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TITLE:

Employing Pressurized Hot Water Extraction (PHWE) to Explore Natural Products Chemistry in the Undergraduate Laboratory

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SHORT ABSTRACT:

Here, we employ a pressurized hot water extraction (PHWE) method, which utilizes an unmodified household espresso machine to introduce undergraduates to natural products chemistry in the laboratory. Two experiments are presented: PHWE of eugenol and acetyleugenol from cloves and PHWE of seselin and (+)-epoxysuberosin from the Australian plant *Correa reflexa*.

LONG ABSTRACT:

A recently developed pressurized hot water extraction (PHWE) method which utilizes an unmodified household espresso machine to facilitate natural products research has also found applications as an effective teaching tool. Specifically, this technique has been used to introduce second- and third-year undergraduates to aspects of natural products chemistry in the laboratory. In this report, two experiments are presented: the PHWE of eugenol and acetyleugenol from cloves and the PHWE of seselin and (+)-epoxysuberosin from the endemic Australian plant species *Correa reflexa*. By employing PHWE in these experiments, the crude

clove extract, enriched in eugenol and acetyleugenol, was obtained in 4–9% w/w from cloves by second-year undergraduates and seselin and (+)-epoxysuberosin were isolated in yields of up to 1.1% w/w and 0.9% w/w from *C. reflexa* by third-year students. The former exercise was developed as a replacement for the traditional steam distillation experiment providing an introduction to extraction and separation techniques, while the latter activity featured guided-inquiry teaching methods in an effort to simulate natural products bioprospecting. This primarily derives from the rapid nature of this PHWE technique relative to traditional extraction methods that are often incompatible with the time constraints associated with undergraduate laboratory experiments. This rapid and practical PHWE method can be used to efficiently isolate various classes of organic molecules from a range of plant species. The complementary nature of this technique relative to more traditional methods has also been demonstrated previously.

INTRODUCTION:

The isolation and identification of natural products are of fundamental importance to the scientific community and society more generally.[1] Bioprospecting, the search for valuable organic molecules found in nature, remains an indispensable process in the discovery of new drug leads and potential therapeutic agents. It is estimated that from 1981–2014, ~75% of all approved small molecule pharmaceutical drugs were natural products, natural product-derived or natural product-inspired.[1] Furthermore, natural products possess enormous structural and chemical diversity. For this reason, they also represent valuable chemical scaffolds that can be directly used in organic synthesis or in the development of chiral ligands and catalysts.[2,3]

Traditionally, relatively time-intensive procedures such as maceration, Soxhlet extraction, and steam distillation have been the mainstay of research focused on the isolation of secondary metabolites from plants.[4] More modern extraction techniques, including accelerated solvent extraction, have focused on reducing extraction times and establishing greener protocols.[4,5] In 2015, an original pressurized hot water extraction (PHWE) method was reported.[6] This technique employed an unmodified household espresso machine to facilitate the rapid and particularly efficient extraction of shikimic acid from star anise. Espresso machines have been specifically designed and engineered to extract organic molecules from appropriately ground coffee beans. To achieve this, these instruments heat water at temperatures up to 96 °C and at pressures of typically 9 bar.[7] With this in mind, it is perhaps not surprising that espresso machines can be utilized to efficiently extract natural products from a range of plant material.

Subsequent studies involving a variety of terrestrial plant species have demonstrated the capacity of this PHWE technique to efficiently extract natural products across a relatively broad polarity range.[6,8–15] Furthermore, compounds containing somewhat sensitive functional groups, such as aldehydes, epoxides, glycosides, and potentially epimerizable stereogenic centers were typically unaffected by the extraction process. The complementary nature of this technique relative to more traditional methods has also been demonstrated.[12,16] This PHWE method has also been employed to isolate multi-gram quantities of natural products, which have been used to prepare novel natural product derivatives and in complex molecule synthesis more generally.[8,11,17]

It was identified that this new PHWE method could serve as a useful teaching tool that could be incorporated in the undergraduate laboratory. This primarily derives from the rapid nature of this technique relative to the traditional extraction methods that are often incompatible with the time constraints associated with undergraduate laboratory experiments. Consequently, this technique supplanted the traditional undergraduate chemistry laboratory experiment focused on the extraction of eugenol from cloves employing steam-distillation at the University of Tasmania.[9,18] Since that time, variations of this experiment have been adopted by other universities and a modified experiment focusing on the PHWE of cloves now features in the undergraduate chemistry laboratory program at the University of Sydney (*vide infra*).

In order to demonstrate the practicality and feasibility of employing this new PHWE approach for teaching purposes, two protocols are presented as part of this study. The first part of this report highlights an experiment on the PHWE of eugenol and acetyleugenol from cloves which is part of the second-year undergraduate laboratory program at the University of Sydney (**Figure 1**). This experiment serves to introduce students to natural products chemistry while developing fundamental practical skills. The second part features an experiment on the PHWE of the endemic Australian plant species *Correa reflexa* which is part of the third-year undergraduate laboratory program at the University of Tasmania (**Figure 2**). This experiment is designed to simulate natural products bioprospecting and reinforce core laboratory techniques.[11]

PROTOCOL:

Note: It is advisable that all procedures are performed in a fume hood. Students must wear appropriate personal protective equipment at all times in the laboratory and the safety data sheets (SDS) associated with each reagent must be consulted before use.

1. PHWE of Cloves: Isolation of Eugenol and Acetyleugenol

1.1. Extraction of eugenol and acetyleugenol from cloves

1.1.1. Place coarsely ground cloves (12.5 g) in a 250-mL beaker.

1.1.2. Add sand (12.5 g) to the clove grinds and mix well.

1.1.3. Collect a portafilter (sample compartment) and load the basket with the entire clove-sand mixture. Lightly compress the sample with the tamper.

NOTE: Do not compress the mixture too much or fluid will not flow through.

1.1.4. Position the portafilter into the espresso machine and place a clean 250-mL beaker beneath it. Add a 30% ethanol/H_2O solution to the water tank of the espresso machine if it is less than half full.

1.1.5. Use the espresso machine to collect 100 mL of the extract.

NOTE: Consult an instructor if the machine appears to be clogged.

1.1.6. Allow the portafilter to finish dripping and then remove it from the espresso machine.

CAUTION: The grinds and surrounding metal areas will be hot.

1.1.7. Using a spatula, remove the clove grinds from the portafilter and discard into the waste bin.

1.1.8. Rinse out the residual solids from the portafilter with H_2O under a tap in the sink and return it for the next person to use.

1.1.9. Cool the clove extract in an ice bath until the temperature has reduced to at least 30 °C.

1.1.10. Place the extract into a 250-mL separatory funnel, add 30 mL of hexane and shake gently.

1.1.11. Place the separating funnel in a ring clamp fitted to a retort stand and allow the aqueous and organic layers to separate then collect the aqueous (lower) layer back into the 250-mL beaker.

NOTE: It can take upto 10 min for the layers to separate. Students are advised to carry out solvent optimization of TLCs while waiting for the first separation to occur (see steps 1.2).

1.1.12. Transfer the organic (top) layer (which contains the product) to a clean 250-mL conical flask, and then pour the bottom (aqueous) layer back into the separatory funnel.

1.1.13. Extract the aqueous layer a further two times with hexane (2 x 30 mL).

1.1.14. Combine the organic (top) layers into the same flask after each extraction.

1.1.15. After the third liquid-liquid extraction, pour the combined organic extract into the separatory funnel and wash with 100 mL of H_2O by shaking vigorously. Collect the organic (top) layer into a clean 250-mL conical flask and dry by adding $MgSO_4$ and swirling the flask.

1.1.16. Filter the ensuing mixture through fluted filter paper contained in a glass funnel into a pre-weighed 250-mL round-bottom flask.

NOTE: The solid residue (hydrated $MgSO_4$) can be discarded in the waste.

1.1.17. Evaporate the solvent (hexane) from the collected filtrate using a rotary evaporator (water bath temperature: 60 °C, vacuum pressure: 350 mbar) and re-weigh the flask containing

the resultant oil.

1.2. Optimization of thin-layer chromatography (TLC) solvent system

NOTE: As a group, students will be assigned a solvent system from 100:0 acetone:cyclohexane to 0:100 acetone:cyclohexane by the demonstrator to identify the ratio that provides the maximum resolution of eugenol from acetyleugenol. These solutions were prepared by lab technicians prior to the lab session.

1.2.1. Obtain a TLC reference solution of pure eugenol and acetyleugenol.

NOTE: The necessary TLC reference solutions of pure eugenol and acetyleugenol were prepared by lab technicians prior to the lab session.

1.2.2. On a TLC plate, mark a *baseline* ~1.5 cm from the bottom with a soft pencil. Mark off three equally spaced points.

1.2.3. Use a TLC spotter to spot one drop of pure eugenol TLC reference solution in one lane, one spot of pure acetyleugenol TLC reference solution in the third lane and a spot of each in the second lane (the co-spot).

1.2.4. Check for the presence of eugenol and acetyleugenol on the TLC plate by viewing the plate under a UV lamp (254 nm) in the TLC viewing cabinet.

NOTE: There should be small (1–2 mm wide) black spots on the plate where the TLC reference solutions were spotted. If there are no spots or the spots appear faint, apply another spot of the appropriate TLC solution until a black spot is observed under UV light.

1.2.5. Add 10 mL of the allocated solvent mixture to a clean, dry TLC jar.

NOTE: Ensure the solvent height in the jar does not exceed ~1 cm.

1.2.6. Using tweezers, place the prepared TLC plate into the TLC jar. Close the lid of the jar.

NOTE: The solvent must lie below the baseline of the TLC plate.

1.2.7. Allow the solvent to travel up the TLC plate. Once the solvent is ~1 cm from the top of the plate, remove the TLC plate from the jar with tweezers and mark the line of the solvent front with a pencil.

1.2.8. Allow the solvent to evaporate from the TLC plate (~1 min) then view the TLC plate under a UV lamp (254 nm). Using a pencil, circle the black spots observed on the TLC plate.

1.2.9. Calculate the retention factor (R_f) of eugenol and acetyleugenol by dividing the distance

traveled by the compound by the distance traveled by the solvent.

1.2.10. Calculate the difference between the R_f values for eugenol and acetyleugenol (ΔR_f).

1.2.11. Share the results with the rest of the class. Record the retention values acquired by other students with other solvent ratios.

1.2.12. Identify which solvent system will be best to analyze the crude eugenol solution and subsequent purification steps.

NOTE: The best TLC solvent ratio will provide the greatest separation between eugenol and acetyleugenol denoted by the largest ΔR_f value. If ΔR_f is plotted against solvent composition, the graph should resemble a bell curve.

1.3. Separation of eugenol and acetyleugenol by liquid-liquid extraction

1.3.1. Add hexane (10 mL) to the crude eugenol-containing extract obtained from step 1.1.17 and pour the ensuing solution into a 250-mL separatory funnel.

1.3.2. Rinse the round-bottom flask with hexane (10 mL) and add this to the separatory funnel.

1.3.3. Extract the hexane solution with 3 M aqueous NaOH (2× 25 mL) via liquid-liquid extraction. Collect and combine the aqueous bottom layers in a 250-mL conical flask from each extraction. Collect the organic layer in a 50-mL conical flask and dry by adding $MgSO_4$ and swirling the flask.

NOTE: NaOH is corrosive. Avoid any contact with skin. Acetyleugenol remains in the organic layer, while eugenol is now in the alkaline aqueous extract (bottom layers).

