EDUCATION LETTER

Determination of antioxidant activities of berries and resveratrol

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(Received 1 June 2011; final version received 5 July 2011)

There has been growing interest in the health benefits of fruits with the emphasis on antioxidants. Berries contain considerable amounts of chemicals referred to as phenolic compounds which have been identified as an important source of antioxidants. The determination of antioxidant capabilities of various berries and resveratrol by reducing 1,1-Diphenyl-2-picrylhydrazyl (DPPH) essay was designed as an experiment for general, analytical, and introductory biochemistry laboratories. The reduced DPPH radical is colorimetrically analyzed by UV-Vis spectrophotometry to determine the IC50, which is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. This experiment acquaints students with free radicals and their scavengers, solution preparation, the extraction of a natural product, and UV-Vis spectroscopy. Unlike so many undergraduate laboratory experiments, the procedure does not utilize any toxic reagents. Students gain an understanding of the overlap among the different fields of chemistry and the concept of green chemistry.

Keywords: antioxidants; berries; resveratrol; 1,1-Diphenyl-2-picrylhydrazyl (DPPH); free radical scavenging; phenolic content; ISustain™ Green Chemistry Index

1. Introduction

Oxygen is essential for the survival for all living things. Approximately 5% of inhaled oxygen is reduced to oxygen derived free radicals in normal physiological and metabolic processes (1). Atoms or molecules that possess unpaired electrons are called free radicals. Some of the free radicals include the hydroxyl radical (OH), the superoxide radical (O2⁻), and hydroperoxyl radical (HO₂). Although free radicals are naturally produced under aerobic conditions, an excess of free radicals can damage all cellular macromolecules including proteins, carbohydrates, lipids, and nucleic acids (2). The free radicals start reactions such as the oxidation of DNA which can ultimately cause mutations in the genetic material and possibly cancer (3). When oxidizing proteins, it has been found that the free radical can inhibit enzymes or cause proteins to denature or degrade (4). The free radicals have also been implicated in the pathogenesis of diabetes, liver damage, atherosclerosis, inflammation, cardiovascular disorders, neurological disorders, and in the process of aging (5,6).

Antioxidants are substances that neutralize the harmful free radicals in our bodies. Antioxidants act as “free radical scavengers” and hence prevent or slow the damage done by these free radicals. Their function is as a reducing agent, which ultimately removes free radical intermediates and prevents further oxidation by being oxidized themselves. Fruits and vegetables are known as good sources of antioxidants, such as retinol (Vitamin A), ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), carotenoids, flavonoids, tannins, and other phenolic compounds (7). Many studies show that daily consumption of fruits and vegetables is associated with reduced risks for degenerative diseases like cancer and cardiovascular diseases. Some antioxidants can be synthesized within the body, but most are obtained through diet or supplements.

Several studies show that strawberry (Rosaceae Fragaria), blueberry (Vaccinium corymbosum L.), raspberry (Rubus idaeus L.), blackberry (Rubus fruticosus), resveratrol (Polygonum cuspidatum), and acai berry (Euterpe Oleracea) generally possess a high level of antioxidant activity which is linked to the levels of polyphenolic compounds such as flavanoids, catechins, and anthocyanins in the fruits (8–10) (Figure 1). These have shown to inhibit human low-density lipoprotein and lipidosome oxidation (11). Resveratrol is the key ingredient in red wine that helps prevent damage to blood vessels, reduces “bad” cholesterol, and prevents blood clots. As an antioxidant, it is believed to help protect them from obesity

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and diabetes, both of which are strong risk factors for heart disease.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging assay is a rapid, simple, and inexpensive colorimetric experiment which determines the percent inhibition of antioxidants. DPPH is widely used to test the ability of compounds to act as free radical scavengers and to evaluate antioxidant activity of foods. DPPH is a stable free radical which is due to the delocalized electron. It has a purple color in methanol solution, and a strong absorption maximum at 517 nm is observed due to the presence of an odd electron \((\cdot N\). As soon as the DPPH free radical is mixed with an antioxidant that can donate a proton, the reduced form will be generated. This can be observed because DPPH in methanol solution changes from purple to yellow color, as the odd electron of DPPH radical becomes paired with hydrogen from the antioxidant to form the reduced DPPH-H \((\cdot N)\) (Figure 2).

