techniques especially those techniques involving the use of equipment not used in the previous Chem 112-114 experiments, namely, measuring pipets and 3-way safety bulbs.

**Materials Available**

- ABTS (2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)
- Potassium persulfate
- Trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)
- Epicatechin, ((2R,3R)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol)
- Sodium carbonate, anhydrous
- Quercetin dihydrate, (2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one dihydrate)
- Ethanol, 100% or equivalent
- ABTS\(^{+}\) that has been incubated for 12 hours
- 25 mL amber vials and 250 mL bottles
- volumetric flasks in 25mL, 50mL, 100mL and 250mL sizes
- 5 mL measuring pipets
- 1000-200 μL and 200-10 μL auto pipets (“micro pipets”)
- 3-way safety bulb
- Cuvets
- Waste Jar

**Waste Disposal**

You should have a beaker designated for waste material on your lab bench. After you have performed the determination of maximum wavelength, the ABTS radical should be disposed of in this beaker. All the glassware used in preparing the solutions for the TEAC assay should be rinsed with a small amount of water from your wash bottle and poured into the waste beaker. You can then empty the beaker into the large waste jar found in the main hood.
Laboratory Procedures

Part I: Spectrophotometric determination of wavelengths of maximum absorbance ($\lambda_{\text{max}}$) for the ABTS$^+$ substrate:

Today you will measure an absorbance spectrum (sometimes called “UV-Vis”) of a completed ABTS$^+$ preparation.

While the spectrophotometer is warming up, feel free to complete some of the solution preparation parts that follow.

Throughout this module, you will be using a square plastic cuvet instead of the glass test tube-like cuvets that you may have used in the past. These cuvets have two clear sides and two ridged sides so it is important that you insert the cuvet into the spectrophotometer with the correct orientation. The cuvets have an arrow molded into the plastic to help you orient the cuvet in the spectrophotometer.

An ABTS$^+$ solution that has already been incubated for 12 hours will be provided. Make 100 mL of a 1/100 dilution of this solution in distilled water. This will be referred to as the ABTS$^+$ substrate. Transfer 3 mL of the ABTS$^+$ substrate into a cuvet and begin making measurements of the absorbance of this solution at 375 nm. For this sample measure the absorbance at intervals of 10 nm through the 375 to 835 nm range. The spectrophotometer must be calibrated for each wavelength using deionized water as the blank.

(Note: For spectrophotometers with scanning capability, intervals of 1 nm are preferred). For future studies, the position of the peak that you find closest to 735 nm will be used for your TEAC analysis. Dispose of the ABTS solution in the waste jar. The cuvets can be thrown away.

Part II: Preparation of Solutions for TEAC Assay

You will make two stock solutions that are stable in the dark for several months, ABTS and potassium persulfate. These two solutions are combined together in equal volumes to create the working ABTS$^+$ that has strong spectrophotometric properties in the visible range and is used in diluted form for the TEAC assay. It requires over 12 hours for the formation of ABTS$^+$ to be complete.

**ABTS$^+$ (radical cation) reagent**

Make 25 mL of a 0.014 M ABTS solution in deionized water. Make this solution in a volumetric flask. (A word of caution when using a volumetric flask: do not fill above the line and then try to remove solvent volume! You can fill almost to the line relatively rapidly, but then add drop wise until the meniscus of your solution is at the line for an accurate solution concentration.) Transfer the solution to an amber vial or a clear vial covered with foil to block light for storage, cap tightly, and store in the refrigerator. Put paraffilm around the cap of the vial for weekly storage to avoid solvent evaporation.

Make 100 mL of 0.0049 M potassium persulfate, $K_2S_2O_8$, in deionized water also in a volumetric flask. Store in a bottle. This solution is not light or temperature sensitive.

To make the ABTS$^+$ reagent, mix 5 mL each of the ABTS and $K_2S_2O_8$ solutions you just made into a foil covered or amber vial. Cap tightly, paraffilm, and store in the
refrigerator. Label all solutions with contents, your name, room number, date and section number. This solution will be ready to use after 12 hours and is stable if kept in the dark up to an additional 10 days.

**Standard Antioxidants: Making Stock Solutions of Trolox, Epicatechin and Quercetin**

Make 25 mL of 2.5 mM Trolox stock solution in ethanol in a volumetric flask. Transfer the solution to an amber vial. Cap tightly, parafilm, and store in the refrigerator. Label all solutions with contents, your name, room number, date and section number. Notice that it would be very difficult to do this in one step because you would need to weigh an extremely small amount of solute (Trolox). However, in a situation such as this, you can make a larger volume and then extract the amount you need. You’ll need to look at the sizes of the volumetric glassware that you have available in order to determine what volume you will scale up to.

Make 25 mL of 2.5 mM epicatechin and 25 mL of 1.0 mM quercetin in ethanol in the same way. Here again, you need to consider the amount of material that you need to weigh in order to make these solutions. One option is to make a more concentrated solution (for which you know the exact concentration) and dilute it. For example, you could make a 25 mM solution of epicatechin in 25 mL, and a 5.0 mM solution of quercetin in 100 mL. Then you can dilute those to end up with the solution concentrations and quantities that you need.

In general, it is important to think about not only the concentration of a solute that you need, but how you will work with the substance when you are making the solution – such as weighing it. You will want to keep this in mind, for lab periods 4-6 when you design your own experimental procedure.

**Preparation of Diluted Trolox, Epicatechin and Quercetin Solutions**

Make dilutions of the stock solutions of Trolox and epicatechin to 25, 50, 100 and 200 μM and quercetin to 20, 40, 80 and 160 μM (see example in figure on the next page). Be sure to use the appropriate solvent for dilutions. Storage for the dilute solutions is the same as for the stocks. When finished, you should have twelve dilute, or “sample”, solutions. Each of these will be run in a separate TEAC measurement in laboratory activity 2.

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The stock solutions of Trolox, epicatechin, and quercetin will be prepared for you. *Always check the bottle for the actual concentration of these and all other solutions.*

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Your solutions will be stored in vials for use in Week 2 of the module. *All* student groups should prepare solutions of Trolox. Half of the groups will also prepare solutions of epicatechin. The other half of the groups will do solutions of quercetin. Check with your TA for your group assignment.
Part III: Preparation of reagents for the total phenolics measurement

Dissolve anhydrous sodium carbonate in water to make 100 mL of a 7.0% solution (w/v) and store in a bottle. This solution is not light or temperature sensitive. Label all solutions with contents, your name, room number, date and section number.

All the glassware used in preparing the solutions should be rinsed with a small amount of water from your wash bottle and poured into the waste beaker. The waste beaker can then be emptied into the large waste jar found in the main hood.

Post-Lab Calculations and Analysis of the results

a. Calculate the concentration of ABTS$^+$ in the solution that you scanned using the absorbance that you obtained and the $\varepsilon$ at 735 nm given earlier.

b. Explain, based on intermolecular forces, the rationale for using water to dissolve potassium persulfate, ABTS, and sodium carbonate rather than ethanol that was used as the solvent for Trolox, epicatechin, and quercetin.

Preparation for Next Week

a. Read next week’s lab.
b. Prepare your notebook with necessary pre-lab information to carry out the lab.
c. Carry out any pre-lab or sample calculations that are necessary.
d. Be prepared to hand in your post-lab from this week.
Laboratory 2: TEAC Activity of Epicatechin, Quercetin and Trolox

Overview of This Activity
In this lab you will follow the reaction between ABTS$^{+}$ and primary antioxidants over time and estimate strength of antioxidant activity for the flavonoids quercetin and epicatechin in comparison to Trolox. You will derive a value for quercetin and epicatechin known as the “Trolox-equivalent antioxidant capacity” or TEAC value. There are two components to this analysis: (1) the calculation to determine the extent of reaction for each antioxidant, and (2) the comparison of the extent of reaction for each antioxidant with that of Trolox to determine antioxidant strength.

