Crafting A More Environmentally Benign Extraction and Analysis of Pharmaceutical Precursors from a Medicinal Plant: A Student-Led Innovation

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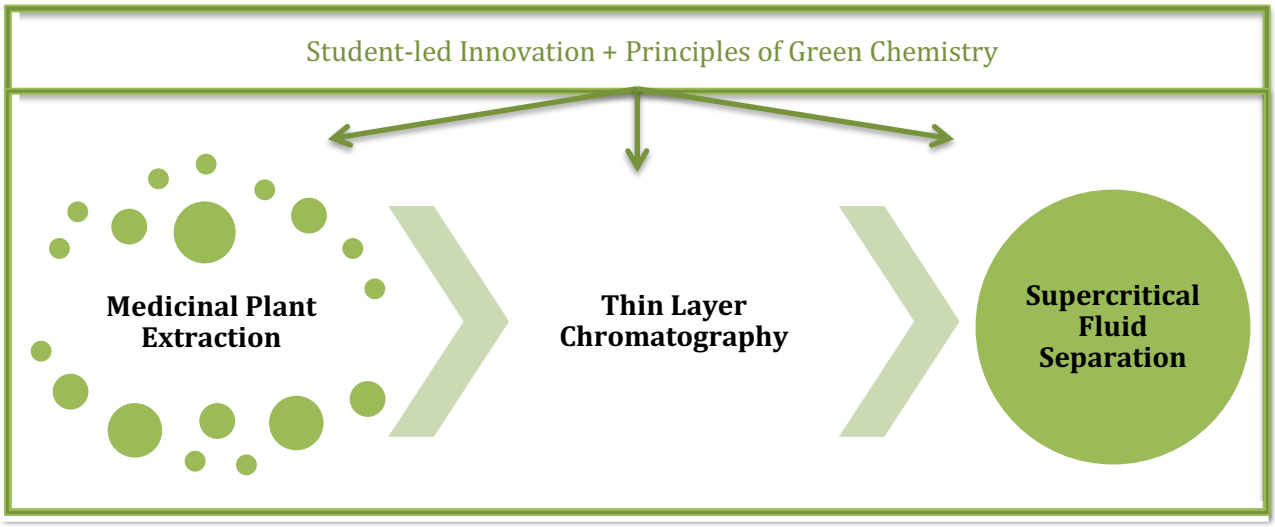
ABSTRACT

A student-led research seminar was utilized to develop and validate an innovative 4-part undergraduate chemistry laboratory module that exposes students to a more environmentally benign method for the extraction and analysis of pharmaceutical-precursor alkaloids from the leaves of a medicinal plant, the Madagascar periwinkle. This plant is well known for its production of valuable pharmaceutical alkaloids but obtaining these compounds in therapeutic amounts has relied on traditional techniques that often ignore environmental impacts. Our student-directed design team has optimized an instructional protocol for extracting alkaloids from leaves by successfully, and for the first time, replacing the traditionally used dichloromethane extraction solvent with cyclopentyl methyl ether, a less environmentally harmful solvent. As a pedagogical exercise in the principles of green chemistry, students work in teams performing extractions with conventional vs. “green” solvents for comparison. We also introduce the student to the concept of the qualitative assay for alkaloid presence. Thin layer chromatography is performed with various solvents to optimize resolution of major alkaloid components, as well as to introduce fundamental principles of chromatography to the students. Finally, supercritical fluid chromatography is utilized as a previously unexplored, and less waste-producing analytical technique for confirmation of the presence of the valuable pharmaceutical precursors vindoline and catharanthine.

GRAPHICAL ABSTRACT



KEYWORDS

First-Year Undergraduate / General, Interdisciplinary / Multidisciplinary, Laboratory Instruction, Inquiry-Based / Discovery Learning, Problem Solving / Decision Making, Green Chemistry, Student-Centered Learning, Plant Chemistry, Natural Products, Drugs / Pharmaceuticals