In this article, we described an experiment to determine the antioxidant activities of berries and resveratrol. The experiment follows the Twelve Principles of Green Chemistry. The starting materials are renewable, water-soluble, and non-toxic. DPPH shows low toxicity. The experiment procedure has minimal energy requirement. This experiment is suitable for the general chemistry, analytical chemistry, and introductory biochemistry laboratory courses and works well for either individual students or pairs of students.

Students were required to evaluate the process via ISustain™ Green Chemistry Index. The ISustain™ Green Chemistry Index is a tool which provides a methodology to generate a sustainability-based score for chemical products and processes \((14)\). The internet version of the ISustain™ is based on the Twelve Principles of Green Chemistry and takes into account such factors as waste generation, energy usage, health and environmental impact of raw materials and products, and safety of processing steps. It was used as a learning tool for students to increase familiarity with the Twelve Principles of Green chemistry and evaluate the overall sustainability of the synthesis.

Bringing everyday concepts such as antioxidant capacity of the fruits will attract students' interest in chemistry. With emphasis on preparation of solutions, extraction of active ingredient from natural sources, utilization of UV-Vis spectrophotometry and data analysis, the experiment offers opportunities for students to experience a number of essential techniques in the chemistry laboratory.

2. Experimental section

Berries were purchased at local farmer’s market or supermarket. Resveratrol and acai berry chews were obtained from a local health store. The fruits and chews utilized were raspberry, blueberry, strawberry, blackberry, resveratrol chews, and acai berry chews.

All measurements were performed in triplicate.
General laboratory safety procedures, including the wearing of safety goggles and gloves, must be followed at all times. The DPPH–ethanol solution should be prepared on the day of the experiment.

**Radical scavenging to determine antioxidant activity by DPPH free radical**

DPPH (0.010 g) was dissolved in 100 mL of 80% ethanol solution. To prepare the extract, 5 g of each berry as an antioxidant was mashed and it was stirred in minimum amount of 80% ethanol for about 15 minutes. Resveratrol and acai berry chews were stirred in 20 mL of 80% ethanol for about 20 minutes. The antioxidant solution was centrifuged at 5000 rpm for 3 minutes. Supernatant was kept as a sample liquid. Individual solutions of antioxidants were prepared at concentrations varying from 1 to 10 mg/mL in 80% ethanol. Two milliliter of DPPH solution was added into the 2 mL of each individual antioxidant solution in vials. The solutions were shaken well and incubated for 30 minutes at room temperature. Two milliliter of 80% ethanol with 2 mL DPPH mixture was used as control measurement. The absorbencies of control and sample solutions were measured at 517 nm by using CARY-100 UV-Vis spectrophotometer. Spectronic 20 instrument can be employed as well since it is more commonly found in teaching labs. The students recorded the absorbencies in the worksheet, which is provided here as a supplementary material.

**Waste disposal**

All solutions should be disposed in an organic waste container. Stubborn stains and sticky residues from berries and chews can be removed either with ethanol or soap followed by rinsing with water. All vials and caps should be air dried and recycled for use in other experiments.

**3. Results and discussion**

Fruits and vegetables containing high concentrations of phenolic compounds have attracted great interest in recent years due to their potential health-promoting effects. The phenolic compounds, particularly in berries, have been identified as an important source of antioxidants that delay or inhibit various diseases, including Alzheimer’s disease and cardiovascular disease. The students investigated the antioxidant properties of resveratrol and five berries, namely, raspberry, blueberry, strawberry, blackberry, and acai berry, by using DPPH free radical-scavenging activity. The DPPH radical, which is a stable organic free radical with an absorption maximum at 517 nm, is a practical reagent for the evaluation of the antioxidant capacity of berries and resveratrol. The antioxidants in the berries and resveratrol reduce the DPPH radical to a yellow compound, diphenylpicryl-hydrazine, and the reaction depends on the hydrogen-donating ability of the antioxidant. Reduction of DPPH by an antioxidant can simply be shown as

\[
\text{DPPH} + A \rightarrow \text{DPPH} - H + A
\]

where A represents an antioxidant.