Introduction
The study of the extent of reactions, or chemical equilibrium, involves measuring the concentration of reactants and products at a point in time when no further observable change occurs. For the reaction between strong primary antioxidants and ABTS$^{+}$ the rate is quite fast, reaching equilibrium in seconds. For weaker antioxidants a much longer time period is required. This reaction can be written in shorthand as Eq. 7, where AH is an antioxidant capable of donating a hydrogen atom.

\[
\text{ABTS}^+ + \text{AH} \rightarrow \text{ABTS}H^+ + A^-
\]

Eq. 7

The extent of the reaction in Eq. 7 is measured in the TEAC assay. The extent of the reaction will be correlated with the antioxidant capacity, or strength, of the antioxidant being tested. In the antioxidant research literature for TEAC, the extent of reaction is determined based on the percentage of reactant, specifically ABTS$^{+}$ substrate, converted to product at defined concentrations of antioxidant. Measurements are taken for each test compound and compared to the extent of the reaction for Trolox, the water-soluble form of the nutrient antioxidant vitamin E.

The first thing you will need to do is measure the extent of reaction of each antioxidant (Trolox, epicatechin or quercetin) with the radical cation. For this objective, you will mix ABTS$^{+}$ substrate with a defined concentration of each antioxidant and their solvent(s) and read the absorbance at 735 nm after 6 minutes. The results of each measurement are first converted to a value of $\Delta A_{\text{ABTS}^+}$ (using absorbance units), then to the percent of ABTS$^{+}$ substrate converted to product, which is expressed as “percent inhibition”. You may wonder why the extent of reaction is represented as percent inhibition. This relates to the functional role of antioxidants in foods and living systems. Consider ABTS$^{+}$ as a type of ROS. In complex systems this ROS would interact with and potentially destroy large macromolecules. Reduction of ABTS$^{+}$ to ABTS$H^+$ inactivates this ROS and prevents this damage. Therefore, antioxidants inhibit damage caused by ROS, and representing the results as percent inhibition not only reflects the extent of the reaction but also the potential functionality of the antioxidant in biological systems. Therefore, a high percent inhibition implies that the antioxidant has inactivated a large amount of the ABTS$^{+}$ substrate ROS.

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The second objective that you will accomplish is to compare the strength of the test antioxidants to Trolox. This will be done by plotting the relationship between percent inhibition and concentration for each antioxidant used in the analysis. From the slope of the straight line that fits this relationship you will determine the value of percent inhibition per unit concentration of the antioxidant. The next step will be to divide the slope of each test antioxidant by the slope obtained for Trolox, as a means to standardize the comparison of different antioxidant preparations to one another. This ratio of slopes is the “TEAC value” for each sample. A larger TEAC value indicates greater antioxidant capacity.

What is the Purpose of a Control?

In all of the techniques you will be using in this module, you will need to carefully use “controls”. The purpose of a control is to ensure that your measurement is yielding the proper values for a known substance. In some cases, this means that you need to know what effect your solvent has on the measurement, so that you can subtract it out. In the first three laboratory periods you will be guided about when and how to use control measurements. For your own research project, you will need to carefully plan how you will incorporate controls into your measurements.

Pre-Lab Requirements:

In addition to writing introduction and experimental sections for this laboratory, as before, you will also need to calculate the volumes needed to make all dilutions for the TEAC assay before coming to lab. Determine if an absorbance measurement at time t=0 is necessary for every aliquot of the ABTS·+ substrate.

Materials Available

- Your previously prepared solutions of quercetin, epicatechin, Trolox, and ABTS·+
- ethanol, 100%
- volumetric flasks in 25mL, 50mL, 100mL and 250mL sizes
- 25mL amber vials
- spectrophotometer
- cuvets
- 5 mL measuring pipets
- 1000-200 μL and 200-10 μL auto pipets (“micro pipets”)
- 3-way safety bulb
- Waste jar

Waste Disposal

You should have a beaker designated for waste material on your lab bench. After you have performed the TEAC procedure, all solutions should be disposed of in this beaker. All the glassware used in preparing the solutions should be rinsed with a small amount of water from your wash bottle and poured into the waste beaker. The waste beaker can then be emptied into the large waste jar found in the main hood.
Laboratory Procedures

TEAC Procedure

Turn on the spectrophotometer and allow it to warm up for at least 30 minutes.

Preparation and dilution of ABTS\(^+\) (radical cation) reagent

Measure 2.5 mL of the ABTS\(^+\) solution into a 250mL volumetric flask and bring to volume with deionized water. This solution will be referred to as the ABTS\(^+\) substrate.

Measurement of substrate quality

Blank the spectrophotometer using water at 735 nm. Add 2.9 mL of ABTS\(^+\) substrate to a clean cuvet. Record the absorbance of the substrate at 735 nm. If the absorbance of ABTS\(^-\) is not above 0.65 then your ABTS\(^-\) has gone bad. You will need to borrow some for this measurement and you will need to remake your stock solution for next week.

Measurement of samples

Next you will measure the absorbance of the Trolox, epicatechin and quercitin dilutions that you made in Lab 1 reacting with ABTS\(^+\). Add 2.9 mL of ABTS\(^+\) and 100 \(\mu\)L of one sample to a clean cuvet, and mix thoroughly. (You can mix by putting a small piece of parafilm over the top of the cuvet, putting your finger tightly over that, and inverting a few times.) Set aside for approximately 6 minutes and take a reading at 735 nm. You will measure two trials of each of your twelve sample dilutions.

You also need to run a control sample. In the TEAC procedure, this means running the TEAC assay with 2.9 mL of ABTS\(^+\) substrate and 100 \(\mu\)L of the solvent of the sample being tested. The purpose of this measurement is to mimic the conditions of the sample measurements exactly except for the presence of the antioxidant. Be sure to use the correct solvent. If you are running more than one sample with the same solvent, you do not need to do multiple controls.

Run at least two trials of each sample and of the control.

Dispose of all solutions in the waste jar provided. The cuvets can be thrown in the trash.

Post-Lab Calculations and Analysis of Results

Calculations and Graphing:

To accomplish the first objective, calculate the change (\(\Delta\)) in [ABTS\(^-\)] as represented by the change in absorbance on \(A_{735}\) described in Eq. 10. Use the data obtained for the 6-minute time point. Repeat for each antioxidant at each concentration. For each sample concentration, average the values of the two trials that you took. Calculate % Inhibition as illustrated in Eq. 11.
ΔA_{ABTS+} \text{ 6 min} = A_{735}Control - A_{735}Test \text{ at } T=6 \text{ min} \quad \text{Eq. 10}

A_{735}Control = \text{Absorbance at 735 nm of the reaction between ABTS}^{\cdot+} \text{ and } [\text{AH}]=0.0
A_{735}Test = \text{Absorbance at 735 nm of the reaction between ABTS}^{\cdot+} \text{ and } [\text{AH}]=x \text{ M}

\% \text{ inhibition} = (\Delta A_{ABTS+} \text{ 6 min} / A_{735}Control ) \times 100 \quad \text{Eq. 11}

Table 3 is an illustration of a spreadsheet calculation in Microsoft Excel© for these results at Time = 6 minutes.

<table>
<thead>
<tr>
<th>[AH] (\mu M)</th>
<th>A(735nm)</th>
<th>\Delta A_{ABTS+} (\mu M)</th>
<th>Avg</th>
<th>%I</th>
</tr>
</thead>
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<tr>
<td>0</td>
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<tr>
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</tbody>
</table>

For each antioxidant, make a graph of % Inhibition versus antioxidant concentration using linear scales for each antioxidant. Using the “add trend line” feature under the chart menu in Microsoft Excel©, fit a straight line to the data. Under the options tab, check “set intercept = 0,” “display equation on chart,” and “display R-squared value on chart.” The R^2 value obtained should be between 0.95 and 1.00 to indicate a good fit of the line to the data†. Figure 8 illustrates a graph of quercetin data.

