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Laboratory Experiment

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Paper Chromatography and UV−Vis Spectroscopy To Characterize Anthocyanins and Investigate Antioxidant Properties in the Organic Teaching Laboratory

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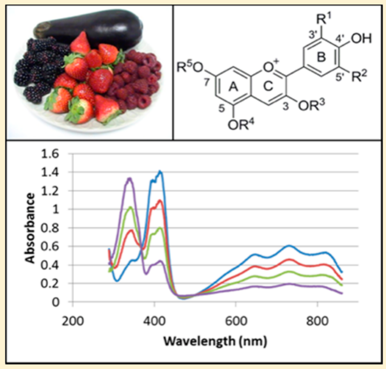
Supporting Information

ABSTRACT: A variety of fruits and vegetables, including raspberries, blueberries, Concord grapes, blackberries, strawberries, peaches, eggplant, red cabbage, and red onions, contain flavonoid compounds known as anthocyanins that are responsible for the blue-red color and the astringent taste associated with such foods. In addition, anthocyanins exhibit a wide range of chemical properties, such as radical scavenging, metal chelation, pH-dependent color changes, and intramolecular stabilization. Two experiments have been developed for anthocyanin-containing extracts isolated from freeze-dried berries to teach students the skills of solid−liquid extraction, paper chromatography, UV−vis spectroscopic characterization, and detection and evaluation of radical scavenging properties.



KEYWORDS: *Second-Year Undergraduate, Organic Chemistry, Laboratory Instruction, Collaborative/Cooperative Learning, Chromatography, Free Radicals, Natural Products, UV-Vis Spectroscopy, Hands-On Learning/Manipulatives, Food Science*

INTRODUCTION

Rapidly evolving research and technology necessitates continued re-evaluation and redesign of laboratory curriculum.[1] In a year-long organic chemistry nonmajors laboratory course, renovation of laboratory rooms prompted modernization of experiments to emphasize real-world connections and the relevance of organic chemistry. Potential benefits of consumption of fruits and berries were integrated into the design of two experiments. Discoveries about antioxidant properties of certain foods have led to investigations to understand how polyphenols, anthocyanins, and related compounds contribute to antioxidant properties and to potential health benefits. Two recent studies documented a decreased risk of myocardial infarction and arterial stiffness in women with a consistent intake of foods high in anthocyanins.[2,3] Anthocyanins have been used to develop laboratory experiments and outreach activities[4−11] and are included in an effort to use natural products to teach chemical analysis.[12] The experiments described herein teach students the techniques of solid−liquid extraction, reflux, paper chromatography, UV−vis spectroscopy, and radical scavenging assays within the context of natural products they frequently ingest.

Two experiments were developed for students to gain experience with anthocyanins. The first experiment involves extraction of anthocyanins from freeze-dried blueberries, blackberries, and raspberries; hydrolysis of the anthocyanin extracts to their anthocyanidins; and characterization and quantification using UV−vis spectroscopy and paper chromatography. These berries are often present in cereal, trail mix, or granola, allowing students to make connections to foods they eat regularly. In the second experiment, students perform an antioxidant assay to investigate the potential of berry extracts to scavenge the stable free radical produced by one-electron oxidation of 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS^{+}). Pairs of students investigate one of three berries, and students pool results to compare findings across all three berries to determine if a correlation exists between anthocyanin content and antioxidant potential. Because the berry extracts contain other soluble antioxidants, not all of the antioxidant potential of the extracts is due to their anthocyanin content. Students must consult the literature to reconcile experimental antioxidant data of the second experiment with anthocyanin concentrations determined in the first experiment. By reconciling the differences between expected results for a pure sample and the data obtained from their real world samples, students have the opportunity to develop inquiry and analysis skills that the Association of American Colleges & Universities (AAC&U) recognizes as vital to higher education.[13]

