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Laboratory Experiment

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An Anesthetic Drug Demonstration and an Introductory Antioxidant Activity Experiment with “Eugene, the Sleepy Fish”

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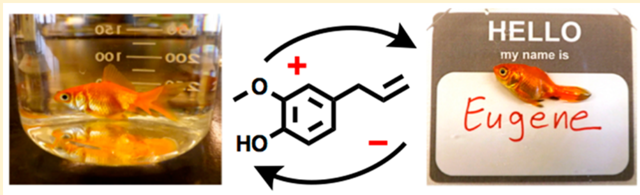
Supporting Information

ABSTRACT: Students are introduced to spectrophotometry in comparing the antioxidant activity of pure eugenol and oil of cloves from a commercial source using a modified ferric reducing antioxidant power (FRAP) assay. The extraction of the essential oil from dried cloves is demonstrated to facilitate discussions on green chemistry. The anesthetic properties of clove oil are also explored by temporarily sedating Eugene, the “sleepy” fish.



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Essential oils make compelling substances for demonstrating the applications of chemistry in everyday life. Not only do they have a long and rich history in traditional medicine, they are also used as antiseptics, preservatives, cosmetics, and in foods. For instance, the flower buds of the clove tree are not only used as spices, but also extracted for the Oil of Cloves. This aromatic oil is used for flavoring foods, as an antifungal agent against *Aspergillus* and *Candida*,[1] and as a potential cancer therapy.[2] Oil of Cloves further possesses an anesthetic property, which has the same efficacy as benzocaine in relieving toothaches.[3] The main component of Oil of Cloves is eugenol, a phenolic compound that is also an antioxidant.[4]

Described herein is an experiment devised for students that introduces them to natural products, antioxidant activity, and drug properties. Aspects of the experiment are linked to green chemistry to further engage student interest. Thus, a demonstration of eugenol extraction from cloves is performed to show students how chemists can harness natural products. Eugenol (Figure 1) from the odoriferous clove spice is palpable and easily compared with the pure substance, as well as with a commercial sample of Clove Oil. Students observe the presence of a brown oil, which has a characteristic scent. Further verification by ^{1}H NMR (Supporting Information) impresses upon them the efficiency of the extraction, and provides further introduction to spectroscopy. The small amount of clove oil from the extracts as well as the time restrictions of the lab, prohibits the preparation of the CO_2 extract for the antioxidant study. However, students may still compare the antioxidant activity of store bought clove oil and a solution of pure eugenol using a modified ferric reducing antioxidant power (FRAP) assay,[5] which introduces students to spectrophotometry and allows them to compare chemical reactivity by plotting data.

Demonstration of the anesthetic property of clove oil using a live fish impresses upon the students the impact chemicals have on living systems, and including toxicity. The anesthetization of Eugene shows how any chemical can be toxic depending on the dose and on the organism, and further demonstrates that what could be a nontoxic dose for humans could be fatal for other animals. Students become eager for Eugene’s recovery from the anesthesia, thus bringing the natural world closer to the chemistry lab. This opens up discussions on the anthropogenic release of chemicals in the environment, such as dichlorodiphenyltrichloroethane (DDT), dioxins, and the Fukushima accidental release of radiochemicals. In these and in other documented ecotoxicology examples, the harm is first evident among wildlife and progresses up the food chain through bioaccumulation.

■ PROCEDURE

Part I: Clove Oil Extraction (20 min)

Students are first shown cloves, store bought clove oil, and pure eugenol to compare the substances by olfaction. Students are asked to smell a bag of cloves and then asked to waft clove oil and then eugenol vapors. Cloves are readily available and are a popular choice for steam-distillation experiments. To facilitate discussions of green chemistry and to expedite the demonstration, liquid CO_2 extraction was adapted for cloves.[6] A plastic centrifuge tube is filled halfway with fresh cloves, and then filled with dry ice.[7] The tube is capped and placed in a beaker of warm water to pressurize the dry ice and produce liquid CO_2, which the students observed. While waiting for the CO_2 to evaporate, carbon dioxide, a byproduct of chemical processes, was discussed in its role as a greenhouse gas and as a green solvent.[8] After all the CO_2 has evaporated, the cloves are emptied from the centrifuge tube and the students are asked to observe and smell the oil droplets that are found at the bottom.

Part II: Antioxidant Titration (90 min)

Stock solutions of acetate buffer (pH 3.6), iron(III) chloride (20 mM), and TPTZ (2,4,6-tri-(2-pyridyl)-1,3,5-triazine, 10

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mM) were prepared in distilled water and sparged with nitrogen gas prior to the experiment, which displaces oxygen that could interfere with the redox reaction. More consistent results were obtained from sparged solutions, and sparging may be done several days in advance, as long as the bottles are sealed airtight. The proper use of pipettes were reviewed with the students, and then they were tasked to prepare the FRAP oxidizing reagent.