† R^2 is a fraction between 0.0 and 1.0, has no units, and indicates how well you can predict the value of Y (%inhibition, in this case) based on a known value of X (concentration). An R^2 value of 0.0 means that knowing X does not help you predict Y and there is no linear relationship between X and Y. When R^2 equals 1.0, all points lie exactly on a straight line with no scatter, so knowing X lets you predict Y perfectly.

To accomplish the second objective, you will use the slope of the best-fit line printed on each graph. You can see that the slope is \Delta \% \text{ Inhibition per } \Delta \mu M antioxidant. Divide the slope for the test antioxidants (quercetin, epicatechin) by that for Trolox to determine the TEAC value.

\text{TEAC value} = (\text{Slope sample}) / (\text{Slope Trolox}) \quad \text{Eq. 12}

Because the units are identical in the numerator and the denominator, this value is a unitless ratio when comparing pure compounds of known concentration.
The TEAC assay may also be used with food products. To find the TEAC value for food products, plot the TEAC results for TE and the food sample incubations for the 6 min reading as percent inhibition versus concentration in the reaction mixture. For the food samples, express this concentration in amount of food per liter of reaction (μL/L). Calculate the TEAC values for the food sample incubations by dividing the slope of the regression line for each by that for Trolox. Convert the units for TEAC for the food products to mM Trolox per L of food sample. In some cases, it may be more telling to also present the TEAC values in mM Trolox per g of food sample.

**Analysis of the Results:**

a. Should there be variation in your control absorption values for the substrate? Was there variation in your experiment? Please explain.

b. What TEAC values were obtained for quercetin and epicatechin? Which is a better primary antioxidant?

c. Why can $A_{ABTS^+}$ be used to represent the change in ABTS$^+$ concentration?

**Preparation for Next Week**

a. Find out which ascorbate quantification method you will be using (HPLC or titration) and plan your procedure accordingly. Find out if your instructor will provide juice samples or if you are to bring your own.

b. Write an introduction and experimental section in your notebook.

c. Carry out the pre-lab calculations before going to lab.

d. Be prepared to turn in your post-lab from this week.

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**Figure 8. TEAC results plot of % Inhibition versus quercetin concentration**

The TEAC assay may also be used with food products. To find the TEAC value for food products, plot the TEAC results for TE and the food sample incubations for the 6 min reading as percent inhibition versus concentration in the reaction mixture. For the food samples, express this concentration in amount of food per liter of reaction (μL/L). Calculate the TEAC values for the food sample incubations by dividing the slope of the regression line for each by that for Trolox. Convert the units for TEAC for the food products to mM Trolox per L of food sample. In some cases, it may be more telling to also present the TEAC values in mM Trolox per g of food sample.

**Analysis of the Results:**

a. Should there be variation in your control absorption values for the substrate? Was there variation in your experiment? Please explain.

b. What TEAC values were obtained for quercetin and epicatechin? Which is a better primary antioxidant?

c. Why can $A_{ABTS^+}$ be used to represent the change in ABTS$^+$ concentration?

**Preparation for Next Week**

a. Find out which ascorbate quantification method you will be using (HPLC or titration) and plan your procedure accordingly. Find out if your instructor will provide juice samples or if you are to bring your own.

b. Write an introduction and experimental section in your notebook.

c. Carry out the pre-lab calculations before going to lab.

d. Be prepared to turn in your post-lab from this week.

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We will only use the HPLC method, not the titration method.
Laboratory Period 3: Determination of Ascorbate (Vitamin C) Concentration and Total Phenolics in Common Foods

Introduction

Ascorbate (or vitamin C) is an essential nutrient that is found in a variety of plant foods. A lack of sufficient amounts of this nutrient in the diet is responsible for the disease known as “scurvy.” Sir James Lind established the link between scurvy and an essential component of plant foods (primarily citrus) in the 1700s. Lind’s experiments determined that limes contained a substance that would prevent the scurvy, so British sailors began consuming limes during long voyages and acquired the nickname “limeys.” This important substance was ascorbate.

Most animal species can synthesize ascorbate, but humans, guinea pigs, fish and fruit bats require dietary sources of the nutrient. In the human body ascorbate serves two main functions. It serves as a cofactor for reactions that lead to the maturation of collagen, and vitamin C is the primary water-soluble antioxidant in the body. Optimal ascorbate concentrations in the blood approach 75 μM, and the requirement of up to 90 mg/day is the largest of all the vitamins. Vitamin C is the most common of the single-nutrient supplements and it is often added to many prepared foods to prevent oxidation. Mega doses of the vitamin have been proposed to cure many diseases including the common cold and cancer. Although most of these types of claims are disputable, the importance of the vitamin for maintaining nutritional health is quite clear.

As an antioxidant, ascorbate can donate two electrons sequentially to terminate radicals; the ascorbate is converted to the oxidized species dehydroascorbate.

\[
\text{Ascorbate} \xrightarrow{-H^+, e^-} \text{Ascorbyl free radical (monodehydroascorbic acid)} \xrightarrow{-e^-} \text{Dehydroascorbic acid}
\]

Eq. 13

In living systems that utilize ascorbate as an antioxidant, enzymatic reactions catalyze the reduction of dehydroascorbate using reducing equivalents supplied by glucose oxidation. This allows the vitamin to be reused multiple times - a common characteristic of essential nutrients. Since ascorbate is the most abundant nutrient antioxidant in fruits and vegetables, you will determine its concentration in the samples that you test as a basis for comparison. Ascorbate present in foods and supplements will contribute to the overall TEAC activity. Two methods are offered to quantify vitamin C content in foods. The first method involves extraction of ascorbate from the food matrix followed by separation and quantification by high-pressure liquid chromatography (HPLC). The second method is a titration method using a dye compound that is reduced by vitamin C to a colorless liquid. Your instructor will determine which method you will use.
A Primer on High Pressure Liquid Chromatography (HPLC)

HPLC is a common analytical technique that is used for many applications including separation, identification, purification, and quantification of various chemical species. It is used in research in the fields of chemistry, biochemistry, biology, toxicology, cosmetics, and pharmaceuticals. The method involves separating molecules dissolved in a solvent that is in motion (mobile phase) at high pressure over a solid support material (stationary phase). This leads to the separation of individual chemical species based on their binding affinities to the solid matrix versus their solubility in the mobile phase. A diagram of the key components of an HPLC system is shown below.

Figure 9. Schematic of a typical HPLC setup

The mobile phase is pumped through a column that can sustain high pressures. The column is packed with support material with chemical characteristics that can be varied. The material commonly used has a strong hydrophobic nature and separates molecules based on dispersion forces. Other column materials have ionic characteristics and separate molecules via dipole forces. This lab is written to use a hydrophobic solid support material which is silica-based but has long aliphatic 18-carbon hydrocarbon chains attached at the functional groups (C\textsubscript{18} material). Another column material, pentafluorophenylpropyl, may also be used. The effluent, or outflow, from the column passes through a detector, which can be a spectrophotometer, electrochemical detector, or other device adapted to function with a stream of flowing solvent. The choice of detection methods depends on the characteristics of the chemical species to be analyzed. Ascorbate is active electrochemically and also exhibits an absorption spectrum with a $\lambda_{\text{max}}$ in the UV range at 267 nm. For this lab you will prepare the samples, standards, and mobile phases. These will be delivered to the HPLC facility where the samples will be run. The data will be provided to you for analysis.
**Determination of Ascorbate Concentration by Titration**

This method is based on the ability of ascorbic acid to reduce the oxidation-reduction dye indicator 2,6-dichloroindophenol to a colorless solution. The reaction is shown in Eq. 14.