BACKGROUND

Anthocyanins are water-soluble, acid stable, pigments found in the vacuoles of many plants.[14,15] They are responsible for the red-blue color of many fruits and vegetables, ranging from strawberries to blackberries, and eggplant to red onion.[16] Anthocyanins are in the flavonoid family, which has long been studied for its potential health benefits.[15] In naturally occurring anthocyanins, hydroxy and/or methoxy substituents are present on all three rings and the hydroxy substituents are often modified by glycosidic linkages or acetyl groups (Figure 1). Over 600 anthocyanins have been identified in nature due to the number of variations possible with mono- or diglycosides of mono-, di-, or trisaccharides.[15,16]

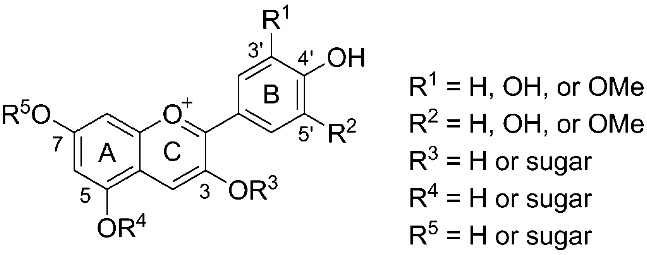


Figure 1. Variability in the structure of the common anthocyanins and anthocyanidins.

The aglycosidic forms of anthocyanins are called anthocyanidins. They are formed by acid catalyzed hydrolysis of the anthocyanins (Scheme S-1 in Supporting Information).[16] Only 17 anthocyanidins are found in nature, 6 of which are common in plants.[15,16] These common anthocyanidins vary by placement of hydrogen, hydroxy or methoxy substituents at the 3′- and 5′-positions of the B ring (Table 1), while all have the hydroxy substituent at the 4′-position.

Table 1. Structural Variability of the Common Anthocyanidins

Anthocyanidin	R^1	R^2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OMe	H
Petunidin	OMe	OH
Malvidin	OMe	OMe

Although anthocyanins are found in many fruits and vegetables, their identities and amounts vary. Cyanidin is present in the majority of anthocyanin-containing foods, while petunidin and malvidin are uncommon.[17,18] Students investigate blueberries, blackberries, and raspberries due to their complementary anthocyanidin profiles (Table 2). Blackberry and raspberry primarily contain different amounts of cyanidin, with smaller amounts of pelargonidin. Blueberry contains all of the common anthocyanidins except pelargonidin.

The colors of anthocyanins and anthocyanidins are pH-dependent.[19,20] In acidic solution, the extended conjugation of the flavylium cation (Scheme 1) is responsible for the substituent-dependent visible absorbance at 465−550 nm, giving rise to variable colors. The color disappears as pH increases and the pH-dependent equilibrium shifts. The pK_a typically ranges from 2.5 to 3.5 depending on substituents.

While anthocyanins do not have the density of aromatic rings or hydroxyl group of true polyphenols, they do share many of the chemical properties of polyphenols, including the ability to scavenge free radicals (Scheme 2).[21−24] Students explore the radical scavenging potential of the berry extracts by performing an antioxidant assay.[22,25]

■ EXPERIMENTAL PROCEDURES

Students work in pairs. Experiment 1 is done in one 3-h lab period and Experiment 2 is done in two 3-h lab periods. Detailed procedures are in the Supporting Information.

Experiment 1: Extraction and Characterization[18,26,27]

Students grind freeze-dried berries to a powder in a mortar and pestle. A solid−liquid extraction is performed on the berry powder with acidic methanol solution. An aliquot of the extract is set aside for analysis and the remaining solution is hydrolyzed with an equal volume of HCl (2 M) at reflux for 45 min. During reflux, students perform paper chromatography on the anthocyanin extract by spotting blueberry, blackberry, and raspberry extracts. Students characterize their sample with paper chromatography before and after hydrolysis.[16,26,28] The colors of anthocyanins and anthocyanidins are used for visualization of spots. Students acquire a UV−vis spectrum of the hydrolyzed extract.

