

The Technique of Distillation

Isolation of Volatile Oils from Spices by Steam Distillation and Bioassay for Biological Activity

Introduction

The spices cloves, cinnamon, ginger, nutmeg, and tumeric will be provided. There are many other possible spices such as caraway, cumin, or thyme. Feel free to bring in ~10 grams of a spice of your choice. There is no guarantee that a particular spice will yield much volatile oil or that the components of this oil will have biological activity, but it will be fun to see if they do. If you don't get anything from your spice, you can continue the procedure with some clove oil that is available.

Activities:

- ✓ Read the Introduction section of the Distillation Experiment found in Chapter 5 of the Lab Guide.
- ✓ **In the first lab (Day One)**, you will use **steam distillation** to separate any volatile oils from your spice of choice; you may work in teams for this distillation only. In preparation for this, read the theory of steam distillation presented below. You will be using a variation of the distillation set-up as in Procedures 1A and 1B in Chapter 5 of the Lab Guide. In place of the liquid mixtures toluene/cyclohexane or ethanol/water, you will use water and your solid spice to carry out steam distillation. However, typical distillation data for simple and fractional distillation is provided below and you will plot these for your Final Report (see last page of this handout). You are responsible for understanding how simple and fractional distillations are done and the advantages and disadvantages of these types of distillations as well as steam distillation.
- ✓ You will inoculate sterile agar plates with *Bacillus Cereus* bacteria and incubate them to test biological activity. The component that you isolated from the spice will be tested for antibacterial activity.
- ✓ You will use GC and GC/MS to characterize your volatile oil. In preparation for product analysis using these methods, read Sections 11.7 and 11.7.1 on gas chromatography, Sections 11.2.1 – 11.2.4 on mass spectral interpretation, and section 11.8 on gas chromatography/mass spectrometry of Chapter 11 in the Lab Guide. If you do not have time to finish the GC and/or GC/MS runs, sign up for time outside of lab time to complete these analyses.
- ✓ **In the second lab (Day Two)**, you will be carrying out Procedure 3 (p.116 of the Lab Guide), a microscale fractional distillation of an unknown mixture; work individually. This second distillation will give you experience with the fractional distillation technique.

PreLab:

Your chemical data table should include the chemicals mentioned on page 116 of your Lab Guide and also eugenol, thymol, and quinine. Hand in a photocopy of the completed **Common Shelf Chemical Date Table** with your PreLab.

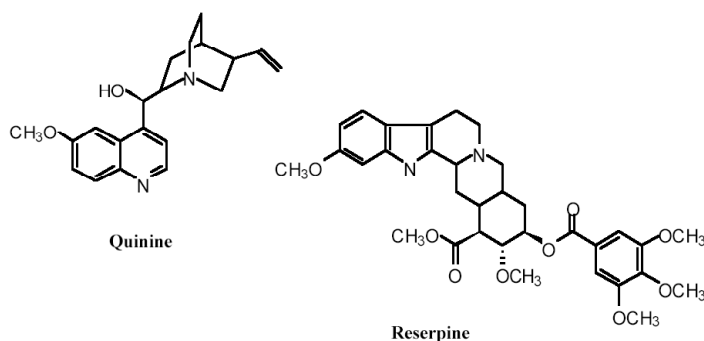
Answer the PreLab questions in Chapter 5 (p 106) of the Lab Guide.

Modeling the Drug Discovery Process

Adapted from William H. Miles, (Department of Chemistry, Lafayette College, Easton, PA 18042; milesw@mail.lafayette.edu) and Patricia M. Smiley (Southern Lehigh High School, 5800 Main Street, Center Valley, PA 18034; smileyp@solehi.k12.pa.us), *J. Chem. Ed.* **2002** 79, 90

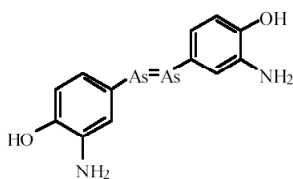
Chemistry and Medicine

Since ancient times, humankind has sought medicines from nature. Quinine, the antimalarial compound isolated from the bark of the cinchona tree, and reserpine, the antidepressant from the Indian plant *Rauwolfia serpentina*, represent two of the more famous natural products used in modern medicine.