INTRODUCTION

Overview

Academic institutions have begun showing increased interest in redeveloping the undergraduate chemistry curriculum to incorporate the principles of green chemistry[1–3], as well as increasing student experiences with real-world industrial and academic research problems[4–6]. Following our department's signing of the 'Green Chemistry Commitment' through the Beyond Benign Institute (http://www.beyondbenign.org/he-whos-committed/) we have undertaken deliberate efforts to transform our curriculum by teaching and practicing green chemistry starting from our introductory

chemistry courses. Additionally, we have acknowledged an increasing student interest at our institution and others in broad subject areas such as environmentally conscious science, pharmaceutical research, and preparation for medical school. In our work described here, we show how student-led design teams were guided to produce and validate an undergraduate laboratory module that demonstrated the process of extracting and analyzing important pharmaceutical precursors (vindoline and catharanthine) from the Madagascar periwinkle *(Catharanthus roseus)*, a medicinal plant that has been the subject of research in our own department[7–10]. In addition, our implementation of peer-driven research teams emphasized the implementation of established principles of green chemistry[11] to produce a more environmentally benign process.

In order to isolate, identify, and quantify levels of alkaloids in the *C. roseus* plant material, labs interested in these compounds must draw on techniques similar to the extraction and analysis procedures that are overviewed in this module. However, these techniques have not previously been reevaluated to apply the principles of green chemistry. Here we describe, for the first time, a more environmentally benign technique that implements alkaloid extraction with the solvent cyclopentyl methyl ether (CPME) and analysis by supercritical fluid chromatography (SFC). The ACS Green Chemistry Pharmaceutical Roundtable has encouraged the use of CPME as a green solvent in part because exposure to it was found to have low toxicity and not cause any mutagenicity or genotoxity[12,13]. The synthesis of this solvent also meets essential principles of green chemistry as it is conducted via the addition of methanol to cyclopentene and is 100% atom efficient[14]. Furthermore, it has been shown to demonstrate low volatility, low water solubility, acidic stability, and to be less prone to peroxide formation[15].

The result of our peer-guided, green chemistry optimization is a laboratory module (**Figure 1**) that accomplished several important pedagogical goals[16]. These objectives show applicability to a more traditional curriculum as well, as listed below:

- Perform and understand the process of extracting alkaloids from a natural source.
- Test for the presence of specific alkaloids with a qualitative reagent, thin-layer chromatography, and super critical fluid chromatography with UV detection.

●Compare and contrast the outcomes of a more environmentally benign procedure with those of a more traditional method.

●Work cooperatively in student teams.

●Gain early exposure to departmental research.

