Experiment 2: ISOLATION AND ANALYSIS OF CITRUS OILS
A GREEN-CHEMISTRY APPROACH

Learning Objectives

- Understand principles behind distillation and gas chromatography (GC)
- Critical analysis of distillation, liquid-liquid separation, and GC technique
- Analyze data to assess percent recovery & composition of essential oil
- Understand the role of intermolecular forces as they relate to volatility and boiling point

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How to Complete this Lab + Assignments

See Canvas Exp 2 Module

BEFORE LAB

1. Read this PDF – background, procedure, safety, pre-lab and in-lab questions
   - Option to listen to **Exp 2.1 & 2.2 Podcasts** = Caitlin reads this lab in 2 parts ☺
2. Attend **lab lecture** and take notes on **templates**
3. Practice the lab online *via* **Slugs@home** - sites.google.com/ucsc.edu/slugshome/home
4. **Pre-lab questions** – prepare your responses to the questions that follow the procedure
   - **Pre-lab quiz** on Canvas, due the Monday before lab – based on pre-lab questions

5. Lab Notebook Preparation – *Required at the start of lab or you'll be sent home :/*

- Refer to the **Lab Notebook Template** to prepare your lab notebook, one day at a time…
- **Purpose:** brief summary of the main lab goals and structures of citrus oil components
- **Reagent Table** – add chemical properties; Wikipedia is a reliable source for chemical info
- **Procedure with Diagrams**
  - Instructions & sketches with all **equipment, chemical names** with **amounts**, & **transfers**
  - Break up text with bullet-points & diagrams. Avoid copying the procedure word-for-word.
  - *Feeling lost?* Go to the **Slugs@home website** - pictures & videos of the whole lab
  - **Cleaning & Safety** – copy the table from the end of the procedure
- **Data** – entries copied from template

DURING LAB

- Check the **safety rules** to dress for lab and arrive a few minutes early to **Thimann Labs**
- **Pre-lab talk:** tips for success and open Q&A
- Show your prepared **lab notebook pages** to your TA
- Perform the experiment with a partner, fill out data & observations in **lab notebook**

AFTER LAB – each partner submits separate, individual assignments

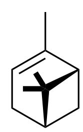
- Upload **Notebook Pages** to Canvas by midnight on lab day – graded on completeness / participation
- Complete & upload the **Lab Report** on GradeScope (GS) – due date on Canvas
  - Responses to questions & prompts - use the **template** at the end of this document

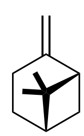
BACKGROUND: CITRUS OIL DISTILLATION AND GAS CHROMATOGRAPHY (GC) ANALYSIS

Essential oils are mixtures of volatile compounds made by plants to communicate with their environments. They are used to attract insects and other animals to help in the fertilization and propagation processes. Some are also herbicides used by plants to defend their territory from aggressive vegetation. In this experiment students will isolate the essential oil of oranges. This same procedure could be used to isolate most other citrus oils and fragrant plants – lemon, grapefruit, lavender, and spearmint! Steam distillation will be used to obtain the oil from citrus peels and gas chromatography (GC) will be used for analysis.

Distillation of freshly grated citrus peels with water will produce a mixture that consists largely of water and small amounts of citrus oil. The oil is immiscible with and less dense than water. The distillate may look cloudy because of the emulsification of the oil in water, but if left to settle, it will separate into two layers: a large aqueous bottom layer and a small oily upper layer. The separation of the oily upper layer from the bottom aqueous layer can be difficult, especially if the volume of oil is small. To circumvent this problem, the oil is usually separated from the water by liquid-liquid extraction with an organic solvent (most commonly, ether). This process is very effective in removing the oil from the water but requires the use of organic solvents, which are usually toxic, expensive, and contribute significantly to the waste stream. This is of great cost to the planet.

Green chemistry is the quest for finding safer and more environmentally friendly chemicals to carry out common laboratory operations. One of the principles of green chemistry is to use safer solvents or eliminate solvents altogether if possible (reduce, reuse, recycle!). It is common industry practice to trap, purify, and reuse solvents and reagents as much as possible. Unfortunately, waste is an inevitable part of research even with extensive recycling efforts. Steam distillation is a green chemistry method for collection of citrus oil without organic solvents. Enough oil is produced that it can easily be separated from water using a 50-mL buret (the same piece of glassware used in titrations). All waste from this procedure (citrus water) is safe to put back into the environment!


