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Extraction and Antibacterial Properties of Thyme Leaf Extracts: Authentic Practice of Green Chemistry

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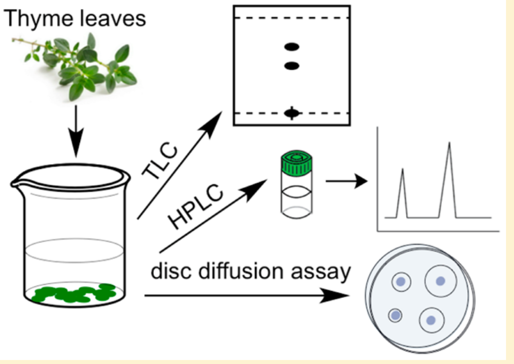
Supporting Information

ABSTRACT: In this undergraduate analytical chemistry experiment, students quantitatively assess the antibacterial activity of essential oils found in thyme leaves (*Thymus vulgaris*) in an authentic, research-like environment. This multiweek experiment aims to instill green chemistry principles as intrinsic to chemical problem solving. Students progress through various techniques including extraction, chromatography (TLC and HPLC), culturing bacteria, and disk diffusion via a process of guided exploration that emphasizes green experimental design. Approximately 600 undergraduate students carried out the experiment and self-reported substantial learning gains.



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Authentic practice, the implementation of research-like experiments that mimic genuine scientific problem solving, has begun to take root in chemistry education.[1−7] This approach allows students to experience a research setting while learning the techniques and theory presented in the traditional analytical laboratory setting.[8−13] There is growing evidence in the literature that authentic practice in laboratory courses improves students' learning of scientific practices, engagement and retention in the subject, ability to think critically, scientifically, and to communicate progress effectively.[1,14−18]

Green chemistry is also an important area for curricula development.[19] Although strides have been made in the education of chemistry students on the tenets and practice of green chemistry, chemical educators must continue to develop and redesign laboratory curricula to reflect these principles.[16,20−23] By making green chemistry central to laboratory-based education, educators have the opportunity to mold a generation of scientists to whom green chemistry is no longer an abstract ideology applied to the practice of chemistry. These scientists will know and practice a chemistry that is inherently green.

We had two goals in developing this experimental module. First, integrate authentic practice and green chemistry into our analytical and general chemistry laboratory curriculum by creating a module on the authentic practice of green chemistry. Second, guide students to rigorously apply a wide range of techniques in analysis and characterization to solve an authentic problem. Although both authentic practice and green chemistry are recognized as exciting components of laboratory curricula, they have yet to be fully implemented at many institutions.[14] Few undergraduate analytical chemistry experiments cover such a dynamic range of techniques in the analysis and characterization of real-world samples as are included within the two module described.[10,23−27] Even fewer accomplish this while emphasizing the practice of green chemistry.[21,23] This experiment, successfully implemented in general chemistry courses serving approximately 600 first-year STEM majors over three years, aims to fill a large gap in current curricula by underscoring the application of a range of analytical techniques in the authentic practice of green chemistry.

One of the challenges of incorporating authentic practice in the laboratory is ensuring that students are able to meet learning objectives of the experiment while still allowing for as much inquiry as possible.[14,28]The experiment accomplishes this task by guiding students through a variety of analytical and microbiological techniques for analyzing thyme leaves (*Thymus vulgaris*), an herb with known antibacterial properties.[29] Thyme oil is found in a variety of green household cleaning products and is touted as an antibacterial agent.[30,31] In order to further explore this claim, students begin with a natural product and are guided through a research-like process with the ultimate

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goal of isolating compounds and quantifying the antibacterial properties of compounds in thyme leaves. The process begins when students explore concepts of solid–liquid extraction by extracting essential oils from thyme leaves in solvents of varying polarity.[32,33] Extracts are analyzed via thin layer chromatography (TLC) and cospotted with three major components reported in the literature: thymol, carvacrol, and cymene.[34,35] When TLC analysis does not fully resolve the isomers thymol and carvacrol, students are guided toward the more powerful techniques of high-performance liquid chromatography (HPLC) and the method of standard addition.[36−39] The experiment also introduces quantitative biology through analysis of the antibacterial activity of student extracts using Kirby–Bauer disk diffusion.[40−42] Students gain an understanding of the design and practice of green chemistry through the application of rigorous analytical techniques in chemistry and microbiology to answer a genuine research question.