![Equation 14](image)

At the endpoint of this analysis addition of unreduced dye to the reaction mixture yields a rose pink color in acid solution. Since this is a colorimetric reaction involving a non-specific dye indicator, or an indicator that will react with many titrants, there are certain food substances that give erroneous results. Foods that contain other compounds capable of reducing the dye can result in erroneously high values. Foods that contain transition metals (iron, copper) that compete with the dye for oxidation of ascorbic acid can result in erroneously low ascorbate values. Highly colored foods interfere with the determination of the endpoint.

**Measurement of the Total Polyphenolic Content of Foods**

As indicated in the introduction to this module, polyphenolic flavonoids are among the most abundant non-nutrient antioxidants in foods. To estimate the contribution that these compounds make to the TEAC value, you will quantify the total amount of polyphenolics in chosen samples. The measurement is based on a method which was originally developed over 100 years ago, was upgraded and improved during the 1960’s and 1970’s, and today is commonly used in the wine industry. This analysis involves the use of a spectrophotometer rather than an HPLC or titration.

**Overview of This Activity**

In this laboratory activity you will prepare tomato and orange juice samples for ascorbate concentration determination and polyphenolic measurements. For either ascorbate concentration determination method you will prepare a stock solution and dilutions of ascorbate for use in generating a standard curve. You will then treat and
dilute samples of tomato and orange juices so that they are suitable for injection into the HPLC or use in the titration method. For the ascorbate analysis by HPLC the standard and test samples will be sent to the instrumentation lab for injection and the results returned to you for later analysis. For the total polyphenolic measurement (and titration analysis) you will conduct the analysis on the same juice samples in the lab today.

Pre-Lab Requirements
In addition to writing introduction and experimental sections for this laboratory, you will also need to calculate the volumes needed to make all dilutions mentioned below. Pay special attention to the volumes and dilutions described in Total Polyphenolics Measurement – you will need to choose the volumes for many of the solutions. NOTE: the balances are not reliable below about 10 mg (0.010g). So, if your calculations show that you need to weigh out less than this amount, you should consider making a more concentrated solution and diluting to the required concentration.

It may be useful to map out the time you will be spending on this lab to determine which parts of the procedure should be attempted first to maximize time efficiency. Some parts require long incubations and should be attempted earlier in the period.

Materials Available
- Professionally prepared HPLC standards
- ascorbic acid
- 5% acetic acid with 0.35 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) – also referred to as acetic acid/TCEP solution
- Folin-Ciocalteau reagent
- 7% (w/v) sodium carbonate solution – previously prepared
- Orange juice
- Tomato juice
- 2,6-dichloroindophenol
- Sodium bicarbonate
- Spectrophotometer
- HPLC autosampler vials (for HPLC analysis only)
- Centrifuge
- Cuvets
- 1 μm syringe filters
- Syringes
- 25 mL, 50 mL, 100 mL and 250 mL volumetric flasks
- 25 mL amber vials
- 5 mL measuring pipets
- 1000-200 μL and 200-10 μL auto pipets (“micro pipets”)Filter paper
- 3-way safety bulb
- 50 mL buret (for titration method only)
- Magnetic stirrers and stir bars (for titration method only)
**Waste Disposal**

You should have a beaker designated for waste material on your lab bench. After you have performed the measurement of Total Polyphenolics, all solutions should be disposed of in this beaker. All the glassware used in preparing the solutions for polyphenolic measurement should be rinsed with a small amount of water from your wash bottle and poured into the waste beaker. The waste beaker can then be emptied into the large waste jar found in the main hood.

**Procedures**

**Preparation of Ascorbate Standards and Juice Samples (for HPLC or Titration Method)**

Record the serving size of the juices as you will need this information for analysis.

You will first make 25 mL of a stock solution of 1.0 mM ascorbate in dilute (5% v/v) acetic acid in 0.35 mM TCEP (this is known as the acetic acid buffer). **NOTE: the balances are not reliable below about 10 mg. So, if your calculations show that you need to weigh out less than this amount, you should consider making a more concentrated solution and diluting to the required 1.0 mM.** This ascorbate solution will serve as the stock concentration for either the HPLC method or the titration method. Store in the cold and protect from light. In our experience the stock is stable under these conditions for about 10 days. Note that the standards for either assay method should be prepared at the same time as the test samples (below). All ascorbate solutions prepared with 5% acetic acid/0.35 mM TCEP are stable up to 10 days if refrigerated and protected from light. Without TCEP, ascorbate solutions are extremely unstable.

**Ascorbate Measurement by HPLC:**

*Note: If you are only performing the titration method, skip this part.*

Each group in the laboratory will be responsible for preparing one HPLC standard. Your teaching assistant will assign your group a concentration. There are five 25 mL standards to be made by diluting the ascorbate stock with the acetic acid buffer to obtain working standards at 25, 50, 100, 150 and 200 μM. Fill an HPLC vial for each solution; you will need to lightly “flick” the top (wide diameter section) of the HPLC vial with your finger (fingernail side) to ensure that there is no air bubble trapped in the narrow tip at the bottom of the HPLC vial. Make sure to flick the vial gently so as not to break it. Make sure to label your sample with sample name, concentration, group name, section number and date (ask your instructor about sample codes for your class). Calibration standards must be prepared on the day of HPLC analysis. A set of professionally prepared ascorbate standards will also be loaded for comparison with the student made standards.
To make the HPLC test samples

(i) Make a 1/2 dilution tomato juice solution with acetic acid-TCEP and mix thoroughly. (This means 1 part tomato juice and 1 part acetic acid-TCEP solution, so that the tomato juice ends up as ½ of the total volume of the solution.) Note – in the next step you will be diluting this solution, so you need to make sufficient quantity to work with. Centrifuge this dilution for five minutes and decant the supernatant.

Dilute the supernatant to 1/5 with acetic acid-TCEP. Filter this diluted supernatant with a 1 μm syringe filter to remove fine particles. To do this, pull the solution into a syringe, then put the filter on the tip, and push the solutions through the filter into a clean container. Throw away the filter and return the syringe after use. Fill an HPLC vial with this solution. Now make additional dilutions of the filtered supernatant with acetic acid buffer to 1/2 and 1/5, making 25 mL of each solution. Fill an HPLC vial with each of these two solutions as before. You should now have three HPLC vials with diluted samples. Make sure they are labeled.

(ii) Make a 1/2 dilution of orange juice with acetic acid-TCEP and mix thoroughly (calculate the amount of orange juice needed to make all of your dilutions). Centrifuge the mixture for 5 minutes and decant, then dilute the supernatant to 1/5 with acetic acid-TCEP and filter using a vacuum filtration apparatus, shown below.

![Vacuum filtration apparatus](image)

**Figure 10:** Vacuum filtration apparatus

Pass the filtrate through a 1 μm syringe filter to remove fine particles. Fill an HPLC vial with this filtered solution, and label your HPLC vial. Make additional dilutions of the filtered solution to 1/2 and 1/5 with acetic acid-TCEP to a final volume of 25 mL of each solution. Fill HPLC vials with these diluted solutions and label the vials.
Your HPLC vials will be delivered to the instrument for HPLC analysis. The samples will be loaded into an autosampler and sequentially injected onto the HPLC column. The output from the column is monitored with a UV or electrochemical detector. The signal from this detector is processed with a computer program and provides several pieces of information. First, a chromatogram, which is a graph of the output from the detector over time, is printed. A chromatogram for each sample will be printed and provided to you. Second, the computer is programmed to integrate the area of each peak on the chromatogram. Using the HPLC protocol described in this module, ascorbate separates from other components in the sample and elutes as a single peak with a retention time of 4.1 min. Retention time is the elapsed time from injection until the maximum height for a single peak passing through the detector.

**Ascorbate Measurement by Titration with 2,6-dichloroindophenol:**

*Note:* If you are only performing the HPLC measurements, skip this part.