Experiment 2: Investigation of Antioxidant Potential[29,30]

Students perform an antioxidant assay with $ABTS^{+\bullet}$ on unhydrolyzed acidic berry extract from Experiment 1. A constant volume (12 mL) of $ABTS^{+\bullet}$ solution (see the Instructor Notes in Supporting Information) is pipetted into each assay tube, followed by variable volumes of diluted (1/100) berry extract (0 (control), 0.5, 1.0, or 1.5 mL), and sufficient acidified methanol to keep the total assay volume constant in each assay tube (15 mL). Details of solution preparation for the assay are provided in Supporting Information Table S-1 and accompanying text in the Instructor Notes. The concentration of $ABTS^{+\bullet}$ is monitored in each assay sample by UV−vis spectroscopy 10 min after sample preparation. Plots of $ABTS^{+\bullet}$ concentration as a function of berry extract volume are prepared and analyzed.

■ HAZARDS

Standard laboratory safety procedures should be followed including wearing gloves and goggles. All operations should be performed in fume hoods. Methanol and butanol are flammable. Hydrochloric and formic acids are caustic and should not be inhaled. ABTS is toxic if inhaled or ingested. Anthocyanins and their aglycones are not dangerous as they are present in many common food items. They are highly colored dyes, but the colors fade with time, so they will not generally leave permanent stains on clothing.

Table 2. Anthocyanidin Content of Berries[17]

Food	Anthocyanidin (mg/100 g of fresh or consumed form)					
	Cyanidin	Delphinidin	Pelargonidin	Peonidin	Petunidin	Malvidin
Wild Blueberry	66.3	141		36.9	87.6	154.6
Blackberry	244		0.7			
Raspberry	90.2		1.9			

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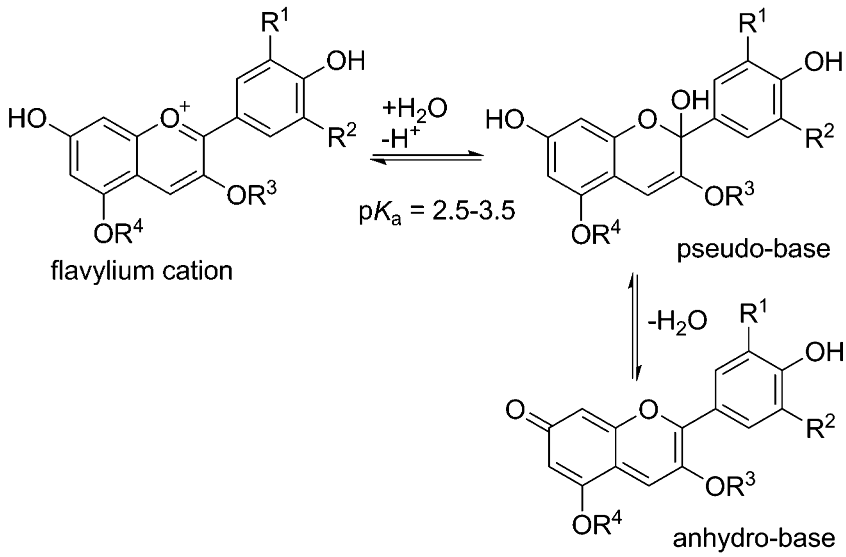
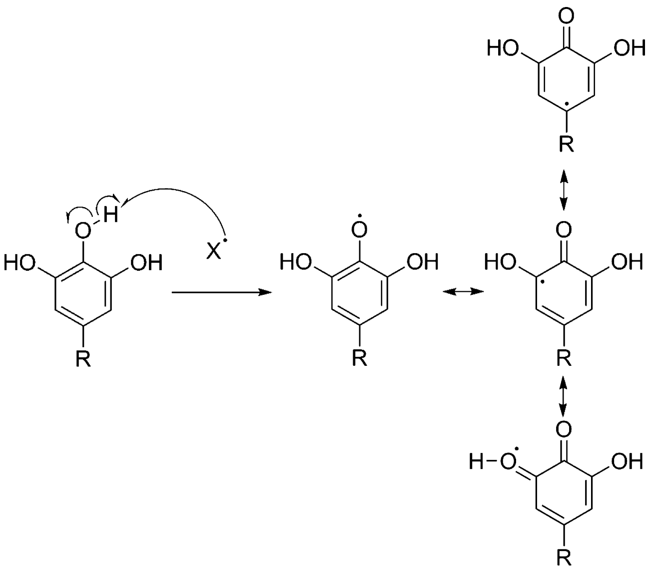
Scheme 1. pH-Dependent Equilibrium of the Flavylium Cation and the Pseudo-Base/Anhydro-Base