METHODS

This module is best delivered over two laboratory periods, each 4 h, affording student pairs ample time to complete experiments and allowing for guided exploration.

Laboratory Period 1: Extraction and HPLC Analysis

Students grind thyme leaves (several samples of 0.10 g leaves, each) with mortar and pestle and perform solid–liquid extractions in 1.00 mL aliquots of heptane, ethyl acetate, water, and a mixture thereof to allow for comparisons of extraction efficiency between different solvents. Normal-phase, silica thin layer chromatography (TLC) is employed and students use standard solutions of thymol, carvacrol, and cymene to determine the identity of extracted compounds. Students also use various TLC mobile phase solvents in order to optimize R_f values and separate compounds. Visualization is achieved via UV lamp and iodine staining.

Students prepare an additional sample of thyme leaves (1.00 g) for extraction by grinding with mortar and pestle. A small volume of methanol is used to facilitate transfer to a volumetric flask and extraction by triplicate 10.00 mL aliquots of methanol, each with stirring at room temperature for at least 10 min, yields 30.00 mL of extract solution to be analyzed by HPLC. Students prepare their extract for HPLC analysis and quantitation using standard addition. Students prepare five volumetric flasks (10.00 mL) each with 5.00 mL of extract and varying amounts of a standard thymol solution. Students submit sample extracts for HPLC analysis, a technician loads the samples into an autosampler, and the data are returned to the students via the course Web site. Reversed-phase HPLC with gradient elution (full parameters can be found in Supporting Information) is used to analyze the samples. Students use data obtained to construct a calibration curve, determine which compounds are present in samples, and determine the percent weight of thymol in thyme leaves.

Laboratory Period 2: Antibacterial Activity Assay

Students assay the antibacterial properties of extracts alongside essential oil standards (Sigma-Aldrich) via Kirby–Bauer disk diffusion.[12,13,42] A bacterial solution of *Escherichia coli* (*E. coli*) is spread evenly across agar plates and antibacterial disks are added to the plates. Students prepare 4 or 5 antibacterial disks to test a hypothesis of their choice (involving synergistic effects, commercial antibacterial products, etc.) in addition to assaying the antibacterial properties of their extracts. These disks are prepared using a standard hole punch and filter paper (Whatman, 40). Students submerge the disks either in standard solutions or thyme leaf extract in ethyl acetate (0.00–1.00 M) and allow the disks to dry for 30 min. Commercial tetracycline disks are used as a positive control and ethyl acetate disks are used as a negative control. Dried disks are applied to the surface of the agar plates. The bacteria are allowed to grow for a week at room temperature or a shorter period if an incubator is available. The following lab period, students measure the zones of inhibition in order to determine minimum inhibition concentration, quantitatively assessing the efficacy of their extracts as antibacterial agents. This measurement only requires a few minutes of time at the beginning of the next lab period.