α-Pinene 155 °C


β-Pinene 165 °C

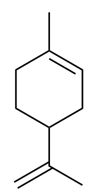
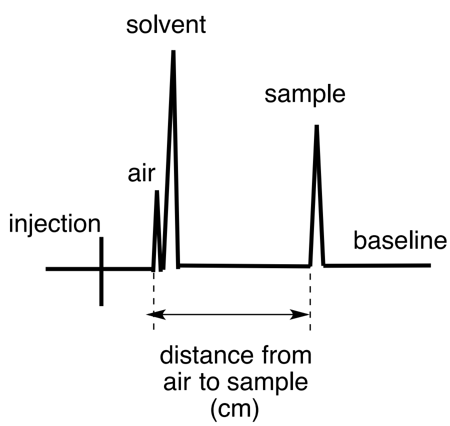

Limonene 175 °C

Figure 1. Terpenes commonly found in citrus oils and their normal boiling points (1 atm)

Citrus oils consist largely of volatile hydrocarbons and small quantities of aldehydes, alcohols, and other oxygenated compounds. Any *essential oil* has a particular combination of chemicals that makes its smell unique and often times easily recognizable. Spearmint, orange, and lemon oils have very similar components but very distinct fragrances! It is common for natural plant extracts to contain compounds having carbon atoms in multiples of five. This broad class of compounds is called **terpenes**. They are biosynthetically derived from two or more *isoprene* units. There are several different ways for plant metabolism to combine two five-carbon isoprene units into a ten-carbon *monoterpene*, hence the occurrence of many different isomers. The most prominent terpenes in citrus oils are **alpha-(α)-pinene, beta-(β)-pinene, and limonene**. The major terpene in citrus oil is **limonene, with 90 – 97% composition** (**Figure 1**).

The isolated oil will be analyzed by gas chromatography (GC) to determine its composition. The components of the oils usually travel through the heated GC column in the order of their boiling points. **More volatile, lower boiling compounds travel fastest and elute first (earliest to exit the column and create a peak on the GC chart).** Less volatile compounds travel slower and elute last. There are exceptions to this relationship so authentic samples of each compound are injected individually. The authentic samples are called **standards**. **Retention times** are calculated and used to compare the standards' relative elution (exit) order from the GC column. The standard retention times are compared to those of the peaks in the citrus oil. The areas under the peaks will be calculated to determine the percent composition of the oil.

At a given set of GC conditions (temperature and type of column) *on the same instrument,* one compound will consistently exit the column in the same amount of time after injection. This is known as the **retention time, t_R** (the amount of time the compound is retained on the column). For example, students inject pure limonene and observe one major peak. There is no timer on the recorder, but it moves the paper at a certain speed (2.5 cm/min). The distance (cm) from the start of the injection to the beginning of the peak can be converted to its retention time in minutes (**eq 1**) then converted into seconds. For better precision, students should calculate **corrected retention times (t_R')**, where the distance is measured using the air peak as the beginning of the run. You can expect there to be several peaks in the chromatogram of citrus oil, which correspond to the various components isolated in the distillation step. If the citrus oil contains any limonene, then expect to see a peak at the exact time as the standard. Once the peaks are identified, calculate the percent composition of each component in the citrus oil using the area under the curves (integration) of each of the peaks (**eqs 2 & 3**).



$$t_R' \text{ (sec)} = \text{distance from air to sample (cm)} \times \underbrace{\frac{1 \text{ min}}{2.5 \text{ cm}}}_{\text{chart speed}} \times \frac{60 \text{ sec}}{1 \text{ min}} \qquad (1)$$

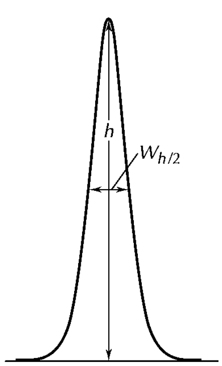


FIGURE 20.13
Determining peak area: h = height; $W_{h/2}$ = width at half-height.