Scheme 2. Radical Scavenging by Polyphenols and Anthocyanins



RESULTS AND DISCUSSION

Experiment 1

Students were assigned to one of two eluting mixtures, butanol/acetic acid/water (4:1:5, upper phase) or a formic acid (formic acid/conc HCl/water, 5:2:3) mixture. These solvents were chosen because of differences in polarities. The more polar formic acid mixture achieves greater separation. Information on similarities and differences between TLC (performed 2 weeks earlier) and paper chromatography were provided in the laboratory manual (Supporting Information).

Pure anthocyanins and anthocyanidins have a single wavelength of maximum absorbance (λ_{max}) in the visible region, with a narrow absorbance band range of 80−100 nm. Mixtures may show multiple λ_{max} and/or wider absorbance bands.[16,26] Because one anthocyanidin represents many anthocyanins, students analyze only the anthocyanidins with UV−vis spectroscopy using 1:10 to 1:50 dilutions of their hydrolyzed sample with acidic methanol. A typical student spectrum is shown in Figure 2. Students recorded λ_{max} in the visible region and calculate an approximate concentration of

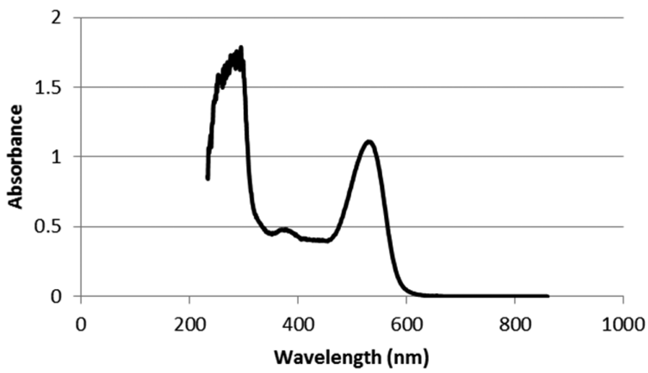


Figure 2. Typical student results for the UV−vis spectrum for blackberry anthocyanidins mixture, λ_{max} = 530 nm. (Note: anthocyanidins have UV absorbance, but the intensity of the UV peak is also attributable to other compounds in the extract.)

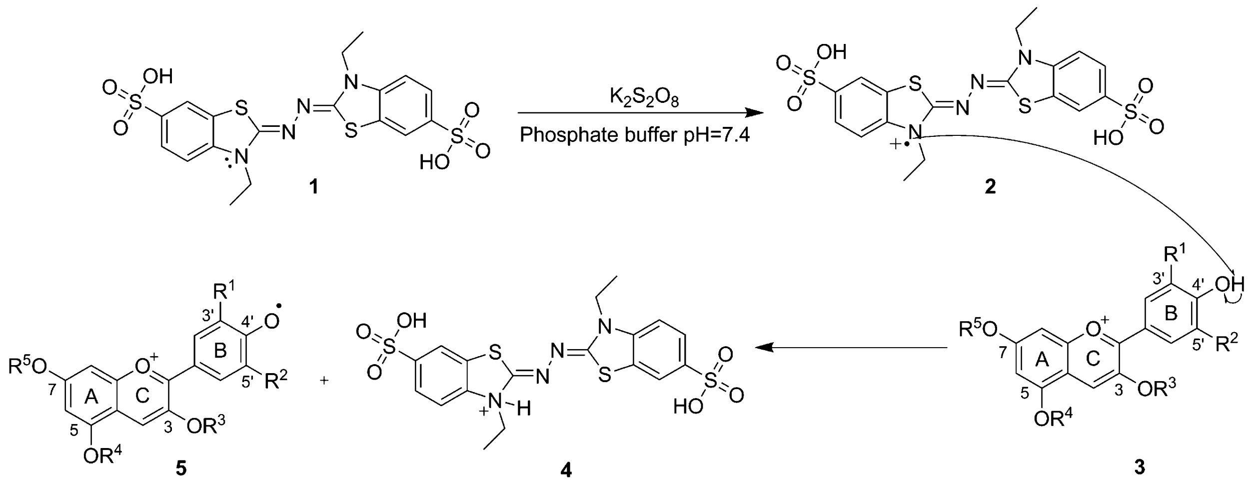
anthocyanidins in their berry using a molar absorptivity coefficient of 3.0 × 10^4 M^{-1} cm^{-1}.[16] Students estimated the moles of anthocyanidin/anthocyanin per gram of freeze-dried fruit based on their dilution factor and mass of berry powder. Typical student results are in the range of 3.0 × 10^{-6} to 2.0 × 10^{-5} mol/g of berry with raspberry extracts containing less anthocyanin than the other two extracts. Calculations are summarized in the Instructor Notes in Supporting Information.