HAZARDS

Thyme oil is harmful if swallowed in quantity and can be a skin, eye, and respiratory irritant. Thymol, carvacrol, and *p*-cymene are potential irritants and should not be ingested. Caution should be taken when visualizing TLC plates with UV light to avoid eye exposure. Iodine is corrosive and an irritant; vapors should not be inhaled. Ethyl acetate and heptane, although less harmful than many other solvents, are both harmful if ingested or if vapors are inhaled. Methanol and tetrahydrofuran are toxins and irritants. Methanol, ethyl acetate, heptane, and tetrahydrofuran are flammable and should be handled in a fume hood if possible. Ethanol is an irritant and flammable; only small volumes should be used during sterilization. Open flame poses burn and fire hazards; flame sterilization should not be performed in proximity to flammable solvents. LB Agar is an irritant and harmful if ingested or inhaled. Tetracycline is an irritant and is hazardous if ingested or inhaled. *E. coli* is a biosafety level one (BSL-1) organism, all appropriate precautions must be taken and institutional biosafety committee (IBC) approval granted. All biological waste should be isolated and autoclaved before disposal in accordance with IBC policy.

RESULTS

Extractions of thyme leaves in ethyl acetate, heptane, and mixtures thereof were the most efficient, yielding robust UV light and iodine visualization via TLC whereas other solvents tested were not as efficient at extraction and resulted in suboptimal visualization. Thymol and carvacrol both have average R_f values of approximately 0.6, making them nearly indistinguishable on the standard 10 cm TLC plates used by the students when run in a 1:1 mixture of ethyl acetate and heptane. Cymene, which is extracted in low concentration, may also be visualized by UV light and has an R_f of approximately 0.7.

Students' HPLC data are plotted and standard addition calibration curves are generated (Figure 1). Cymene, though detected by TLC, results in a weak signal by HPLC coupled with a diode array detector. The concentration of thymol in thyme leaves was determined experimentally for a class of 120 students. Results varied depending on extraction technique and sample of thyme leaves used. The bulk of the student data clustered at 2.11% (w/w). This is in close agreement with values cited in literature and obtained by instructors and laboratory support staff.[33,43]

Students recorded the minimum inhibitory concentration (MIC), the minimum concentration at which growth of bacteria is visibly inhibited, and determined thymol to have the greatest antibacterial activity per unit concentration (Figure

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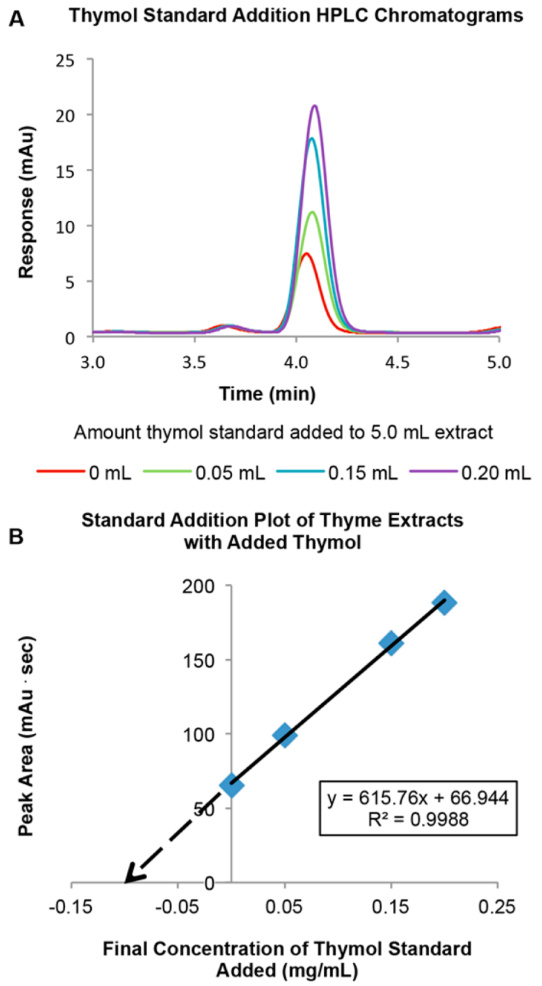

Figure 1. (A) Sample student HPLC chromatograms of thyme extracts with varying amounts of thymol standard added using the method of standard addition. (B) Student data of standard addition calibration curve of HPLC peak area for thymol. Data are used to determine the amount of thymol in the extracts, then in thyme leaves by weight.