Integration: Peak Area = $h \times w_{h/2}$ **(2)**

$$\text{\% Composition of A} = \frac{\text{Area of component A}}{\text{Total area of all components}} \qquad (3)$$

PROCEDURE, PART 1 - ISOLATION OF CITRUS OILS

1. At Home: Prepare peels before lab. Use either **5 oranges, 6 grapefruit, 8 lemons, OR 8 limes** per group. **Select your citrus** using the guidance below, or just bring what's available to you!

- Stronger citrus fragrance = higher terpene content = better recovery ☺
- No smell means low terpene content and little-to-no recovery
- Fruit fresh from the tree gives best results, if you happen to know a guy.
- Caution! Clementines or "cuties" with thin peels tend to give very low recovery ☹

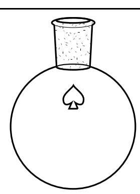
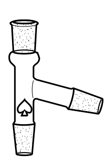
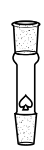
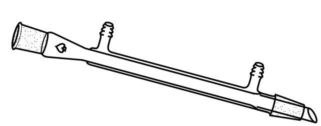
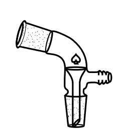
Chop or grate your citrus. Read bullet points below, then write what applies to you in your lab notebook.

- We strongly encourage you to **prep peels at home**, if at all possible.
  - **Only one grater** will be provided in each lab.
  - *Unable to bring citrus peels?* Please fill out the **Citrus Peel Request form** (Canvas Exp 2 module) by the **Friday before Exp 2.**
- **Prepare the peels the night before** and store in the fridge if necessary, but the fresher the better!
  - Transport peels to lab in a leak-proof plastic bag (no Tupperware).
- **OPTION 1, Grate:** It is ideal for the colorful part of the peel to be *coarsely* grated the morning of lab, leaving behind the pith (white part) to the best of your ability. It doesn't *need* to be a small zester, but that's ok too.
- **OPTION 2, Chop with sharp knife**: carefully cut the colorful peel away from the white pith, then further chop to approximately 1 cm^2 pieces.

2. Distillation Assembly: Tare a large beaker on the balance and transfer the prepared citrus peels into it. Record the mass of peels. (The mass should be no less than 100 g and no more than 150 g.) Transfer the peels into a 500-mL round bottom flask (RBF) set on a cork ring using a wide-mouth plastic funnel and stir rod to poke the peels through. **Add 150-175 mL of distilled water** (depending on your mass of peels) to the beaker, swirl to pick up any bits of peel, and gradually transfer into the 500-mL RBF. Assemble a simple distillation apparatus, beginning with the 500-mL RBF (see **Figure 2** and more instructions on next page).

Caution!

- The peels *and water* should already be in the flask *before* clamping it to the ring stand.
- If you forget to add the distilled water before heating, the orange peels will scorch the flask ☹
- Do NOT transfer orange peels over the heating mantle. That would create burning orange peels later ☹

Round-bottom flask (RBF)	Distillation Head	Thermometer adapter	Water-jacketed condenser	Take-off adapter
				