Students pool spectroscopy results and discuss and compare their results with published anthocyanidin content for each berry. The paper chromatography data is pooled both by berry and by eluting solvent to facilitate observations before and after hydrolysis.

Experiment 2

Neutral ABTS (**1**, Scheme 3) is colorless. One electron oxidation of **1** by potassium persulfate yields the radical cation $ABTS^{+•}$ (**2**). Solutions of **2** are a blue/teal color and absorb in the UV and also in the visible range at 415, 645, 734, and 815 nm (Figure 3).[22,29,30] Students compare a UV−vis spectrum of **2** with the anthocyanidin spectra collected during experiment 1

Scheme 3. Reaction Scheme for the Antioxidant Assay



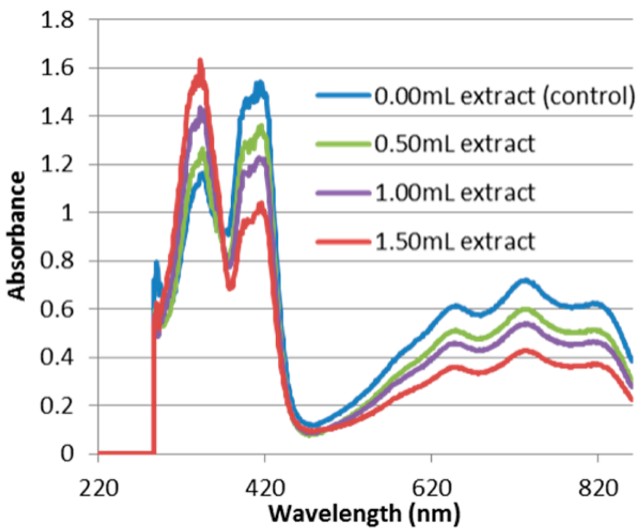


Figure 3. Typical student results for the antioxidant assay with blackberry extract.