A number of modifications were made to the literature procedures to minimize the hazards and adapt the experiment to undergraduate laboratories (15,16). Dimethyl sulfoxide (DMSO) or methanol is commonly used as a solvent in DPPH assay. However, DMSO easily penetrates skin and might result in accidental poisoning. On the other hand, methanol is volatile and requires adequate ventilation to avoid the excessive exposure which can cause headache and nausea. Therefore, the students replaced the DMSO and methanol solvent with 80% ethanol which is much less toxic. We have chosen to provide students with the stock solution of DPPH–ethanol, but they could also prepare the solution. The literature recommends incubation of solutions at 37°C for one hour. The students changed the conditions to 30 minutes at room temperature which is more environmentally benign. Owing to limitations of the number of UV-Vis spectrophotometer and time, each student or group was assigned a fruit or a chew and their results were compared at the end of the laboratory period but this is also a matter for the instructor to decide. The gathered data on different berries and resveratrol usually generate a stimulating discussion on the antioxidant capacities of fruits.

Figure 3 shows the photograph of the various concentration of DPPH–blueberry solution prepared by the students after 30 minutes of incubation. Colors change from dark purple to yellow. While purple solutions are a sign of low or no antioxidant activity, yellow solution indicates high antioxidant activity.

![Figure 3. Photograph of DPPH–blueberry solutions at various concentrations.](image-url)
The spectrophotometric method for assessing the total antioxidant capacity of the fruit is based on the absorbance decrease of the DPPH radical in the presence of antioxidants. The antioxidant capacity of the fruits was expressed as inhibitory concentration, IC\textsubscript{50}. The IC\textsubscript{50} is the concentration of an antioxidant at which 50\% inhibition of free radical activity is observed. The lower IC\textsubscript{50} value indicates the greater overall effectiveness of the antioxidant. The IC\textsubscript{50} of the fruits was measured by spectrophotometric method at the \( \lambda \text{max} \) of DPPH, 517 nm. The percent inhibition was calculated by the following equation:

\[
\text{Inhibition}(\%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \( A_{\text{control}} \) is the absorbance of the control reaction.

Table 1 indicates the IC\textsubscript{50} values of berries and resveratrol measured by CARY-100 UV/Vis spectrophotometer. The results indicate that while blueberry and raspberry have the highest antioxidant activity, blackberry is a very strong antioxidant as well. The obtained low IC\textsubscript{50} value shows the good free radical scavenging activity of those berries. Strawberry and resveratrol have reasonable degree of antioxidant activity due to high IC\textsubscript{50} value. However, utilizing actual acai berry fruit instead of the chew might alter the results.

While CARY-100 UV-Vis spectrophotometer was used here, more commonly found spectrophotometers such as Spectronic 20's can be employed for the experiment. If it is available, high throughput UV-Vis plate readers can be used as well.

4. Conclusion

The antioxidant activities of fruits, especially berries, are promoted in the news, and students are very interested in the subject. This experiment provides students with an opportunity to understand the connection between chemistry and nutrition. Students also gain knowledge of and practice with laboratory techniques such as solution preparation, extraction of antioxidants from fruits, and spectral measurements. We chose DPPH assay because DPPH testing determines accurately, conveniently, and rapidly the antioxidant activities of berries and resveratrol. It is reasonable to expect that high antioxidant foods have greater potential to reduce free radicals in the body than do low antioxidant foods. An exciting outcome of the laboratory experiment was the inspiring classroom discussions after each student or group had different antioxidant capacity for the berries.