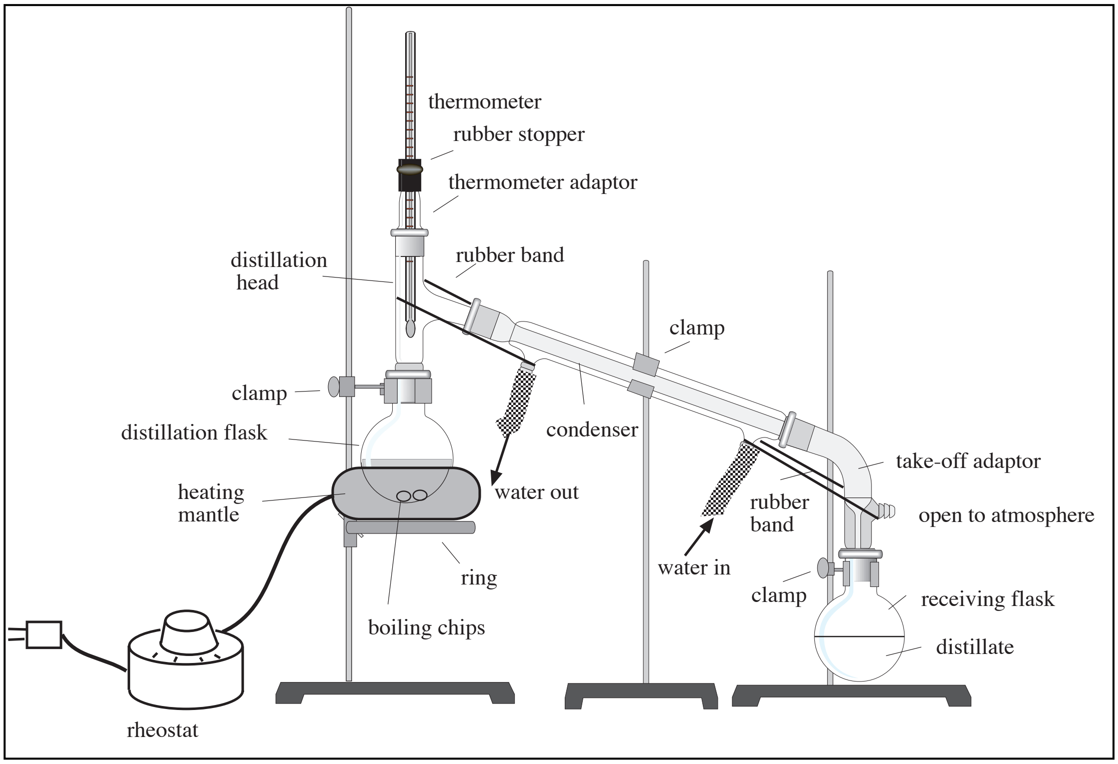


Figure 2. A 'simple distillation apparatus' used for steam distillation (omit boiling chips).

** See also the **Exp 2 class note templates** for step-wise diagrams to set up the distillation apparatus. Copy these labeled diagrams by hand into the procedure portion of your notebook. Your TA will demonstrate the distillation setup at the beginning of lab.

In addition to the supplies in your drawer, obtain **three ring stands, 2 rubber bands, 3 clamps, 1 support ring, 4 clamp holders,** and **2 hose clamps**. Center the ring stands in front of the **water hoses** at your bench. Secure the 3 **clamps** and **support ring** onto separate clamp holders. Clamp the support ring approximately halfway up the ring stand. This must be secure enough to hold the weight of the **heating mantle** and boiling orange peel suspension. Attach one of the clamps to the same ring stand above the heating handle, leaving plenty of room for the **500-mL RBF**. Hold the RBF *containing orange peels and water* with one hand and pinch the clamp around the neck of the RBF with the other hand. Keeping the pressure on the clamp, let go of the RBF and securely tighten the wing nut of the clamp. The flask should be steady and there should be no space in between the clamp, clamp holder, and ring stand.

Carefully raise the heating mantle by loosening the clamp holder from the ring stand. Leave a **small air gap (1 mm)** in between the flask and the heating mantle. Tighten the clamp holder to the ring stand. It may be necessary to adjust the position of the clamp horizontally (closer or farther from the ring stand). **Have your TA check your setup**, then turn the heating mantle to LOW while assembling the rest…

Add a *small dab* of **grease** to the joint of the **distillation head**. Attach the distillation head to the clamp and turn to spread the grease. Separately, insert a **thermometer** into the red **rubber thermometer adaptor** (not a stopper) using water as a lubricant (NOT grease), then secure the rubber adaptor around the **glass thermometer adaptor**. Lightly grease and connect the adapter joint to the **distillation head** and spin to spread the grease. Loosely drape two **rubber bands** around the **condenser** *in between the hose adaptors*. Connect the '**water in**' hose (labeled blue) to the bottom **water inlet** using pressure and a circular motion, then secure with a small wire clamp. The hose must be at least over two bumps on the hose adapter. Repeat with the '**water out**' hose (labeled red) and the top **water outlet**. The rubber bands should still be in between the hoses on the condenser.