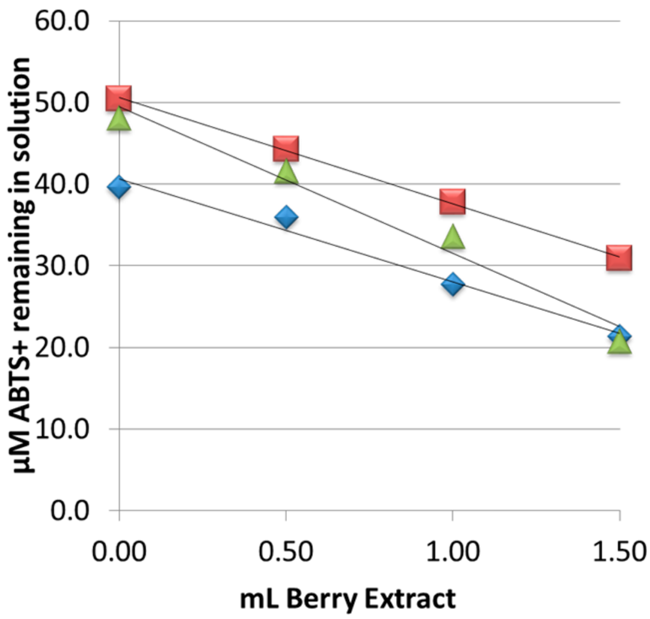


Figure 4. Typical student plot of μM ABTS$^+$ in solution versus volume of berry extract; Blueberry (♦); Blackberry (■); Raspberry (▲).

to choose a wavelength to monitor the assay. To measure changes in concentration of **2** accurately without interference from anthocyanin, students should choose a longer wavelength to monitor. For the experiment, **2** is prepared by the instructor to have an absorbance around 0.7 at 734 nm to be concentrated enough for students to measure changes upon addition of the anthocyanin (see Instructor Notes in Supporting Information). The molar absorptivity coefficient is 12867 M^{-1} cm^{-1} at 734 nm.[30]

After acidic extraction of the berries as in experiment 1, an antioxidant assay is performed with **2** on the unhydrolyzed extract. Upon addition of an antioxidant such as anthocyanin **3** to the solution of **2**, the radical abstracts hydrogen from the antioxidant and the blue/teal color fades as the radical is reduced to **4**, the conjugate acid of **1**. The antioxidant strength of the berry extracts are determined by the extent of decolorization of **2** as measured by absorbance changes.

Figure 3 shows typical student spectra for blackberry. Beer's Law is used to calculate the concentration of **2** remaining in solution. Calculations are summarized in the Instructor Notes.

A plot of concentration of **2** remaining in solution vs the volume of berry extract is prepared with a least-squares trend line, and data is pooled for all berries. Figure 4 shows a typical student plot for each berry. Additional plots are in the Supporting Information. Students discuss the meaning of the trend line and its slope, including comparisons across berries, and the relevance to the results of the previous experiment where the approximate anthocyanin content of each berry extract was determined.

■ TYPICAL STUDENT OUTCOMES

During fall 2012 and 2013, these experiments were performed by 270−290 students in a first-semester organic chemistry nonmajors laboratory course. The first experiment was completed during one 3-h laboratory class around midterm. Students wrote a formal report describing the purpose of the experiment and relating their data to the published literature. Many students had difficulty interpreting the paper chromatography for the anthocyanins because multiple anthocyanins in each berry caused the spots to smear and run together. Students were challenged by quantitative interpretation of the UV−vis spectra because of various dilutions used for the extracts. Overall, students were able to interpret their results accurately and reach reasonable conclusions.

The second experiment was the last experiment of the semester. Students were given two 3-h lab periods to complete it. The second period was available for students to re-do the experiment if their plot did not exhibit linearity with $r^2 \geq 0.95$. Approximately 15% of student pairs re-ran their assay. Also, the second week was used for in-class discussion facilitated by teaching assistants (TAs) regarding student results, comparison across berries, and comparisons with results from the first experiment (see Supporting Information). At first, students struggled with the meaning of the slope of the trend line and possible causes for a berry with relatively low anthocyanin content (raspberry) to be a strong radical scavenger.[22,31] The room temperature acidic methanol extraction technique was optimized to extract anthocyanins rather than higher molecular weight polyphenols, but smaller water-soluble antioxidants, such as ascorbic acid, can be co-extracted with anthocyanins.[32] TAs did not directly inform students of the other antioxidant contributors in the berry extracts. Instead, TAs were instructed to ask guiding questions, and students were encouraged to read the literature (leading references[5,14−19,26−31] are provided in the laboratory manual) and to practice inquiry and analysis skills as called for by the AAC&U.[13] Most students recognized that there was not a strong correlation between radical scavenging strength and anthocyanin concentration of the three berry extracts, and they were able to come to reasonable conclusions concerning contributions from other antioxidants present in the extracts. Students wrote a formal report for this experiment, also.

