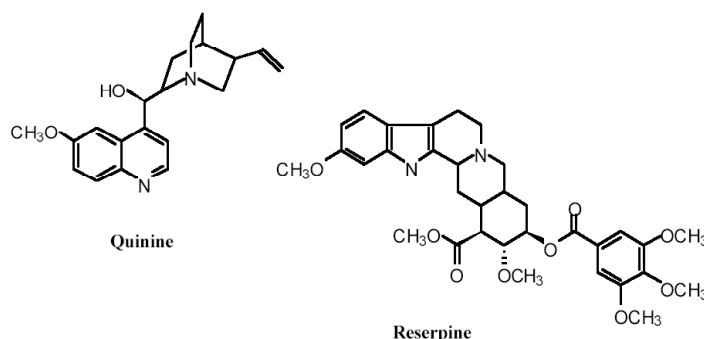


STEAM DISTILLATION OF A SPICE

INTRODUCTION

Chemistry and Medicine

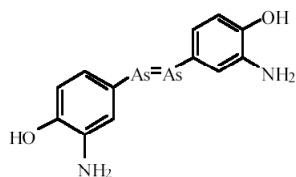
Since ancient times, humankind has sought medicines from nature. Quinine, the antimalarial compound isolated from the bark of the cinchona tree, and reserpine, the antidepressant from the Indian plant *Rauwolfia serpentina*, represent two of the more famous natural products used in modern medicine.



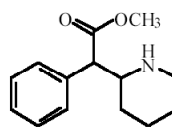
Today, pharmaceutical companies use several approaches for the discovery of new compounds to treat human illness, including the screening of natural products derived from bacteria, fungi, plants, or animals (not puppies or even mice, but animals like slugs or sponges, simple multi-cellular, bottom-dwelling 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

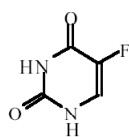
synthesized in the laboratory and have become useful drugs. For example, Ritalin is used for the treatment of attention deficit disorder (ADD), 5-fluorouracil is a potent anti-cancer drug, and L-DOPA is used to treat Parkinson's disease.



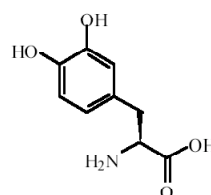
Compound 606
(Salversan)



Ritalin



5-Fluorouracil

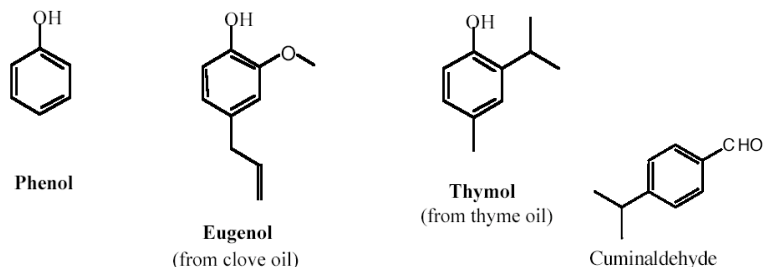


L-DOPA

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 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 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

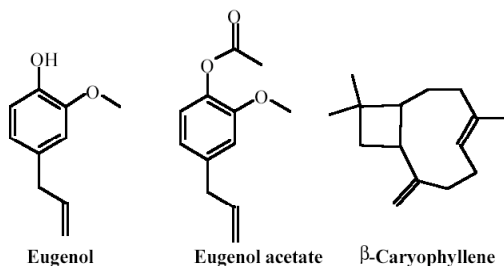
The testing of compounds for biological activity is the first step in the determination of their potential as a drug in medicine. In most cases, the compound is tested against an organism (bacteria, fungi, or virus) *in vitro*, that is, outside a living organism in a controlled environment such as a 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

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. In other 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.

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

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, separating into liquid water with small amounts of immiscible eugenol. 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.

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This will be a two day lab. On the first day you will follow procedure 1, steam distillation of spices. You can choose your spice: cloves, nutmeg, or tumeric. On day two you will be following procedure 2, biological analysis.

PRELAB EXERCISE

Name some other compounds that can be isolated by steam distillation.

PROCEDURE 1: STEAM DISTILLATION OF SPICES

Distillation Set-up

Assemble the apparatus for steam distillation as shown in Figure 1. Start by placing your magnetic stirrer on the base of a ring stand and then obtain a 250 mL electric heating mantle from the stock room. Attach the heating mantle (**without sand in it**) to the ring stand so it rests on top of the stirrer. Plug the heating mantle into the Varistat, a variable voltage controller, on the side of the hood and plug the Varistat into the socket above your bench. Place a 1" stirring bar in a dry, 250 mL, 3 way round-bottomed flask (*Stock Room*) and clamp this to the ring stand so that it fits snugly in the heating mantle. Stirring promotes even boiling and avoids "bumping". As you assemble the apparatus, lightly grease each ground joint by putting a very small dap of grease on the inner joint and spreading it over the surface of the joint (do not use excess grease as it will contaminate the product). When pressed into the outer joint and twisted, the connection should appear more transparent. Put a 100-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.



