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

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Supporting Information

Table of Contents

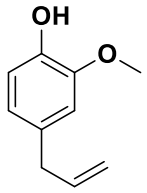
I. Extraction of Eugenol and Acetyleugenol from Cloves

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.

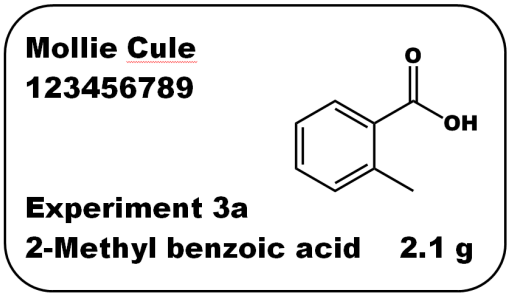
Once you have finished your chromatographic separation, allow the remaining solvent in the column to drain into a flask or beaker by leaving the tap open. Once the solvent has flowed through the column, dispose of the solvent in the "Non-halogenated organic waste" bottle. Turn the chromatography column 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*

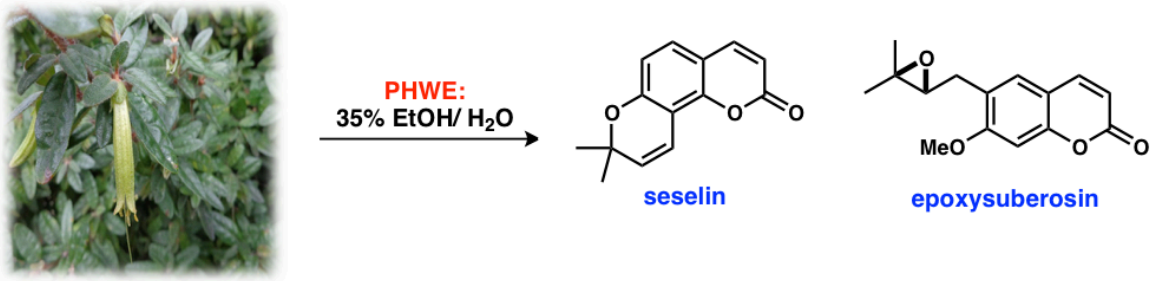
TAKEN FROM LAB MANUAL (University of Tasmania)

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).

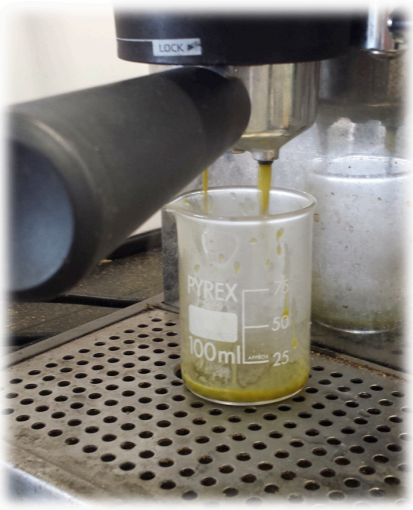
Part B. Extraction of Other Plant Material

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).