Melting Point Experiment

Carry out the procedure described in the Lab Guide and in addition have each person in your group do one additional melting point of one of the following: brown sugar, pure sugar, and gelatin. Make sure your group has at least one melting point determination for all three of these compounds.

Also in Procedure 5, instead of using a 0.01% solution of methylene blue each of you will decolorize a solution of colored sugar. On the common shelf you will find containers of red and green colored sugar. Place enough colored sugar in 1 mL of water to produce a colored solution. Follow the instruction for decolorization using this solution.

In your final report, give the structure of table sugar (sucrose) and a segment of gelatin and discuss the mp behavior of each.
The Technique of Distillation

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

Introduction
The spices cloves, 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:
✓ In the first lab, you will use steam distillation to separate any volatile oils from your spice of choice. In preparation for this, read the theory of steam distillation presented below. Also read the introductory information in the Procedure section on pages 120-127 of your lab guide. You will be using a variation of the distillation set-up shown in Figure 5.3. 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. 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.
✓ In the second lab, you will be carrying out Procedure 3, page 125 of your lab guide a microscale fractional distillation of an unknown mixture that will give you experience with the fractional distillation technique.
✓ You will use prepared 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.
✓ Read the introduction to the Liquid/Liquid Extraction laboratory (Introduction through Drying Agents), because the essential oil will be isolated using liquid/liquid extraction.
✓ You will use GC and GC/MS to characterize your volatile oils. In preparation for product analysis using these methods, read Sections 11.2, 11.7, and 11.8 on gas chromatography in the Chem 36 Lab Guide.

PreLab:
Your chemical data table should include the chemicals mentioned in Table 5.1 on page 125 of your Lab Guide and also eugenol, thymol, and quinine. Hand in a copy of the completed Common Shelf Chemical Date Table with your PreLab.
Prelab Questions:

Answer the prelab questions asked in the lab guide for the Distillation experiment, page 115 questions 1-3.

4. After reviewing the assay, your essential oil shows antibiotic activity, how would you determine which compound(s) in the oil is (are) the active ingredient?

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

Chemical Relevance: 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.

![Quinine](image1.png)  
![Reserpine](image2.png)

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.

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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 that 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.

![Chemical structures of Phenol, Eugenol, Thymol, and Cuminaldehyde](image)

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 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.

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. In this laboratory experiment we will use the whole oil without further separation. If you were to identify which component(s) in the oil exhibited antibiotic behavior you would need to carry out a separation that would probably include acid/base liquid/liquid extraction methods and extensive column chromatography.

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

1. A sample of the initial organic extract of the steam distillate containing. Dichloromethane, CH₂Cl₂, will be used for this extraction.
2. A control sample that does not contain the extracted oil.
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.

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

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, Kriz, and Engel, Saunders College Publishing, 1990, pg. 91 and 688.)

Spices Used in this experiment: Cloves, 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-watt connector with some glass wool to insulate it so that the steam vapors 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

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”.

Isolation of the “TOTAL OILS” mixture: After drying over anhydrous sodium sulfate for at least 10 min, a small amount the CH$_2$Cl$_2$ 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 you 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:
Each member of your research group will analyze your “total oils” mixture using the Hewlett Packard 5890 GCs. You will need to take your small vial containing the dichloromethane and the oil mixture to the instrument room for analysis. You should have signed up for time on the instrument and you will need to do the CALIOPE GC tutorial on the computer adjacent to the GC. The tutorial is interactive so it will show you how to the do the analysis, while you go through it. The tutorial will not provide you with a temperature program so use the following: 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* 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

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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.
that have been sprayed with ethanol 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 copper loops 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.

![Image of bacterial growth plates](image)

Test disks (clockwise from top left) of: control, 2 mg of clove oil, 2 mg of neutral compounds from clove oil, and 2 mg of eugenol from 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
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. Tape the Data Table below 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 Questions:

1. The liquid boiling in a simple distillation flask consists of 80 mole percent of hexane and 20 mole percent of n-propylbenzene. At 79°C a small amount of distillate is collected. The standard vapor pressures of pure hexane is 812 mm and pure n-propylbenzene is 560 mm Hg, respectively, at 79°C. Calculate the percentage of each of the two components in this fraction of distillate. Show your calculations. Hint: Use the formulae in the introduction.

2. Liquids that have boiling points greater than 200°C are usually distilled under vacuum (<20 torr). What effect would this have on the distillation, and give a reason why this might be advantageous.

3. What criteria would you use to decide which type of distillation technique is appropriate for the purification of a reaction mixture.
References:

Distillation Data for Ethanol/Water

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Technique of Thin-Layer Chromatography

Experiment Title: Applying TLC As A Method to Monitor the Multistep Synthesis of Aspirin

Introduction
Aspirin will be synthesized from methyl salicylate in two steps. Thin layer chromatography will be used to monitor the reaction for both steps of the synthesis. The first step requires the saponification of methyl salicylate to salicylic acid. The second step is the acetylation of salicylic acid with acetic anhydride to produce acetylsalicylic acid (aspirin).