Dilute the ascorbate stock in acetic acid buffer to obtain 25 mL of working standards at 500 μM and 1.0mM. The 2 mM stock solution will also be used with this method. These three concentrations (500 μM, 1.0mM and 2.0mM) will make up the calibration curve. Prepare three trials of all points to be used in the calibration curve. Measure enough juice to make all of your dilutions and centrifuge for at least five minutes. Decant the supernatant and dilute each juice sample 1/2 with acetic acid buffer and mix thoroughly. If a precipitate forms, remove it by either centrifugation or filtration. Make dilutions of the resulting clear supernatant with acetic acid buffer 1/2, 1/5, and 1/10. Make a final volume of 25 mL of each solution.

[Note: the indophenols standard will be provided for you by the prep lab]. If you were to make the indophenol standard solution yourself, you would dissolve 50 mg 2,6-dichloroindophenol sodium salt in 50 mL water to which has been added 42 mg NaHCO₃. Mix thoroughly and, when the salt is dissolved, dilute to 1000 mL with deionized water. Filter and store in an amber bottle. The solution breaks down upon exposure to light. Avoid prolonged periods of exposure in clear glassware.

To carry out the determination, place 5 mL of standard ascorbate solution into a 125 mL Erlenmeyer flask containing 5 mL of acetic acid buffer. Fill a 50 mL buret with indophenol solution. Titrate with indophenol solution to an endpoint of distinct light rose color. Record the amount of solution used. Repeat for all solutions.

**Total Polyphenolics Measurement**

Dilute the previously prepared epicatechin stock to achieve three or more standards in the range of 25 to 200 μM in ethanol. Dilute the concentrated commercially prepared Folin-Ciocalteau reagent 1 part in 10 with deionized water. Always prepare this fresh daily.

**Procedure:** Measure all test samples (tomato and orange juices from Part 1) and standards in duplicate. To each tube add 225 μL water (which will serve as the control) or sample or epicatechin standard. Then add 1.5 mL of diluted Folin-Ciocalteau reagent, mix thoroughly and let set at room temperature for 7 minutes. Add 1.5 mL of 7% (w/v)
sodium carbonate solution, mix thoroughly and allow to sit for 30 min. Read and record absorbance in a spectrophotometer with wavelength set at 750 nm.

Dispose of all solutions in the waste jar provided. Cuvets can go in the trash.

Post-Lab Calculations and Analysis of the Results

Ascorbate Measurement by HPLC

The instrument lab will provide two pieces of information from your ascorbate analysis by HPLC. The first will be a profile (similar to Fig. 11) showing the elution of ascorbate from the HPLC column and the resolution of the peak from surrounding peaks. You can use this to verify that the peak quantified as ascorbate was correct and that the quantification of the peak was not compromised by other peaks eluting in close proximity.

Figure 11. Typical HPLC profiles for ascorbate separation and quantification.
(Top figure)
Ascorbate standards at four different concentrations.
(Bottom figure)
Orange juice at 1/10 and 1/100 dilution.
The upper figure in Fig. 11 is an overlay profile showing an example of ascorbate standards injected sequentially from low (~25µM) to high concentration (~200µM). The x-axis is time and the y-axis is absorbance at 268nm. Notice that there are two peaks which elute sequentially from the column. The first set of peaks is the flow-through containing material in the sample that is not retained by the column. For these injections the acid (used to dilute the standards) is providing this response. The second set of peaks eluting at 4.17 min corresponds to the ascorbate standard. Notice the symmetrical shape, perfect overlap, and increasing size of the peak corresponding to the greater concentrations injected. These observations provide a qualitative affirmation of the success of the ascorbate standards analysis. This will be verified by the quantitative analysis that you will complete in the next section. (Note: you will get separate chromatograms for each sample, they will not be overlayed like this figure.)

The lower chromatogram in Fig. 11 is an example of an overlay of the orange juice analysis. Notice two major peaks are observed along with several smaller peaks not present in the chromatograms of the standards. Notice the first peak, the flow-through, exhibits a relatively constant size which suggests that the solvent containing acid contributes to this peak. Other components of the sample with absorbance at 268 nm may also be contributing to this peak. The peak eluting at 4.17 min is a symmetrical peak that exhibits a larger peak area for 1/10 diluted sample and a much smaller peak area for the 1/100 diluted sample. The peak corresponding to 1/1000 is too small to be visible. Since this peak elutes at the same time, has the same shape, and does not overlap with other adjacent peaks, we assume it is ascorbate and will use the quantification of the area of this peak in comparison to the standard peak areas to determine the concentration of ascorbate in the juice samples.

The second piece of information that will be provided is a table with areas and retention times for each peak present in the chromatogram. The absorbance values measured as the eluting sample passes through the UV detector are converted to an electronic signal in units of mV. The computer program used to analyze the results will integrate the area under each peak and report it. The example that follows is focused on the peak identified as ascorbate. Table 4 and Table 5 below summarize this information for the standards and the two juice samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Description</th>
<th>Concentration µM</th>
<th>Dilution</th>
<th>Peak Area</th>
<th>Retention Time min</th>
</tr>
</thead>
<tbody>
<tr>
<td>STND1</td>
<td>Ascorbate Standard</td>
<td>26.125</td>
<td></td>
<td>5711390</td>
<td>4.167</td>
</tr>
<tr>
<td>STND2</td>
<td>Ascorbate Standard</td>
<td>52.250</td>
<td></td>
<td>13732536</td>
<td>4.167</td>
</tr>
<tr>
<td>STND3</td>
<td>Ascorbate Standard</td>
<td>104.500</td>
<td></td>
<td>27619134</td>
<td>4.167</td>
</tr>
<tr>
<td>STND4</td>
<td>Ascorbate Standard</td>
<td>209.000</td>
<td></td>
<td>55852793</td>
<td>4.167</td>
</tr>
</tbody>
</table>
Table 5: Summary of HPLC peak integration and retention times for juice samples.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Description</th>
<th>Dilution</th>
<th>Peak Area</th>
<th>Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP10A</td>
<td>Apple Juice</td>
<td>10</td>
<td>51632744</td>
<td>4.167</td>
</tr>
<tr>
<td>AP10B</td>
<td>Apple Juice</td>
<td>10</td>
<td>53506976</td>
<td>4.167</td>
</tr>
<tr>
<td>AP100A</td>
<td>Apple Juice</td>
<td>100</td>
<td>6339594</td>
<td>4.167</td>
</tr>
<tr>
<td>AP100B</td>
<td>Apple Juice</td>
<td>100</td>
<td>5954836</td>
<td>4.167</td>
</tr>
<tr>
<td>AP1000A</td>
<td>Apple Juice</td>
<td>1000</td>
<td>598612</td>
<td>4.167</td>
</tr>
<tr>
<td>AP1000B</td>
<td>Apple Juice</td>
<td>1000</td>
<td>540179</td>
<td>4.167</td>
</tr>
<tr>
<td>OR10A</td>
<td>Orange Juice</td>
<td>10</td>
<td>35111848</td>
<td>4.167</td>
</tr>
<tr>
<td>OR10B</td>
<td>Orange Juice</td>
<td>10</td>
<td>34649980</td>
<td>4.167</td>
</tr>
<tr>
<td>OR100A</td>
<td>Orange Juice</td>
<td>100</td>
<td>3890611</td>
<td>4.133</td>
</tr>
<tr>
<td>OR100B</td>
<td>Orange Juice</td>
<td>100</td>
<td>3750404</td>
<td>4.200</td>
</tr>
<tr>
<td>OR1000A</td>
<td>Orange Juice</td>
<td>1000</td>
<td>359440</td>
<td>4.133</td>
</tr>
<tr>
<td>OR1000B</td>
<td>Orange Juice</td>
<td>1000</td>
<td>272837</td>
<td>4.167</td>
</tr>
</tbody>
</table>

Use the graph function in Excel to evaluate the fit for the standard curve. The example that follows illustrates a very good fit of the data to a linear regression.