1.3.4. Retain the organic layer (organic solution A) for later analysis.

1.3.5. Swirl the conical flask that contains the alkaline aqueous fraction from step 1.3.3 in an ice-water bath and slowly add 10 M aqueous HCl until a white emulsion is formed; check its acidity with Congo red paper, using a pipette to transfer a drop of the solution onto the pH paper (it should turn blue).

CAUTION: HCl is corrosive. Avoid any contact with skin. Addition of HCl can cause vigorous bubbling, HCl should be added carefully, keeping the conical flask on ice. A total of 20–30 mL of HCl (10 M of an aqueous solution) will be required.

1.3.6. Extract the milky aqueous emulsion with hexane (2× 30 mL), using liquid-liquid extraction in a 250 mL separating flask. Make sure that the temperature of the aqueous extract is at room temperature or below before adding the hexane. Combine the two hexane extracts into a clean 100 mL conical flask.

NOTE: The eugenol will now be in the combined organic (top) layers (organic solution B).

1.3.7. Add $MgSO_4$ to dry organic solution B.

1.3.8. Filter organic solution B through fluted filter paper into a pre-weighed 250-mL round-bottom flask. Discard the solid residue (hydrated $MgSO_4$) in the waste.

1.3.9. Remove the solvent from the round-bottom flask using a rotary evaporator (water bath temperature: 60 °C, vacuum pressure: 350 mbar).

1.3.10. Add diethyl ether (5 mL) to the ensuing sample in the round-bottom flask and transfer the purified eugenol into an unlabeled, pre-weighed vial using a funnel.

1.3.11. Rinse the flask with further diethyl ether (5 mL) into the vial. Evaporate the solvent using a rotary evaporator (water bath temperature: 50 °C, vacuum pressure 800 mbar) with a vial attachment. Record the yield and label the vial appropriately.

1.3.12. Analyze organic solution A, organic solution B, pure eugenol TLC reference and acetyleugenol TLC reference by TLC using the optimized TLC solvent ratio identified in the previous session.

Note: Aqueous solutions can be poured down the sink for disposal. Hexane and ether waste should be disposed of in the non-chlorinated organic waste bottles.

2. PHWE of *Correa reflexa*: Isolation of Seselin and (+)-Epoxysuberosin

2.1. Session 1. PHWE of *Correa reflexa*

2.1.1. Grind *Correa reflexa* leaves (10 g) in an electric spice grinder and then transfer the ground plant material to a 100-mL beaker.

NOTE: Grinding should take 20–30 s.

2.1.2. Add ~ 2 g of coarse sand to the beaker containing plant material.

2.1.3. Mix and pack into the basket of the portafilter (sample compartment). Compress the sample with the tamper.

NOTE: Do not pack the sample too tightly.

2.1.4. Add ~300 mL of 35% ethanol/H_2O solution to the espresso machine tank.

2.1.5. Position the portafilter into the espresso machine and place a clean 250-mL beaker

beneath it.

2.1.6. Collect ~100 mL of extract, wait for ~1 min and then collect a further 100 mL.

CAUTION: The machine and extracts will be hot at this point.

2.1.7. Cool this mixture in the ice bath and evaporate the ethanol using a rotary evaporator (water bath temperature: ~40 °C).

2.1.8. Transfer the aqueous extract to a separatory funnel and extract with ethyl acetate (4x 50 mL).

NOTE: Time may be needed to allow emulsions to separate between extractions.

2.1.9. Combine the organic extracts, dry by adding $MgSO_4$ and swirling the flask, filter using a sintered glass funnel, and evaporate using a rotary evaporator (water bath temperature: ~35 °C) to provide the crude extract.

2.1.10. Obtain an ^1H nuclear magnetic resonance (NMR) spectrum (see the instructor for assistance).[11]

2.1.11. Perform TLC analysis of the crude extract to determine an appropriate solvent system to isolate the compounds that have been extracted.

NOTE: TLC analysis is performed by analogy to procedures outlined in step 1.2.

2.2. Session 2. Separation of seselin and (+)-epoxysuberosin by flash column chromatography[11,19]

NOTE: The following protocol involves the use of flash column chromatography for the separation of organic compounds. Please consult an instructor to demonstrate how to pack a flash silica gel column.

2.2.1. Place the column (~30 mm in diameter) in a clamp fitted to a retort stand. Place a 100-mL conical flask underneath the column.

2.2.2. Fill the column with silica gel (60 μm flash grade) to a level of ~10 cm and then add hexanes (~100 mL) to the column.

2.2.3. Place a glass stopper in the column, remove the column from the clamp and shake to obtain a slurry. Place the column in the clamp and then allow the mixture to settle.

2.2.4. Open the tap of the column and, using a gas adaptor attached to a compressed air line, empty the column to leave ~2mm of solvent above the bed of silica gel. Remove the gas

adaptor then close the tap.

2.2.5. Use the hexanes collected in the conical flask (~5 mL) to wash down any silica gel from the walls of the column with a Pasteur pipette fitted with a rubber septum.

2.2.6. Repeat step 2.2.4 then add a small layer of sand (~1 cm) to the column.

2.2.7. Add dichloromethane (~ 1mL) to the flask containing the crude extract from step 2.1.8. Carefully load the ensuing solution onto the column using a Pasteur pipette fitted with a rubber septum. Open the tap of the column and allow the sample to adsorb onto the silica gel.

2.2.8. Repeat step 2.2.7 a further two times.

2.2.9. Carefully add hexanes (~20 mL) to the column. Repeat step 2.2.4.

2.2.10. Carefully add (~180 mL of 15% ethyl acetate/hexanes solution). Open the tap of the column and, using a gas adaptor attached to a compressed air line, empty the column to leave ~2 mm of solvent above the bed of silica gel, collecting the fractions into 10-mL test tubes.

NOTE: This will allow seselin to be isolated.

2.2.11. Combine the test tube fractions containing seselin in a 250-mL round bottom flask and evaporate using a rotary evaporator (water bath temperature: ~35 °C).

NOTE: TLC analysis is used to determine this and is performed by analogy to procedures outlined in step 1.2.

2.2.12. Carefully add (~75 mL of 25% ethyl acetate/hexanes solution). Open the tap of the column and, using a gas adaptor attached to a compressed air line, empty the column to leave ~2 mm of solvent above the bed of silica gel, collecting the fractions into 10-mL test tubes.

NOTE: This will allow (+)-epoxysuberosin to be isolated.

2.2.13. Combine the test tube fractions containing (+)-epoxysuberosin in a 250-mL round bottom flask and evaporate using a rotary evaporator (water bath temperature: ~35 °C).

NOTE: TLC analysis is used to determine this and is performed by analogy to procedures outlined in step 1.2.

2.2.14. Samples of the isolated compounds are analyzed using NMR spectroscopy.[11]

Note: NMR spectroscopy experiments are performed by a lab technician.

REPRESENTATIVE RESULTS:

PHWE of cloves. When attempting to perform the liquid-liquid extraction step, students often encountered emulsions (the addition of brine was typically not effective). At this stage, students were instructed to allow the mixture to stand in the separating funnel while they explored the effects of eluent composition on the separation of eugenol and acetyleugenol by TLC. It should be noted that hexane can be substituted with either heptane or dichloromethane in the liquid-liquid extraction step.[9] Students were allocated a TLC solvent ratio of acetone and cyclohexane and provided with pure standards of eugenol and acetyleugenol and then performed TLC analysis (**Figure 3**). Their results were tabulated on a whiteboard, and the effects of solvent composition on the retention factor (R_f) and the optimum eluent were considered in a group discussion (**Table 1**). The optimum solvent compositions identified by students typically ranged from 5–20% acetone/cyclohexane with a ΔR_f between 0.1–0.2.

Following TLC eluent optimization, students returned to their eugenol extractions. The crude clove extract (consisting mainly of eugenol and acetyleugenol) was isolated in 4–9% w/w. In the second session of this experiment, students exploited the different acid-base properties of the two major organic molecules to separate them by liquid-liquid extraction. Typically, eugenol was isolated in a yield of 45–65% w/w of the crude extract while acetyleugenol was isolated in a yield of 5–10% w/w of the crude extract. Students then utilized the optimized eluent (identified as outlined above) to determine the success of their liquid-liquid extraction by comparison of their extracts to the pure reference samples by TLC (**Figure 3**). Students also analyzed their crude clove extract, and their purified eugenol and acetyleugenol samples by performing Fourier-transform infrared (FTIR) spectroscopy.[9] Solvent or water peaks were occasionally observed in IR spectra due to poorly executed work-up procedures (or poor sample preparation).

Advanced students committed approximately half of their isolated crude oil to the liquid-liquid extraction described above and subjected the other portion to flash column chromatography (more information is provided in the supporting information). Although completing the liquid-liquid extraction and flash column chromatography steps in a single four-hour session may appear rather ambitious this was achievable for most of the advanced students undertaking this experiment. The complete separation of eugenol from acetyleugenol by flash column chromatography was rarely achieved due to their close retention factors (**Figure 4**). However, students were generally able to collect a few fractions containing pure eugenol. Advanced students were then asked to comment on the two different purification techniques as part of their report.

PHWE of *Correa reflexa.* Students performed the PHWE of *Correa reflexa* with minimal assistance from the laboratory instructor. During the liquid-liquid extraction step emulsions typically formed and students often were required to allow the mixture to stand in the separatory funnel (~0.25 h) with periodic agitation of the mixture with a glass rod. The chromatographic purification of the crude extract was comfortably completed within the four-hour laboratory session by students. Seselin and (+)-epoxysuberosin were isolated in yields of up to 1.1% w/w and 0.9% w/w, respectively and isolated samples of both compounds were

analyzed by ^{1}H and ^{13}C NMR and FTIR spectroscopy (**Figure 2**). While students undertook the FTIR spectroscopy experiments and prepared samples for NMR spectroscopy, lab technicians performed NMR spectroscopy experiments. The results obtained by students were consistent with the previously published work.[11]

Although this has not been presented in this report, in practice, this experiment also features a second part that challenges students to perform the extraction a plant species that has not been studied employing PHWE (more information is provided in the supporting information).

FIGURE AND TABLE LEGENDS:

Figure 1. PHWE of cloves.[9]

Figure 2. PHWE of *Correa reflexa.*[11]

Figure 3. A representative TLC plate prepared by a student. (10% acetone/ cyclohexane elution). Lane 1 (E): eugenol standard; lane 2 (crude): crude clove extract; lane 3 (A): acetyleugenol standard).

Table 1. Table of retention factors outlining the effect of eluent composition on R_f.

Figure 4. Representative TLC plates prepared by students. Left: A TLC plate analyzing the outcome of the liquid-liquid extraction step (10% acetone/ cyclohexane elution). Lane 1 (E): eugenol reference standard; lane 2 (LEB): eugenol-containing organic extract; lane 3 (A): acetyleugenol reference standard; lane 4 (LEN): acetyleugenol-containing organic extract. Right: A TLC plate analyzing the outcome of flash column chromatography step (10% acetone/ cyclohexane elution). Numbers on the TLC plate relate to the test-tube fraction number.