Stock solutions of the clove oil and pure eugenol (6.5 mM) were prepared separately in ethanol prior to the experiment. Clove oil contains 70−90% eugenol, and to produce a solution of similar concentration as the standard, the UV absorption was used to adjust the concentration (λ_{max} = 280 nm, ε = 2407 M^{-1} cm^{-1}).[9] The stock solutions were sparged with N_2 prior to the experiment. Aliquots were provided to the students when they are ready to run the experiment.

Students were shown how to operate a spectrophotometer to measure the absorbance of Fe^{2+}-TPTZ at 594 nm. Once proficient in its operation, students began the experiment by using the FRAP oxidizing reagent as a blank. The students added eugenol solution (300 μL) to a 10 mL portion of the FRAP solution using a syringe. A graduated pipet may be used; however, a syringe provides students greater control, which is imperative for minimizing errors in handling small volumes. The mixture immediately changed into a blue color and the timer was started. The reaction was carefully shaken, and an aliquot was transferred into a clean cuvette. The absorbance was measured 4 min after the reaction started, and then every 2 min thereafter for 10 data points. The blank was inserted prior to each measurement.

After performing the FRAP assay for eugenol, the experiment was repeated for clove oil. Solutions from the experiments are discarded as aqueous waste, and the students were tasked to clean their glassware and cuvettes. Afterward, students plotted the data and obtained the slope and the correlations from the straight lines.

Part III: Sleepy Fish Demonstration (10 min)

A small 2-in. fish was placed in a crystallizing dish containing water from its holding tank. Clove oil was added (10−20 drops) to the water, and the fish began to exhibit decreased respiration and muscle tone. As the fish starts to lose equilibrium, the handler kept the fish upright to maintain the fish's swim bladder position. The swim bladder is an internal organ that allows the fish to swim upright. Complete sedation occurred within 1−2 min, and the unresponsive fish was taken out carefully and shown to the students. Care was taken not to leave the fish too long in the crystallizing dish to prevent euthanasia. To restore normal activity, the fish was replaced in its holding tank and kept upright by the handler until it regained equilibrium.

■ HAZARDS

Clove oil is a GRAS (generally recognized as safe) substance by the U.S. Food and Drug Administration (FDA) when used as food. However, when used as an anesthetic for fish, it is an unapproved drug. Nonetheless, many hobbyists use clove oil to safely handle fish that require medical attention. Eugenol and ethanol are volatile and flammable compounds. Iron(III) chloride and TPTZ are listed as irritants.

Students must be reminded not to pipet using the mouth. Students must also be shown how to remove air bubbles from syringes and to take care if a needle is attached. Only two syringes are needed, and they must be accounted for at the end of each experiment. Needles must be discarded in a sharps container. Dry ice can cause burns; never touch it with bare hands.

■ STUDENT RESULTS

FRAP Assay

Antioxidant assays normalize activity against known standards;[10] however, the experiments herein are constrained to introduce students to the procedure. Within this design, the FRAP reaction is not heated and the absorbance measurements were not limited to 4 min. Extension of the FRAP assay to 20 min allows students ample time to measure data, and even plot it in real time. It was found that the results are consistent among different groups, provided that the solutions are sparged with nitrogen.

Representative student data are shown in Figure 1 for both the clove oil and eugenol FRAP assay. The antioxidant activities

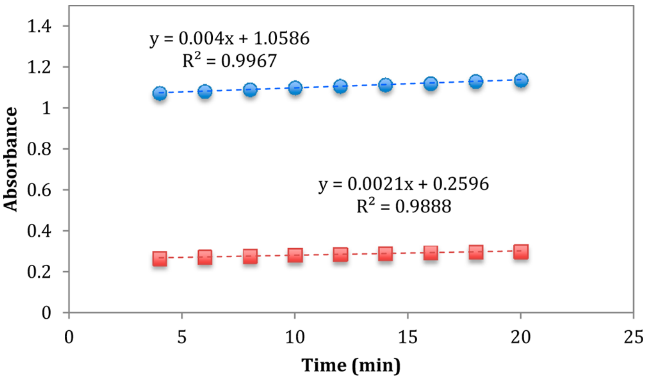

Figure 1. Representative student data for the FRAP assay for eugenol (circles) and clove oil (squares). The antioxidant activity is higher for eugenol, as indicated by the slope.

were compared by determining the slope, which represents the rate of the reduction, $\Delta[Fe^{2+}TPTZ]$/min from the increase in absorbance. Invariably, experimental results for the antioxidant activity of eugenol are higher than that of the clove oil. Many students account for the difference in the slope as the variable concentration of eugenol in the pure sample and in the clove oil. This is a reasonable conclusion without having measured the UV absorption comparison between the clove oil and the pure eugenol solutions, since the spectrophotometers in our teaching labs are limited to the visible range. The concentration of the eugenol in clove oil was estimated to be equal that of pure eugenol by UV during sample preparation (Supporting Information). A more reasonable explanation is that there are other UV-absorbing compounds in clove oil that do not have antioxidant properties. Another explanation is that there are other compounds in clove oil that could chelate iron, causing an apparent decrease in the antioxidant activity. Many students highlight that the impurities in clove oil may interfere with the FRAP assay in their responses. The eugenol activity was found to be twice as much compared to clove oil, and the difference in reduction was easily observed qualitatively by the color of the solution, and quantitatively by looking at the slope of the FRAP assay.