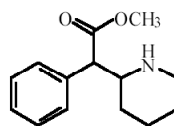


Today, pharmaceutical companies use several approaches for the discovery of new compounds to treat human illness, including the screening of natural products derived from bacteria, fungi, plants, or animals (not puppies or even mice, but animals like slugs or sponges, simple multi-cellular, bottom-dwelling animals called “Porifera”). For example, extraction of a marine sponge with an organic solvent can yield a complex mixture of organic compounds, which can then be tested for biological activity or are “bioassayed”. If the mixture shows promising biological activity, the components of the complex mixture may be separated and purified. When further testing identifies the active component, its chemical structure can be determined using modern spectroscopic techniques (IR, NMR, Mass Spectrometry, X-ray crystallography). Even if the new compound has side effects that render it unsuitable as a drug candidate, the pharmaceutical chemists will use the compound as a guide for the synthesis of new drug candidates with similar structures.

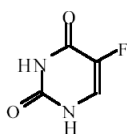
The synthesis of organic compounds, whether of natural products or of “unnatural products” (compounds that are not found in nature), has been a primary goal of organic chemists. Some of the first synthetic compounds produced industrially were dyes, fragrances and flavorings. It was not until the twentieth century that synthetic drugs were successfully developed. The synthesis of the antisyphilitic drug arsphenamine, Compound 606, by Ehrlich in 1910, was a dramatic demonstration of the power of chemistry to attack a medical problem. (Yes, arsphenamine contains arsenic. Not good stuff, but remember, you’re dying of syphilis, so you can’t be fussy.) Since then, many new compounds, including biologically active compounds found in nature, have been synthesized in the laboratory and have become useful drugs. For example, Ritalin is used for the treatment of attention deficit disorder (ADD), 5-fluorouracil is a potent anti-cancer drug, and L-DOPA is used to treat Parkinson’s disease.



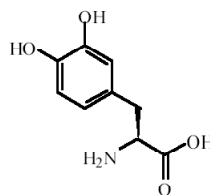
Compound 606
(Salvarsan)



Ritalin



5-Fluorouracil

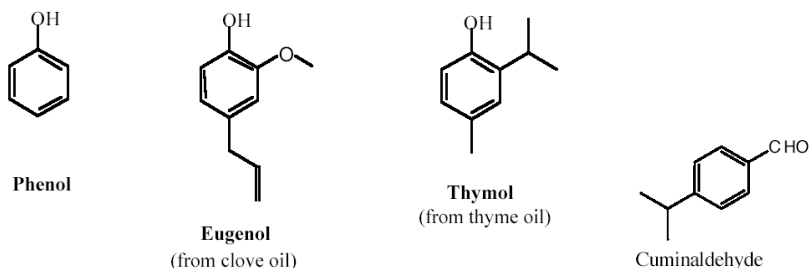


L-DOPA

Essential oils, which are isolated by steam distillation of volatile organic compounds present in plants and animals, have a long and rich history in medicine. Typically, complex mixtures of organic compounds, essential oils, have been used to treat a variety of illnesses for thousands of years. In some cases these essential oils do have therapeutic utility by modern medical standards. The essential oils of cloves and thyme contain phenolic compounds (organic molecules that have an OH group attached to an aromatic ring), which are effective antiseptics (germ-killers). Even before the establishment of the germ theory, the antiseptic power of both thyme oil and clove oil was recognized. Joseph Lister, whose name is immortalized by the mouthwash *Listerine*, used synthetic phenol (carbolic acid) to prevent infection during surgical procedures in the mid-nineteenth century,

revolutionizing the medical treatment of wounds. He recognized that antiseptic chemicals can prevent infection and proved the value of some of the old herbal remedies. Phenolic compounds, both synthetic phenol and thyme oil, were used as antiseptics until World War I.

The clove tree produces flower buds which, when harvested and dried, become the familiar spice of the same name. It has long been known that cloves contain an ingredient with an anesthetic property that is particularly effective against dental pain. Before modern medicine developed better remedies, people often chewed on cloves to dull the pain of a toothache. The active compound responsible for this effect is found in the essential oil of cloves which makes up approximately 16-18% of the clove by weight. The major component of this oil is the compound *eugenol*. Eugenol has a boiling point of 254° C. It would be difficult to isolate this oil by simple distillation since this high temperature could lead to its decomposition as well as the decomposition of other components of the clove. However, eugenol can be isolated relatively easily from cloves using the technique of steam distillation. Once the aqueous distillate containing the clove oil has been obtained, the oil can be separated from the water by extraction into the organic solvent dichloromethane.