DISCUSSION:

The classical procedure for isolating eugenol from cloves by steam distillation has been part of the intermediate chemistry laboratory program at the University of Sydney for decades but was modernized to employ PHWE methodology in 2016 (**Figure 1**).[9,18] This provided a number benefit. Firstly, utilizing household espresso machines in the laboratory environment immediately fascinated and engaged students by illustrating the application of a non-classical, alternative method to affect a traditional scientific study. In addition, this new method reduced the time taken to complete the extraction and enabled the incorporation of additional exercises into this new iteration of the experiment. Specifically, this allowed thin-layer chromatography (TLC) to be introduced (and flash column chromatography for advanced students).

The experiment focusing on the PHWE of cloves was designed as an introductory laboratory experience for second-year undergraduate chemistry students and for this reason, it features expository teaching methods.[9] This more prescriptive, recipe-style procedure allows students with somewhat limited experience in organic chemistry to efficiently complete the extraction of eugenol from cloves. In this experiment, concepts such as acid-base extraction of acidic

compounds, utilizing TLC to identify suitable eluent composition for chromatography, and the use of a rotary evaporator are introduced. In complementary components undertaken during the two allocated sessions, students in the advanced stream of intermediary chemistry also separated eugenol and acetyleugenol by column chromatography and determined the identity of the extracted components using TLC. In the second session, students could critically compare the two separation methods. In general, students were able to complete the overall experiment within the allocated two four-hour periods with minimal instruction.

The experiment focusing on the PHWE and isolation of seselin and (+)-epoxysuberosin from *Correa reflexa* was developed for more experienced students third-year undergraduate chemistry students. Notably, this learning exercise was a result of a study originating in the research laboratory.[11] The first iteration of the experiment was incorporated into the third-year undergraduate chemistry laboratory program at the University of Tasmania in 2015. After two years of revisions and re-evaluation, this experiment was performed by a third-year undergraduate class for the third time in 2017.

This experiment was specifically designed as a guided-inquiry-based activity that strives to simulate some of the approaches employed in natural products research laboratories and features minimal written instructions. This is a student-directed learning experience and the laboratory instructor plays a key role in assisting students as they work through the experiment by providing direction as required. In this experiment, students develop key laboratory skills in chromatography and employ NMR spectroscopy to perform structure elucidation. This laboratory experience reinforces the concept of bioprospecting which is presented to students in the classroom and this can be extended to studies on previously unstudied plant material to provide a more representative experience of natural products bioprospecting. *C. reflexa* is an endemic Australian plant species, however, this sample can be substituted for appropriate leaf material from other terrestrial plant species in this experiment.

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DISCLOSURES:

The authors have nothing to disclose.

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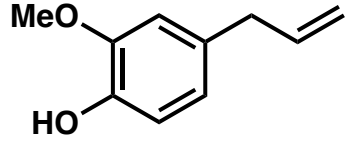
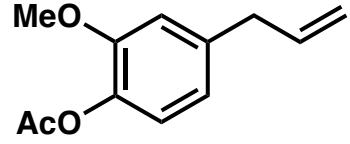
cloves

PHWE:
30% EtOH/ H_2O



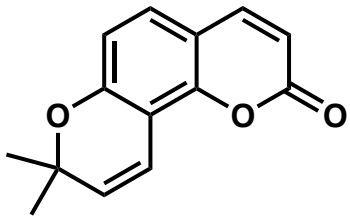
eugenol



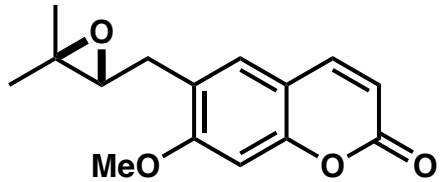
acetyleugenol

Correa reflexa

PHWE:
35% EtOH/ H_2O



seselin



(+)-epoxysuberosin

Figure 3

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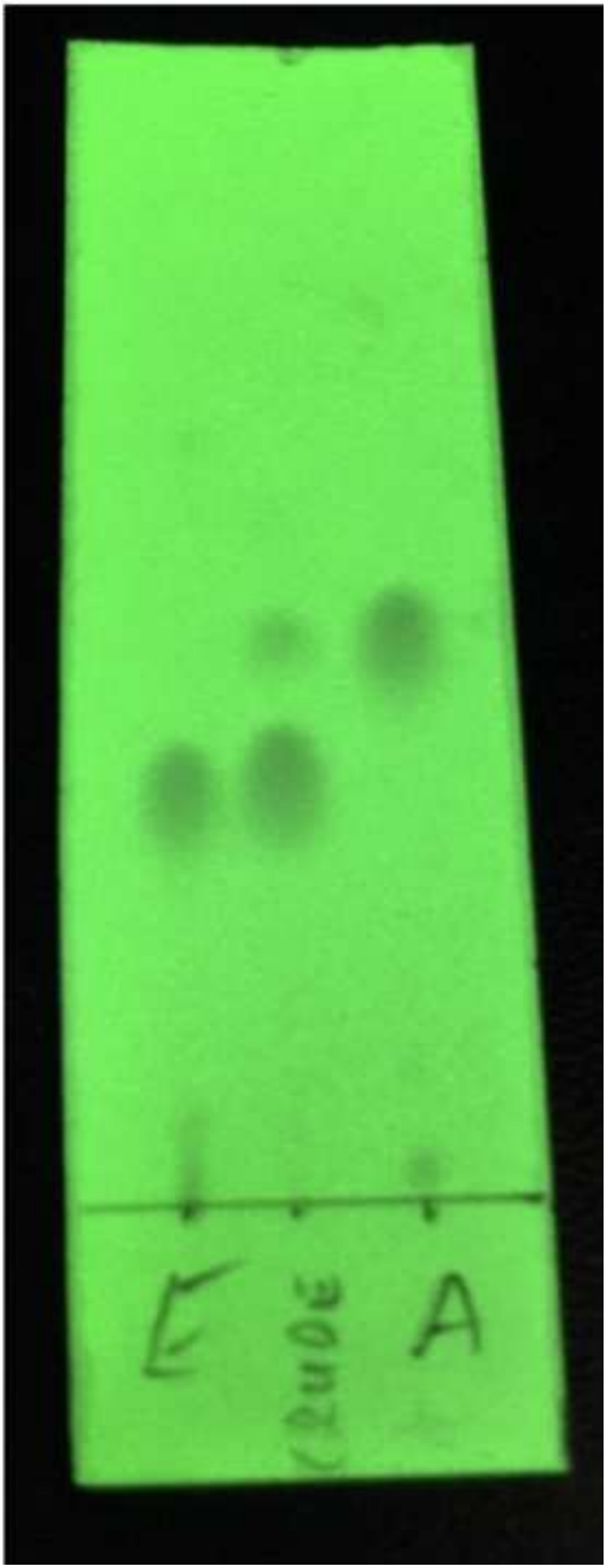

Figure 4

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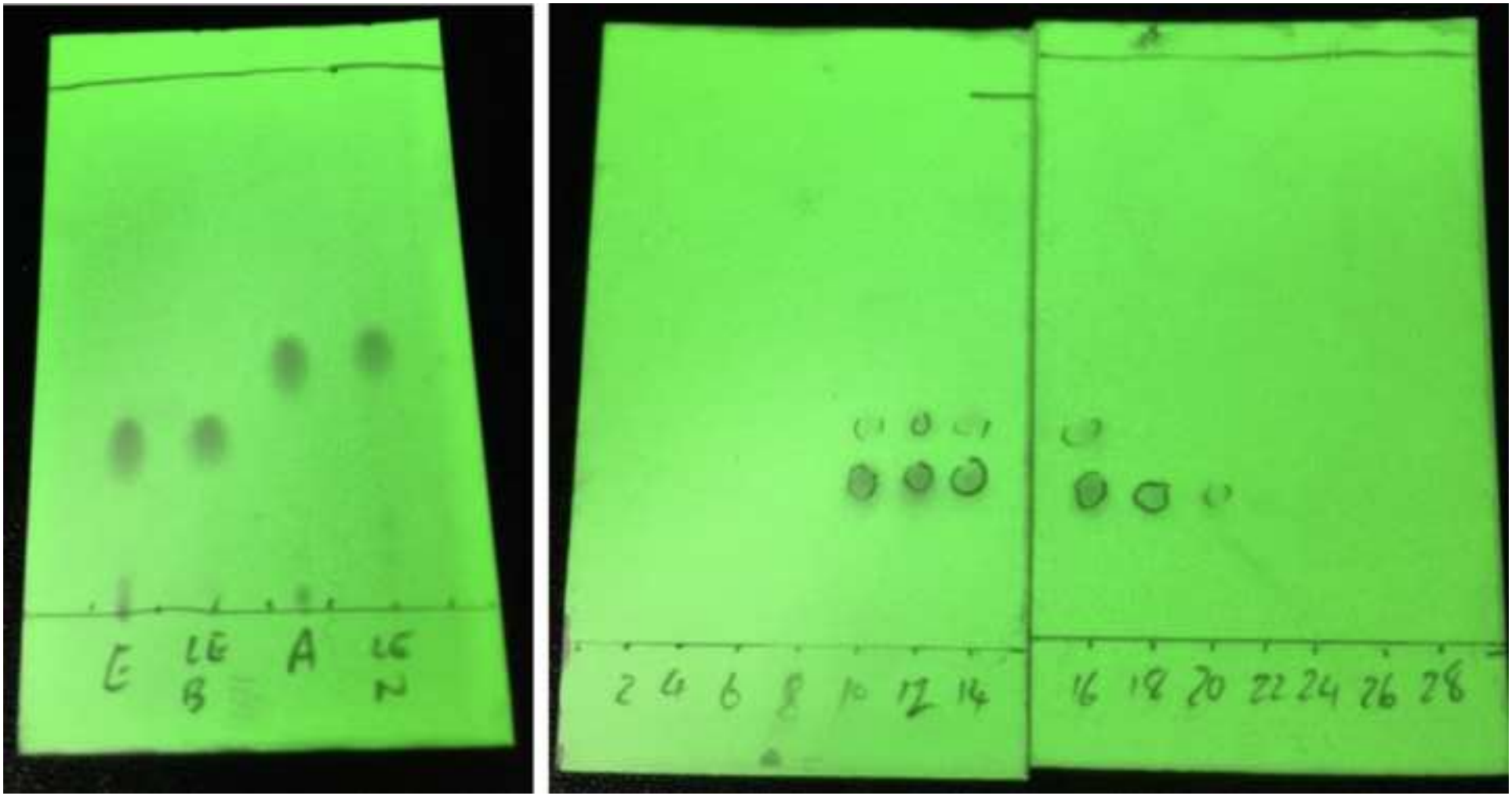

Table 1. Left: Table of retention factors outlining the effect of eluent composition on R_f.

acetone/ cyclohexane (%v/v)	mean acetyl-eugenol R_f	acetyl-eugenol R_f (σ)	mean eugenol R_f	eugenol R_f (σ)	mean ΔR_f	number of TLC analyses
0	0.06	0.08	0.04	0.06	0.02	12
5	0.34	0.11	0.27	0.09	0.07	15
10	0.45	0.07	0.34	0.05	0.12	20
20	0.51	0.07	0.41	0.06	0.10	20
30	0.58	0.10	0.49	0.12	0.10	19
40	0.63	0.08	0.56	0.08	0.07	16
50	0.76	0.08	0.73	0.08	0.03	17
60	0.77	0.13	0.73	0.15	0.04	12
70	0.84	0.13	0.81	0.13	0.03	11
80	0.90	0.06	0.87	0.08	0.02	10
90	0.88	0.06	0.87	0.05	0.01	11
100	0.87	0.13	0.86	0.14	0.02	6

Materials Table

Click here to download Table of Materials JoVE_Materials.xlsx

Name of Material/ Equipment	Company
espresso machines	Breville/Sunbeam
rotary evporators	Buchi and Heidolph
cloves (plant material)	Dijon Food Pty Ltd
Correa reflexa (plant material)	sample obtained in Tasmania
sand	Ajax
ethanol	Redoc Chemicals
hexanes	Ajax
magnesium sulfate	Ajax
diethyl ether	Merck
silica on aluminium TLC plates	Merck
eugenol	Merck
eugenyl acetate	Aldrich
acetone	Redox Chemicals
cyclohexane	ChemSupply
silica gel 60	Trajan
Congo red paper	ChemSupply
32% hydrochloric acid	Ajax

Catalog Number

Breville espresso machine model 800ES / Sunbeam EM3820 Café Espresso II

Sample collected from mature shrubs in the Thomas Crawford Reserve at the University of T

1199

E95 F3

251

1548

1009215000

1055540001

1069620100

W246905

Aceton13

CA019

5134312
IS070-100S
256

Comments/Description

Cloves must be ground in a food processor for students.

asmania

40 - 63um (230-400mesh)

1 Alewife Center #200
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3 July 2018
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I hope that you find this revised manuscript to be suitable for publication in *JoVE*.