■ CONCLUSIONS

These experiments gave students experience with extraction and characterization of anthocyanins from freeze-dried berries, as well as an investigation of their antioxidant potential, a property that has potential health benefits. The experiments provided an introduction to solid−liquid extraction, reflux, paper chromatography and UV−vis spectroscopy. Students were able to complete the experiments within the allotted time and demonstrated successful outcomes through formal reports and class discussions. Pooling data permitted students to make comparisons across the berries used in the experiments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

CAS registry numbers of chemicals, notes on hazards, additional background material, instructor notes, student procedures formal report guidelines, and student and instructor results. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Hofstein, A.; Lunetta, V. N. The Laboratory in Science Education: Foundations for the Twenty-First Century. *Sci. Educ.* **2004**, *88*, 28.

(2) Cassidy, A.; Mukamal, K. J.; Liu, L.; Franz, M.; Eliassen, A. H.; Rimm, E. B. High Anthocyanin Intake Is Associated with a Reduced Risk of Myocardial Infarction in Young and Middle-Aged Women. *Circulation* **2013**, *127*, 188−196.

(3) Jennings, A.; Welch, A. A.; Fairweather-Tait, S. J.; Kay, C.; Minihane, A.; Chowienczyk, P.; Jiang, B.; Cecelja, M.; Spector, T.; Macgregor, A.; Cassidy, A. Higher Anthocyanin Intake Is Associated With Lower Arterial Stiffness and Central Blood Pressure in Women. *Am. J. Clin. Nutr.* **2012**, *96*, 781−790.

(4) Curtright, R. D.; Emry, R.; Markwell, J. Student Understanding of Chromatography: A Hands-On Approach. *J. Chem. Educ.* **1999**, *76*, 249−252.

(5) Curtright, R. D.; Rynearson, J. A.; Markwell, J. Fruit Anthocyanins: Colorful Sensors of Molecular Milieu. *J. Chem. Educ.* **1994**, *71*, 683−684.

(6) Edionwe, E.; Villarreal, J. R.; Smith, K. C. How Much Cranberry Juice Is in Cranberry−Apple Juice? A General Chemistry Spectrophotometric Experiment. *J. Chem. Educ.* **2011**, *88*, 1410−1412.

(7) Garber, K. C. A.; Odendaal, A. Y.; Carlson, E. E. Plant Pigment Identification: A Classroom and Outreach Activity. *J. Chem. Educ.* **2013**, *90*, 755−759.

(8) Lech, J.; Dounin, V. *JCE* Classroom Activity #110: Artistic Anthocyanins and Acid-Base Chemistry. *J. Chem. Educ.* **2011**, *88*, 1684−1686.

(9) Rossi, H. F.; Rizzo, J.; Zimmerman, D. C.; Usher, K. M. Extraction and Quantification of FD&C red Dye #40 from Beverages Containing Cranberry Juice: A College-Level Analytical Chemistry Experiment. *J. Chem. Educ.* **2012**, *89*, 1551−1554.

(10) Smestad, G. P.; Gratzel, M. Demonstrating Electron Transfer and Nanotechnology: A Natural Dye-Sensitized Nanocrystalline Energy Converter. *J. Chem. Educ.* **1998**, *75*, 752−756.

(11) Soares, M. H. F. B.; Ramos, L. A.; Cavalheiro, É. T. G. Spectrophotometric Determination of Total Sulfite in White Wine Samples Using Crude Extracts from Flowers. *J. Chem. Educ.* **2002**, *79*, 1111−1113.

(12) Hartwell, S. K. Exploring the Potential for Using Inexpensive Natural Reagents Extracted from Plants to Teach Chemical Analysis. *Chem. Educ. Res. Pract.* **2012**, *13*, 135−146.

(13) *The LEAP Vision for Learning: Outcomes, Practices, Impact, and Employers' Views*; American Association of Colleges and Universities: Washington, DC, 2011.