Wrap the first rubber band around the **distillation head**, attach the condenser to the distillation head, and turn to spread the grease. Position a clamp *loosely* around the condenser with the second ring stand. This clamp is a **secondary support and the condenser should rest on the clamp**, rather than the clamp being secured tightly around the condenser. Wrap the second rubber band around the **take-off** adaptor and connect this to the condenser with grease. The rubber bands should be securing the set-up as shown below. Loosen the water clamp to begin water flow.

Pour 70 mL of water into a **250-mL RBF** and mark the level of the liquid with a marker. Pour the water out and use this as the **receiving flask** for the distillate. Attach to the take-off adaptor then clamp the flask once in position. Your TA must OK your apparatus before increasing the heat. Heat the distillation flask using a heating mantle, starting at a medium setting. When the **first drop of distillate** enters the receiving flask, **record this initial temperature** on the thermometer, estimating one decimal place (ex. 97.0 °C). Thank you for your patience - it may take about 20 minutes for that first drop of distillate (mostly water, tiny bit-o-terpenes).

**** During the distillation down-time, carry out the GC calculations (see Part 2).**

- **Chromatograms (graphs) will be provided in the lab.**
- Prepare before lab - copy the GC data tables from the Exp 2 Lab Notebook Template.

[Back to distillation] Adjust the heat supply so that the liquid distills at an approximate rate of **one drop per second**. Distilling too quickly with too high heat will severely decrease recovery of oil and becomes dangerous. Collect approximately 70 mL of distillate. Record the **final temperature**, estimating 1 decimal place (ex. 99.0 °C). **Stop the distillation:** turn off and unplug the heating mantle, then carefully lower the ring support for the heating mantle. You will need two sets of hands to accomplish this safely. You may take off the receiving flask; place a **beaker** under the take-off adapter to collect any further distillate.

Wait for the entire system to cool before disassembling. Refer to the **Clean-Up & Safety Table** for taking apart the cooled apparatus.

3. Separation and Collection of Citrus Oil: Fasten a 50-mL buret to a ring stand using a buret clamp. Use a funnel to add distilled water to leave about 5-mL of space above the liquid. *Slowly* add a few milliliters of the orange distillate to the buret, being careful not to overflow. Allow the liquid to settle for 1-2 minutes, then drain to the 45 mL mark, catching the liquid in a 125-mL Erlenmeyer flask. Repeat the add – settle – drain process until all of the distillate has been transferred. Add 10 mL of water to the flask, swirl to dislodge any oil, and transfer the liquid portion-wise to the buret. Repeat this operation with another 10-mL portion of water. This process should limit the formation of emulsions, allowing the orange oil to neatly rest on top of the water.

Let the system settle for at least 5 minutes, or until phase separation of oil on top of water is apparent. (There may be some air bubbles or oil droplets stuck along the buret walls, however, we have found that attempting to collect this oil has an overall negative effect on recovery.) The level of the liquid should be near the 50-mL marking so that you can collect this top layer of citrus oil by pipet. Label a small screw-cap vial with your name and the name of the oil. Weigh it and keep it handy.

Use a glass pipet and pipet bulb to carefully collect the layer of oil from the top of the buret, avoiding the water as much as possible. You will likely not be able to collect all of the oil so just do your best! Students can expect to recover anywhere from 0.5 to 5 mL of citrus oil. Transfer the oil to the pre-weighed vial. Carefully remove any visible droplets of water in the bottom of the vial using a pipet. Weigh the labeled vial with the oil and determine the mass by difference. Calculate the **% recovery** of citrus oil from citrus peels. Cap the labeled vial and store in your drawer for GC analysis (Day 2). **Share your peel observations** (3 descriptors – color, size, and fragrance), as well as **% recovery with a neighboring group**. You'll need this for the lab report!

PROCEDURE, PART 2 – GAS CHROMATOGRAPHY (GC) ANALYSIS OF CITRUS OIL

* *Time permitting*, TAs will guide students in the use of the **GC during distillation down-time**.

* **Sample charts** will be provided to ensure everyone gets GC data for the lab report (standards & citrus oil).

* Bring a **calculator**; take off gloves when using it. Do not contaminate your phone in the lab!