![Standard Curve for Ascorbate](image)

**Figure 12.** Ascorbate standard curve

You can use the trend function in Excel to calculate the concentration of each unknown sample. Choose the dilutions of juice which fall into the range of the standard curve. Once this is complete, calculate the concentration of ascorbate in the juice by multiplying the concentration of each sample injected by the dilution factor.

Table 5 does not include the 1/2 initial dilution of the juice samples and this should be accounted for in the calculation. Thus for sample OR10A, the calculation is:

$$[\text{Ascorbate in OJ}] = (132 \mu M) \times \frac{20 \text{ volume juice dilutions}}{1 \text{ volume juice}} = 2640 \mu M$$

The first value, 132 µM, comes from the graph in Fig. 12 and its linear regression. The concentration value corresponding to peak area for sample OR10A in Table 5 is 132 µM.
The number 20 results because the OR10A sample is a 1/10<sup>th</sup> dilution, and there was a 1/2 dilution in the procedure for the juice samples, such that 1/2 x 1/10 = 1/20.

Converting this concentration to a number that can be compared to the information given on the juice nutrition information label can be done as follows:

\[
\text{Ascorbate F.W.} = 176.13 \text{ g/mol}^{-1} \quad 0.240 \text{ L = 1 cup}
\]

\[
\frac{0.00264 \text{ mol ascorbate}}{1 \text{ L juice}} \times \frac{0.24L}{1 \text{ cup}} \times \frac{176.13 \text{ g ascorbate}}{1 \text{ mol ascorbate}} = 0.1116 \text{ g / cup}
\]

or 111.6 mg / cup ascorbate

The standard for food labels for vitamin C is 60 mg per serving. The label on a typical orange juice container indicates that the amount per cup is 120% of the RDA. What do the data indicate about the amount of vitamin C in your juice sample? Discuss explanations for any possible discrepancies you find.

Which HPLC dilutions of juices produced usable results? If you were to take another measurement, what concentration should it be? If you decide to use the HPLC method in your research (Part 4 of this module), it would be useful for you to know what dilutions produce usable results. Furthermore, some foods have more vitamin C than others, so in the context of your research project, you will want to think about the relative amount of vitamin C your food product has before deciding on dilution factors.

**Ascorbate Concentration Measurement by Titration**

Construct a standard curve for ascorbate plotting concentration versus volume of indophenol titration solution. Determine the concentration for samples using the best fit straight line for the standards as described above. Note that this method is much less sensitive than the HPLC method.

**Total Polyphenolics**

Construct a standard curve for epicatechin plotting concentration versus absorbance at 750 nm similar to the graph in Figure 11. Determine the concentration for samples using the best fit straight line for the standards in a similar way as for ascorbate described in the previous section. How do these two juice samples compare in terms of the quantity of total phenolics? Does this result differ from the ascorbate results?

**Post-Lab Questions and Analysis of Results**

a. What is the purpose of using 5% acetic acid as a solvent for the ascorbate solution? Why is it also needed as a solvent for the orange and apple juices?

b. Did the dilutions used in this period produce usable results? If not, what changes can you make in the future to your dilutions to produce results that will be more valuable?

**Preparation for Next Week**

a. There is substantial reading for the next period, so give yourself ample time.

b. Prepare your own unique experimental design based on the techniques you have learned. Be prepared to discuss your design and procedures.
Laboratory 4 - 6: Original Research Project

Introduction
In this laboratory period you will go through the process of hypothesis testing in antioxidant research using an example of citrus fruits which are rich in vitamin C. Science is the process by which we try to understand nature by continually testing our theories and refining explanations based on observation. The scientific method is the means by which we try to make this process reliable and consistent. Generally there are several key components: observation and description of phenomena, formulation of a hypothesis to explain the phenomena, performance of experiments to test the hypothesis, rejection or modification of a hypothesis inconsistent with experimental results, and application of a validated hypothesis to explain new observations. Observations can include descriptions but also quantifiable data, which are useful because comparisons can be made and trends observed. A hypothesis which explains observations must be testable and constructed to address one factor at a time. Testable means that the hypothesis statement is written in a way that one can determine it to be incorrect by experimentation. Experiments are procedures carried out generally involving an independent variable that is purposefully manipulated and a dependent variable that is observed or measured. Good experimental design attempts to minimize the effect of bias on the results. Several factors must be considered to be successful here. These include a representative sample, accuracy, precision, and reproducibility. A representative sample is one that truly reflects and encompasses the independent and dependent variables. Accuracy is how close a measured value is to the “true” or “real” value. Precision is the spread in the data of repeated measurements or trials. Reproducibility indicates that the results of an experiment are the same when repeated again at a later time or by another individual or research group. This lab will apply the scientific method to a question about which chemical species is responsible for the total antioxidant activity that can be measured in one particular vitamin C-rich food.

Pre-Lab Requirements
You will need to prepare your own unique experimental design and procedure to share with your group during lab. In addition, look up any pertinent information about the products you are interested in studying to see if some tests would be more telling than others and design your experiment accordingly.

Plan ahead for the mixing of your ABTS$^-$ as it will need to be made at least 12 hours but not more than 10 days before your next lab period.

Finally, think about the products you will be using with respect to the appropriate dilution factors. What did you learn about dilutions from previous experiments that could be useful in determining where to start? Are there dilutions that are too dilute or too concentrated when trying to use the line of concentration versus signal from your standards? It may take more than one try to get the dilutions right.

Overview of this Research Activity
We have described many observations concerning oxidative stress, its role in health and protection by antioxidants in this module. Also, we have introduced various
types of antioxidants in foods, and you have learned how to measure the total amount of antioxidant activity as well as the concentration of ascorbate, a key nutrient antioxidant, commonly found in foods. Based on these observations we can formulate a hypothesis concerning total antioxidant activity in ascorbate-rich foods. A testable hypothesis could be stated as follows “fruit samples stored at room temperature for a week will exhibit a 50% greater loss in antioxidant activity compared to similar samples stored at 4°C.” The techniques that you have learned so far, TEAC, HPLC or ascorbate titration, and polyphenolics measurements, can be used to assess this hypothesis. The TEAC assay assesses total antioxidant activity. HPLC, the ascorbate titration, and the polyphenolics measurements are more specific in their assessments. Each of these methods can have a place in examining this hypothesis: TEAC can evaluate changes in total antioxidant capacity, while HPLC, the ascorbate titration, and the polyphenolics measurement can assess changes in specific flavonoid classes. To isolate a specific variable, it is important that as many other factors as possible be kept constant between the samples. For this reason, this experiment will require that the samples are drawn from the same batches of solution, kept in the same kinds of vials, either with or without parafilm (but the same for both conditions), and with the same amount of contact with sunlight and motion. An example experimental design is as follows:

- Make solutions to be tested
- Measure antioxidant activity of the samples using TEAC and HPLC
- Fill two sets of identical amber vials, one for room temperature, one for 4°C
- Put parafilm on the vials
- Store the room temperature vials in a drawer to minimize contact with sunlight and risk of being knocked over; store 4°C vials in the refrigerator, taking precautions if there is direct sunlight
- Note the time
- 7 days later, return and remove the samples at approximately the same time from their conditions, opening them in the same way
- Measure antioxidant activity of the samples using TEAC and HPLC
- Compare antioxidant activities and changes in antioxidant activity

What is Currently Known and Unknown About Antioxidants in Foods?
A search of the peer-reviewed literature reveals many papers published that measure some indicator of total antioxidant activity in food and biological samples. Because our focus is on health benefits, I will use the example of PubMed, the main government-financed database used by researchers in the health field to survey the literature. If one does a search on PubMed using the search terms that describe total antioxidant activity (TEAC, ORAC, & TRAP), 275 references are obtained. (See Appendix B for tips and information about reading scientific research articles – it’s not as hard as it looks!) When these terms are combined with the term “food,” 106 references are found. Many of these papers report on total antioxidant activity of commonly consumed fruits and vegetables, teas, spices, various grains, food additives, wine, and chocolate. Also, some papers report analysis of uncommon plant parts which may have a basis in traditional medicine. A few papers (not many) have been published testing the effects of processing, storage or digestion of fruits and vegetables on antioxidant activity. A few studies have explored the effects that mixing high antioxidant and high protein
foods has on total antioxidant activity. Very few published reports have tested whether antioxidant-rich foods exhibit synergy, whether more highly palatable foods containing fruits or vegetables exhibit antioxidant activity, whether treatments that simulate the digestive process diminish antioxidant activity, or whether common cooking methods (grilling, boiling, sautéing in butter, baking, deep frying, etc.) significantly decrease antioxidant activity.