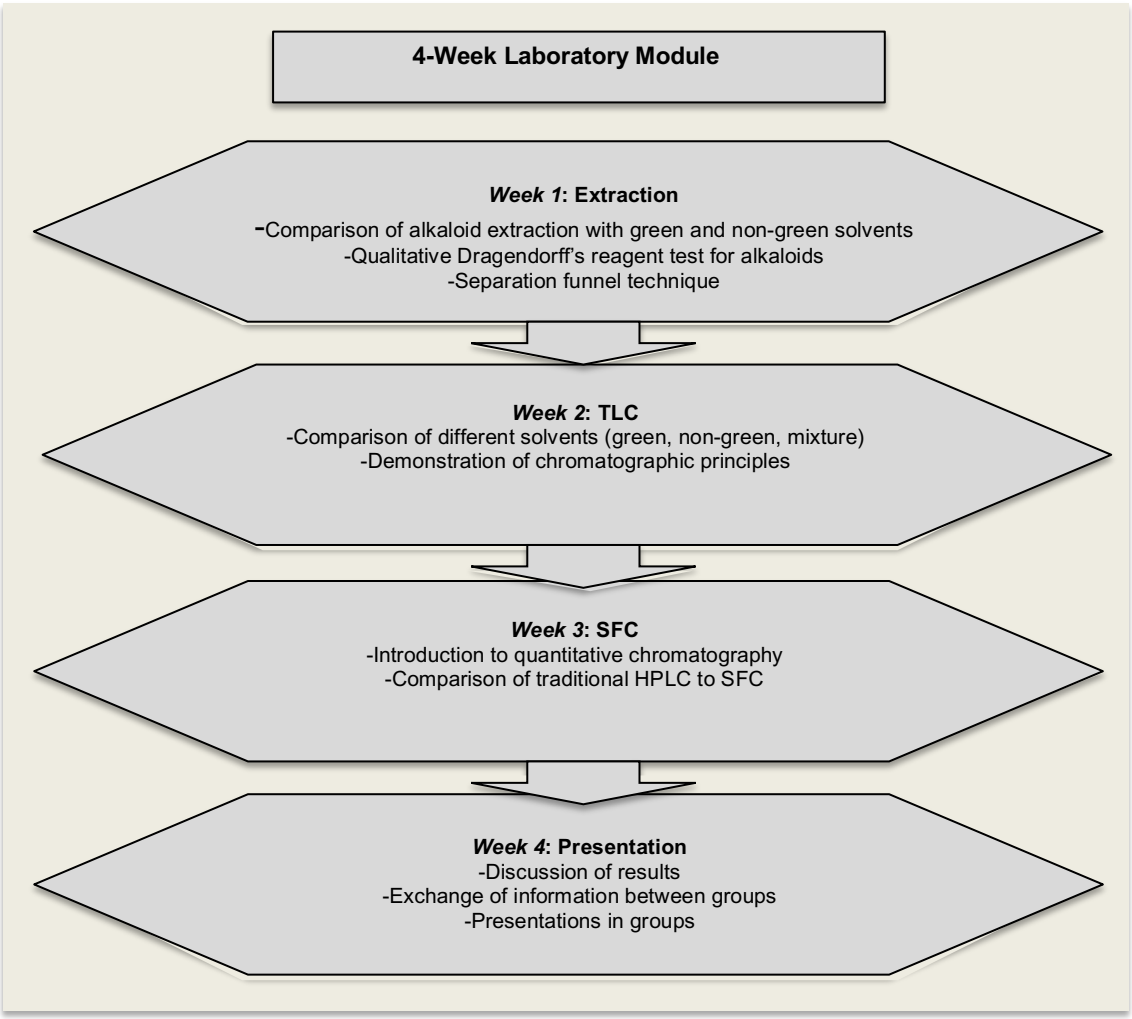


Figure 1. Module Workflow

EXPERIMENTAL OVERVIEW

Summary

The specifics of the laboratory experiment were optimized by peer-guided research teams of 5 to 8 students and each session of the experiment was repeatedly tested by multiple undergraduate student pairs for consistency and validation of results over the course of approximately one year in research seminar sessions. Each weekly session is approximately 3 hours in length. In **session one**, student

pairs compare the use of both the traditionally used dichloromethane (DCM) as well as a recognized "greener solvent" CPME[14] (Fischer Scientific, CAS# 5614-37-9) to extract and work up the alkaloid fraction from the leaves of *C. roseus*. With both extraction solvents, this fraction contains the important pharmaceutical precursors catharanthine and vindoline. In order to initially validate the presence of these compounds, the students perform thin layer chromatography (TLC) in **session two**. TLC mobile phases of solvents such as DCM, ethanol, ethyl acetate, and toluene are tested by students for good separation and contrasted based on their environmental impact. Below we describe an ideal separation with the relatively benign solvent, ethyl acetate. **Session three** was used to perform SFC to give further evidence for the presence of these two compounds. **Session four** is used for the group presentation and discussion of findings.

C. roseus leaves for these experiments were grown in the greenhouses at Brown University (see acknowledgements) but the plant is commonly found in large hardware stores and online. In addition, the dried leaves can be purchased as a tea online from several sources including:

http://www.tropilab.com/periwinkletea.html

Session One

Preparation of Dragendorff's Reagent:

Dragendorff's reagent is used to test for the presence of alkaloids. If added to a small amount of extract, the formation of a yellow-beige precipitate indicates that alkaloids are present. A stock solution was made by mixing 850 mg bismuth subnitrate (Fischer Scientific, CAS# 1304-85-4) with 10 mL of glacial acetic acid and 40 mL of water. This was mixed with 50 mL of a 50% m/v potassium iodide solution, stirred until dissolved, and stored in a dark bottle. A working solution was made with 10ml of stock, 20ml glacial acetic acid and 70ml of water and stored in a dark bottle.

