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Laboratory Experiment

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Identification and Analysis of Bioactive Components of Fruit and Vegetable Products

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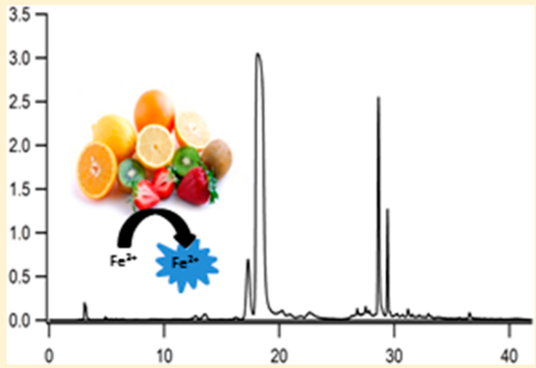
Supporting Information

ABSTRACT: Many small-molecule antioxidants found in whole fruits and vegetables are analyzed and identified in this laboratory module for upper-division biochemistry courses. During this experiment, students develop their knowledge of the bioactivity of fruit and vegetable products while learning techniques to identify vitamins and nutritionally derived secondary metabolites. Students first develop skills by analyzing well-studied fruit and vegetable juice products prior to development of a multicomponent fruit and vegetable superjuice displaying maximal antioxidant capacity. Antioxidant capacity is measured using ascorbic acid standardized ferric reducing antioxidant power (FRAP) assay, and both aqueous and lipophilic small molecules are identified using reverse-phase, high-performance liquid chromatography. Final analyses include correlation of whole fruit and vegetable products with antioxidant capacity and vitamin content using basic statistical analysis, and identification and characterization of antioxidant compounds derived from whole produce. This laboratory experiment is designed to be performed by groups of three or four students in three, 4 h laboratory periods; however, the modular nature of the experiment allows for completion of the components separately. This laboratory is easily adapted for use with thin layer chromatography to allow maximum resource flexibility.



KEYWORDS: *Upper-Division Undergraduate, Biochemistry, Problem Solving/Decision Making, Vitamins, Natural Products, Laboratory Instruction*

The importance of fruits and vegetables in the diet is a common popular science topic covered in both the scientific and news media communities. Even so, the average American eats 1.1 servings of fruit and 1.6 servings of vegetables per day,[1] which lies far below the recommended daily allowance.[2] Furthermore, 36−37% of Americans consume fruits and/or vegetables less than once per day.[1] Many factors seem to contribute to higher consumption of produce, including proximity to quality produce and education level.[3,4] Benefits of fruit and vegetable consumption include consumption of antioxidants, vitamins, and vitamin precursors. Though dietary supplementation had once been considered appropriate for acquisition of vitamins and antioxidants,[5,6] recent investigations demonstrate that supplementation often has results ranging from ineffective to harmful.[7] It appears that whole foods achieve health benefits that supplements fail to achieve, possibly due to a synergistic effect of the interaction of the small molecules.[2,8] In any case, consumption of fruits and vegetables is important for human health, and multiple programs are in place to encourage increased consumption. One such program involves the addition of a nutrition curriculum to medical training, as surveys of incoming medical school students indicate deficiencies in their basic nutrition education, including that of vitamins and antioxidants.[9] As such, a hands-on experiment in upper-division biochemistry courses during undergraduate education may help students understand and identify the bioactive small molecules found in whole fruits and vegetables, thus increasing awareness of the nutritive content.

Antioxidant content is a popular laboratory topic in chemistry. Previously published general chemistry activities have largely focused on quantifying or screening antioxidant capacity or activity in single food items.[10,11] Organic chemistry activities focus on the synthesis and characterization of antioxidant compounds.[12,13] Upper-division chemistry laboratories are available for analytical[14] and biochemistry. Previous laboratories in biochemistry focus on methods of detection for bioactive antioxidant compounds including nuclear magnetic resonance spectroscopy and enzyme-based detectors.[15,16] This laboratory activity offers a novel approach to characterization and identification of antioxidants for upper-division biochemistry curriculum.

In this experiment, students focus on the bioactivity and small molecule content of fruits and vegetables in juice preparations. The learning objectives are to quantify antioxidant capacity and identify antioxidant molecules in a fruit or vegetable sample, use statistical analysis to evaluate antioxidant capacity from different sources of antioxidants, and synthesize a

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unique fruit and vegetable juice product that demonstrates maximal antioxidant capacity. To facilitate acquisition of these objectives, students report their results from the initial characterization of a single fruit or vegetable juice sample to the instructor, who then collates and redistributes the information for statistical analysis by the entire class. Students utilize this information to develop a novel multicomponent "superjuice" which they then produce and assay. Finally, students report their results to the instructor, who then distributes the final ranking of superjuices with information on the antioxidant capacity, small molecule antioxidant content, and ingredients. Student achievement of the learning objectives is evaluated by a postlaboratory quiz and a formal laboratory report. This laboratory experiment has been performed in three, 4 h laboratory periods with students working in groups of 3 or 4 for three years with 3 or 4 laboratory sections consisting of 16−18 students per section (48−64 students annually).