Sincerely,

Alex C. Bissember

Employing Pressurized Hot Water Extraction (PHWE) to Explore Natural Products Chemistry in the Undergraduate Laboratory

Curtis C. Ho,[1] Bianca J. Deans,[1] Jeremy Just,[1] Gregory G. Warr,[2] Shane Wilkinson,*[,2] Jason A. Smith,*[,1] Alex C. Bissember,*[,1]

1. School of Natural Sciences – Chemistry, University of Tasmania, Hobart, Tasmania, Australia
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Supporting Information

Table of Contents

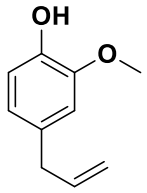
I. Extraction of Eugenol and Acetyleugenol from Cloves

TAKEN FROM LAB MANUAL (The University of Sydney)

Natural product extraction and separation

This experiment will take place over two weeks, and will involve the extraction of a mixture of two natural products from cloves (part a), followed by their separation into individual compounds by two different methods (part b). All sections of this experiment are to be completed individually.

Eugenol is a main component of cloves, responsible for their odour. Eugenol makes up the bulk of the "oil of cloves" obtained by steam distilling the flower buds. As well as flavouring, eugenol is also used in dental preparations, perfumes and as an insect attractant.



Eugenol
MW 164.2
bp 254°C, density 1.066 gmL^{-1}

Cloves contain 14–20% by weight of essential oil, and about half of this can be isolated. As well as eugenol, clove oil also contains a significant amount of its acetyl derivative *acetyl eugenol*. In this experiment, you will extract eugenol from cloves, and will investigate two methods of separating eugenol from acetyleugenol.

1. LEARNING OUTCOMES

After undertaking this experiment, you will have the following laboratory skills:

- Pressurized hot extraction of natural products
- Solvent extraction and filtration
- Drying of organic solvents / solutions
- Liquid-liquid extraction
- Analysis of purity by thin-layer chromatography
- Isolation of compounds by column chromatography

After undertaking this experiment and writing the lab report, you will understand:

- Glassware and chemical handling
- Liquid-liquid extraction
- Acid-base equilibria
- Chromatography equilibria and separation
- Retention factor (R_F) values
- Solvent and compound polarities

2. CHEMICALS USED AND SAFETY DATA

Cloves	**Not a hazardous substance**
Ethanol	**Highly flammable**
Hydrochloric acid (3M)	**Corrosive**
Sodium hydroxide (3M)	**Corrosive**
Hexanes	**Flammable**
Magnesium sulphate	**Irritant**
Diethyl ether	**Highly flammable, highly irritant**
Silica	**Irritant**
Cyclohexane	**Flammable, irritant**
Acetone	**Highly flammable, irritant**

3. ORAL PRESENTATION

At the start of this lab, you will present your results from Experiment 2.

4. PROCEDURE

Week a

Part 1: Extraction of eugenol and acetyleugenol from cloves

Demonstrators will show groups of students how to set-up the experiment at the start of the session.

Collect coarsely ground cloves (12.5 g) in a 250 mL beaker. Add sand (12.5 g) to the clove grinds and mix well. Collect an espresso portafilter and load the filter basket with the entire clove-sand mixture. *Lightly* compress it with the tamper – *do not compress the mixture too much or it will prevent the fluid from flowing through it*. Position the portafilter brewing piece into the machine and place a clean 250 mL beaker beneath it. Add 30% aqueous ethanol to the espresso machine water tank if it is less than half full. Turn the espresso machine knob to the right and extract 100 mL of fluid. Consult a demonstrator if the machine appears to be clogged. Stop the extraction process by turning the knob back to the vertical position. Allow the portafilter to finish dripping and then remove it from the espresso machine (*Caution: the grinds and surrounding metal areas will be hot)*. Using a spatula, remove the clove grinds from the portafilter into the waste bin. Rinse the portafilter with water and return it to the espresso machine for the next person to use. Cool the cloves extract in an ice bath until the temperature has reduced to at least 30 °C.

Figure 1. Sample of cloves (top); sample of cloves that is too finely ground (bottom left); sample of cloves that is appropriately ground (bottom middle); sample of cloves that has not been ground sufficiently (bottom right).

Place the extract into a 250 mL separatory funnel, add hexane (30 mL) and shake gently. (***Ask a demonstrator*** if you're not sure of the correct technique. *More information about using a separating funnel can be found in Appendix 4.*) Allow the layers to separate (this can take up to 5 minutes) then run-off the aqueous (lower) layer back into the 250 mL beaker – *do not throw it away*! Transfer the organic (top) layer (which contains the product) to a clean 250 mL conical flask, then pour the bottom (aqueous) layer back into the separatory funnel. Repeat this extraction twice more on the aqueous layer using fresh hexane (30 mL) each time. The organic (top) layers can be combined into the same flask after each extraction. After the 3rd liquid-liquid extraction, pour the combined organic extract into the separatory funnel, and wash with water (100 mL) by shaking thoroughly. Collect the organic (top) layer into a clean 250 mL conical flask and dry over magnesium sulphate. Filter the dried hexane solution through a fluted filter paper into a 250 mL round bottom flask. Evaporate off the hexane using a rotary evaporator.

Add diethyl ether (5 mL) to dissolve the crude eugenol/acetyl eugenol mixture and transfer the solution into a pre-weighed vial using a funnel (*Note: do not attach a label to the vial yet as it will fall off in the water bath. Record the vial's tare weight in your lab book*). Rinse the round bottom flask with more diethyl ether (5 mL) and add it to the vial. Evaporate off the solvent using a rotary evaporator using the vial adaptors.

Dispose of residual hexane and diethyl ether in the "Non-halogenated organic waste" bottles. Aqueous layers can be disposed of down the sink.

Weigh your vial and record the mass of extracted product in your laboratory notebook.

Week a Part 2: Solvent Optimisation for Chromatographic Separation

Thin Layer Chromatography (TLC) is a rapid and convenient method for detecting impurities, as well as for optimising conditions for separating mixtures on larger scale by column chromatography, which you will do in week b. The composition of the solvent or *eluent* can affect the quality of your results quite significantly, so you will spend some time understanding this effect and optimising conditions in preparation for week b. Refer to Appendix 6 for detailed instructions.

In order to separate acetyl eugenol / eugenol mixtures you will be using acetone/cyclohexane mixtures as your eluent. Your demonstrator will assign you some solvent compositions (%v/v) to examine, and the results for the group will be pooled to determine the optimal conditions for separation. For each TLC plate and each solvent mixture, you should prepare samples by spotting only the pure eugenol and acetyl eugenol standards. Before development, check your TLC plates using the UV lamp provided; If either spot does not fluoresce, you need to spot some more. Then develop your plates in each of your assigned solvent mixtures, visualise them under UV, and record the result in your lab notebook. You should sketch the plate or paste a photo in your lab notebook. Determine the retention factors (R_F, which are expressed as a fraction between 0 and 1) of both compounds in each mixed solvent.

After examining the combined results for the group, identify the solvent composition which gives the greatest *difference* between R_F values, ΔR_F. Note this in your lab book. This mixture should be used to analyse your crude product today, and will also be used for TLC analysis in week b. Record the appearance of the developed plate in your notebook and confirm whether both eugenol and acetyl eugenol are present in your crude extract.

Hand your sample of crude product labelled with your name, Experiment 5a, SID and day of attendance, in to the Service Room before you leave the lab.

Week b: Separation of eugenol from acetyleugenol

Collect your vial of crude product from the Service Room. With a pipette, transfer half of your crude eugenol extract to a second vial, recording the mass transferred.

Method 1: Liquid-liquid extraction

Prepare a TLC with 5 lanes (Eugenol standard, acetyl eugenol standard, crude eugenol, organic solution A and organic solution B). Spot the standard eugenol and acetyl eugenol in their respective lanes. Add hexane (10 mL) to one of your eugenol vials, then spot this into the "crude eugenol" lane of your TLC (Note: *it will be quite concentrated so you will not need to spot much – just a single dab should do it*). Put this aside until you have completed the liquid-liquid extraction.

Pour your crude eugenol-hexane solution into a 250 mL separating funnel. Rinse the vial with a second 10 mL of hexane, and add this to the separating funnel. Extract the hexane solution with sodium hydroxide solution (3 M, 2 × 25 mL). Collect the aqueous (bottom) layers in a 250 mL conical flask. Collect the organic layer in a 50 mL conical flask and add magnesium sulphate to dry this solution. Acetyl eugenol remains in the organic layer, while eugenol is now in the alkaline aqueous extract (bottom layers). Retain the organic layer (organic solution A) for later analysis.

Do this next step in a fumehood. Swirl the conical flask that contains the combined sodium hydroxide solutions in an ice-water bath and slowly add concentrated hydrochloric acid (collect 10 mL from a dispenser in the front fumehood in a flask and add in portions) until the solution is acidic: check its acidity with congo red paper, using a pipette to transfer a drop of the solution onto the pH paper (it should turn blue). A total of 20-30 mL of conc. hydrochloric acid will be required, and you should see a white precipitate. ***Caution: Addition of acid can cause vigorous bubbling, so you should add the acid carefully, keeping the conical flask on ice.***

Extract the milky aqueous emulsion with hexane (2 × 30 mL), using the same procedure as above. Make sure that the aqueous extract is at room temperature or below before adding the hexane. Collect your organic layers in a 100 mL conical flask. The eugenol will now be in the combined organic (top) layers (organic solution B). Add anhydrous magnesium sulphate to dry this solution. You will now need to assess the purity by thin layer chromatography (TLC) using the optimal solvent mixture you determined in week a. Spot the organic solutions A and B from your liquid-liquid extractions onto your prepared TLC plate. Run your TLC plate, and visualise it using the UV lamp provided and circle any spots you observe with a pencil. Take a photograph of your TLC plate, or redraw it, so that you have a record in your laboratory notebook.

Whilst you are waiting for your TLC plate to finishing running, filter your organic solution B through fluted filter paper into a 100 mL round bottom flask. Use a rotary evaporator to evaporate the solvent. Add diethyl ether (5 mL) to the round bottom flask and transfer your purified eugenol into an *unlabelled*, *pre-weighed* vial using a funnel. Rinse the round bottom flask with further diethyl ether (5 mL) into your flask. Evaporate the solvent using the rotary evaporator with vial attachment. Record the yield and label your vial appropriately.

