THE FRAP ASSAY: ALLOWING STUDENTS TO ASSESS THE ANTI-OXIDIZING ABILITY OF GREEN TEA AND MORE

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ABSTRACT

Dietary sources of polyphenols receive significant public attention due to their many health benefits and speculated preventative medical applications. It has been previously established that total reducing capacity can be linearly correlated to its antioxidant power and thus, this benefactor on polyphenols stems from its reducing ability. While undergraduate students are possibly aware of the potential benefits of antioxidants compounds found naturally in materials such as green teas and berries, they may not have yet considered its chemical mechanism. Although the chemical mechanism by which natural materials act as antioxidants varies, many use polyphenol structures to perform these redox reactions. Therefore, the antioxidizing power of various materials such as green tea leaves, coffee beans, and berries can be compared by quantifying the concentration of polyphenols in these materials. In this study, we have developed an experiment in which undergraduate organic chemistry students will use the “Ferric Reducing Ability of Plasma” assay (FRAP) to directly measure the reducing capacity of green tea leaves and to look at the natural antioxidant factors from dietary sources. This experiment thus helps students gain an appreciation for the relevance and diversity of electrochemical reactions in natural materials, as well as introduces them to Green Chemistry principles. Students will use the FRAP assay to assess the viability of safe, natural, reducing agents.

INTRODUCTION

Green tea, berries, coffee, and several spices are well established sources of dietary polyphenols; a class of reducing compounds linked to anti-aging properties, cardiovascular disease prevention, and anti-cancer potentials. The reducing ability of these dietary polyphenols stems from their free-radical scavenging abilities; through a hydrogen donation they are believed to terminate potentially dangerous superoxide or hyper-oxide chain reactions in the body initiated by reactive oxygen species. In these dietary sources the most significantly reducing polyphenolic compounds which have previously been identified include flavonoids, flavones, and catechins. Catechins specifically have been identified as the main polyphenolic constituent of perhaps the most well-established dietary antioxidant: green tea leaves.
Although there are many different polyphenolic compounds in various relative concentrations in not only green tea, but also in berries, citrus fruits, spices, the antioxidant power of these materials has been found to be directly proportional to the total polyphenol concentration. The various dietary polyphenols have a range of antioxidant efficacy which require more difficult, costly methods to specifically quantify in plant materials, palthrough generally total antioxidant ability of the individual polyphenols has been found to increase linearly with respect to the extent of hydroxylation of the backbone structure. However, the interest mainly lies on many cases only the total reducing ability of a given material, rather than the reductive strength and relative concentrations of all unique polyphenols present.

Despite the variety of specific polyphenolic compounds in green tea leaf, berries, and other plants, their antioxidant potential can be easily compared by measuring their total reductive capacity. This total reducing capacity, or antioxidant power, of a substance can be measured quickly, reliably, and inexpensively by students in this experiment using the Ferric Reducing Ability of Plasma (FRAP) assay. The easily visualized FRAP assay allows both quantitative analysis through spectrophotometry as well as, and subsequent comparison of the concentration of reduction products to determine the relative antioxidant power of the compounds studied. Therefore use of the FRAP method in this experiment provides a straightforward means of comparison of the reducing ability of the aforementioned dietary antioxidants which also introduces students to the potential uses of electrochemical reactions in developing a more natural and greener synthesis.

MATERIALS AND METHODS

The FRAP reagent is made from a 1:1:10 mixture of three solutions: 20mM FeCl₃, 10mM TPTZ, and 0.3M acetate buffer at pH 3.6. FRAP reagent should have a light blue-purple color and may be used for one day. To make the extracts of the natural samples (ie green tea leaves) three portions of 0.05g of the sample (green tea leaves, herbs, etc) are added to 3 separate vials placed in a parallel reactor at 60°C for 90 minutes. The extinction coefficient (ε) for Fe(II) is obtained from a standard curve using a 2 mmoL⁻¹ FeSO₄ solution. FeSO₄ solution is prepared at a specific concentration by dissolving 0.112g FeSO₄·7H₂O in 200mL degassed water. For the calibration curve 2500uL FRAP reagent, 275uL distilled water, and 60uL of the FeSO₄ solution was added to a 3 mL cuvette, or in a 500:55:12 ratio respectively. After extracts have been in the parallel reactor 90 minutes, 3mL FRAP reagent, 330uL water, and 70uL of the extract are added to a 5 mL glass vial or in ratios of 66:14:1 respectively and inverted to ensure mixing. The extracts are then left for 30 minutes before absorbance is measured. The generation of the indigo Fe²⁺-TPTZ complex in the FRAP reagent from the clear Fe³⁺-TPTZ complex can be easily observed by students at this stage. The spectrometer is calibrated using a blank cuvette of 3 mL FRAP reagent and 330 uL of distilled water. A diluted blank cuvette is prepared with 3.060 mL distilled water and 330 uL FRAP reagent or in other words, with a 100:11 mix of FRAP reagent and distilled water. It is used for extracts which required a 0.1 dilution factor to be read the spectrometer. After 30 minutes the 20 mL vials of FRAP reagent and extract were obtained. Absorbance of solutions containing extract and FRAP solution were measured at 595 nm.