■ EXPERIMENTAL METHODS

Skill Development: Antioxidant Capacity of Common Juices

Cranberry juice is classically considered rich in phenolic compounds, and tomato juice is a good source of lycopene.[17] Fresh, unopened commercial samples are provided (Campbell's tomato juice and Ocean Spray 100% cranberry juice were utilized), and students quantify the antioxidant capacity of the filtered juice sample using the modified ferric reducing antioxidant power (FRAP) assay using inexpensive and easily available L-ascorbic acid.[18] The FRAP assay measures the reduction of the ferric ion to the ferrous ion by the antioxidant molecule. This reaction is stabilized by 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), the complex of which produces a blue color detectable at 593 nm. The absorbance of this reaction is compared to that with a standard 30 μM ascorbic acid sample, and the FRAP value is calculated using eq 1,[18] where A indicates absorbance, and Df indicates the dilution factor of the juice sample with an absorbance closest to, but not to exceed, the 30 μM ascorbic acid standard at 4 min. The FRAP value describes the antioxidant capacity of a sample relative to the ascorbic acid standard.

$$\mathrm{FRAP} = \frac{A(4\ \text{min fruit juice}) - A(0\ \text{min fruit juice})}{A(4\ \text{min}\ 30\ \mu\text{M ascorbic acid}) - A(0\ \text{min}\ 30\ \mu\text{M ascorbic acid})} \times 30\ \mu\text{M} \times \mathrm{Df}$$

After completion of the assay, the instructor collects and compiles this information for the class in order to facilitate simple statistical analysis (q test, Student's t test) of antioxidant capacity of the two juices. Additionally, students can compare their values to those found in the literature.[17]

Skill Development: Antioxidant Compounds from Common Juices

Students separate and identify antioxidant small molecules from the cranberry and tomato juices using high performance liquid chromatography (HPLC) via two methods: lipophilic liquid−liquid extraction and HPLC separation allows the detection and identification of carotenoids at 460 nm, while acid hydrolysis utilizing 5 M hydrochloric acid/10% methanol at 100 °C for 30 min allows for separation of phenolic glycosides and protonation of acids for separation and detection at 280 nm. Identification of compounds occurs by comparison of retention time to authentic standards and comparing peak areas within each chromatograph, thus allowing students to describe relative (though not absolute) quantities of each compound. This lab has also been successfully completed using thin layer chromatography (TLC) against fewer standards. While carotenoids are naturally pigmented, visualization of phenolic aglycosides on the TLC plate is possible using 2% $FeCl_3$ in ethanol and heating to 100 °C.

Analysis and Synthesis: Development of a Multicomponent Superjuice

In the initial skill development modules, students cultivate skills described in the first learning objective. They analyze their FRAP value data using common statistical analysis techniques, and they identify the antioxidant compounds found in the juices via HPLC. This facilitates connections between antioxidant compounds and antioxidant capacity. The final module is designed to require analysis of data from the skill development activities in the design of a novel fruit and/or vegetable juice product with maximal antioxidant capacity. When developing the superjuice recipe, students consider the results from previous experiments, as well as consulting literature sources. Students construct a juice using a juicer or blender, and characterize the antioxidant capacity and antioxidant molecules found in the juice using the skills developed in the first two modules. Information on the composition of the juice, FRAP value, and antioxidant compounds observed is collected and collated by the instructor. The instructor then makes this information available for students to analyze in the production of a formal laboratory report.

A detailed description of the experiment is in the Supporting Information.

■ HAZARDS

Students should wear personal protective equipment including goggles, gloves, closed-toe shoes, and long sleeves. Solvents such as ethyl acetate, hexanes, and methanol pose fire hazards. Hexanes, methanol, hydrochloric acid, acetic acid, ferric chloride, ethyl acetate, chloroform, and formic acid are all irritants and permeators. Many are toxic to the central nervous (n-hexane) and renal systems. Instructors should take care when preparing solutions of TPTZ, ascorbic acid, carotenoid standards, and phenolic standards. Hazards for these specific compounds are found in the Supporting Information.