Acidified solid/liquid extraction:

Approximately 200 mg of *C. roseus* leaves were ground to a fine powder in a grinder (Krups F203 Electric Spice and Coffee Grinder). (N.B. we tested from 200 mg to 1000 mg of dried, ground leaves and found acceptable results from all extracts.) The leaf powder was thoroughly mixed with approximately 20 mL of 0.5 M H_2SO_4 in a 50 mL Erlenmeyer flask. This mixture was gently heated at

approximately 205°C (mixing approximately every 3 minutes to prevent boiling) for 15 minutes to extract alkaloids. Plant material was then filtered out with vacuum filtration and discarded. A small amount of filtered solution (approx. 0.5 mL) was pipetted into a separate test tube to perform a Dragendorff's test for alkaloids. This is performed by adding a few drops of reagent to the test tube and looking for the appearance of a yellow-beige precipitate (positive for alkaloids).

Liquid/liquid separatory funnel extraction:

NH_4OH was added to the extract until a pH strip indicated the solution was alkaline and then transferred to a separatory funnel. 2 mL of CPME (or DCM) was added so that there were two well-defined layers: a green/opaque aqueous layer on bottom, and a clear organic CPME layer on the top (DCM will be on the bottom). The funnel was gently shaken to facilitate the movement of alkaloids to the nonpolar layer. After settling for 3-5 minutes, the bottom layer was drained and set aside for reextraction Extraction was repeated a total of 5 times. A small amount of the aqueous layer (approx. 0.5 mL) was pipetted into a separate test tube to perform a Dragendorff's test for alkaloids. The nonpolar layer was tested as well. Anhydrous sodium sulfate was added to the extracted organic layer to remove water that may have been carried over. This layer will be saved and ubjected to TLC and SFC in the next sessions.

Session Two

Thin-layer chromatography (TLC):

Students were encouraged to attempt TLC separation with a variety of common laboratory solvents (DCM, ethanol, ethyl acetate, toluene) influenced by combinations previously published in the literature[17]. Following this strategy, our TLC experimentation yielded good resolution on 7.5 cm by 2.5 cm plates with ethyl acetate plus one drop of NH_4OH as the mobile phase (36 ml ethyl acetate to one drop of NH_4OH) as previously demonstrated by Hernandez, et al. Approximately 5 uL of extract was spotted on the TLC plate. Exposure to shortwave UV (254 nm) showed two clear spots, corresponding to vindoline and catharanthine (**Figure 2**).

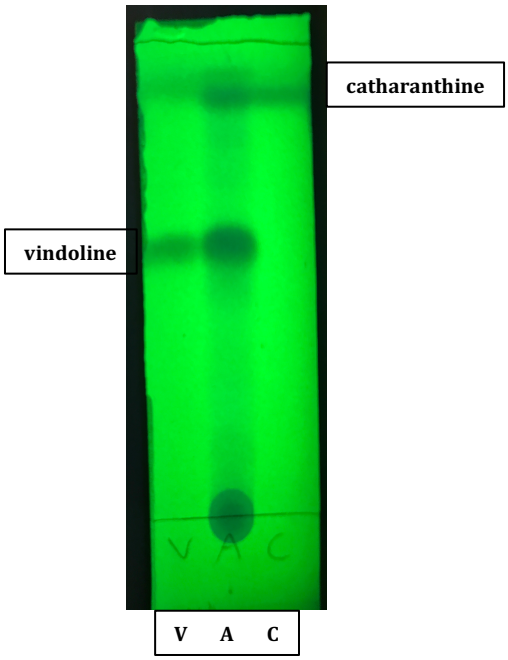


Figure 2. **Thin layer chromatography plate retention factor verification of the identity of vindoline and catharanthine in the cyclopentyl methyl ether extract of *Catharanthus roseus* leaves.** Left, V is the starting point for the vindoline standard. Right, C is for the catharanthine standard. Center, A is for the CPME extract of *C. roseus* leaves. Vindoline and catharanthine are both metabolites from different biosynthetic pathways but are found in dried leaf extracts at high levels. Visualized with a shortwave UV lamp (254 nm).