The dried fruit of cumin (*Cuminum cyminum* L.), a small shrub cultivated in eastern Europe and India, is an important seasoning in curries, goulashes, and sausages. It is also a major component of chili powder used in Mexican food. The major volatile constituent of cumin is *p*-isopropylbenzaldehyde (cuminaldehyde). Cumin oil also contains limonene and other compounds which contribute to the aroma of this condiment.

The Isolation of Organic Compounds

As you know, functional groups give the molecules distinctive chemical reactivity, as well as play a role in the physical properties of compounds. Different physical properties allow the separation of one component from a mixture. In some cases, separations can be based on the solubility of the compound in a given organic solvent, so that a compound can be recrystallized, as you learned in the recrystallization technique experiment. As you will learn in this technique experiment, organic compounds with appreciable vapor pressure can be separated by distillation at atmospheric pressure, or if their vapor pressure is low, steam distillation or vacuum distillation at reduced pressure can be used. In some cases, as in the Liquid/Liquid Extraction Technique Experiment, separations can be done based on the acid/base chemistry of the organic molecules. Many separations are based on chromatographic techniques, which you'll study later.

Biological Testing

The testing of compounds for biological activity is the first step in the determination of their potential as a drug in medicine. In most cases, the compound is tested against an organism (bacteria, fungi, or virus) *in vitro*, that is, outside a living organism in a controlled environment such as a Petri dish. One method for testing antibiotic activity is to apply the compound onto a sterile paper disk and place the disk on an agar plate containing a culture of bacteria. If the compound is active, then a zone of inhibition will appear around the disk; other areas of the Petri dish will show evidence of bacterial growth. The size of the zone indicates the potency of the drug. Adjusting the amount of compound applied to the disk allows a crude dosage effect of the compound to be seen.

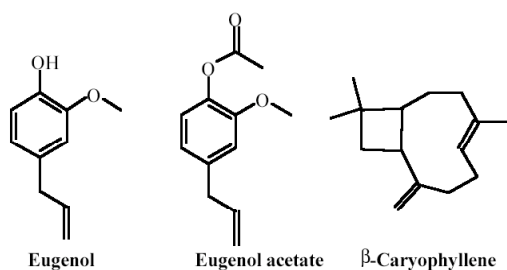
If the pharmaceutical companies discover a promising lead, the next step is to investigate the potency of the drug *in vivo*. Animal studies determine the effectiveness of the drug as well as provide information about the toxicity, metabolic pathway of decomposition, and side effects of the drug. After exhaustive animal studies, the drug will then undergo human trials. Human trials start with Phase I clinical trials, in which the toxicity and dosage response of the drug is determined with healthy volunteers. In Phase II clinical trials, the drug is tested on people (100-300) who have the disease. In Phase III clinical trials, the compatibility of the new drug with other medications is determined in several clinics and hospitals with as many as a thousand patients. If no

complications or serious side effects are found, then the company can apply for Food and Drug Administration (FDA) approval for this drug in the treatment of a given disease. If FDA approval is granted, then the drug is available for physicians to prescribe. Since problems may appear even after such extensive testing, the pharmaceutical companies continue to monitor the use of their drugs.

This process is long and expensive. The average cost has been estimated at 250 million dollars, with at least a ten-year span from the initial testing of the drug to final FDA approval. It has been estimated that only one compound out of ten thousand compounds prepared by a pharmaceutical company receives final FDA approval. Although the research and development costs are considerable in the pharmaceutical industry, the rewards are significant; a blockbuster drug like Prozac has sales in the range of two billion dollars a year.

Modeling the Drug Discovery Process

The separation of a biologically active compound such as eugenol from a mixture of organic compounds obtained from a natural source (cloves in the case of eugenol), and biological testing of this compound (as well as the other compounds) using a simple bioassay for antibiotic behavior, models many aspects of the drug discovery process. You will separate, isolate and test the antibiotic activity of the initial organic extract of the steam distillate using *Bacillus cereus* growing on agar plates, which you will prepare.



Steam Distillation

Distillation is a common method for the separation and purification of organic compounds. These are usually liquids at room temperature although numerous lower melting solids can be distilled at higher temperatures, say above 150°C. However, many organic substances decompose at these higher temperatures. By distilling at low pressure (~ 1 torr or 1/760 of an atmospheric pressure), the material boils at lower temperatures and decomposition is minimized. The vacuum pump carts you see in the lab are used for this purpose.