■ RESULTS

In the initial module, students developed skills in assaying antioxidant capacity using the FRAP assay. One important observation occurred early in that laboratory: students were asked to centrifuge their juice sample briefly prior to constructing dilutions for the FRAP assay. Those assigned cranberry juice observed that this was unnecessary; however, those assigned tomato juice observed much of the juice pigment is found in the pellet after centrifugation. As the FRAP assay requires soluble components, it was common for students to predict that tomato juice would have lower antioxidant capacity than cranberry juice. However, in the three years of execution of this laboratory, this prediction has not been supported. Typically, cranberry juice has a slightly higher, but statistically insignificant, average FRAP value when compared to tomato juice.

Following the results of the FRAP assay, the HPLC results were useful in providing information on the specific antioxidant compounds found in the juices. Analysis of lipophilic separation

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revealed that tomato juice contained lycopene and β-carotene in appreciable amounts, while cranberry juice contained lutein and unknown lipophilic compounds (Figure 1). Extraction and separation of the phenolic and acid components revealed unique divergent chromatographs (Figure 2). Notably, tomato juice contained ascorbic and citric acids, as well as unidentifiable peaks. Cranberry juice extracts showed high concentrations of chlorogenic acid and lower concentrations of ascorbic acid, citric acid, benzoic acid, and quercetin. Students also explored the likely identities of peaks that did not match standards using literature resources describing the antioxidant molecules found in these juices. Proposed chemicals included α-carotene, zeaxanthin, cinnamic acid derivatives, and various flavonoids and flavonols, all of which are found in tomato and cranberry juices in appreciable quantities.[17]

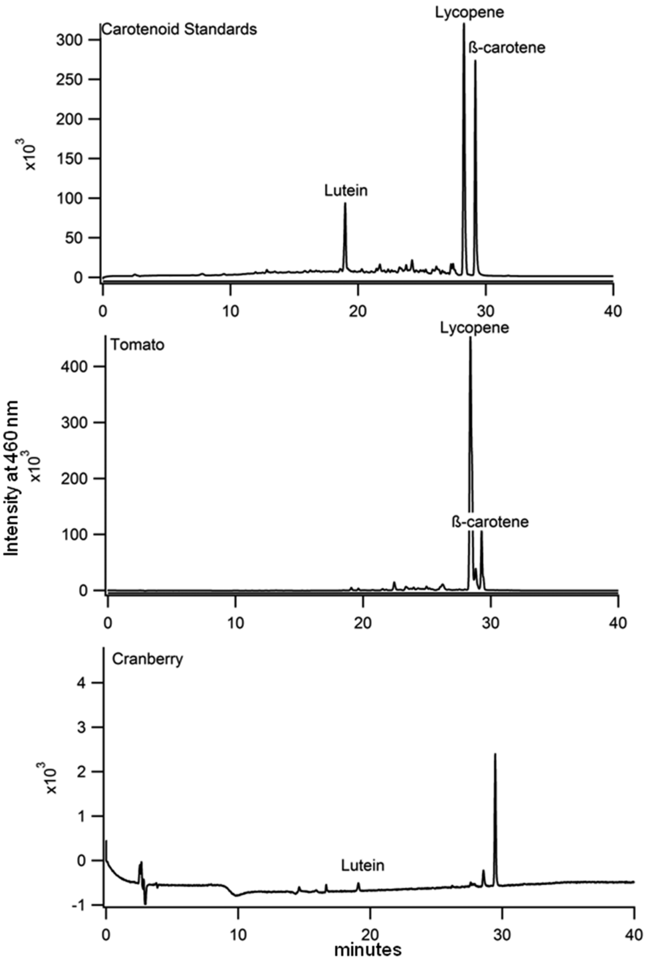

Figure 1. Representative student separation of carotenoids using HPLC. Lipophilic extracts of cranberry and tomato juice were compared to authentic standards (Sigma-Aldrich, Hy-Vee).