Filter your organic solution A through fluted filter paper into a 100 mL round bottom flask and use a rotary evaporator to evaporate the solvent. Add diethyl ether (5 mL) to the round bottom flask and transfer your product (which should be acetyl eugenol) into an *unlabelled*, *pre-weighed* vial using a funnel. Rinse the round bottom flask with further diethyl ether (5 mL) into your flask. Evaporate the solvent using the rotary evaporator with vial attachment. Record the yield and label your vial appropriately.

Waste disposal: Aqueous solutions can be poured down the sink for disposal. Hexane and ether waste should be disposed of in the "Non-chlorinated organic waste" bottles.

<u>Method 2: Column chromatography</u>

Pack the column according to the instructions in Appendix 7. You will be using 3% acetone in cyclohexane as the eluent. (Note that this will not be exactly the same composition as you found for your TLC analysis, due to differences in experimental conditions.)

Load your sample onto the column, and run it with the prepared eluent. Collect 12 fractions in test tubes (fill to about 2 cm from the top). After you have collected the fractions, spot each separate fraction onto a TLC plate (spot 6 fractions per TLC plate). Observe the TLC plate under the UV lamp to determine which fractions contain one or both products. If there is a UV-active spot still visible for the 12th fraction, set your TLC up to run in a TLC jar (using your solvent composition

determined in week a) and then collect *an additional 6 fractions whilst the TLC is running*. Spot these 6 fractions onto another TLC and determine whether the UV-active spot has finished eluting from the column *before* you run the TLC. Continue to collect fractions until no UV-active spots are observed on TLC.

If you see faint spots or are unsure whether all your sample has eluted, you can visualise your TLC with permanganate stain. Ask your demonstrator to show you this.

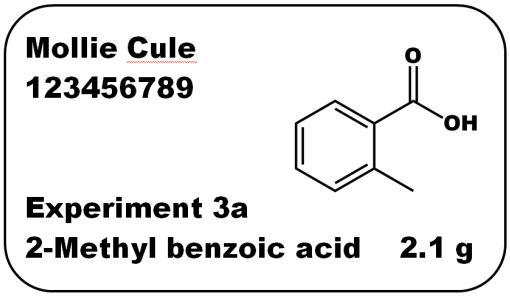
Once you have finished your chromatographic separation, allow the remaining solvent in the column to drain into a flask or beaker by leaving the tap open. Once the solvent has flowed through the column, dispose of the solvent in the "Non-halogenated organic waste" bottle. Turn the chromatography column upside down over the emptied flask/beaker. *Do not attempt to empty the column yourself. Overnight, the silica powder will fall into the flask/beaker and Service Room technicians will dispose of it.*

If you have time (check with your demonstrator), collect and combine the fractions that contain the purest eugenol and acetyl eugenol into separate round bottom flasks, and evaporate the solvent using a rotary evaporator. Add diethyl ether (5 mL) to your oily residual and transfer it to an *unlabelled, pre-weighed* vial using a funnel. Rinse the round bottom flask with additional diethyl ether (5 mL) into the vial. Evaporate the solvent using the rotary evaporator. Record the yield and label your vial appropriately.

Dispose of residual hexane and diethyl ether in the "Non-halogenated organic waste" bottles.

5. SAMPLE HAND-IN

Hand in your purified eugenol and acetyl eugenol samples to the service room following the example label shown. Also submit your TLC plates showing (i) liquid extraction layers A and B compared with eugenol and acetyl eugenol standards, and (ii) column chromatography fractions. Make sure that you have taken a record of the TLC plates before you do so.



6. REPORT

This experiment will be assessed by a full report (see Appendix 1). In the results section of your report, you should include the following:

- A description of the appearance of your products.
- A table of R_F values for eugenol and acetyl eugenol for different solvent compositions.
- A sample calculation of R_F for both compounds at one solvent composition based on a correctly-labelled figure showing a reproduction of a TLC plate, clearly identifying each spot.

- The maximum mass of eugenol you could obtain, assuming that your starting materials contains 17 wt% essential oil. Use this to calculate the yield of your crude product (week a) as a weight percentage.
- The resultant yields of eugenol and acetyl eugenol obtained by liquid-liquid extraction as both masses and percentage yields based on the quoted essential oil content of cloves.
- The percentage (%w/w) composition of eugenol and acetyl eugenol in your crude extract, based on your final yields.
- A narrative tying together the various results reported here.

Make sure that you pay attention to the number of significant figures which you use.

In the discussion section of your report, you should include answers to the following:

- Describe how the retention factors of eugenol and acetyleugenol depend on solvent composition. Which of these compounds moves faster on the TLC plate? Why is this?
- Method 1 uses the process of liquid-liquid extraction to separate eugenol from acetyleugenol. Draw a figure (using ChemSketch or a similar program, and with an appropriate caption) showing the chemical structure of eugenol in acidic and basic solutions, and explain why it moves into the aqueous layer upon basification.
- Compare and contrast the two purification methods that you used, considering factors such as the ease of the technique, the efficacy, and how easily it could be used for other purifications. (If you isolated products from the column chromatography method, compare the yield with that obtained from liquid-liquid extraction.)

II. Extraction of Seselin and Epoxysuberosin from *Correa reflexa*

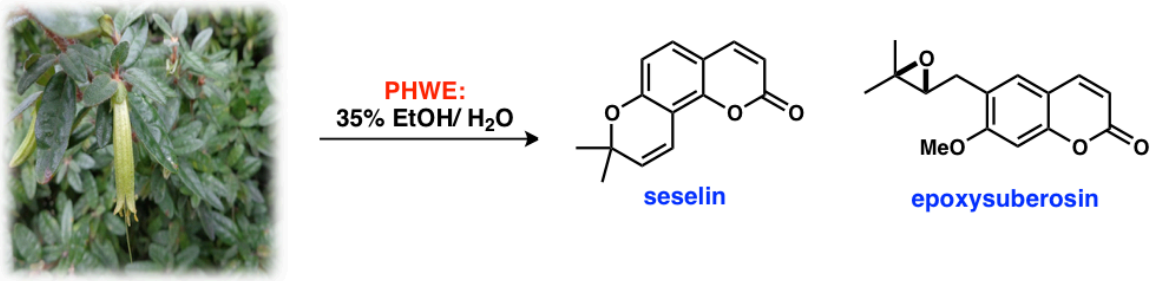
TAKEN FROM LAB MANUAL (University of Tasmania)

Experiment 3B: Natural Products Isolation and Bioprospecting

Introduction

The isolation and identification of natural products remains of fundamental importance. Bioprospecting, the search for valuable organic molecules found in nature, remains an indispensable process in the discovery of new drug leads and potential therapeutic agents. For example, it is estimated that, from 1981–2014, ~75% of all approved small molecule pharmaceutical drugs were natural products, natural product-derived or natural product-inspired.[1]

In Part A, you will perform an isolation of a natural product using a new extraction technique developed at the University of Tasmania.[2] You will isolate seselin and epoxysuberosin from the leaves of the plant *Correa reflexa* by using hot pressurised water extraction to obtain the crude extract followed purification via column chromatography.[3] All isolated compounds will need to be characterised via ^{1}H and ^{13}C NMR and IR spectroscopy.



Correa reflexa

In Part B, you will use this method to attempt the isolation of natural products from some other plant species. Consult Jason Smith or Alex Bissember about the types of plants that might be good candidates to investigate, which could be guided by the University of Tasmania natural products research group or the scientific literature. You may need to perform extractions on 2 or 3 other specimens to determine if any have natural products that can be isolated by using this method. All isolated compounds will need to be characterised via ^{1}H and ^{13}C NMR and IR spectroscopy.

Please note that Part B is designed to simulate a small research project and that not all natural product extraction studies may proceed as planned. These should not be considered a failure as often more is learnt when research does not proceed as anticipated.

Part A. Extraction of *Correa reflexa*

Take 10 g of ground *Correa reflexa* and add ~ 2 grams of course sand. Mix and pack into the espresso machine sample compartment (note: do not pack the sample too tightly). Prepare a 35% ethanol/water solution (~300 mL) and pour it into the coffee machine tank. Secure the sample compartment into the machine, place a beaker under the filter and collect ~100 mL of extract. Wait for ~1–2 minutes and then collect a further 100 mL. Cool this mixture in ice bath and then evaporate

1. Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2016**, *79*, 629–661.
2. Just, J.; Deans, B. J.; Olivier, W. J.; Paull, B.; Bissember, A. C.; Smith, J. A. *Org. Lett.* **2015**, *17*, 2428–2430.
3. Deans, B. J.; Just, J.; Chetri, J., Burt, L. K.; Smith, J. N.; Kilah, N. L.; de Salas, M.; Gueven, N.; Bissember, A. C.; Smith, J. A. *ChemistrySelect* **2017**, *2*, 2439–2443.

the ethanol using a rotary evaporator. Transfer the aqueous extract to a separatory funnel and extract with EtOAc (4x 50 mL). Note that you may need a little time for the emulsions to separate between extractions. Combine the organic extracts, dry ($MgSO_4$), filter using a sintered glass funnel and evaporate to give the crude extract. Prepare an NMR sample and obtain a ^1H NMR spectrum (see your demonstrator for assistance). Carry out a TLC on the extract to determine an appropriate solvent system to isolate the compounds that have been extracted. Perform flash column chromatography and isolate pure fractions if the two major products and obtain ^1H and ^{13}C NMR and IR spectroscopic data to determine the perform structure elucidation and determine the purities of your products.

Figure 1. Sample of *Correa reflexa* (left); sample of *Correa reflexa* that is appropriately ground (left).

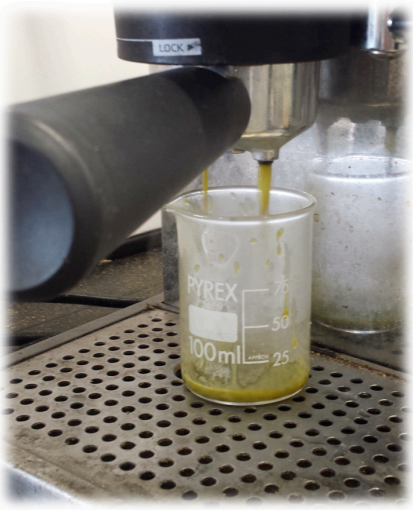
Part B. Extraction of Other Plant Material

Obtain ~100–200 g of fresh plant material and air dry. Grind ~ 20 g of the dry materiel using a spice grinder and transfer into the coffee filter (if the sample is ground too fine a small amount of sand can be added so that the filter does not block). Obtain an extract as described above and record the mass of the extract. Prepare an NMR sample and obtain a ^1H NMR spectrum and perform TLC analysis. In consultation with you demonstrator, analyse the spectroscopic and TLC data associated with the crude extract. Based on that assessment decide whether to proceed to the attempt the isolation of the key components by flash column chromatography or perform an extraction on a different plant sample.

Hazard Warning

This experiment should be undertaken in a fume hood. Wear all standard personal protective equipment in the laboratory (lab coat, safety glasses and gloves). Ethanol is flammable, and should be diluted (35 % v/v in water) to make a less flammable solution prior to use in the coffee machine. The espresso machine operates at temperatures of ~90–95 °C and should be used in a fume hood, away from other flammable solvents, and possible ignition sources. Organic solvent waste should be transferred to the appropriate solvent waste container.