### References

1. Yu, B.P. *Physiol. Rev.* **1994**, *74*, 139–162.
2. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44–84.
3. Nakabeppu, Y.; Sakumi, K.; Sakamoto, K.; Tsuchimoto, D.; Tsuzuki, T.; Nakatsu, Y. *Biol. Chem.* **2006**, *387*, 373–379.
4. Stadtman, E. *Science* **1992**, *257*, 1220–1224.
5. Marx, J.L. *Science* **1987**, *235*, 529–531.
6. Verma, N.; Khosa, R.L.; Pathak, A.K. *Pharmacology online* **2008**, *3*, 206–215.
7. Huang, D.; Ou, B.; Prior, R.L. *J. Agric. Food Chem*. **2005**, *53*, 1841–1856.
8. Vinson, J.A.; Su, X.; Zubik, L.; Bose, P. *J. Agric. Food Chem*. **2001**, *49*, 5315–5321.
9. Sellappan, S.; Akoh, C.C.; Krewer, G. *J. Agric. Food Chem*. **2002**, *50*, 2423–2438.
10. Beaver, B. *J. Chem. Ed.* **1999**, *76*, 1108–1112.
11. Häkkinen, S.; Heinonen, M.; Käärenpää, S.; Mykkänen, H.; Raushan, J.; Törönen, R. *Food Research Int.* **1999**, *32*, 345–353.
12. Alamed, J.; Chaiyasit, W.; McClements, D.J.; Decker, E.A. *J. Agric. Food Chem*. **2009**, *57*, 2969–2976.
13. Miliauskas, G.; Venskutonis, P.R.; van Beek, T.A. *Food Chem*. **2004**, *85*, 231–237.
14. (https://www.isustain.com)
15. Berger, J.M.; Roshniben, J.R.; Javeed, H.; Javeed, I.; Schulien, S.L. *J. Chem. Ed.* **2008**, *85* (3), 408–410.
16. Tominaga, H.; Kobiyashi, Y.; Goto, T.; Kasemura, K.; Nomura, M. *Yakugaku Zasshi* **2005**, *125*, 371–375.
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SUPPLEMENTARY MATERIAL

Experimental Procedure-Student Handout

Objective: The main objective of this work is the determination of the antioxidant capacities of resveratrol, raspberry, blueberry, strawberry, blackberry and acai berry, by using DPPH free radical-scavenging activity method.

All the calculations necessary for the preparation of solutions should be done before the laboratory period and shown to the instructor in the beginning of the class.

1. Measure 5.0 g of assigned berry and mashed the fruit by mortar and pestle. If a resveratrol or acai berry chew was assigned, break the chew into smaller pieces.
2. Add 10 mL 80% ethanol and stir on a magnetic stir plate for 15 minutes. Then, centrifuge the fruit mixtures for 3 minutes.
3. While waiting for the stirring process, dissolve 10 mg of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) in 100.0 mL of 80% ethanol in a 100 mL volumetric flask.
4. Prepare 18 vials and place 2 mL of DPPH solution into each vial.
5. Label three of these vials as “Control” and add 2 mL of 80% ethanol to each.
6. Take next three vials and add 2 mL of most dilute solution (1 mg/mL) of the first assigned antioxidant. Label each vial with the name of the antioxidant and the concentration.
7. Repeat step #6 for the other concentrations of the antioxidant.
8. If there are more than one fruit assigned, repeat steps #5 and 6 for the new antioxidant.
9. Cap the vial and lightly shake them. Allow the vials to stand for 30 minutes at room temperature.
10. Measure each absorbance of 3 vials of “Control” solutions in UV-vis spectrophotometer at 517 nm and record them. Use 80% ethanol as blank solution.
11. Measure and record each absorbance of other prepared antioxidant-DPPH solutions.
12. Calculate the averages of absorbencies for “Control” solutions (A_{control}).
13. Calculate the averages of absorbencies for each solution for specific concentration (A_{sample}).
14. Calculate the Inhibition (%) for a specific concentration of an antioxidant by using: Inhibition (%): \( \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \)
15. Graph inhibition (%) in the Y axis and concentration in the X axis in Microsoft Excel® program. Calculate IC_{50} of the assigned antioxidant by determining X value when Y = 50%.