Figure 1. Set-up for steam distillation

Water enters the condenser at the connection nearest the *distillation receiver*. Because of the large heat capacity of water only a very slow flow is needed; too much water pressure will cause the tubing to pop off, causing a flood. The condenser should be clamped to a separate ring stand. Rubber bands (*Common Shelf*) should be used to hold the angled connecting tube to the condenser.

Steam Distillation

Weigh out 5 to 6 g of ground spice (either clove, nutmeg or tumeric; your choice) and transfer to the flask using the weighing paper as a funnel. Add 80 mL of distilled water to the round bottomed flask. Clamp an addition flask or separatory funnel to the flask, and cover the remaining hole with a septum. Wrap the top of the 250-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 (Figure 1). Start stirring and heating the mixture with a Varistat setting of 60. Lower the heating voltage if foaming is a problem. 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, or addition funnel so that it roughly matches the rate of distillate collecting in your 100-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 distillate should be cloudy when the natural products are co-distilling with the water. When the distillate drops becomes clear you can remove the heat from the round bottom flask.

Isolation of Oil

Remove the 100-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 100-mL round bottom flask 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. Extract the distillate with three 10-mL portions of CH_2Cl_2 . Collect the organic layers into a 125-mL Erlenmeyer flask. Dry the organic layer for 10 min over anhydrous sodium sulfate (Na_2SO_4). Extract can be decanted into a *tared* and labeled 25-mL Erlenmeyer flask. Allow the dichloromethane to evaporate until the next lab period. Alternatively, if time permits one could blow off the dichloromethane with a stream of nitrogen while immersing the flask in a beaker of warm water.

PROCEDURE 2: BIOASSAY OF SPICE OIL

As stated earlier, essential oils extracted from natural sources may exhibit antibiotic behavior. You will bioassay your isolate oil on a cultured plate of bacteria. (There are many bacterial cultures commercially available, but *Bacillus cereus* is preferred because it is non-pathogenic, requires no special medium for growth, and grows at room temperature in a short time. 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.) Agar culture plates are prepared in sterile Petri dishes or can be purchased; they will be ready for your use. These plates are stored in the refrigerator.

Please try to coordinate your bioassay time with your hoodmate or a labmate's bioassay time; so, share a plate with another student. Be sure to label your side of the plate with your name and label the two discs (one will be a test disc sterilized by soaking in ethanol then drying; the other will be the oil-absorbed test disc); use a wax pencil for labeling. Figure 2 shows an example of a plate in which two students shared one Agar plate. As you can see two compounds show no bacteria growth surrounding it while the other two show bacteria surrounding them (Figure 2).

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 bacteria, because your hands can easily contaminate the agar.

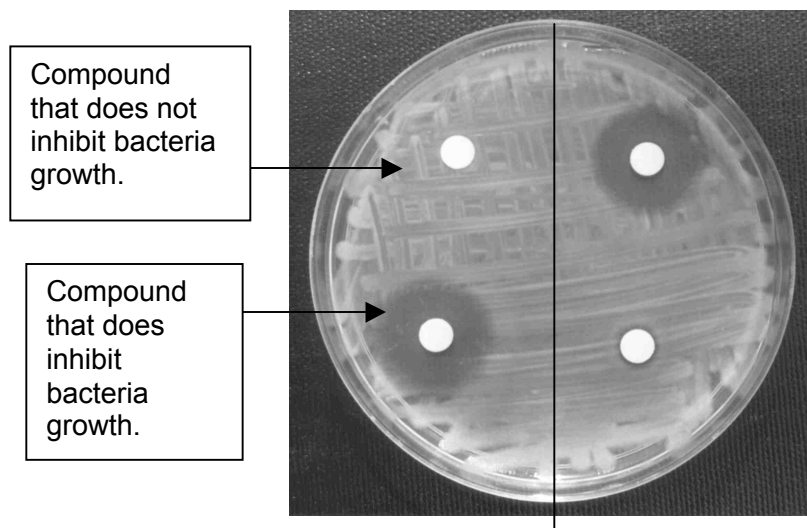


Figure 2. An agar plate with two sets of compounds from two different students after incubation.

Bioassay Using Agar Medium Bacterial Growth Plates

Use a sterile swab to transfer the bacteria from the starter culture plate to a previously prepared sterile agar culture plate. 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.

Add the required volume of acetone to your oil sample to make 5 to 10% (wt/vol) solution 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 test discs. Two workers can use one Agar plate. Be sure to label your plates properly and draw one line down the middle of the plate to separate each worker's disks from each other (Figure 2). Label the bottom of the culture plate with your name and label the blank disc and the oil-absorbed disc. Place plate in an undisturbed area of your hood or locker. The results of the assay can be assessed after twenty-four hours. Record observations and discuss them in our final report.

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 non-halogenated waste 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. The stockroom ladies can dispose of the plates properly for you.

POSTLAB QUESTIONS

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.

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