Session Three

Supercritical fluid chromatography procedure:

An Agilent, 1260 Infinity II SFC System with dual UV detection (280 and 330 nm) was used to perform this separation (**Figure 3 and supplemental figures**). The mobile phase was CO_2 with an organic modifier of methanol containing 20 mM ammonium acetate. The modifier was isocratic at 5% for 1 minute, then a gradient from 5% to 40% at 2% per min. The pressure was 150 bar, the temperature was 40°C, and the flow rate 2.0 mL/min[18,19]. 5 uL of extract was injected onto a column of 3.0 µm particles of 2-ethylpyridine silica with a pore size of 60 Angstroms (Princeton Chromatography). Column dimensions were 25 cm x 4.6 mm internal diameter.

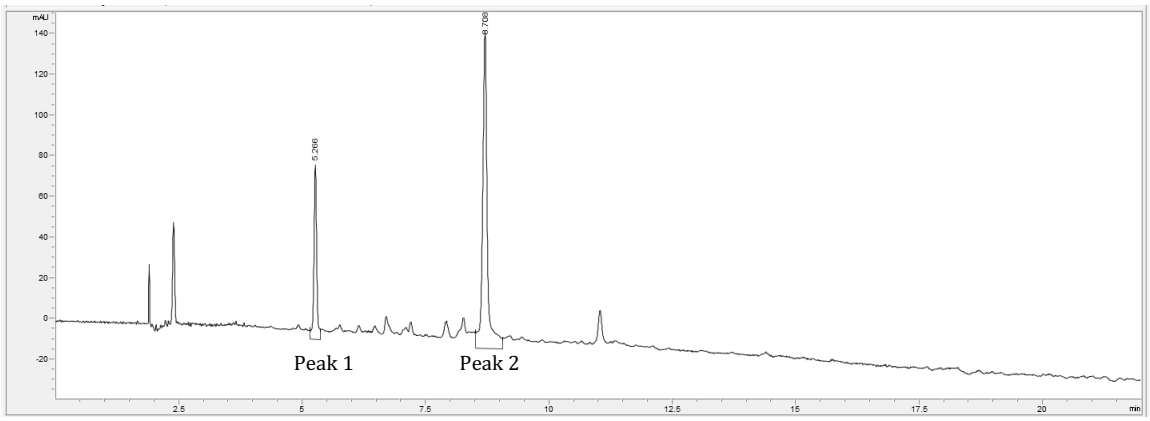


Figure 3. **Supercritical fluid extraction chromatography of cyclopentyl methyl ether extracted** *C. roseus* leaves. UV detection at 280 nm. Peak 1 at 5.266 min was verified as vindoline with a standard (Sigma-Aldrich, CAS# 2182-14-1); peak 2 at 8.708 min was verified as catharanthine with a standard (Sigma-Aldrich, CAS# 2468-21-5).

Session Four

Student presentation and discussion of results:

Student groups were instructed to prepare short presentations to lead a discussion for sharing their data using photos, short videos, graphics, data from scientific papers, and figures displaying their results. Brief guidance was given for preparing their slideshow to include an introduction, their results, and their conclusions. The results of this work were utilized to create a laboratory project for the first semester laboratory of general chemistry. As a result, the project has been used for the last four weeks of the first semester labs to help students better understand intermolecular forces and polarity differences amongst different chemical structures and how chemists use these differences in properties to separate and identify compounds. In addition, this project offers an opportunity for students to apply the 12 principles of green chemistry in their work by critically evaluating their results and ultimately choosing the most efficient and green method of extraction and characterization of the key compounds from the plant. This project also offers the opportunity to learn extraction and TLC techniques, which are subsequently used in our second semester general chemistry laboratory on biofuels and in our second year organic laboratories.

HAZARDS

It is recommended that all steps of this procedure be performed under a fume hood. CPME is extremely volatile and therefore any container should remain covered whenever possible. In a concentrated solution, both ammonium hydroxide and sulfuric acid are very corrosive. Gloves are

therefore necessary throughout this experiment. Anhydrous sodium sulfate and CPME are also potentially dangerous upon contact with the skin or eyes.