Steam distillation is another way to distill high boiling substances and is useful for the isolation of oils, waxes, and some complex fats. Any organic liquid that is immiscible with water can be distilled at a temperature around 100°C, the boiling point of water. For example, eugenol boils at 254°C and would decompose extensively if we tried to distill it at this temperature. We could use vacuum distillation or steam distillation. How does steam distillation allow the vaporization of such a high boiling substance as eugenol at ~ 100°C? Unlike the normal distillation of two miscible liquids, in steam distillation, each component of an immiscible liquid mixture contributes to the total vapor pressure as if the other component were not present. Another words, the total vapor pressure of the mixture is the sum of the vapor pressure of the water plus the vapor pressures of each of the organic components in the mixture.

$$P_{\text{total}} = P_{\text{component 1}}^0 + P_{\text{component 2}}^0 + P_{\text{component 3}}^0 + P_{\text{component n}}^0 + P_{\text{water}}^0$$

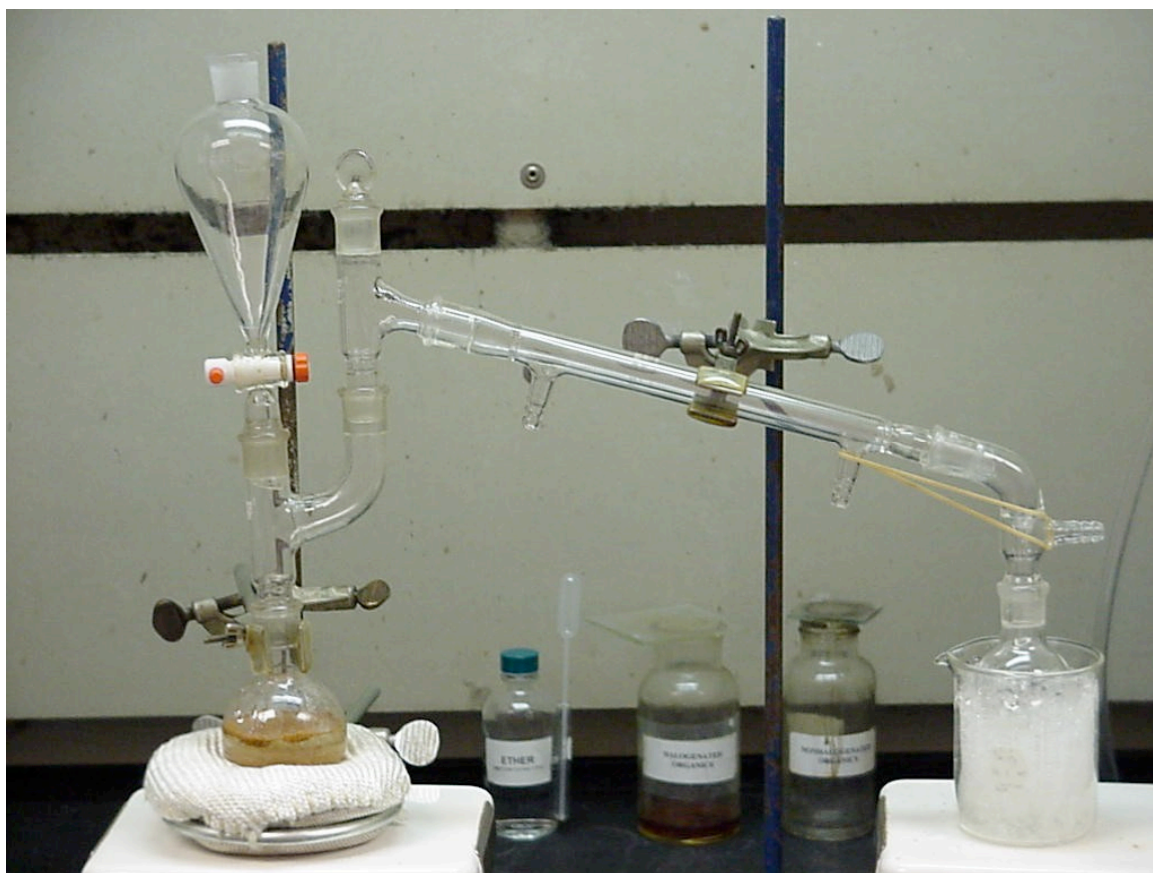
A liquid or liquid mixture boils when the total vapor pressure of the liquid is equal to atmospheric pressure. The vapor pressure of water is 760 torr at 100°C. The presence of any immiscible organic material contributes to the total vapor pressure and thus causes the boiling point of water to drop below 100°C. While eugenol only has a vapor pressure of about 20 Torr at 100°C, the eugenol vapor is swept out of the boiling flask by the boiling water vapor or steam into the condenser where everything condenses, separating into liquid water with small amounts (~ 20/760's) of immiscible eugenol condensing out also. This yields a cloudy distillate of water and eugenol (plus other volatile organic substances). Since it takes a lot of water vapor to sweep out the higher boiling organic oils, it is often necessary to add water to the distilling flask at frequent intervals throughout the distillation until the organic compounds have been removed from the mixture. The liquid in the condenser will become clear when the organic compounds have all be isolated from the starting mixture. The water is added via separatory funnel, burette or pipet. The addition rate should be equal to the collection rate of drops.