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Green Chemistry

Students were able to recognize the green chemistry facets of the experiment. For the eugenol extraction, they retained from the demonstration that CO_2 is a byproduct of many industries and using it as a solvent is a form of recycling. They are less able to relate to the use of organic solvents, since they have little experience or awareness of their environmental impact. They also readily pointed out that clove oil is a natural product that may be used as a sustainable chemical or as a natural antioxidant, as opposed to synthetic antioxidants.

Few students recognized the green chemistry aspects of the FRAP titration, such as the use of iron, a benign metal, and water, a benign solvent. The concentrations of the reagents are also very low (mM), yet the color change is palpable during the FRAP assay. In the "sleepy fish" demonstration, many students recognized the toxicity and drug effects of chemicals, and were surprised that something that is benign to humans could be harmful to aquatic organisms. This highlights that the careless release of chemicals could have devastating effects to other organisms and the ecosystem.

■ DISCUSSION

Eugenol from cloves has been studied extensively in teaching laboratories.[11] This experiment builds on a similar approach, so that clove oil is extracted from the raw material using a green teaching method.[7] The commercial clove oil used for the comparison study demonstrates to students the chemistry behind over-the-counter drugs as well as herbal remedies. Antioxidants make engaging topics for undergraduate instruction, and various methods have been employed in teaching laboratories such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method,[12] and trolox equivalent antioxidant capacity (TEAC) method.[13] Phenols have also been studied using the ferric ammonium sulfate (FAS) and Folin-Ciocalteau (F−C) methods.[14] These methods, along with the FRAP, have been extensively used to study both natural and synthetic antioxidants.[4b,15] The FRAP assay has been used to study clove oil[16] and other phenols.[17] Modifications of the FRAP assay described herein extends its application to the undergraduate teaching lab and allows undergraduates to study other antioxidants of interest (Supporting Information).

■ CONCLUSION

The laboratory activities described herein explore the natural essential oil eugenol, starting with its extraction from cloves, examination of its antioxidant activity using the FRAP assay, and a demonstration of its anesthetic effect on fish. The experiments are suitable for introducing general chemistry students to green chemistry, spectrophotometry, and antioxidant activity determination.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Student handout; instructor notes. The Supporting Information is available on the ACS Publications website at DOI: 10.1021/ed5008469.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Pinto, E. n.; Vale-Silva, L. s.; Cavaleiro, C.; Salgueiro, L. g. Antifungal activity of the clove essential oil from Syzygium aromaticum on Candida, Aspergillus and dermatophyte species. *J. Medi. Microbiol.* **2009**, *58* (11), 1454−1462.

(2) Jaganathan, S. K.; Supriyanto, E. Antiproliferative and Molecular Mechanism of Eugenol-Induced Apoptosis in Cancer Cells. *Molecules* **2012**, *17*, 6290−6304.

(3) Alqareer, A.; Alyahya, A.; Andersson, L. The effect of clove and benzocaine versus placebo as topical anesthetics. *J. Dentistry* **2006**, *34* (10), 747−750.

(4) (a) Viuda-Martos, M.; Ruiz Navajas, Y.; Sánchez Zapata, E.; Fernández-López, J.; Pérez-Álvarez, J. A. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour Fragrance J.* **2010**, *25* (1), 13−19. (b) Thaipong, K.; Boonprakob, U.; Crosby, K.; Cisneros-Zevallos, L.; Hawkins Byrne, D. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Compos. Anal.* **2006**, *19* (6−7), 669−675. (c) Srinivasan, K. Antioxidant Potential of Spices and Their Active Constituents. *Crit. Rev. Food Sci. Nutr.* **2012**, *54* (3), 352−372. (d) Kranl, K.; Schlesier, K.; Bitsch, R.; Hermann, H.; Rohe, M.; B√∂hm, V. Comparing antioxidative food additives and secondary plant products, Äì use of different assays. *Food Chem.* **2005**, *93* (1), 171−175. (e) Shan, B.; Cai, Y. Z.; Sun, M.; Corke, H. Antioxidant Capacity of 26 Spice Extracts and Characterization of Their Phenolic Constituents. *J. Agric. Food Chem.* **2005**, *53* (20), 7749−7759.