DISCUSSION

Pharmaceuticals from a Plant: The Madagascar Periwinkle

The Madagascar periwinkle, or *Catharanthus roseus*, naturally produces a subset of drugs called the vinca alkaloids, which contain the second-most used class of cancer drugs in the United States[20,21]. Two of the most important of these anticancer compounds are vinblastine and vincristine. However, *C. roseus* only produces those compounds at very low yields leading to bottlenecks in production. Fortunately, the plant produces certain precursor compounds at higher levels, which can be extracted and then coupled to produce vinblastine or vincristine. In this experiment, we demonstrate a method to isolate and analyze two of those important biosynthetic precursors— vindoline and catharanthine— in a new, more environmentally benign manner.

Vindoline and catharanthine are naturally formed in the plant from a common precursor of their own, strictosidine, and both can typically be found in the dried leaves of *C. roseus*. When under attack by a pest, the living plant upregulates enzymes in this pathway which ultimately leads to an enzymatic coupling reaction that bring vindoline and catharanthine together to form 3, 4-anhydrovinblastine, which is then converted to vincristine and vinblastine[22] (**Figure 4**). These compounds bind tubulin to stop cell division which serves the ecological function of hindering pests. In humans, they serve as potent anti-cancer drugs by arresting rapidly dividing cancer cells. A synthetic direct coupling reaction of these two natural product precursors has also been described for the production of vinblastine with an 80% yield[23,24]. Current research is focused on increasing production of these valuable compounds[20].

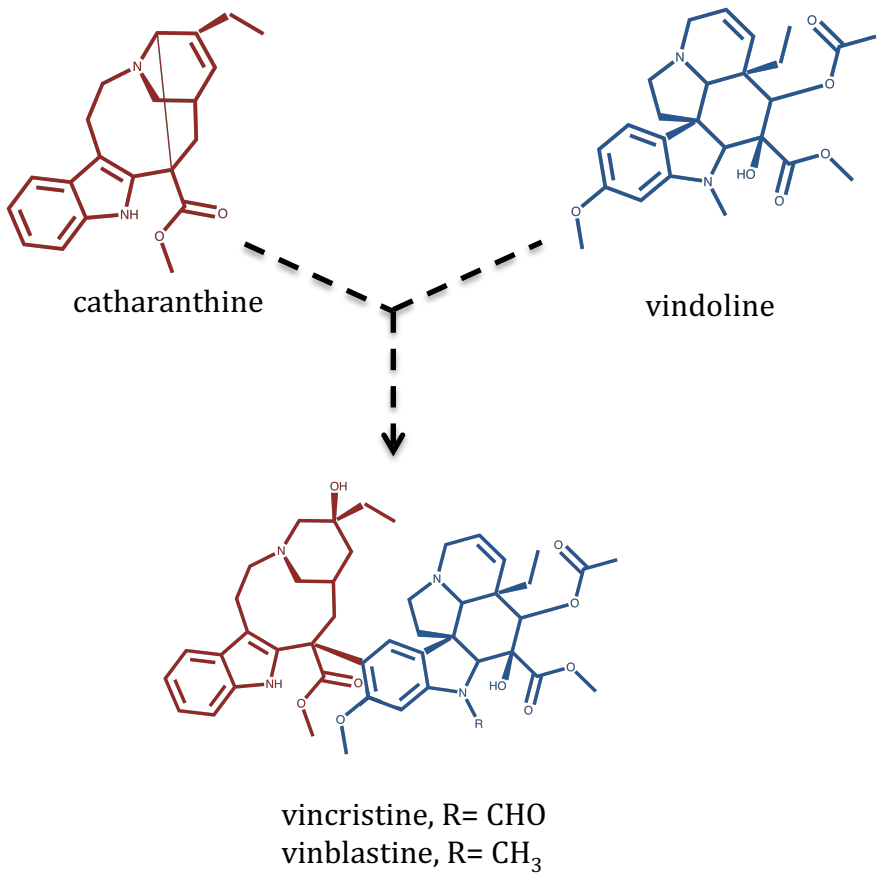


Figure 4. **The biosynthetic pathway for the production of vincristine and vinblastine.** Through an enzymatic coupling reaction, these two precursors are brought together to form 3, 4-anhydrovinblastine, which is then converted by the plant to the tubulin-binding drugs, vinblastine and vincristine. By mimicking nature, this coupling reaction has also been recreated through a synthetic reaction.