Procedure for Steam Distillation

(Procedure adapted from Introduction to Organic Laboratory Techniques A Microscale Approach by Pavia, Lampman, Kris, and Engel, Saunders College Publishing, 1990, pg. 91 and 688.)

Spices Used in this experiment: Cloves, Cinnamon, Ginger, Nutmeg, Tumeric or a spice of your choice.

Distillation Set-up: Clamp a 100-mL heating mantle (Do NOT fill the heating mantle with sand) to a ring stand above a magnetic stirrer and clamp a 100-mL round bottom flask to the ring stand; flask should be sitting in the heating mantle. Weigh out 5 to 6 g of ground spice and transfer to the flask using the weighing paper as a funnel. Fill the flask half full of distilled water and drop in a 1 in. magnetic stir bar. Complete the distillation set-up as shown below. Put a 50-mL round bottom flask on the connecting tube or vacuum adapter to collect the distillate. The thermometer is unnecessary as the temperature will always equal approximately the boiling point of water. Make sure you securely clamp the round bottom flask and the condenser. Also, make sure to use a rubber band to hold the vacuum adapter on the end of the condenser. Start stirring and heating the mixture with a variostat setting of 60. Lower the heating voltage if foaming is a problem. [Acknowledgements go to Marisa Cases and Alexandra Mieczkowski for allowing a photograph to be taken of their steam distillation set-up (shown below). Thank you!]



Steam Distillation: Turn on the cooling water. Make sure that water is flowing through the condenser. You only need to turn the water valve slightly; no matter how much you turn the valve the water flow will remain close to a trickle, which is fine. You do not want to heat the mixture too intensely or it will bump over. Wrap the top of the 100-mL round bottom flask and the 3-way connector with some glass wool to insulate it so that the steam vapors don't condense there. You should try to adjust the heating so that distillate drips at a rate of one drop every 2-5 seconds. Begin adding the water in small amounts via the separatory funnel so that it roughly matches the rate of distillate collecting in your 50-mL round bottom collection flask. Replenish the ice in the beaker that is used to cool your collection flask as necessary. Use a Pasteur pipet to remove some of the water before adding more ice. The vapor should be cloudy when the natural products are co-distilling with the water. When the vapor becomes clear you can remove the heat from the round bottom flask.

Isolation of Oil: Extract the distillate (collected in receiving flask) with dichloromethane (3 x 10 mL portions). Dry the organic layer over Na₂SO₄, filter, and divide into two portions: one portion in a shorty vial, capped (used for GC and GC/MS analysis) and one portion put in a 20-mL vial. The 20-mL vial portion should be concentrated down (with a light stream of nitrogen gas) then dissolved in acetone. This portion will be used for the biological assay.

Gas Chromatography (GC) and GC Mass Spectrometry (GC-MS) Analysis:

As a class, determine what spices were steam distilled and select a group of samples such that each person analyzes a different sample by GC and GC-MS. In other words, for every spice, we will want the GC and GC-MS analysis of the essential oils mixture. Prepare your designated sample for GC analysis and sign up to run it on GC#1 or GC#2. Use a column temperature program from 40 to 280°C at 10°/min. Inject 1 µL of solution. After obtaining your chromatogram, show it to your TA before running the GC-MS.