The absorbance was measured using a ThermoScientific Genesys 20 UV-VIS spectrometer at 595nm was measured after each Fe²⁺ addition increments. The absorbance and Beer-Lambert Law are used to determine the extinction coefficient. The proportion of Fe³⁺ reduced to Fe²⁺ concentration is then calculated.

RESULTS

The FRAP assay results were used to determine and compare the total antioxidant capacity of commercially available green tea, decaffeinated orange pekoe tea, blueberries, cilantro, and coffee grounds. Using the FRAP assay it was determined that green tea leaves are by far the most powerful reducing agents of the five materials studied, reducing double the amount of Fe(III) to Fe(II) as black tea leaves, the second most powerful reductant (figure 1).

By observing the reduction of the pale blue Fe³⁺-TPTZ complex in the FRAP reagent to an indigo colored Fe²⁺-TPTZ complex which absorbs strongly at 595nm we measured the total non-specific reducing capacity of the sample was measured, and from this
value, the antioxidant potential was inferred. The Fe²⁺-TPTZ concentration was determined from absorbance values and calibration of the absorbance against a solution of known Fe²⁺ concentration. Then, by using the Beer-Lambert Law to relate the absorbance to the concentration, the concentration of reduced Fe²⁺ particles in solution was determined.

Using the absorbance values measured from the FRAP assay for the various samples are then used to calculate the concentration of reduced Fe²⁺ particles in solution using the Beer-Lambert Law.

We found that the FRAP assay can easily be used for a wide variety of freeze or oven dried plant, tree, and other natural samples. The FRAP assay enables this observation of the vivid reduction of Fe³⁺-TPTZ to Fe²⁺-TPTZ using everyday plants and food products, providing an appreciation for the relevance and diversity of electrochemical reactions, and making the FRAP assay ideal for use in an undergraduate laboratory setting. We found this standardized FRAP assay to be a quantitative, yet highly visual approach which could easily be used by beginner scientists to easily quantify and compare the antioxidant capabilities of any number of materials of interest.

**DISCUSSION**

**Applications for Undergraduate Chemical Education**

The FRAP assay provides a visual, yet quantitative approach to students to study antioxidants found in their day to day lives. Here we established that various dried leaves, berries, etc can be safety used by students to produce accurate FRAP assay results. As an undergraduate assignment, students can use the FRAP assay to study a variety of potential antioxidants of their choosing. Students may prepare extracts and measure absorbance of their material of choice in the same manner as described in the above procedure, and directly compare overall antioxidant potential without any other modifications to the procedure. Students should also be encouraged to suggest novel materials which may possess antioxidant abilities and, if appropriate test these materials using the FRAP assay as previously described. By comparing the strength of these natural antioxidants to traditional, more hazardous reductants (such as sodium borohydride) students will explore green chemistry alternatives in electrochemical processes.
Limitations

The FRAP reagent provides a useful tool for quick, inexpensive analysis of a given material's reducing capacity; here the FRAP assay is used to compare concentrations of reducing polyphenols in various extracts from natural antioxidants. However, the main limitation of this method is that it cannot distinguish between reducing agents, as the Fe²⁺ complex forming a blue solution after reduction from Fe³⁺ does not detect how the reduction occurred. This allows for simple comparison of the reducing potential of various substances, but in order to identify the reducing agents and determine their relative concentrations, different analytical methods must be employed.

CONCLUSION

This experiment encourages students to explore various natural sources of polyphenols and use of the FRAP assay allows quantitative comparison of the total reducing capacity of these materials. The FRAP method produces fast, easily repeatable results and is thus ideal for exposing students at the high school or undergraduate level to green chemistry principles. This approach bridges the basic principles of electrochemistry taught to students with common industry claims of health-promoting antioxidants, allowing students to apply their science education to everyday situations. FRAP is used in many fields, from the biologist's expertise to green engineering as well. The FRAP assay and comparison of natural antioxidants also introduce students to the principles of green chemistry by suggesting alternative reducing agents to more common, but also more hazardous, alternatives.

ABBREVIATIONS

FRAP Ferric Reducing Ability of Plasma
TPTZ 2,4,6-Tris(2-pyridyl)-s-triazine

KEY WORDS

Chemistry; education; antioxidant; FRAP

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