Chemical Background

Any given sample of plant material may contain thousands of compounds with various chemical and physical properties. With that in mind, it would seem quite a daunting task when we consider that any given mass of a crude extract of *C. roseus* leaves may only contain a fraction of a percent of the medicinal compounds (or their precursors) that we desire[25]. However, since these compounds are alkaloids, we can first enrich the entire alkaloid fraction.

Alkaloids derived from plants often have medicinal or bioactive properties—morphine (from the seed pod of opium poppy), quinine (from the bark of the cinchona tree), and caffeine (from the seed of the coffee plant), and of course the vinca alkaloids are a few accessible examples. The alkaline nature of alkaloids allows us to form their salts in the presence of an acid—a concept that students are exposed to when taught acid-base neutralization reactions. In this case, if we subject plant material containing alkaloids to an aqueous acidic environment, alkaloid salts will form and thus will be more

soluble in the aqueous solution. If we then raise the pH of that extracted solution, the alkaloids will return to their free base form, which are more soluble in nonpolar organic solvents. Traditional methods have relied on chlorinated solvents that have been recognized as environmentally problematic[26].

As an instructional tool, we utilize both solid/liquid and liquid/liquid extraction to isolate the alkaloid fraction. Most students are very familiar with solid/liquid extractions of alkaloids—for example, when one runs water over coffee beans to brew coffee, the alkaloid caffeine is extracted into the water. To make decaffeinated coffee, merchants in the early 1900's extracted coffee with benzene to remove the caffeine. While decaffeinating coffee with benzene has since been discontinued due to benzene's carcinogenic properties, it is still an example of environmentally harmful chemistry that will surely capture a student's attention.

In this experiment, we extract alkaloids from plant tissue (solid) into a dilute sulfuric acid mixture (liquid) to produce alkaloid sulfates. The mixture is then heated in order to make the extraction more efficient. We then raise the pH to form freebase alkaloids, which tend to be more soluble in nonpolar solvents. A separatory funnel is used to move the alkaloids from the aqueous extract (liquid) to CPME (liquid). We found CPME to be an efficient replacement for DCM for the extraction of alkaloids from *C. roseus* leaves. Since basified free alkaloids from the plant material will be more soluble in CPME than water, they move from the aqueous layer to the organic layer, which we collected for further analysis. In our optimization, CPME formed an easily visualized partition with water and effectively extracted alkaloids. We tested all stages of this experiment with the traditionally used dichlormethane (DCM), as well as another, more environmentally benign solvent 2-Methyltetrahydrofuran (2-MeTHF). While DCM also provided an excellent partition in the liquid/liquid extraction and effectively extracted alkaloids, it is a chlorinated solvent recognized as an environmental contaminant. 2-MeTHF was difficult for students to work with as it must be aliquoted under pressure and quickly formed intractable emulsions in the separatory funnel. Therefore, CPME was chosen as the "greener" alternative to DCM.

For analysis of the extracted compounds, we first utilize a qualitative test (Dragendorff's) to test the nonpolar layer to ensure that we successfully extracted alkaloids. Thin layer chromatography is then performed against known standards to give an initial verification that there are specific *C. roseus*

alkaloids present (vindoline and catharathine). An environmentally-benign method of supercritical fluid chromatography is employed as a final step to confirm the presence of vindoline and catharanthine.

Student Experience

This laboratory experiment was developed by a student-led team of undergraduate science majors (chemistry, chemical engineering, and biology) ranging from freshman to seniors, as well as a graduate student leader. The team met weekly for approximately 6 months to conceptualize all aspects of the experiment. Once developed, the team optimized each aspect of the experiment over the course of 6 months. Optimization was completed to reduce environmental impact and material usage, as well as to streamline the instructional aspects of the experiment. Some of the students participated in the writing and development of the project handouts, prelab modules and auxiliary media and learning material to be used from students enrolled in the general chemistry laboratory course (honors and chemistry majors cohort) for which the material is intended. Currently the module is implemented for 96 students enrolled in an honors general chemistry laboratory course.

Nuances of our Experimental Optimization

This project was undertaken with two main explicit goals in the design process:

1. Utilize outcomes of work performed by a cooperative team of undergraduate students, graduate students, and faculty who work together to create and optimize a project for use in a freshman undergraduate lab.