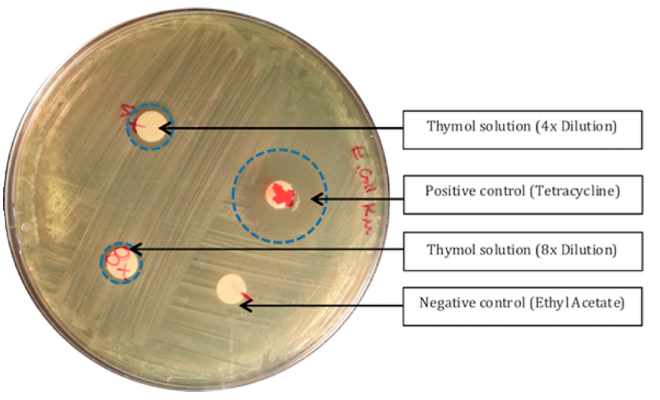

Figure 2. Sample student Kirby–Bauer disk diffusion plate is shown after *E. coli* bacterial growth. Disks prepared with varying concentrations of thymol extract solution, a tetracycline positive control, and an ethyl acetate negative control were loaded onto the agar plate prior to incubation. Zones of inhibition (as marked) are measured and used to determine the minimum inhibitory concentration of thymol on *E. coli* bacteria.

2). MIC values for thymol and carvacrol standards were determined to be 0.041 ± 0.005 M and 0.070 ± 0.014 M, respectively, when representative data were included from three independent laboratory sections (approximately 25 students per section). Students also used the assay to answer a question of their own design. There were a wide variety of explorations proposed by students from the thyme leaf extract itself, bleach, hand sanitizer, turmeric, antibacterial ointments, and possible synergistic effects of coupling two compounds on one disk. Results of these studies varied widely and in many instances the students indicated further testing was needed to determine concentrations of active ingredients in the materials they tested.

■ DISCUSSION

This laboratory experiment successfully incorporates the multidisciplinary character of modern chemical research into the undergraduate curricula while highlighting the importance and practice of green chemistry in the research setting. Importantly, this laboratory demonstrates that incorporating authentic practice and green chemistry into laboratory curricula does not limit the range of classic analytical techniques used but rather can seamlessly contextualize this material within a meaningful, modern, research question. This laboratory module emphasizes the authentic practice of green chemistry without compromising the learning objectives of the traditional analytical laboratory course (Table 1).

As students begin the first module they explore the efficacy of various green extraction solvents, taking note that harsh organic solvents such as dichloromethane are not necessary for many extractions. Students then optimize the ratio of heptane and ethyl acetate in the TLC mobile phase (changing elution strength) in attempt to resolve the thymol and carvacrol spots by optimizing R_f. As students utilize ethyl acetate, a biodegradable solvent, they are introduced to the importance of considering waste streams and degradation products. The first module also simulates an authentic research experience as students recognize that TLC does not have the resolving power necessary to separate the extracted compounds and are guided toward a more powerful alternative, HPLC.

As students progress into the second module, they continue to explore themes within green chemistry in an authentic research setting. In performing the disk diffusion assays, students consider the use of green solvents and renewable feedstocks. An introduction to basic principles within toxicology is particularly valuable as it highlights the importance of considering the biological activities of chemical products and waste streams. Students explored a variety of independent hypotheses regarding the efficacy of commercial products including hand sanitizer, air freshener, hand wipes, bleach, and lemon juice. A few groups even compared the commercial tetracycline control disks with tetracycline solution supplied by a graduate student in the department. This aspect of the laboratory was enjoyed by students and emphasized the use of positive and negative controls in exploring scientific questions.