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### Worksheet

**Name:** ____________________________  
**Date:** ____________________________

**Antioxidant:** ____________________________

|          | Absorbance 1 | Absorbance 2 | Absorbance 3 | Average Absorbance | % Inhibition |
|----------|--------------|--------------|--------------|--------------------|-------------|
| Control  |              |              |              |                    |             |
| 1.0 mg/mL|              |              |              |                    |             |
| 2.5 mg/mL|              |              |              |                    |             |
| 5.0 mg/mL|              |              |              |                    |             |
| 7.5 mg/mL|              |              |              |                    |             |
| 10.0 mg/mL|             |              |              |                    |             |

**Antioxidant:** ____________________________

|          | Absorbance 1 | Absorbance 2 | Absorbance 3 | Average Absorbance | % Inhibition |
|----------|--------------|--------------|--------------|--------------------|-------------|
| Control  |              |              |              |                    |             |
| 1.0 mg/mL|              |              |              |                    |             |
| 2.5 mg/mL|              |              |              |                    |             |
| 5.0 mg/mL|              |              |              |                    |             |
| 7.5 mg/mL|              |              |              |                    |             |
| 10.0 mg/mL|             |              |              |                    |             |
iSustain Green Chemistry Index-Student Handout

The iSustain™ Green Chemistry Index is a learning tool for the students to gain knowledge of the Twelve Principles of Green chemistry. It helps the students to understand the factors within their control that can affect the overall sustainability of their laboratory experiments. The index provides a methodology to generate a sustainability-based score for chemical products and processes. The internet version (https://www.isustain.com) of the iSustain™ takes into account such factors as waste generation, energy usage, health and environmental impact of raw materials and products, safety of processing steps.

The student generates a scenario in the iSUSTAIN™ Index for the experiment. The scenario contains information on the materials going into a process (the Bill of Materials In or BOM In), the materials out of a process (the Bill of Materials Out or BOM Out) and the conditions used for the various steps in a process.

1. Go to https://www.isustain.com and register the web-site. Click on the “Scenarios” link at the top of the page to go to the scenarios listing. Click on the button for “Add New Scenario” to create a scenario entitled “New Scenario”.

2. Hover the mouse pointer over the icon in the “Actions” column on the left, choose the “Edit Details” selection, enter a name and description for the scenario and click on the “Save Changes” icon. All list entries in the iSUSTAIN™ Index work this way.

3. Choose the “Data Input” link at the top of the page. Most of the information required for a scenario may be entered on this page.

4. You will see a blank Bill of Materials In (BOM In), a Bill of Materials Out (BOM Out) and a Process Steps table. All metrics in the iSUSTAIN™ application range from 0 to 100, with zero being low sustainability and 100 being the highest and most desirable. All scores are also rounded to the nearest 5 in recognition of the expected variability in the process.

5. To enter the materials into the BOM In table: Click on the “Add New” button. You will find a material selection dialog box with alphabetic and “Chemical Abstract Service Registry Number” (CAS) listings. Start typing in part of the name of the desired chemical into the entry box at the top and choose the material from the list. A new row will be created for that material. Then click on the “Save Changes” icon. Other BOM In materials are entered in the same manner.

6. To input materials into the BOM Out table: first click on the Edit icon for the Product line and enter the Product name, weight and “% Diluents” and click on the “Save Changes” icon. Now click on the “Add New” link below the BOM Out table and enter a name for this waste stream, a total weight for it and then click on the Update icon.

7. You will enter the steps in the Process Steps and click on the “Save Changes” icon.

8. Click on the “Scenarios” link at the top of the page. Under the “Actions” column on the left, choose the “Export to Excel”. You can print the report out and submit it along with your worksheet.