2. Allow greater involvement of undergraduates in the development process from the bench work to the development of pedagogical material.

This framework allows for a two-fold benefit to the student directly involved in the development process and to the students who will interact with the developed material in the laboratory classroom[27]. Our explicit goal was to make the resulting project best suited for the students by asking their peers to be highly involved in its preparation and optimization. Moreover, this process prepared the undergraduate researchers to ultimately become peer facilitators for the laboratory module implementation process because of their high familiarity with the developed material[28]. By testing, optimizing, and helping write this module – and fitting it within the parameters of green chemistry –

undergraduate researchers were effectively trained to work cooperatively within a design team. After internalizing the goals of the curriculum they become ready to co-facilitate the implementation with teaching assistants and faculty. In addition to these outcomes, the introduction of "greener" projects (specifically natural product extraction and identification) are an ideal platform for teaching green chemistry and how it is applied in research efforts found in our department. Our module therefore adds to the desired skill set in our undergraduate students by providing opportunities for: cooperative learning, experimental design, understanding toxicity, and the 12 principles of green chemistry. In addition, it exposes students to real-world research in chemistry and explicit research in our institution.

A Real-World "Greener" Approach

Traditional solvents used to extract plant-derived pharmaceuticals (often chlorinated and in very large volumes) are far from environmentally benign. In this designed module, students have the opportunity to experience the process of extraction and identification using those traditional solvents (e.g. DCM) and are then, after being exposed to the 12 principles of green chemistry, tasked with improving this extraction process. In this case they are specifically extracting important pharmaceutical precursors used for global health. With the newly-learned principles in mind, they create their own best design based on their experimental evidence and are then tasked with defending and rationalizing their conclusions. In the design process they are ultimately faced with answering the question of whether there is, in fact, a more environmentally benign way to complete this extraction. Such realistic experience gives an explicit opportunity to the students to apply green chemistry in a real situation and have ownership of the design process.

Learning Lab Outcomes

This laboratory was replicated more than 10 times by our student-led green chemistry optimization team in various groups of 2-4 students. From the design to the implementation process we focused in creating an environment where the students had the opportunity to **a)** Understand the basic concepts, techniques and instrumentation related to their work within the designed prelab module and quiz process **b)** Work cooperatively **c)** Engage in experimental design **d)** Use evidence to support their argument for 'what is the greenest and most efficient extraction process?' **e)** Critically think about

what is green chemistry, how it is applied to our project, and our departments research efforts. Additional pedagogical aims and how they are assessed are available in our supplemental pre and post lab questions, handouts, rubrics for peer assessment, report and presentation assessment (see Supporting Information).

CONCLUSION

Accomplishments and Possibilities for Customization

Our work shows that a team of undergraduate students in collaboration with a graduate teaching assistant and faculty support can successfully develop a laboratory module for use in introductory general chemistry labs. Further, this module was used to teach basic extraction, TLC techniques, and SFC for the analysis of pharmaceutical precursors from Madagascar periwinkle leaves. This experience was used to connect student work to concepts of intermolecular forces, polarity, and the 12 principles of green chemistry through the successful development of a green extraction protocol. We also observed that deep undergraduate involvement in the development and implementation process of the laboratory curriculum can be a very successful way of undertaking such efforts.

We recognize several benefits of offering this valuable research experience while also having students involved in the module-development process. This platform creates a sense of ownership while also preparing students for the mentorship of incoming undergraduates. Those students will then encounter this project in an introductory laboratory course geared to teach cooperative learning, green chemistry, experimental design, basic extraction and identification techniques. In our case, this module also creates familiarity with our departmental research.

We have undertaken to continue this workflow for additional modules with revolving teams of undergraduates. These teams are currently engaging in this circular process to help the development of a set of “green chemistry”-themed projects while they also support the complete reevaluation of the introductory laboratory modules in our program. Further plans are underway to assess the pedagogical benefits and learning gains of the implemented curriculum in our institution with the combined support of our undergraduate mentors, graduate teaching assistants, and faculty.

ASSOCIATED CONTENT

Supporting Information

Student handout with post lab questions, Prelab quiz, Grading rubric for oral project presentations. Also, additional SFC analysis of standard solutions of vindoline and catharanthine used for comparison.

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