Exp’t 610

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

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Introduction

Activities:

✓ You will use steam distillation to separate any volatile oils. You will also carry out liquid/liquid extraction of the steam distillate to separate the neutral, acidic, and basic components.

✓ You may need to prepare sterile agar plates and inoculate them with Bacillus Cereus bacteria and incubate them to test biological activity. Samples of the components you isolated will be tested for antibacterial activity.

✓ You will use GC and GC/MS to characterize your volatile oils.

Modeling the Drug Discovery Process

Chemistry and Medicine

Since ancient times, humankind has sought medicines from nature. Quinine, the antimalarial 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 (No, 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 “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.
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 cloves. 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 cynimum 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 compounds have. Different physical properties allow the separation of one organic molecule from another. 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 techniques 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 and this 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.

To review, certain organic functional groups (carboxylic acids and phenols) are relatively acidic, some are basic (amines), but most (ketones, esters, alkanes, alkenes, etc.) are neutral. The solubility of organic compounds in water, especially higher molecular weight compounds, is poor, since the non-polar intermolecular interactions between the organic molecules are not readily broken by water, a very polar solvent. Organic acids and bases, however, can be converted into ionic compounds that are soluble in water. The addition of base will convert the carboxylic acids and phenols into their conjugate bases, which are soluble in water but relatively insoluble in the organic solvent. Also, the addition of acid will convert amines to their conjugate acids, which are soluble in water but relatively insoluble in the organic solvent. If you extract an organic solution with an aqueous base solution, an organic acid will dissolve in the aqueous phase as its conjugate base. In a similar way, if you extract with an aqueous acid solution, an organic base will dissolve in the aqueous phase as its conjugate acid. The organic compounds that are not acidic or basic will remain in the organic phase. Neutralizing the aqueous phase and extracting with an organic solvent can then recover the organic acids and the organic bases. This method of separating organic acids, bases and neutral compounds from one another is referred to as a "chemically active extraction". This procedure is the basis for the separation of eugenol, an acidic phenol, from the neutral compounds found in clove oil.

![Acid/Base Chemistry of Phenol](image)

**Extraction Scheme for Eugenol**

**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. The separation of essential oils from plant materials and spices is readily accomplished by a combination of steam distillation and acid/base liquid/liquid extraction methods described earlier. For example, eugenol, a phenolic compound, is easily separated from the neutral components in clove oil, eugenol acetate and β-caryophyllene.

You will separate, isolate and test the antibiotic activity of the following samples using Bacillus cereus growing on agar plates which you will prepare.

1. The initial organic extract of the steam distillate containing everything, neutral, acidic and/or basic components. Dichloromethane, CH$_2$Cl$_2$, will be used for this extraction.
2. The acidic components of that dichloromethane extract separated by extraction with the base NaOH. Since these components probably will not crystallize out as solids when we acidify with acid, we have to extract them back into dichloromethane.
3. The basic components of that dichloromethane extract as separated by extraction with the acid HCl. Since these components probably will not crystallize out as solids when we acidify with acid, we have to extract them back into dichloromethane.
4. The neutral components that remain unextracted from the dichloromethane layer.

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.

\[
P_{\text{total}} = P^0_{\text{component 1}} + P^0_{\text{component 2}} + P^0_{\text{component 3}} + \cdots + P^0_{\text{component n}} + P^0_{\text{water}}
\]

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 about 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 your 100-mL heating mantle (You should NOT fill the heating mantle with sand) clamped to a ring stand above a magnetic stirrer and clamp a 100-mL round bottom flask to the ring stand and 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 in Figure 5.2 of the Lab Guide except replace the thermometer with Pasteur pipet or a separatory funnel or a burette to allow water addition. Also, 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 varistat setting of 60. Lower the heating voltage if foaming is a problem. Also, if material starts to foam into the condenser, you can put a Claisen adapter into the apparatus as shown below.

**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-wat connector with some glass wool to insulate it so that the steam vaporami don’t condense there very much. Once the distillation begins, record the distillation temperature (it should be just below 100 °C). 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 through the thermometer opening 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.

**Liquid/Liquid Extraction**

This is a complicated procedure. Be sure you know which layer is which and label the extract containers as described.

**Separation of the mixture of essential oils from water:** Remove the 50-mL round bottom flask containing your essential oil and water. Use your 125-mL separatory funnel to extract the essential oils in your distillate into dichloromethane. Making sure the stopcock is closed, transfer the contents of the 50-mL round bottom flask to the empty 125-mL separatory funnel. Remember a stopcock can leak so always double check your stopcock to make sure it is tight and always have an Erlenmeyer flask or beaker under the funnel to catch any possible leaks. Add 10-mL of dichloromethane (CH$_2$Cl$_2$) to the separatory funnel and follow the extraction procedure you learned in the liquid/liquid extraction technique experiment. Repeat this process two more times with 10-mL additions of dichloromethane and combine all the organic layers into a 125-mL Erlenmeyer flask labeled “TOTAL OILS”.

**Extraction of basic components with acid:** Rinse out the separatory funnel and, with stopcock closed, pour approximately two-thirds the “TOTAL OILS” dichloromethane extract into it.