4. GC Sample Preparation: Record the chart speed and all other conditions posted on the bulletin board above the instrument (oven temperature, etc.). Use care when handling the sharp, delicate needles. Rinse the syringe three times with the sample before each run to treat the syringe and remove air bubbles. Load 0.2 μL of sample into the syringe and pull back to the 10 μL mark with air. Write the sample name on the chart paper then turn on the chart recorder. Turn the nob on the recorder to mark the beginning of the run and quickly inject the sample with air into the HOT injection port. The air peak will be used as the starting point for the **corrected retention time**. Rinse the syringe three times with acetone and clean the outside of the needle with a Kim wipe to remove traces of liquid after each injection to avoid cross-contamination.

5. GC Data Collection: Injection of Standard Terpenes & Citrus Oil

(time permitting; see notes on previous page)

Inject the terpene standard: **α-pinene, β-pinene,** and **limonene (1:1:1 mixture)**. Allow all the components to come out before performing another injection. Measure and calculate the **corrected retention times** of each standard (see page 3, **eq. 1**) *before* injecting your citrus oil. Although not ideal, **standard peaks** can still be interpreted if the peaks level off at the top (flat), because only the retention time is needed.

Ask your TA to inspect your oil for potential water contaminants. Then, **inject 0.2 μL of your citrus oil** into the **same GC instrument used to inject terpene standards**. Since the pinenes are such minor components in citrus oil, the tallest terpene peak (limonene) should be about 75% of the chart height. Repeat the citrus injection to reveal minor components, if needed – complete no more than 3 injections and consult your TA. Often, **simply repeating an injection at the same volume** will provide data that is easier to interpret. **Do NOT increase sample volume beyond 0.2 μL.** It is relatively common for limonene to be the only peak in the citrus oil chromatogram.

Calculate the **corrected retention time** of each sample peak in your citrus oil chromatogram. Determine which **terpenes are in your citrus oil** by comparing to the corrected retention times of the terpene standards. Measure the height (**h**) and the width-at-half-height ($\mathbf{w_{h/2}}$) of each sample peak. Calculate the **area** of each peak (integration) from these measurements (page 3, **eq. 2**). Calculate the **percent composition** of the oil (page 3, **eq. 2**).

Set yourself up for success: *Copy the examples and equations from page 3 into your notebook. These calculations must be performed during this lab period and checked with your TA.*

**** Take a picture of your terpene standard chromatogram (chart)** and your best **citrus oil chromatogram** to include in your lab report. We cannot grade your results without your GC chromatograms! **

Table 1. Clean-up and Safety – Exp 2 - Distillation

Clean-up	**Safety**
Allow the distillation apparatus to cool completely before disassembling. All ring stands, clamps, etc. should be put back in an organized manner. **Taking the hoses off of the condenser:** disconnect the condenser on both ends, clamp the hoses to stop water flow, then carefully remove the hoses OVER THE WATER TROUGH with a back-and-forth motion	
Strain the distilled orange peels through a strainer into the sink, using water to aid the transfer with shaking. When the strainer becomes full, transfer to the specified compost container.	Use **caution with the heating mantle and distillation apparatus.** Slowly increase heat, which should never go past a **medium setting**.
Dispose of the aqueous layer from the buret down the drain (orange water is safe for fishies!).	α-pinene and β-pinene are **irritants**. Limonene is a possible **carcinogen**.
	Minimize chemical exposure by wearing a **lab coat, goggles, and gloves** during the entire experiment.
Dispose of **pipets** in the glass waste box.	

Table 2. Clean up and safety, Part 2 - GC

Clean Up	**Safety**
Rinse syringes with acetone three times after each injection and clean needle with Kimwipe. Dispose of the citrus oil in its labeled vial in the waste container bag provided, after all GC analysis is complete.	GC Needles are sharp, delicate, and expensive – handle with care.
Keep the instrument room clean and free of personal belongings. No more than 6 students should be in the instrument room at any given time. GC kits should be kept clean and organized. Cap the markers after completing all GC runs.	

Experiment adapted from Palleros, D. R. "Recrystallization of Acetanilide," *Experimental Organic Chemistry*, Wiley: New York, **2000**.

Pre-Lab Questions

The following questions are incorporated into the Canvas Exp 2 pre-lab quiz. **Prepare your responses before starting this individual quiz.** There is a 20-minute time limit with two attempts, and the highest score is recorded in Canvas.