III. PHWE Using an Espresso Machine in Pictures



IV. Notes to Laboratory Instructors & Espresso Machine Maintenance

Detailed information can be found in the supporting information of our previous publication:

Just, J., Bunton, G. L., Deans, B. J., Murray, N. L., Bissember, A. C., & Smith, J. A., Extraction of Eugenol from Cloves Using an Unmodified Household Espresso Machine: An Alternative to Traditional Steam Distillation.
Journal of Chemical Education. **93,** 213–216 (2016).

TITLE:

Employing Pressurized Hot Water Extraction (PHWE) to Explore Natural Products Chemistry in the Undergraduate Laboratory

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KEYWORDS:
Chemical Education, Pressurized Hot Water Extraction, Natural Products, Undergraduate Laboratory, Bioprospecting, Organic Chemistry

SHORT ABSTRACT:
Here, we employ a pressurized hot water extraction (PHWE) method, which utilizes an unmodified household espresso machine to introduce undergraduates to natural products chemistry in the laboratory. Two experiments are presented: PHWE of eugenol and acetyleugenol from cloves and PHWE of seselin and (+)-epoxysuberosin from the Australian plant *Correa reflexa*.

LONG ABSTRACT:
A recently developed pressurized hot water extraction (PHWE) method which utilizes an unmodified household espresso machine to facilitate natural products research has also found applications as an effective teaching tool. Specifically, this technique has been used to introduce second- and third-year undergraduates to aspects of natural products chemistry in the laboratory. In this report, two experiments are presented: the PHWE of eugenol and acetyleugenol from cloves and the PHWE of seselin and (+)-epoxysuberosin from the endemic Australian plant species *Correa reflexa*. By employing PHWE in these experiments, the crude

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clove extract, enriched in eugenol and acetyleugenol, was obtained in 4–9% w/w from cloves by second-year undergraduates and seselin and (+)-epoxysuberosin were isolated in yields of up to 1.1% w/w and 0.9% w/w from *C. reflexa* by third-year students. The former exercise was developed as a replacement for the traditional steam distillation experiment providing an introduction to extraction and separation techniques, while the latter activity featured guided-inquiry teaching methods in an effort to simulate natural products bioprospecting. This primarily derives from the rapid nature of this PHWE technique relative to traditional extraction methods that are often incompatible with the time constraints associated with undergraduate laboratory experiments. This rapid and practical PHWE method can be used to efficiently isolate ~~an array of~~various classes of organic molecules from a range of plant species. The complementary nature of this technique relative to more traditional methods has also been demonstrated previously.

INTRODUCTION:

The isolation and identification of natural products are of fundamental importance to the scientific community and society more generally.[1] Bioprospecting, the search for valuable organic molecules found in nature, remains an indispensable process in the discovery of new drug leads and potential therapeutic agents. It is estimated that, from 1981–2014, ~75% of all approved small molecule pharmaceutical drugs were natural products, natural product-derived or natural product-inspired.[1] Furthermore, natural products possess enormous structural and chemical diversity. For this reason, they also represent valuable chemical scaffolds that can be directly used in organic synthesis or in the development of chiral ligands and catalysts.[2,3]

Traditionally, relatively time-intensive procedures such as maceration, Soxhlet extraction, and steam distillation have been the mainstay of research focused on the isolation of secondary metabolites from plants.[4] More modern extraction techniques, including accelerated solvent extraction, have focused on reducing extraction times and establishing greener protocols.[4,5] In 2015, an original pressurized hot water extraction (PHWE) method was reported.[~~5~~–6] This technique employed an unmodified household espresso machine to facilitate the rapid and particularly efficient extraction of shikimic acid from star anise. Espresso machines have been specifically designed and engineered to extract organic molecules from appropriately ground coffee beans. To achieve this, these instruments heat water at temperatures up to 96 °C and at pressures of typically 9 bar.[~~6~~–7] With this in mind, it is perhaps not surprising that espresso machines can be utilized to efficiently extract natural products from a range of plant material.

Subsequent studies involving a variety of terrestrial plant species have demonstrated the capacity of this PHWE technique to efficiently extract natural products across a relatively broad polarity range.[~~56~~,~~78~~–~~13~~–145] Furthermore, compounds containing somewhat sensitive functional groups, such as aldehydes, epoxides, glycosides, and potentially epimerizable stereogenic centers were typically unaffected by the extraction process. The complementary nature of this technique relative to more traditional methods has also been demonstrated.[~~11~~12,~~14~~–~~15~~6] This PHWE method has also been employed to isolate multi-gram quantities of natural products, which have been used to prepare novel natural product derivatives and in complex molecule synthesis more generally.[~~78~~,~~10~~11,~~15~~167]

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It was identified that this new PHWE method could serve as a useful teaching tool that could be incorporated in the undergraduate laboratory. This primarily derives from the rapid nature of this technique relative to the traditional extraction methods that are often incompatible with the time constraints associated with undergraduate laboratory experiments. Consequently, this technique supplanted the traditional undergraduate chemistry laboratory experiment focused on the extraction of eugenol from cloves employing steam-distillation at the University of Tasmania.[89,16,178] Since that time, variations of this experiment have been adopted by other universities and a modified experiment focusing on the PHWE of cloves now features in the undergraduate chemistry laboratory program at the University of Sydney (*vide infra*).~~universities. For example, a modified experiment focusing on the PHWE of cloves now features in the undergraduate chemistry laboratory program at the University of Sydney.~~

In order to demonstrate the practicality and feasibility of employing this new PHWE approach for teaching purposes, two protocols are presented as part of this study. The first part of this report ~~Part A 1~~ highlights an experiment on the PHWE of eugenol and acetyleugenol from cloves which is part of the second-year undergraduate laboratory program at the University of Sydney **(Figure 1)**. ~~.~~ This experiment serves to introduce students to natural products chemistry while developing fundamental practical skills. The second part ~~Part B 2~~ features an experiment on the PHWE of the endemic Australian plant species *Correa reflexa* which is part of the third-year undergraduate laboratory program at the University of Tasmania **(Figure 2)**. ~~.~~ This experiment is designed to simulate natural products bioprospecting and reinforce core laboratory techniques.[10,11]

PROTOCOL:

Note: It is advisable that all procedures are performed in a fume hood. Students must wear appropriate personal protective equipment at all times in the laboratory and the safety data sheets (SDS) associated with each reagent must be consulted before use.

1. ~~Part A –~~ PHWE of cloves: isolation of eugenol and acetyleugenol

~~1.~~1.1. ~~Session 1a.~~ Extraction of eugenol and acetyleugenol from cloves

~~1.1.~~1.1.1. Place coarsely ground cloves (12.5 g) in a 250-mL beaker.

~~1.2.~~1.1.2. Add sand (12.5 g) to the clove grinds and mix well.

~~1.3.~~1.1.3. Collect a portafilter (sample compartment) and load the basket with the entire clove-sand mixture. Lightly compress ~~it~~ the sample with the tamper.

NOTE: Do not compress the mixture too much or fluid will not flow through.

~~1.4.~~1.1.4. Position the portafilter into the espresso machine and place a clean 250-mL

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Coarsely ground .. number of cloves using mortar and pestle. …

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beaker beneath it. Add a 30% ethanol/H_2O solution to the water tank of the espresso machine if it is less than half full.

~~1.5.~~1.1.5. Use the espresso machine to collect 100 mL of the extract.

NOTE: Consult an instructor if the machine appears to be clogged.

~~1.6.~~1.1.6. Allow the portafilter to finish dripping and then remove it from the espresso machine.

CAUTION: The grinds and surrounding metal areas will be hot.

~~1.7.~~1.1.7. Using a spatula, remove the clove grinds from the portafilter and discard into the waste bin.

~~1.8.~~1.1.8. Rinse out the residual solids from the portafilter with H_2O under a tap in the sink and return it for the next person to use.

~~1.9.~~1.1.9. Cool the clove extract in an ice bath until the temperature has reduced to at least 30 °C.

~~1.10.~~1.1.10. Place the extract into a 250-mL separatory funnel, add 30 mL of hexane and shake gently.

~~1.11.~~1.1.11. ~~Sit~~Place the separating funnel in a ring clamp fitted to a retort stand and ~~Allow~~~~wait for~~allow the aqueous and organic layers to separate ~~(this can take up to 10 minutes)~~ then ~~add~~ collect the aqueous (lower) layer back into the 250-mL beaker.

~~Note~~NOTE: It can take to 10 minutes for the layers to separate. Students are advised to carry out solvent optimization of TLCs while waiting for the first separation to occur (see ~~Part~~ steps 1.2).

~~1.12.~~1.1.12. Transfer the organic (top) layer (which contains the product) to a clean 250-mL conical flask, and then pour the bottom (aqueous) layer back into the separatory funnel.

~~1.13.~~1.1.13. Extract the aqueous layer ~~for~~ a further two ~~more~~ times with hexane (2x 30 mL).

~~1.14.~~1.1.14. Combine the organic (top) layers into the same flask after each extraction.

~~1.15.~~1.1.15. After the third liquid-liquid extraction, pour the combined organic extract into the separatory funnel, and wash with 100 mL of H_2O by shaking vigorously. Collect the ~~top~~ organic (top) layer into a clean 250-mL conical flask and dry by adding $MgSO_4$ and swirling the flask.

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1.1.16. Filter the ensuing mixture through fluted filter paper contained in a glass funnel into a pre-weighed 250-mL round-bottom flask.

~~The solid residue (hydrated $MgSO_4$) can be discarded in the waste.~~

NOTE: The solid residue (hydrated $MgSO_4$) can be discarded in the waste.

~~1.16.~~

~~1.17.~~1.1.17. Evaporate the solvent (hexane) from the collected filtrate ~~hexane~~ using a rotary evaporator (water bath temperature: 60 °C, vacuum pressure: 350 mbar) and re-weigh the flask containing the resultant oil.

~~2.~~1.2. ~~Session 1b.~~ Optimization of thin-layer chromatography (TLC) solvent system

~~2.1.~~ ~~Note~~NOTE: As a group, ~~students~~ students will be assigned a solvent system from 100:0 acetone:cyclohexane to 0:100 acetone:cyclohexane by the demonstrator to identify the ratio that provides the maximum resolution of eugenol from acetyleugenol. These solutions were prepared by lab technicians prior to the lab session.

~~2.2.~~1.2.1. ~~Collect~~ Obtain a TLC reference solution of pure eugenol and acetyleugenol.

NOTE: The necessary TLC reference solutions of pure eugenol and acetyleugenol were prepared by lab technicians prior to the lab session.

1.2.2. On a TLC plate, mark a *baseline* ~1.5 cm from the bottom with a soft pencil. Mark off three equally spaced points.

1.2.3. Use a TLC spotter to spot one drop of pure eugenol TLC reference solution in one lane, one spot of pure acetyleugenol TLC reference solution in the third lane and a spot of each in the second lane (the co-spot).

~~2.3.~~1.2.4. Check for the presence of eugenol and acetyleugenol on ~~your~~the TLC plate by viewing ~~your~~the plate under a UV ~~light~~lamp (254 nm) in the TLC viewing cabinet. ~~There should be small (1-2 mm wide) black spots where you spotted the TLC reference solutions. If there are no spots or the spots appear faint, apply another spot of the respective TLC solution until a black spot is observed under UV light.~~

NOTE: There should be small (1~~–~~–2 mm wide) black spots ~~where you spotted~~on the plate where the TLC reference solutions were spotted. If there are no spots or the spots appear faint, apply another spot of the ~~respective~~appropriate TLC solution until a black spot is observed under UV light.