(14) Castaneda-Ovando, M.; Pacheco-Hernandez, M.; Paez-Hernandez, M.; Rodriguez, J. A.; Galan-Vidal, C. A. Chemical Studies of Anthocyanins: A Review. *Food Chem.* **2009**, *113*, 859−871.

(15) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, FL, 1993; pp 1−25.

(16) Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press: London, 1967; pp 1−36.

(17) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardy, S. E.; Prior, R. L. Concentrations of Anthocyanins in Common Foods in the United States and Estimation of Normal Consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069−4075.

(18) Wu, X.; Prior, R. L. Systematic Identification and Characterization of Anthocyanins by HPLC-ESI-MS/MS in Common Foods in the United States: Fruits and Berries. *J. Agric. Food Chem.* **2005**, *53*, 2589−2599.

(19) Brouillard, R.; Dubois, J. Mechanism of the Structural Transformation of Anthocyanins in Acidic Media. *J. Am. Chem. Soc.* **1977**, *99*, 1359−1364.

(20) Brouillard, R.; Iacobucci, G. A.; Sweeny, J. G. Chemistry of Anthocyanin Pigments 9 UV-Visible Spectrophotometric Determination of the Acidity Constants of Apigeninidin and Three Related 3-Deoxyflavylium Salts. *J. Am. Chem. Soc.* **1982**, *104*, 7585−7590.

(21) Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High Molecular Weight Plant Polyphenolic (Tannins) as Biological Antioxidants. *J. Agric. Food Chem.* **1998**, *46*, 1887−1892.

(22) Rice-Evans, C. A.; Miller, N. J.; Pagandga, G. Antioxidant Properties of Phenolic Compounds. *Trends Plant Sci.* **1997**, *2*, 152−159.

(23) Santos-Buelga, C.; Scalbert, A. Review: Proanthocyanidins and Tannin-like Compounds—Nature, Occurrence, Dietary Intake, and Effects on Nutrition and Health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(24) Clifford, M. N. Review: Anthocyanin—Nature, Occurrence, and Dietary Burden. *J. Sci. Food Agric.* **2000**, *80*, 1063−1072.

(25) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and Biotransformations of Various Dietary Anthocyanins in Vitro. *Eur. J. Nutr.* **2006**, *45*, 7−18.

(26) Takeoka, G.; Dao, L. Anthocyanins. In *Methods of Analysis for Functional Foods and Nutraceuticals*; Hurst, W. J., Ed.; CRC Press: Boca Raton, FL, 2008; pp 247−276.

(27) Wu, X.; Gu, L.; Prior, R. L.; McKay, S. Characterization of Anthocyanins and Proanthocyanidins in Some Cultivars of *Ribes*, *Aronia*, and *Sambucus* and Their Antioxidant Capacity. *J. Agric. Food Chem.* **2004**, *52*, 7846−7856.

(28) Gupta, S. B. Thin Layer Chromatographic Separation of Anthocyanins and Anthocyanidins in *Medicago* (Papilionaceae). *J. Chromatogr.* **1968**, *36*, 115−119.

(29) Re, R.; Pellegrini, N.; Proteggente, A. P.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying An Improved ABTS Radical Cation Decolorization Assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(30) Riedl, K. M.; Hagerman, A. E. J. Tannin-Protein Complexes as Radical Scavengers and Radical Sinks. *J. Agric. Food Chem.* **2001**, *49*, 4917−4923.

(31) Pantelidis, G. E.; Vasilakakis, M.; Manganaris, G. A.; Diamantidis, G. Antioxidant Capacity, Phenol, Anthocyanin and Ascorbic Acid Contents in Raspberries, Blackberries, Red Currants, Gooseberries and Cornelian Cherries. *Food Chem.* **2007**, *102*, 777−783.

(32) Khoddami, A.; Wilkes, M. A.; Roberts, T. H. Techniques for Analysis of Plant Phenolic Compounds. *Molecules* **2013**, *18*, 2328−2375.

dx.doi.org/10.1021/ed400520n | *J. Chem. Educ.* XXXX, XXX, XXX−XXX