Antibiotic Behavior of Spice Essential Oils:

As stated earlier, essential oils extracted from natural sources may exhibit antibiotic behavior. You will be bioassaying your extracts with *Bacillus cereus*¹ to test for its antibiotic behavior. Agar culture plates are prepared in sterile petri dishes. These plates are stored in the refrigerator in Room 216 Whitmore. Whenever you handle the sterile agar petri dishes try to minimize any exposure to random forms of bacteria or dirt in the laboratory. Always wear gloves when handling the plates and the *Bacillus cereus*, because your hands can easily contaminate the agar. Open the cover of the dish a few inches vertically so that you have just enough room to swab the plate with a known strain of bacteria or insert a testing disk and immediately cover the agar to reduce the risk of outside contamination. You will use a pair of forceps to handle the test disk and sterile swabs to streak the plates with the *Bacillus cereus*. After preparing your assay you will observe the growth of bacteria on the plate. If the bacteria do not grow in the area surrounding the disk treated with your essential oil then one or more of the compounds in this isolated mixture is exhibiting antibiotic behavior.

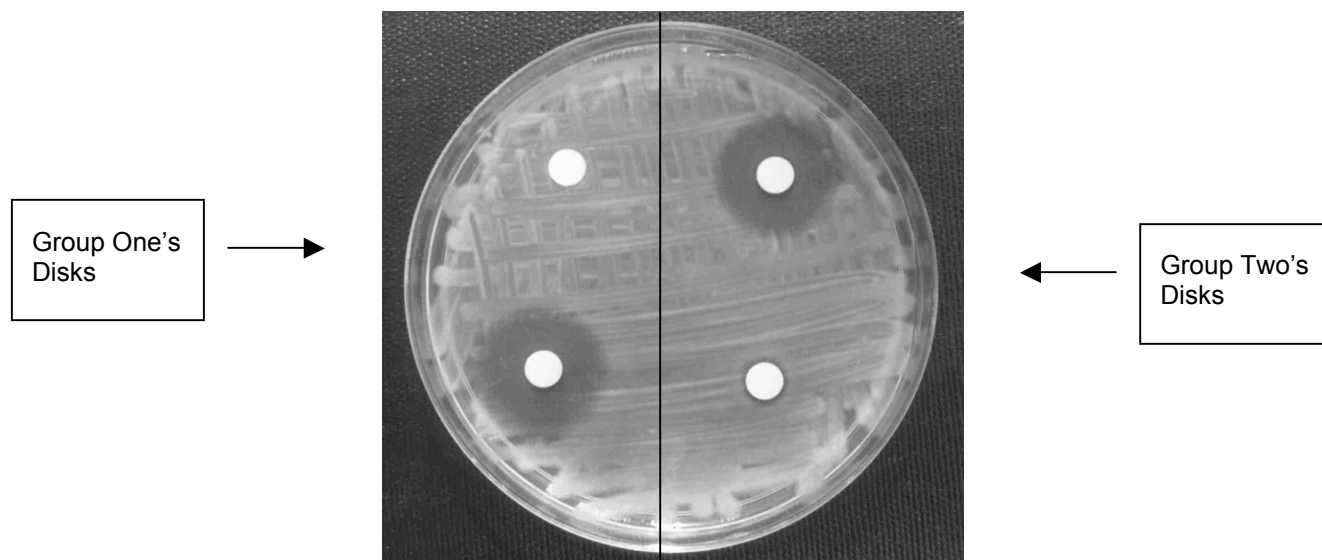
Bioassay using agar medium bacterial growth plates

Sterilize a copper wire loop by heating in a flame. Once cool, use the loop to transfer the bacteria from the starter culture plate to a previously prepared sterile agar culture plate **as demonstrated**.

Add the required volume of acetone to your 4 samples to make 5 to 10% (wt/vol) solutions of your isolated oil in acetone. Using a pair of forceps cleaned by wiping with an ethanol-soaked tissue, pick up a sterile disk and dip it into the acetone solution. Allow the disk to soak for 10 minutes then remove the disk and lean it against a clean glass-stirring rod on a paper towel to air dry. Lift the lid on the agar plate just enough to insert the disk into one quadrant of the plate using clean tweezers (see figure below). Two groups can use one agar plate. Label the bottom of the culture plate with your name and the each disk's identity so you will know which disk is which and tape the cover to the bottom. Be sure to label your plates properly and draw one line down the middle of the plate to separate group's disks from each other.