Any of these questions is a valid research question to test for this module because there is not a lot of information available yet on them, so you would be doing work that could contribute to the known research in this topic. HOWEVER, Dr. Burgess is currently working on the antioxidants in green teas, and in green tea drinks that are mixed with a juice that contains ascorbate (such as lemon juice) that can be found in many supermarkets and convenience stores. He is particularly interested in what effects digestion (i.e. processing through the human digestive tract) has on the antioxidant capacity of these teas and whether the juice makes a difference in this process. You can focus your efforts on this topic for now. (See Appendix A for an in-lab procedure that simulates digestion.)

**Design and Conduct a Research Project on Antioxidants in Food**

Your goal is to design an experimental procedure that actually will test a hypothesis. Review the introduction to this laboratory, to remind yourself about the use of controls and about reproducibility. Also, think about what you plan to test and what each of the techniques you learned in weeks 1-3 actually measures? Do you need to use all of them for the hypothesis you want to test? If not, which one or which ones will you use, and why?

Once a research question has been agreed upon, design an experimental protocol and discuss this as a group. Think about the steps you will be taking, and what you will do with your samples. Consider and plan for the procedures, timing, etc., that will be necessary for you to conduct a valid research study. Consult with your peer leader and/or TA about the research question that your group would like to address. Review the recent peer-reviewed literature to help you refine your research question/hypothesis. Refine your hypothesis or procedures if necessary – it is OK to do this when you do research.

Write up the experimental procedures and a list of supplies. Keep in mind that your supplies will be limited to those that you have already used in laboratory periods 1-3. Some additional items can be provided, as long as you include them in your list of supplies. These additional items include: blender, hot plate, cutting board. The food items will be limited to those provided by your instructor (ask your instructor for a list.) It may be possible for you to carry out research on food items that you provide. You must check with your instructor to see if this will be allowed.

**For Laboratory Period 4:** You should come to lab with an experimental procedure already in mind. In lab, you will meet and discuss this with your group, and with your TA, to finalize a procedure that you will work on for the next 3 lab periods.
Your Contribution to the Research of Dr. Burgess

This portion of the module will culminate with data that you can give to the researcher, thus contributing your work to the body of research. Keep in mind that Dr. Burgess would like to be able to use your data for publication! Therefore, you should conduct and report your experimental procedures and results carefully. For this module, you will provide data that includes the following things:

- Your hypothesis for your research.
- Detailed protocol of your experimental design for testing your hypothesis, including the specific food products researched and the conditions to which they were subjected.
- List of tests performed and procedures carried out.
- Results of the tests, such as numerical values and copies of your spectra.

You will be provided with detailed instructions on how to enter your data into a web-based form that will be sent to Dr. Burgess in electronic form. Be sure to label your data (and all your samples) sufficiently (your group name or number will not mean much to the researcher, so include information about what you studied and when the study was performed and what the data or sample is).

Most of all: have fun!
Appendix A: Procedure to Simulate Digestion

Example of a Test Question: will green tea with lemon preserve antioxidant activity of tea through simulated digestive process?

Ingredients: Lemon Juice. Freshly brewed green tea – two 250 mL cups

Sample Preparation:
- green tea as prepared (brewed)
- green tea with 2 Tbsp (or around 5-6 mL) of lemon juice per cup
- water with 2 Tbsp (or around 5-6 mL) of lemon juice (as a control)

Reserve 25mL in an amber bottle for TEAC and polyphenolics. Samples must be tested for ascorbate immediately. Each preparation must be filtered through 1μm filter, diluted and put into HPLC vials.

Set aside another 10mL of each treatment for Burgess/Ferruzzi test follow-up. Label these as pre-digested or undigested samples and store in freezer. Labeled boxes will be available for students to put their samples in.

In vitro digestion protocol: (Note: If tea is freshly brewed it must be cooled to room temperature to measure the pH. You can cool by putting in an ice bath and monitoring the temperature as it drops.)

1. 30 mL of each test mixture to be tested should be transferred to an 125mL Erlenmeyer flask. Wrap the flask with a single layer of aluminum foil to protect the solution from light. (Consider whether you will have time for duplicates? Will this help with the reproducibility of your experiment?)
2. check the pH of your mixture and if it is above 2.5 adjust the pH to 2.5 by the dropwise addition of 1.0 M HCL,
3. add 2.0 mL of porcine pepsin mixture (40 mg/mL in 0.1 M HCl, supplied),
4. flush the flask with nitrogen and stopper,
5. transfer mixture to shaking water bath at 37°C and incubate for 15 min at 95 rpm
6. next raise the pH of mixture to 5.0 by the addition of 2.0-2.5 mL of 0.9 M sodium bicarbonate,
7. add 9 mL pancreatin enzyme mix (2.0 mg/mL in 0.1 M sodium bicarbonate, supplied) and mix,
8. increase pH of mixture to 6.5 by the addition of 1M sodium hydroxide, dropwise
9. flush with nitrogen and stopper tightly,
10. incubate at 37°C with shaking for 45 min,
11. filter the mixture using a Buchner funnel and P5 filter paper.
12. Transfer two 2.0 mL aliquots of the filtrate to new tubes labeled (post digestion) Burgess lab for potential follow-up catechin quantification. Transfer the remaining filtrate to a clean tube for analysis the following week. Store all of these samples in the freezer.

After Digestion: TEAC and polyphenolic assay should be performed on each sample. Compare Results of TEAC after simulated digestion versus before to determine potential impact of lemon on green tea antioxidant. Dilution factor will need to be considered in before/after comparison.
Appendix B: Reading and Interpreting a Research Paper

A Paper about Processing Effects on Antioxidants in Food Sources

Consider the paper cited below (published in 2005) testing the effects of fermentation and heat treatment on the antioxidant activity of cowpea flours. The purpose of reviewing this paper is not to get bogged down in the details but to review the key characteristics of a study design. Hopefully this will help you to develop your research question and design your own study using the tools and skills you have learned so far in this module.


Cowpea (Vigna sinensis L. var. Carilla) flours were obtained by fermentation with inoculum Lactobacillus plantarum (PF) or with the natural microorganisms present in the flour (NF) and subsequently heat treated in an autoclave. The flours were prepared to study the effect of fermentation on the antioxidant vitamin content and on the antioxidant capacity. Bacterial counts and pH values, vitamins C and E, carotenoids, glutathione (GSH), superoxide dismutase-like activity (SOD-like activity), peroxyl radical-trapping capacity (PRTC), lipid peroxidation in unilamellar liposomes, and Trolox equivalent antioxidant capacity (TEAC) were evaluated in raw and processed cowpea flours. Gamma-Tocopherol and delta-tocopherol were found in raw cowpea, whereas vitamin C and carotenoids were not detected. An increase in the vitamin E activity was observed in PF, whereas vitamin C and carotenoids were not detected in fermented cowpea flours. Fermentation or heat treatment in an autoclave after fermentation produced processed cowpea flours with lower PRTC, glutathione content, and SOD-like activity than those of the raw seeds. However, those processes increased the capacity to inhibit the lipid peroxidation in unilamellar liposomes and TEAC. According to the results obtained in this study, the fermentation of cowpeas (naturally or with L. plantarum) and fermentation and subsequent heat treatment in an autoclave are good processes to obtain functional cowpea flours having higher antioxidant capacity than the raw legume.