(5) (a) Benzie, I. F. F.; Szeto, Y. T. Total Antioxidant Capacity of Teas by the Ferric Reducing/Antioxidant Power Assay. *J. Agric. Food Chem.* **1999**, *47* (2), 633−636. (b) Benzie, I. F. F.; Strain, J. J., [2] Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In *Methods in Enzymology*; Lester, P., Ed. ;Academic Press: New York, 1999; Vol. *299*, pp 15−27. (c) Benzie, I. F. F.; Strain, J. J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of, AAntioxidant Power: The FRAP Assay. *Anal. Biochem.* **1996**, *239* (1), 70−76.

(6) Chatterjee, D.; Bhattacharjee, P. Supercritical Carbon Dioxide Extraction of Eugenol from Clove Buds. *Food Bioprocess Technol.* **2013**, *6* (10), 2587−2599.

(7) Buckley, H. L.; Beck, A. R.; Mulvihill, M. J.; Douskey, M. C. Fitting It All In: Adapting a Green Chemistry Extraction Experiment for Inclusion in an Undergraduate Analytical Laboratory. *J. Chem. Educ.* **2013**, *90* (6), 771−774.

(8) Ramsey, E.; Sun, Q.; Zhang, Z.; Zhang, C.; Gou, W. Mini-Review: Green sustainable processes using supercritical fluid carbon dioxide. *J. Environm. Sci.* **2009**, *21* (6), 720−726.

(9) Goldschmid, O. Determination of Phenolic Hydroxyl Content of Lignin Preparations by Ultraviolet Spectrophotometry. *Anal. Chem.* **1954**, *26* (9), 1421−1423.

(10) Nenadis, N.; Lazaridou, O.; Tsimidou, M. Z. Use of Reference Compounds in Antioxidant Activity Assessment. *J. Agric. Food Chem.* **2007**, *55* (14), 5452−5460.

(11) (a) Sousa, A. M.; Waldman, W. R. Antimicrobial Properties of Spices: An Activity for High School or Introductory Chemistry or Biology. *J. Chem. Educ.* **2013**, *91* (1), 103−106. (b) O'Shea, S. K.; Von Riesen, D. D.; Rossi, L. L. Isolation and Analysis of Essential Oils from Spices. *J. Chem. Educ.* **2012**, *89* (5), 665−668. (c) Smiley, P. M.; Miles, W. H. Modeling the Drug Discovery Process: The Isolation and Biological Testing of Eugenol from Clove Oil. *J. Chem. Educ.* **2002**, *79* (1), 90. (d) Ntamila, M. S.; Hassanali, A. Isolation of oil of clove and

separation of eugenol and acetyl eugenol. An instructive experiment for beginning chemistry undergraduates. *J. Chem. Educ.* **1976**, *53* (4), 263.

(12) (a) Garrido, J.; Garrido, E. M.; Borges, F. Studies on the Food Additive Propyl Gallate: Synthesis, Structural Characterization, and Evaluation of the Antioxidant Activity. *J. Chem. Educ.* **2011**, *89* (1), 130−133. (b) Canelas, V.; Teixeira da Costa, C. Quantitative HPLC Analysis of Rosmarinic Acid in Extracts of Melissa officinalis and Spectrophotometric Measurement of Their Antioxidant Activities. *J. Chem. Educ.* **2007**, *84* (9), 1502.

(13) Hoch, M. A.; Russell, C. n. B.; Steffen, D. M.; Weaver, G. C.; Burgess, J. R. Assessment of Antioxidant Capacities in Foods: A Research Experience for General Chemistry Students. *J. Chem. Educ.* **2009**, *86* (5), 595.

(14) Shaver, L. A.; Leung, S. H.; Puderbaugh, A.; Angel, S. A. Two Methods of Determining Total Phenolic Content of Foods and Juices in a General, Organic, and Biological (GOB) Chemistry Lab. *J. Chem. Educ.* **2011**, *88* (4), 492−495.

(15) (a) Apak, R.; Gorinstein, S.; Böhm, V.; Schaich, K. M.; Özyürek, M.; Güçlü, K. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure Appl. Chem.* **2013**, *85* (5), 957−998. (b) Huang, D.; Ou, B.; Prior, R. L. *J. Agric. Food Chem.* **2005**, *53*, 1841. (c) Prior, R. L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food Chem.* **2005**, *53* (10), 4290−4302.

(16) Gulcin, I.; Elmastas, M.; Aboul-Enein, H. Y. Antioxidant activity of clove oil - A powerful antioxidant source. *Arabian J. Chem.* **2012**, *5* (4), 489−499.

(17) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant Activity of Dietary Polyphenols As Determined by a Modified Ferric Reducing/Antioxidant Power Assay. *J. Agric. Food Chem.* **2000**, *48* (8), 3396−3402.

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