Approximately 600 students (both chemistry majors and other STEM majors) in advanced general chemistry and quantitative analysis classes at UC Berkeley have successfully conducted this experiment. These courses are taught at an honors level at UC Berkeley and would make a suitable capstone experience at other institutions. We observed that students were more engaged when presented with a genuine scientific problem and allowed to explore hypotheses of their choosing within the framework of the module (particularly so

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Table 1. Incorporating Green Chemistry into Learning Goals

Experiment	Learning Goals	Green Chemistry
Extraction of essential oils from thyme	Solid–liquid extraction; solvent polarity and extraction efficacy	Use of renewable feedstocks
TLC of thyme extract	Chromatography basics; mobile phase optimization	Safer solvents and auxiliaries; design for degradation
HPLC of thyme extract	Reverse phase chromatography; standard addition; chromatogram analysis; calibration curves	Safer solvents and auxiliaries; prevention
Kirby–Bauer disk diffusion	Microbiology techniques; calibration curves	Use of renewable feedstocks; safer solvents and auxiliaries; Introduction to toxicology

during the antibacterial assay). It was also noted that experiments of this type generally required students to think more critically about laboratory procedures and aided in student understanding of analytical techniques presented in the lecture hall.

To evaluate these observations and validate the efficacy of the curricula, we conducted surveys to gather student self-assessments of learning gains. These surveys were conducted at the start and finish of the laboratory component of a semester-long lab course in general chemistry and quantitative analysis. Though the survey included questions relating to multiple laboratories with similar themes, we have isolated those relevant to the module described in this paper.

This experiment is one of many with green chemistry applications used at UC Berkeley. A description of the course curriculum can be found in the Supporting Information. The learning gains were measured with entrance and exit surveys and reflect the total curriculum, not gains due to this experiment alone. In a class of chemistry majors in 2015, students reported statistically significant gains in all items relating to experimental design, green chemistry and quantitative analysis concepts. On specific aspects of experimental design emphasized by authentic practice, the chemistry majors showed gains in forming a hypothesis ($N = 100$, $p = 0.0011$), developing an experiment to test a hypothesis ($N = 100$, $p < 0.0001$), data collection and analysis ($N = 100$, $p < 0.0001$), and using the results to support or refute their hypothesis ($N = 100$, $p = 0.0001$). For the class of nonchemistry STEM majors in 2015, the prepost data showed no significant gains in regards to experimental design but did show significant gains in structure property relationships, chromatography, and performing dilutions. The greater gains made by chemistry majors is not surprising given that the chemistry majors pursue their own research project during the latter half of the semester whereas the nonmajors perform only assigned laboratories that have some elements of inquiry. In spring 2014, however, nonchemistry majors reported gains in understanding chromatography ($N = 50$, $p < 0.0001$), understanding of green chemistry ($N = 50$, $p < 0.0001$), and using calibration curves for instrument analysis ($N = 73$, $p = 0.0000$). We suspect that the instructor of the course may have an effect on the overall gains because instructors for the nonmajors course switched from 2014 to 2015. Although we cannot attribute these significant learning gains to this laboratory experience alone, these data are promising. Specific survey questions and statistical analysis are provided in the Supporting Information.

■ CONCLUSIONS

Students successfully applied techniques in analytical chemistry and biology to solve a problem involving the composition and antibacterial properties of a real-world sample, thyme leaves. Through a process of guided exploration, in which students formulate and test hypotheses within the guiding framework of the weekly module, students developed critical reasoning and analytical skills in a research-like environment.

The laboratory experiment described integrates the authentic practice of green chemistry into the undergraduate chemistry laboratory. It was observed that not only were students engaged in solving an authentic research problem but that the experiment resulted in students better understanding the analytical techniques employed and the practice of green chemistry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00891.

Students handout provided to students in preparation for the laboratory; tips for instructors; assessment data and questions; notes for stockroom preparation; description of the HPLC method used; statistical analysis of student learning gains are provided. This information should allow for adoption at other institutions that work within the available resources while maintaining the tenets of green chemistry and guided inquiry. (PDF, DOCX)

CMaterials and cost information for the experiment. (XLSX)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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