What is the main question addressed in this paper?
Answer: What is the effect of fermentation and subsequent heat treatment on the antioxidant activity present in a bean flower (cowpea)

Why is this important?
Answer:
- Antioxidant capacity is an aspect of food that may provide health benefits
- Cowpea a staple of many cultures especially those in the developing world
- Fermentation is believed to improve nutritional quality of processed legume food products, and several examples (soy sauce, tempeh, miso, natto, etc.) are now being consumed more commonly in the West
- Heat treatment after fermentation is used to reduce antinutritional factors derived from this legume
• Cowpea is a type of legume which possesses many nutritional and health promoting properties but its antioxidant activity has not been characterized

What was the hypothesis that the authors tested?

*Answer:* The authors did not clearly state a hypothesis, but based on the extensive introductory material one could write a hypothesis statement for this research. We’ll call this an implied hypothesis: Fermentation will improve the antioxidant capacity of cow pea flour.

How was the research question addressed?

*Answer:*

- Measurements were taken of the sample of interest before the treatment
- Each step in the process of preparing the final product was analyzed, i.e., raw seeds, flour, fermentation (two alternatives were tested), and heat treatment by autoclaving
- Enough replications (trials) were conducted to do statistical analysis
- Multiple tests were conducted to improve confidence in the results. These included
  - Measurement of the concentration of four-six different antioxidants in each sample
  - Using three different methods to estimate total antioxidant capacity
- Careful attention was paid to the completeness of the methods used. An example of this is table 5 in which the authors carefully extracted the sample with multiple solvents starting by using a very hydrophilic solvent and ending with a very hydrophobic solvent. Each extract was then tested for antioxidant capacity.

What was the interpretation of the results?

*Answer:* Fermentation of cow pea flours increased antioxidant capacity as indicated by two of the methods used, but decreased this capacity as measured by a third. The effect of heat treatment by autoclaving the fermented products resulted in variable effects (increase or decrease) depending on the method of measurement. Both fermentation and heat treatment decreased the concentration of the known antioxidant nutrients (tocopherols and glutathione) in the cow pea flour. The implication here is that other non-quantified components in the cow peas is responsible for the antioxidant activity and that some of these compounds actually increase in concentration due to fermentation and possibly heat treatment. The authors of the paper discuss this idea and refer to other published work that implicates certain flavonoid species. The authors conclude: “According to the results obtained in this study, the fermentation of cowpeas (naturally or with *L. plantarum*) and fermentation and subsequent heat treatment in an autoclave are good processes to obtain functional cowpea flours having higher antioxidant capacity than the raw legume.”
Appendix C. Preparation Techniques for Different Foods

Methods for Extraction of Food Using Homogenization, Precipitation and Extraction to Obtain Experimental Samples for Study

Obtaining a representative sample is key to conducting a valid experiment to test a hypothesis. Many food samples are quite complex mixtures of material possessing both characteristics of solutions and colloids. It can be quite difficult to obtain a representative sample from a whole food. Below are listed several sample preparation protocols that can help guide you in obtaining adequate representative samples for your research project. The emphasis is on obtaining samples with good recovery of the more water-soluble antioxidant compounds, including most of the flavonoids. Although the TEAC assay has been tested on fat-soluble antioxidants, its use for testing these compounds is much less common. Some sample protocols follow.

General Supplies:
- Knife
- Cutting board
- Cheesecloth
- Syringe and syringe filters
- 5% acetic acid with 0.35 mM TCEP
- Ethanol, 100%
- Filter paper
- Hot plates
- #5 stoppers
- Aluminum foil
- Shaker/oven
- pH meter
- Nitrogen gas source

Spices and Herbs:
Weigh a little more than 10g of sample and grind (even if using pre-ground) in mortar with pestle. Carefully weigh 10g and dissolve in 100mL of appropriate solvent. If you are unsure whether you’re isolating a water soluble or insoluble antioxidant or both, divide the sample and dissolve 5g in 50mL of buffer and the other 5g in 50mL of ethanol. Stir sample mixture with magnetic stirrer for 30-60 minutes. After stirring, centrifuge the sample for fifteen minutes. Decant and perform syringe filtration on the supernatant. Make dilutions for testing; multiple dilutions over a large range will maximize the possibility that one of the dilutions will fall in the range of the standard curve.

Dried, Frozen, and Canned Fruits
Drain the fruit with cheesecloth, if needed. Weigh a little more than 10g of sample and cut it into small pieces, as necessary. Carefully weigh 10g and dissolve in 100mL of appropriate solvent. If you are unsure whether you’re isolating a water soluble
or insoluble antioxidant or both, divide the sample and dissolve 5g in 50mL of buffer and the other 5g in 50mL of ethanol. Blend in blender for 30-60 seconds. Stir sample mixture with magnetic stirrer for 30-60 minutes. After stirring, centrifuge the sample for fifteen minutes. Decant and vacuum filter supernatant. Perform syringe filtration on the filtrant. Make dilutions for testing; multiple dilutions over a large range will maximize the possibility that one of the dilutions will fall in the range of the standard curve.

### Dry Solid Sample Preparation

<table>
<thead>
<tr>
<th>Step 1: Dietary Supplements, Dried Herbs, and Spices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulverize ~10g of sample using a mortar and pestle</td>
</tr>
</tbody>
</table>

- **If isolating water soluble antioxidants:**
  - Dissolve 10g in 100 mL of acetic acid buffer *
  - Stir with magnetic stir rod 30-60 min or until dissolve (whichever comes first)

- **If isolating water insoluble antioxidants:**
  - Dissolve 10g in 100 mL of 100% ethanol *
  - Stir with magnetic stir rod 30-60 min or until dissolved (whichever comes first)

<table>
<thead>
<tr>
<th>Step 2: Centrifuge for 15 minutes</th>
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</thead>
<tbody>
<tr>
<td>Step 3: Decant supernatant into beaker</td>
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<tr>
<td>Step 4: For HPLC only: Perform syringe filtration on supernatant</td>
</tr>
<tr>
<td>Step 5: Dilute in acetic acid buffer or ethanol</td>
</tr>
<tr>
<td>Step 6: Perform appropriate tests</td>
</tr>
<tr>
<td>Step 7: Remember to run standards for comparison</td>
</tr>
</tbody>
</table>
High Moisture Food Sample Preparation

Fresh, Frozen, or Canned Fruits and Vegetables

Drain food in cheesecloth if necessary

Cut into small pieces as necessary

Weigh 10g of sample

If isolating water soluble antioxidants:
Blend sample with 1:10 w/w acetic acid buffer
Blend for 30-60 seconds

If isolating water insoluble antioxidants:
Blend sample with 1:10 w/w 100% ethanol
Blend for 30-60 seconds

Stir sample with magnetic stir rod 30-60min

Centrifuge sample for 15 minutes

Decant and filter supernatant using vacuum filtration

For HPLC only:
Perform syringe filtration on filtrant

Dilute filtrate with either acetic acid buffer or ethanol
Perform appropriate tests
Remember to run standards for comparison
Liquid Food Sample Preparation

Juice and Tea

If isolating water soluble antioxidants:
Dilute 1:1 with acetic acid buffer
Mix well

If isolating water insoluble antioxidants:
Dilute 1:1 with 100% ethanol
Mix well

If juice has large particulate matter:
Centrifuge 5 minutes, decant
Centrifuge supernatant 15 min if not clear

If juice has small particulate matter:
Centrifuge 15 minutes

For HPLC only:
Decant, perform vacuum filtration
Perform syringe filtration on filtrate

Dilute supernatant in acetic acid buffer or ethanol
Perform appropriate tests
Remember to run standards for comparison
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Bibliography