1. What is a **terpene** and what is the approximate percent composition of each of the terpenes from **Figure 1** in orange oil?

2. What is “green chemistry”? Describe how the distillation procedure used in this lab is a more “green” approach than liquid-liquid extraction with organic solvents.

3. Why should the distillation apparatus have an opening to the atmosphere at the end?

4. How are terpenes collected in this steam distillation, even though their boiling points are significantly higher than water?

5. Define the following concepts and terms as they relate to gas chromatography.
   a) Mobile Phase, AKA Carrier Gas
   b) Stationary Phase
   c) Integration
   d) Corrected Retention Time

6. Look up the **boiling points** of the terpenes commonly present in citrus oil. Predict the order in which these compounds should exit the GC column: **β-pinene, limonene, α-pinene.**

Full Name: **Lab Section:** [replace with day, time, room]

Lab Partner Name: **TA Name:**

EXPERIMENT 2 LAB REPORT TEMPLATE, Page 1 of 4

Download the “Lab Report Template” from Canvas and edit the MS Word doc; or **recreate** the template in a new word processing document. [Keep these instructions]

- Responses must be on the on **same part of the same page as this template** for grading.
- For each question / prompt, provide **TYPED, numbered** responses in **complete sentences**.
- Insert images of **hand-drawn diagrams** and **calculations**, where applicable.

1. Report the **temperature range of distillation** during the collection (temperature at which first and last drop of distillate was collected). [Top of page 1]

2. Was the **initial** distillation temperature **higher or lower** than water’s boiling point?
Explain why the **presence of the peels** affects the **boiling temperature of the water molecules**.
[Middle of page 1]

3. Report the **mass (mg)** and calculate the **% recovery of citrus oil** from the peels. **Show your work** with units on each value and state these results in a complete sentence.
[Bottom of page 1]

% Recovery = [(mass of oil) / (mass of peels)] x 100%

EXPERIMENT 2 LAB REPORT TEMPLATE, Page 2 of 4

4. Report the **observations** of your citrus peels (at least 3 characteristics – ex. size, color, aroma).
[Top of page 2]

5. Report a different group's peel observations and % recovery. [Middle of page 2]

- Or compare your result to the "Slugs@home" remote lab: calculate the % yields for chopped or grated peels using **150.00 g** as the initial mass of peels. See links in Canvas Exp 2 module.

6. Consider how the **terpene molecules** are released from the **peels** and **explain** the observed **relationship between <u>peel size</u> and <u>% recovery</u>**. [Bottom of page 2]

EXPERIMENT 2 LAB REPORT TEMPLATE, Page 3 of 4

7. Gas Chromatography – Standard Terpene Analysis

Note: use the *GC data obtained in the lab OR the sample charts provided*; don't mix & match! Retention times of standards and citrus oil will only match if they're run on the **same instrument**.

(a) Conditions: Report the **GC Name** and how data was obtained (**sample charts or new injections**).
[Top of page 3]

(b) Standards: Report the **corrected retention times (t_R')** for each terpene standard, table format below.
[Middle of page 3]

Standard (pure) GC Corrected Retention times, t_R'

Sample	**t_R' (s)**
α-Pinene standard	
β-Pinene std.	
Limonene std.	
γ-Terpinene std.	

(c) Show any one **sample calculation** for t_R' of a terpene standard. [Bottom of page 3]

[insert image of GC standard chart analyzed]

EXPERIMENT 2 LAB REPORT TEMPLATE, Page 4 of 4

8. GC Analysis of Citrus Oil

(a) Citrus Oil: Using the table format below, Report the **corrected retention time, integration (area), and % composition** for each peak in the GC chromatogram of citrus oil. Assign a number to each sample peak. [Top of page 4]

(add as many rows as needed)

Peak #	**Peak (Terpene) Identification**	**t_R' (s)**	**Integration (cm^2)**	**% Composition**

(b) Show one **sample calculation** each for **integration (area)** and **percent composition**. [Middle of page 4]

5. What is the **major component** of the citrus oil you analyzed? What are the **minor components**, if any? How does this compare with the expected composition? *Hint: everyone should have the same major component!* [Bottom of page 4]