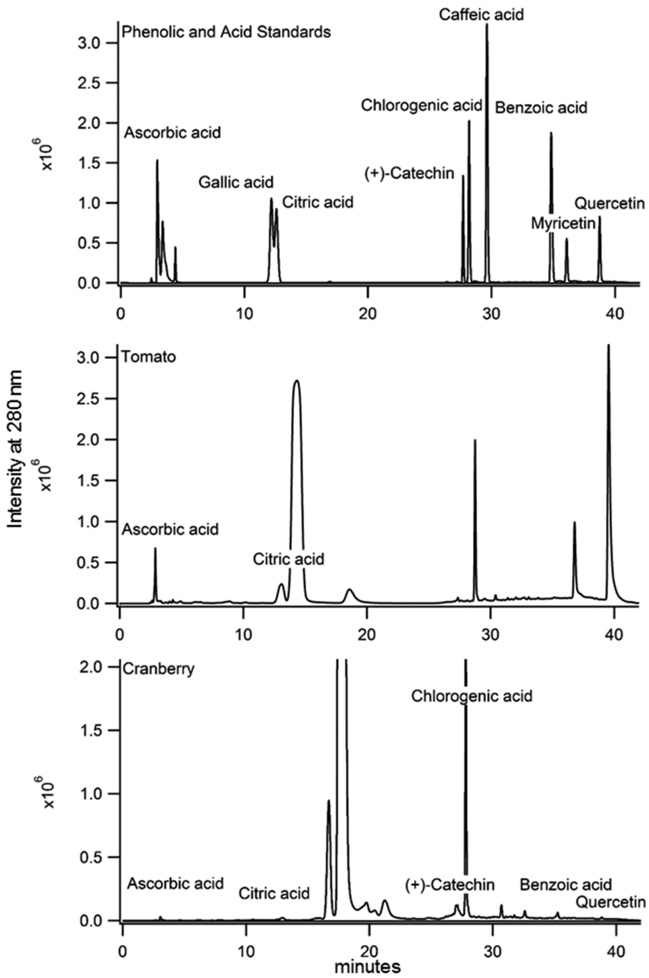

Figure 2. Representative student HPLC separation of hydrolysis of tomato and cranberry juices as compared to authentic standards (Sigma-Aldrich).

Student superjuice results were diverse due to variability in composition. Superjuices with high antioxidant capacity usually contained berries, such as blueberries and blackberries, paired with citrus and acidic fruits, such as pineapple and oranges. Common vegetable components included kale, spinach, and carrot. FRAP values and antioxidant compounds varied widely, however some antioxidant compounds were commonly found in fruits and vegetables and authentic standards for these compounds simplified identification. Typically, ascorbic acid, β-carotene, chlorogenic acid, citric acid, myrecitin, and quercetin were abundant in the superjuices (Figure 3).

■ DISCUSSION AND CONCLUSIONS

Evaluation of the learning objectives occurred via two mechanisms. One week after the completion of the laboratory activity, students completed a postlaboratory quiz. This quiz assessed basic skills in recognition and analysis from the laboratory activity (see Supporting Information) and confirmed skills described in the first learning objective. Additionally, all students wrote formal laboratory reports. In this report, students described the rationale for selection of components of their superjuice and interpreted the results of the antioxidant characterization from the superjuice product by utilizing the compiled class data, as described in the third learning objective. A final requirement for the report involved suggestion of changes to the experiment that may result in more optimal results, which completed the self-evaluation of their learning objectives for this activity. Typically, student performance has been above average on formal laboratory reports for this activity as compared to average scores for remaining assignments (91.2% vs 88.8%, n = 138).

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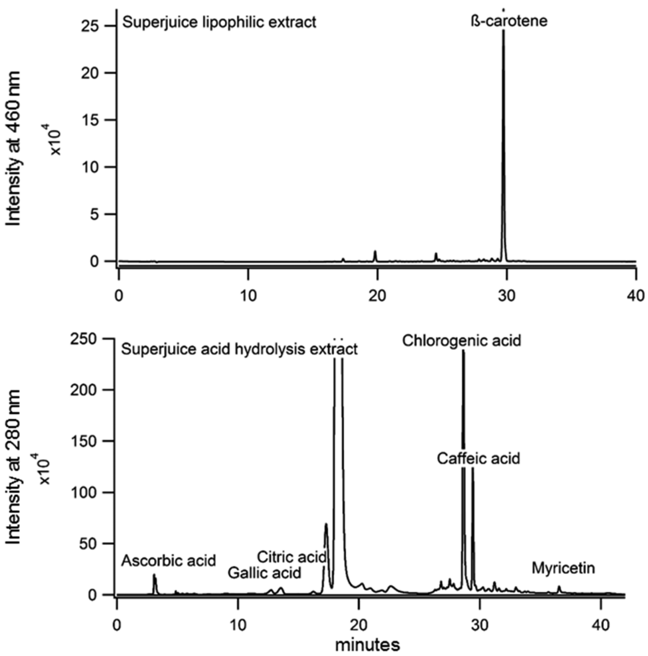

Figure 3. Acid hydrolysis and lipophilic extract of student superjuice sample revealed several common antioxidant compounds as determined by comparison to authentic standards.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Student laboratory procedure; instructor notes, materials, and methods; example postlab quiz. 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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