1.2.5. ~~Prepare~~ Add 10 mL of the allocated solvent mixture ~~and pour in~~to a clean, dry TLC jar. ~~Ensure the solvent height in the jar does not exceed 1 cm.~~

NOTE: Ensure the solvent height in the jar does not exceed ~1 cm.

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1.2.6. Using tweezers, ~~Lower~~place ~~your~~the prepared TLC plate into the TLC jar ~~so the region below yoursuch that the baseline sitsof the TLC plate lies in theabove the solvent mixture~~. ~~The baseline should NOT be submerged in this solvent.~~ Close the lid of the jar.

NOTE: The solvent ~~line~~ must lie ~~above~~below the baseline of the TLC plate~~baseline should NOT be submerged in this solvent~~.

1.2.7. Allow the solvent to ~~climb~~move travel up the TLC plate. ~~-~~ Once the solvent is ~~-~~1 cm from the top of the plate, ~~the lid of the jar,~~ remove the TLC plate from the jar with tweezers and mark the ~~position of~~line of the solvent front ~~(how far the solvent's got to)~~ with a pencil.

1.2.8. Allow the solvent to evaporate from ~~your~~the TLC plate (~1 min) then view ~~your~~the TLC plate under a UV ~~light~~lamp (254 nm). ~~-~~Using a ~~soft~~ pencil, circle the black spots observed on the TLC plate.

1.2.9. Calculate the retention factor (R_f) of eugenol and acetyleugenol ~~and their difference,~~ by dividing the distance travelled by the compound by the distance travelled by the solvent.

1.2.10. Calculate the difference between the R_f values for eugenol and acetyleugenol (~~-~~ Δ ~~-~~ ΔR_f).

~~2.4. and run the TLC in this solution.~~

~~2.5.~~1.2.11. ~~Record and s~~Share the results with the rest of the class. ~~-~~ Record the retention values acquired by other students with other solvent ratios.

1.2.12. Identify which solvent system will be best to analyze the crude eugenol solution and subsequent purification steps. ~~The best TLC solvent ratio will provide the greatest separation between eugenol and acetyleugenol denoted by the largest ~~-~~ R_f value.~~

~~2.6.~~ NOTE: The best TLC solvent ratio will provide the greatest separation between eugenol and acetyleugenol denoted by the largest ΔR_f value. If ΔR_f is plotted against solvent composition, the graph should resembles~~s~~ a bell curve.

~~3.~~1.3. ~~Session 2.~~ **Separation of eugenol and acetyleugenol by liquid-liquid extraction**

~~3.1.~~1.3.1. Add hexane (10 mL) to the crude eugenol-containing extract~~,~~ obtained from step 1.1.17 ~~as described~~ immediately above, and pour the ensuing solution into a 250-mL separatory funnel.

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3.2.1.3.2. Rinse the round-bottom flask with hexane (10 mL) and add this to the separatory funnel.

3.3.1.3.3. Extract the hexane solution with 3 M aqueous NaOH (2× 25 mL) ~~using~~via liquid-liquid extraction. Collect and combine ~~the~~ the aqueous bottom layers in a 250-mL conical flask from each extraction. Collect the organic layer in a 50-mL conical flask and dry by adding $MgSO_4$ and swirling the flask.

CAUTION: NaOH is corrosive. Avoid any contact with skin.

NOTE: Acetyleugenol remains in the organic layer, while eugenol is now in the alkaline aqueous extract (bottom layers).

1.3.4. ~~Acetyleugenol remains in the organic layer, while eugenol is now in the alkaline aqueous extract (bottom layers).~~ Retain the organic layer (organic solution A) for later analysis.

~~3.4. NOTE: Acetyleugenol remains in the organic layer, while eugenol is now in the alkaline aqueous extract (bottom layers).~~

~~NOTE: Perform the following step in a fume hood.~~

3.5.1.3.5. Swirl the conical flask that contains the ~~combined NaOH~~alkaline ~~solutions~~ aqueous fraction from step 1.3.3 in an ice-water bath and slowly add 10 M aqueous HCl until a white emulsion is formed; check its acidity with Congo red paper, using a pipette to transfer a drop of the solution onto the pH paper (it should turn blue).

CAUTION: HCl is corrosive. Avoid any contact with skin. Addition of HCl can cause vigorous bubbling, HCl should be added carefully, keeping the conical flask on ice.

NOTE: A total of 20–30 mL of HCl (10 M of an aqueous solution) will be required.

1.3.6. Extract the milky aqueous emulsion with hexane (2× 30 mL), using ~~the same procedure as above~~liquid-liquid extraction in a 250 mL separating flask. Make sure that the temperature of the aqueous extract is at room temperature or below before adding the hexane. Combine the two hexane extracts into a clean 100 mL conical flask.

~~3.6.~~ NOTE: The eugenol will now be in the combined organic (top) layers (organic solution B).

3.7.1.3.7. ~~Collect the organic layers in a 100-mL conical flask. The eugenol will now be in the combined organic (top) layers (organic solution B).~~ Add $MgSO_4$ to dry ~~this solution~~organic solution B.

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~~3.8.~~1.3.8. Filter organic solution B ~~the ensuing mixture solution~~ through fluted filter paper into a pre-weighed 250-mL round-bottom flask. Discard the solid residue (hydrated $MgSO_4$) in the waste.

~~3.9.~~1.3.9. Remove the solvent from the round-bottom flask using a rotary evaporator (water bath temperature: 60 °C, vacuum pressure: 350 mbar).

~~3.10.~~1.3.10. Add diethyl ether (5 mL) to the ensuing sample in the round-bottom flask and transfer the purified eugenol into an unlabeled, pre-weighed vial using a funnel.

~~3.11.~~1.3.11. Rinse the flask with further diethyl ether (5 mL) into the vial. Evaporate the solvent using a rotary evaporator (water bath temperature: 50 °C, vacuum pressure 800 mbar) with a vial attachment. Record the yield and label the vial appropriately.

~~3.12.~~1.3.12. Analyze organic solution A, organic solution B, pure eugenol TLC reference and acetyleugenol TLC reference by TLC using the optimized TLC solvent ratio identified in the previous session.

Note: Aqueous solutions can be poured down the sink for disposal. Hexane and ether waste should be disposed of in the ~~Non~~non-chlorinated organic waste bottles.

2. ~~Part B —~~PHWE of *Correa reflexa*: isolation of seselin and (+)-epoxysuberosin[~~18~~]

~~**Part B — PHWE of *Correa reflexa*: isolation of seselin and (+)-epoxysuberosin**~~

~~**4.~~*2.1.*** **Session 1. PHWE of *Correa reflexa***

2.1.1. ~~Take~~ Grind ~~10 g of ground~~ *Correa reflexa* leaves (10 g) in an electric spice grinder and ~~add ~ 2 g of coarse sand~~then transfer the ground plant material to a 100-mL beaker.

NOTE: Grinding should take 20–30 seconds.

~~1.1.~~2.1.2. Add ~ 2 g of coarse sand to the beaker containing plant material.

~~1.2.~~ Mix and pack into the basket of the portafilter (sample compartment).

2.1.3. Compress the sample with the tamper.

NOTE: Do not pack the sample too tightly.

~~1.3.~~2.1.4. Add ~300 mL of 35% ethanol/H_2O solution to the espresso machine tank.

~~1.4.~~2.1.5. Position the portafilter into the espresso machine and place a clean 250-mL beaker beneath it.

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~~1.5.~~2.1.6. Collect ~100 mL of extract, wait for ~1~~–2~~ minute~~s~~ and then collect a further 100 mL.

CAUTION: The machine and extracts will be hot at this point.

~~1.6.~~2.1.7. Cool this mixture in ice bath and evaporate the ethanol using a rotary evaporator (water bath temperature: ~40 °C).

~~1.7.~~2.1.8. Transfer the aqueous extract to a separatory funnel and extract with ethyl acetate (4x 50 mL).

NOTE: Time may be needed to allow emulsions to separate between extractions.

~~1.8.~~2.1.9. Combine the organic extracts, dry by adding $MgSO_4$ and swirling the flask, filter using a sintered glass funnel, and evaporate using a rotary evaporator (water bath temperature: ~35 °C) to provide the crude extract.

~~1.9.~~2.1.10. Obtain an ^1H nuclear magnetic resonance (NMR) spectrum (see the instructor for assistance).[~~10~~1]

~~1.10.~~2.1.11. ~~Carry out~~Perform TLC analysis of the crude extract to determine an appropriate solvent system to isolate the compounds that have been extracted.

NOTE: TLC analysis is performed by analogy to ~~steps~~procedures outlined in s~~Part~~tep 1.2.

~~2.~~2.2. Session 2. ~~Session 2.~~ Separation of seselin and (+)-epoxysuberosin by flash column chromatography[~~11~~1,~~19~~8]

NOTE: The following protocol involves the use of flash column chromatography for the separation of organic compounds. Please consult an instructor to demonstrate how to pack a flash silica gel column.

2.2.1. Place the column (~~XX~~30 mm in diameter) in a clamp fitted to a retort stand. Place a 100-mL conical flask underneath the column~~Students perform flash chromatography to isolate seselin and (+)-epoxysuberosin~~.

2.2.2. Fill the column with silica gel (60 µm flash grade) to a level of ~15~~0~~2 cm and then add hexanes (~100 mL) to the column.

2.2.3. Place a glass stopper in the column, remove the column from the clamp and shake to obtain a slurry. Place the column in the clamp and then allow the mixture to settle.

2.2.4. Open the tap of the column and, using a gas adaptor attached to a compressed air line,

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empty the column to leave ~2mm of solvent above the bed of silica gel. Remove the gas adaptor then close the tap.

2.2.5. Use the hexanes collected in the conical flask (~5 mL) to wash down any silica gel from the walls of the column with a Pasteur pipette fitted with a rubber septum.

2.2.6. Repeat step 2.2.4 then add a small layer of sand (~1 cm) to the column.

2.2.7. Add dichloromethane (~ 1mL) to the flask containing the crude extract from step 2.1.8. Carefully load the ensuing solution onto the column using a Pasteur pipette fitted with a rubber septum. Open the tap of the column and allow the sample to adsorb onto the silica gel.

2.2.8. Repeat step 2.2.7 a further two times.

2.2.9. Carefully add hexanes (~~5~~20 mL) to the column. Repeat step 2.2.4.

2.2.10. Carefully add (~~10~~80 mL of a 15~~20~~% ethyl acetate/hexanes solution). Open the tap of the column and, using a gas adaptor attached to a compressed air line, empty the column to leave ~2 mm of solvent above the bed of silica gel, collecting the fractions into 10-mL test tubes.

NOTE: This will allow seselin to be isolated.

Combine the test tube fractions containing seselin in a 250-mL round bottom flask and evaporate using a rotary evaporator (water bath temperature: ~35 °C).

2.2.11.

NOTE: TLC analysis is used to determine this and is performed by analogy to procedures outlined in step 1.2.

2.2.12. Carefully add (~~100~~75 mL of a ~~30~~25% ethyl acetate/hexanes solution). Open the tap of the column and, using a gas adaptor attached to a compressed air line, empty the column to leave ~2 mm of solvent above the bed of silica gel, collecting the fractions into 10-mL test tubes.