Place plate *upside down* in one of the incubator ovens in 216 Whitmore. The results of the essay *must* be assessed after twenty-four hours.

¹ There are many bacterial cultures commercially available, but we chose *Bacillus cereus* because it is non-pathogenic, requires no special medium for growth, and grows optimally at room temperature in a short period. Cultures of *E. coli* not only have to be handled with care due to their potential pathogenicity, but their optimal growth is at 37°C, requiring an incubator for optimal growth.



Test disks (clockwise from top left) of: control, 2 mg of clove oil, control, 2 mg of clove oil.

Cleaning Up

It is important to properly dispose of the chemical and biological wastes. The aqueous acid and base solutions can be poured down the drain with plenty of running water. The acetone solutions of clove oil, eugenol, and the neutral components can go in the NHO container. The dichloromethane solutions used for GC/GC-MS should go in the HO container. If there is an autoclave available, then the agar plates and contaminated materials should be autoclaved for at least 1 h. If an autoclave is not available, then the agar plates and contaminated materials should be soaked in bleach for several days.

Final Report

Record data and observations in your notebook as you work (see the section **The Laboratory Notebook: Observations and Data** section). For the *Results and Discussion* section of your final lab report, follow the point distribution on the grading sheet for this experiment and be sure to include:

- (1) Distillation curves of simple and fractional distillation data supplied to you below for the distillation of ethanol/water. Plot both simple and fractional distillation curves so that you have two graphs that look like Fig 5.1 from the data below. Adjust observed boiling point temperatures to what they would be if corrected to standard atmospheric pressure of 760 Torr, then plot boiling point versus volume of distillate (drops or mL) of the type shown in the curves in the Introduction of Chapter 5 (Lab Guide). Tape the Data Table (last page of this handout) in your Notebook.
- (2) The distillation curve for your Procedure 3 unknown with boiling points adjusted to 760 Torr.
- (3) Give the identities of your two liquids in your distillation unknown.
- (4) Discussion of simple versus fractional distillation. Do the results support predictions?
- (5) Interpret the mass spectra for two compounds detected by you or others from GC-MS.
- (6) Discuss the antibacterial activity you observed for each of your four spice essential oil extracts.
- (7) **Postlab Question:**

What criteria would you use to decide whether distillation is an appropriate technique for the purification of a reaction product and what type of distillation method – simple, fractional vacuum or steam – should be used.

References:

- (1) Lab Guide for Chemistry 36, 36B, 36H, Introductory Organic Laboratory, Robert Minard, Katherine Masters and Tracy Oriskovich Halmi, Kenneth Williamson, The Pennsylvania State University, 2004.

- (2) Introduction to Organic Laboratory Techniques A Microscale Approach, Donald L. Pavia, Gary M. Lampman, George S. Kriz, Randall G. Engel, Saunders College Publishing, pgs. 489, 667.
- (3) William H. Miles, and Patricia M. Smiley, *J. Chem. Ed.* **2002** 79, 90
- (4) Palleros, D. R. *Experimental Organic Chemistry*; John Wiley & Sons, Inc.: New York, 2000.
- (5) Lehman, J. W. *Operational Organic Chemistry: A Problem-Solving Approach to the Laboratory Course*, 3rd ed.; Prentice Hall: Saddle River, New Jersey, 1999.
- (6) Ntamila, M. S.; Hassanali, A. *J. Chem. Ed.* **1976**, 53, 263.

Distillation Data for Ethanol/Water

Distillation Data for the Simple and Fractional Distillation of Ethanol/Water at 727 Torr					
Simple, Distillation			Fractional, Distillation		
Volume mL	Temperature °C	Adjusted Temp °C	Volume mL	Temperature °C	Adjusted Temp °C
0	86		0	77.5	
2	91		1	77.5	
3	92		2	78.5	
4	94		3	79.5	
5	94		4.0	81.5	
6	96		4.5	84.5	
8	97		5	88.5	
10	97		5.2	93.5	
12	97		5.4	95.5	
14	97		5.5	97.5	
16	99		5.8	98.5	
18	99		6.0	99.5	
20	99		6.5	99.5	
22	99		7	99.5	
24	99		8	99.5	
25	99		9	99.5	
			10	99.5	
			12	99.5	
			14	99.5	
			16	99.5	