To the one-third of the CH$_2$Cl$_2$ extract remaining in the flask, use a scoopula to add enough anhydrous sodium sulfate to cover the bottom. Set aside, but swirl occasionally as you go on with this procedure.

Extract the CH$_2$Cl$_2$ in the separatory funnel twice with 5 mL of 5% HCl [Caution, strong acid, use plastic pipet to transfer to 10-mL graduated cylinder], combining the acidic extracts in a small beaker marked “HCl”. You will have to drain out the lower CH$_2$Cl$_2$ layer into a beaker before draining out the aqueous layer extract, then pour the CH$_2$Cl$_2$ back into the sep. funnel.

**Extraction of acidic components with base:** Pour the dichloromethane layer into the separatory funnel and extract it twice with 5 mL of 1 M NaOH [Caution, strong alkalai, use plastic pipet to transfer to 10-mL graduated cylinder], combining the basic extracts in a small beaker marked “NaOH”. You will have to drain out the lower CH$_2$Cl$_2$ layer into a beaker before draining out the aqueous layer extract, then pour the CH$_2$Cl$_2$ back into the sep. funnel.

**Isolation of the neutral components:** The CH$_2$Cl$_2$ layer now contains only neutral components. Pour it into the separatory funnel and wash it twice with 10-mL water. Drain the CH$_2$Cl$_2$ layer into a 125-mL flask containing a thin layer of solid anhydrous sodium sulfate, just enough to cover the bottom of the flask. Swirl occasionally for 10 minutes, then decant a small amount of the dried dichloromethane solution into a labeled shorty vial and the remainder into a labeled tared 20-mL vial, both labeled “Neutral”. Cap the shorty vial, but leave the 20-mL vial with the cap off in you locker to allow the dichloromethane to evaporate until the next lab period. (Alternatively, if time permits, one could blow off the dichloromethane from the 20-mL vial with a stream of nitrogen while immersing the flask in a beaker of warm tap water.)

**Isolation of the acidic components:** Add 6 M HCl dropwise to the beaker labeled “NaOH” until the pH is definitely acidic as indicated by pH test paper. Pour this solution into your rinsed out separatory funnel and rinse the beaker with two 5-mL portions of CH$_2$Cl$_2$, adding these to the separatory funnel. Stopper, shake to extract, and separate the CH$_2$Cl$_2$ layer into a 50-mL Erlenmeyer. Extract a second time with 10 mL of CH$_2$Cl$_2$. Dry the combined extracts over a small amount of anhydrous sodium sulfate as above and a small amount of the dried dichloromethane solution into a labeled shorty vial and the remainder into a labeled and tared 25-mL Erlenmeyer, both labeled “PHENOLS & ORG. ACIDS”. Cap the shorty vial, but leave the 20-mL vial with the cap off in you locker to allow the dichloromethane to evaporate until the next lab period. (Alternatively, if time permits, one could blow off the dichloromethane from the 20-mL vial with a stream of nitrogen while immersing the flask in a beaker of warm tap water.)

**Isolation of the basic components:** Add 1 M sodium bicarbonate solution to the beaker labeled “HCl” until the pH is basic. Pour this solution into your rinsed out separatory funnel and rinse the beaker with two 5-mL portions of CH$_2$Cl$_2$, adding these to the separatory funnel. Stopper, shake to extract, and separate the CH$_2$Cl$_2$ layer into a 50-mL Erlenmeyer. Extract a second time with 10 mL of CH$_2$Cl$_2$. Dry the combined extracts over a small amount of anhydrous sodium sulfate as above and a small amount of the dried dichloromethane solution into a labeled shorty vial and the remainder into a labeled and tared 25-mL Erlenmeyer, both labeled “AMINES”. Cap the shorty vial, but leave the 20-mL vial with the cap off in you locker to

Distillation – page 6
allow the dichloromethane to evaporate until the next lab period. (Alternatively, if time permits, one could blow off the dichloromethane from the 20-mL vial with a stream of nitrogen while immersing the flask in a beaker of warm tap water.)

Isolation of the “TOTAL OILS” mixture: After drying over anhydrous sodium sulfate for at least 10 min, a small amount the CH₂Cl₂ extract can be decanted into a labeled shorty vial and a labeled and tared 20-mL vial, both labeled “TOTAL OILS”. Cap the shorty vial, but leave the 20-mL vial with the cap off in your locker to allow the dichloromethane to evaporate until the next lab period. (Alternatively, if time permits, one could blow off the dichloromethane in the 20-mL vial with a stream of nitrogen while immersing the flask in a beaker of warm tap water.)

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 and if there are duplicates of these, then either the 1) amine components, or 2) carboxylic acid & phenolic components, or 3) the neutral components can be analyzed. 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 uL 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 as demonstrated by the instructor or your TAs. 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. Label the cover of the culture plate with you name and the each disks identity so you will know which disk is which and tape the cover to the bottom. Place in one of the incubator ovens in 216 Whitmore. The results of the essay can be assessed after twenty-four hours or longer.

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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.
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.

References:


Distillation Data for Ethanol/Water

## Distillation Data for the Simple and Fractional Distillation of Ethanol/Water at 727 Torr

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