NOTE: This will allow (+)-epoxysuberosin to be isolated.

~~5.1.~~2.2.13. Combine the test tube fractions containing (+)-epoxysuberosin in a 250-mL round bottom flask and evaporate using a rotary evaporator (water bath temperature: ~35 °C).

NOTE: TLC analysis is used to determine this and is performed by analogy to procedures outlined in step 1.2.

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5.2.2.2.14. Samples of the isolated compounds are analyzed using NMR spectroscopy.[10][1]

Note: NMR spectroscopy experiments are performed by a lab technician.

REPRESENTATIVE RESULTS:

PHWE of cloves. ~~**Part 1A.**~~ When attempting to perform the liquid-liquid extraction step, students often encountered emulsions (the addition of brine was typically not effective). At this stage, students were instructed to allow the mixture to stand in the separating funnel while they explored the effects of eluent composition on the separation of eugenol and acetyleugenol by TLC. It should be noted that hexane can be substituted with either heptane or dichloromethane in the liquid-liquid extraction step.[8–9] Students were allocated a TLC solvent ratio of acetone and cyclohexane and provided with pure standards of eugenol and acetyleugenol and then performed TLC analysis (**Figure ~~1~~3**). Their results were tabulated on a whiteboard, and the effects of solvent composition on the retention factor (R_f) and the optimum eluent were considered in a group discussion (**Table 1**). The optimum solvent compositions identified by students typically ranged from 5–20% acetone/cyclohexane with a ~~Δ~~ΔR_f between 0.1–0.2.

Following TLC eluent optimization, students returned to their eugenol extractions. The crude clove extract (consisting mainly of eugenol and acetyleugenol) was isolated in 4–9% w/w. In the second session of this experiment, students exploited the different acid-base properties of the two major organic molecules to separate them by liquid-liquid extraction. Typically, eugenol was isolated in a yield of 45–65% w/w of the crude extract while acetyleugenol was isolated in a yield of 5–10% w/w of the crude extract. Students then utilized the optimized eluent (identified as outlined above) to determine the success of their liquid-liquid extraction by comparison of their extracts to the pure reference samples by TLC (**Figure ~~1~~3**). Students also analyzed their crude clove extract, and their purified eugenol and acetyleugenol samples by performing Fourier-transform infrared (FTIR) spectroscopy.[9][8] Solvent or water peaks were occasionally observed in IR spectra due to poorly executed work-up procedures (or poor sample preparation).

Advanced students committed approximately half of their isolated crude oil to the liquid-liquid extraction described above and subjected the other portion to flash column chromatography (more information is provided in the supporting information). ~~,~~ Although completing the liquid-liquid extraction and flash column chromatography steps in a single four-hour session may appear rather ambitious this was achievable for most of the advanced students undertaking this experiment. The complete separation of eugenol from acetyleugenol by flash column chromatography was rarely achieved due to their close retention factors (**Figure ~~2~~4**). However, students were generally able to collect a few fractions containing pure eugenol. Advanced students were then asked to comment on the two different purification techniques as part of their report.

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PHWE of *Correa reflexa.* Students performed the PHWE of *Correa reflexa* with minimal assistance of the laboratory instructor. During the liquid-liquid extraction step emulsions typically formed and students often were required to allow the mixture to stand in the separatory funnel (~0.25 h) with periodic agitation of the mixture with a glass rod. The chromatographic purification of the crude extract was comfortably completed within the four-hour laboratory session by students. Seselin and (+)-epoxysuberosin were isolated in yields of up to 1.1% w/w and 0.9% w/w, respectively and isolated samples of both compounds were analyzed by ^{1}H and ^{13}C NMR and FTIR spectroscopy (~~Scheme~~ Figure **2**). While students undertook the FTIR spectroscopy experiments and prepared samples for NMR spectroscopy, lab technicians performed NMR spectroscopy experiments. The results obtained by students were consistent with previously published work.[~~10~~ 11]

Although this has not been presented in this report, in practice, this experiment also features a second part that challenges students to perform the extraction a plant species that has not been studied employing PHWE (more information is provided in the supporting information).

FIGURE AND TABLE LEGENDS:

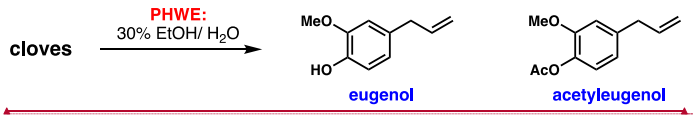

Figure 1. PHWE of cloves.[~~8~~9]

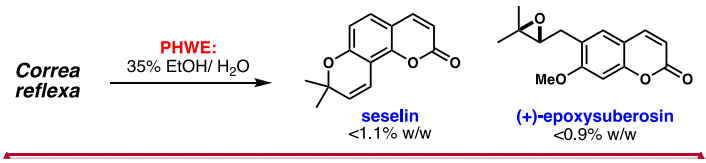

Figure 2. PHWE of *Correa reflexa.*[~~10~~1]

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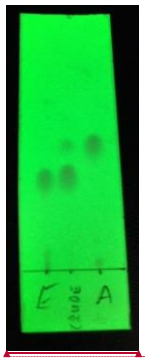

Figure 3. A representative TLC plate prepared by a student. (10% acetone/ cyclohexane elution). Lane 1 (E): eugenol standard; lane 2 (crude): crude clove extract; lane 3 (A): acetyleugenol standard).

Table 1. Table of retention factors outlining the effect of eluent composition on R_f.

acetone/ cyclohexane (%v/v)	mean acetyl-eugenol R_f	acetyl-eugenol R_f (σ^2)	mean eugenol R_f	eugenol R_f ($\sigma\sigma$)	mean $\Delta\Delta R_f$	number of TLC analyses
0	0.06	0.08	0.04	0.06	0.02	12
5	0.34	0.11	0.27	0.09	0.07	15
10	0.45	0.07	0.34	0.05	0.12	20
20	0.51	0.07	0.41	0.06	0.10	20
30	0.58	0.10	0.49	0.12	0.10	19
40	0.63	0.08	0.56	0.08	0.07	16
50	0.76	0.08	0.73	0.08	0.03	17
60	0.77	0.13	0.73	0.15	0.04	12
70	0.84	0.13	0.81	0.13	0.03	11
80	0.90	0.06	0.87	0.08	0.02	10
90	0.88	0.06	0.87	0.05	0.01	11
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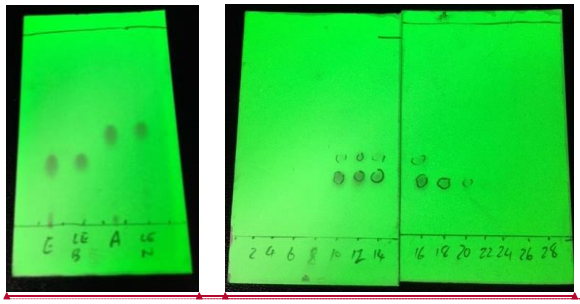

Figure 4. Representative TLC plates prepared by students. Left: A TLC plate analyzing the outcome of the liquid-liquid extraction step (10% acetone/ cyclohexane elution). Lane 1 (E): eugenol reference standard; lane 2 (LEB): eugenol-containing organic extract; lane 3 (A): acetyleugenol reference standard; lane 4 (LEN): acetyleugenol-containing organic extract. Right: A TLC plate analyzing the outcome of flash column chromatography step (10% acetone/ cyclohexane elution). Numbers on the TLC plate relate to the test-tube fraction number.

~~**Scheme 1.** PHWE of cloves.~~

~~**Figure 1. A student's representative TLC plate from optimization of TLC solvent system (Section 1.2).** (10% acetone/ cyclohexane elution). Lane 1 (E)€: eugenol standard; lane 2 (crude): crude clove extract; lane 3 (A): acetyleugenol standard).~~

~~**Table 1.** Table of retention factors outlining the effect of eluent composition on R_f.~~

~~**Figure 2. Representative TLC plates from the separation of eugenol and acetyleugenol by liquid-liquid extraction (left) and column chromatography (right).** Left: A student's representative TLC plate analyzing the outcome of the liquid-liquid extraction step (10% acetone / cyclohexane elution). Lane 1 (E): eugenol reference standard; lane 2 (LEB): eugenol-containing organic extract; lane 3 (A): acetyleugenol reference standard; lane 4 (LEN): acetyleugenol-containing organic extract. Right: A student's representative TLC plate analyzing the outcome of flash column chromatography step (10% acetone / cyclohexane elution). Numbers on the TLC plate relate to the test-tube fraction number.~~

~~**Scheme 2.** PHWE of *Correa reflexa*.~~

DISCUSSION:

The classical procedure for isolating eugenol from cloves by steam distillation has been part of the ~~University of Sydney's~~ intermediate chemistry laboratory program at the University of Sydney for decades but was modernized to employ PHWE methodology in 2016 (~~**Scheme**~~ **Figure 1**).[~~8,~~9,~~16,~~17,~~8] This provided a number benefits. Firstly, ~~the~~ utilizing household espresso machines in the laboratory environment immediately fascinated and engaged students by illustrating the application of a non-classical, alternative method to ~~e~~affect a traditional scientific study. In

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addition, this new method reduced the time taken to complete the extraction and enabled the incorporation of additional exercises into this new iteration of the experiment. Specifically, this allowed thin-layer chromatography (TLC) to be introduced (and flash column chromatography for advanced students).

The experiment focusing on the PHWE of cloves was designed as an introductory laboratory experience for second-year undergraduate chemistry students and for this reason it features expository teaching methods.[~~8~~ 9] This more prescriptive, recipe-style procedure allows students with somewhat limited experience in organic chemistry to efficiently complete the extraction of eugenol from cloves. In this experiment, concepts such as acid-base extraction of acidic compounds, utilizing TLC to identify suitable eluent composition for chromatography, and the use of a rotary evaporator are introduced or reinforced by a combination of on-line pre-lab video training and in-person demonstrations. In complementary components undertaken during the two allocated sessions, students in the advanced stream of intermediary chemistry also separated eugenol and acetyleugenol by column chromatography and determined the identity of the extracted components using TLC. In the second session, students could critically compare the two separation methods. In general, students were able to complete the overall experiment within the allocated two four-hour periods with minimal instruction.

The experiment focusing on the PHWE and isolation of seselin and (+)-epoxysuberosin from *Correa reflexa* was developed for more experienced students third-year undergraduate chemistry students. Notably, this learning exercise was a result of a study originating in the research laboratory.[~~10~~ 11] The first iteration of the experiment was incorporated into the third-year undergraduate chemistry laboratory program at the University of Tasmania in 2015. After two years of revisions and re-evaluation, this experiment was performed by a third-year undergraduate class for the third time in 2017.

This experiment was specifically designed as a guided-inquiry-based activity that strives to simulate some of the approaches employed in natural products research laboratories and features minimal written instructions. This is a student-directed learning experience and the laboratory instructor plays a key role in assisting students as they work through the experiment by providing direction as required. In this experiment, students develop key laboratory skills in chromatography and employ NMR spectroscopy to perform structure elucidation. This laboratory experience reinforces the concept of bioprospecting which is presented to students in the classroom and this can be extended to studies on previously unstudied plant material to provide a more representative experience of natural products bioprospecting. *C. reflexa* is an endemic Australian plant species, however, this sample can be substituted for ~~other~~ appropriate leaf material from other terrestrial plant species in this experiment.

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DISCLOSURES:

The authors have nothing to disclose.

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