Activities:
- Read the Introduction, Theory, Spotting the TLC Plate, Development, Visualization, and Rf Values sections of Experiment 7 in the Lab Guide, page 163-173.
- In the first part of the experiment you will determine an appropriate solvent system to separate methyl salicylate, salicylic acid and acetyl salicylic acid using standard solutions.
- Then you will saponify methyl salicylate with NaOH to produce salicylic acid. You will follow the progress of this experiment by TLC.
- You will isolate the crude salicylic acid and purify it by recrystallization before proceeding to the next step.
- You will then acylate the salicylic acid with acetic anhydride to produce acetylsalicylic acid. This reaction will be monitored by TLC.
- You will purify the acetylsalicylic acid and report a final melting point and % yield.
- You will obtain a 400 MHz 1H NMR spectrum of the acetylsalicylic acid.

PreLab Exercise
Do Questions 1 and 2 from the PreLab exercises of the Thin-Layer Chromatography Experiment 7 in your lab guide, page 163.

3. What is the white solid that is formed when the NaOH is added to the methyl salicylate before the reaction mixture is heated?

4. What is the purpose of adding the concentrated phosphoric acid to the reaction mixture in the synthesis of aspirin?

5. Reaction mechanisms for both steps of the synthesis must be written out with arrows showing the direction of electron flow.
It is extremely important that you read the introductory material from Chapter 7 of the Lab Guide on thin-layer chromatography, because you will be using TLC to monitor the two-step synthesis of aspirin.

Analgesics are substances that relieve pain. The most common of these is aspirin, a component in more than 100 nonprescription drugs. Aspirin has wide spread use in medicine and over 30 million pounds of it are consumed each year in the United States. Willow leaves and bark have been used for centuries for their pain relieving and fever-reducing properties, however the active ingredient in these home remedies is salicylic acid, which over time irritates the stomach lining. In 1893 a German chemist Felix Hofmann synthesized acetyl salicylic acid that offered similar medicinal properties without the stomach irritation. Since the contents of the stomach are acidic, aspirin passes through unchanged and does not get absorbed until it reaches the basic environment of the intestines. Salicylic acid is a white crystalline compound that is commonly used in ointments and plasters for the removal of warts.

Aspirin is synthesized by acetylated salicylic acid to produce the corresponding acylated carboxylic acid. You will begin with methyl salicylate a component of oil of wintergreen that is used in flavoring candies. In the first of this two-step synthesis of aspirin, a methyl ester (methyl salicylate) is saponified to produce the corresponding carboxylic acid (salicylic acid). In this second step the phenolic functional group of salicylic acid is acetylated with acetic anhydride to produce a new ester (acetyl salicylic acid). Keep in mind that anhydrides decompose readily with the moisture in the air, so open and close the acetic anhydride bottle quickly and cover the containers used to transfer this reagent. Use all anhydrides in the hood and use gloves when handling them. You will be monitoring the progress of both reactions by TLC and identifying an appropriate solvent system for the separation of the starting material, intermediate product and final product of this multi-step synthesis.

To identify an unknown by TLC, the usual strategy is to find a stationary phase/mobile phase combination that will separate all the compounds you are analyzing. In this experiment, you will not vary the stationary phase or the silica gel, but will vary the polarity of the mobile phase by using differing ratios of the solvents hexane (nonpolar) and ethyl acetate (polar) to develop TLC plates spotted with standard solutions of the starting material and the two products in the two-step synthesis of aspirin. Once you and your lab group determine the optimal mobile phase that will give the best separation of these, you will use this composition to run a chromatogram of the reaction mixture to monitor the progress of both reactions. From the chromatogram, you should be able to determine whether or not the products have been produced by matching Rf’s of the standards to the compounds in the reaction mixture. You will need at least a 50% reaction yield after isolation and purification of the salicylic acid to carry out the second step in the synthesis.

**Procedure for TLC.**

You will be working in your research groups of 4 or 5 so choose the solvent composition(s) found below for the appropriate number of members in your group. Each group member will prepare two solvent compositions to be used as mobile phases for the separation of the methyl salicylate, salicylic acid, and acetylsalicylic acid.
Table 1. Mobile phase compositions for 5 group members

<table>
<thead>
<tr>
<th>Mobile Phase Mixture Composition Number</th>
<th>%Ethyl Acetate</th>
<th>% Hexane</th>
<th>R_f Methyl salicylate</th>
<th>R_f Salicylic Acid</th>
<th>R_f Acetyl Salicylic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
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<tr>
<td>2</td>
<td>90</td>
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<td>3</td>
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<td>10</td>
<td>0</td>
<td>100</td>
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</tbody>
</table>

Table 2. Mobile phase compositions for 4 group members

<table>
<thead>
<tr>
<th>Mobile Phase Mixture Composition Number</th>
<th>%Ethyl Acetate</th>
<th>% Hexane</th>
<th>R_f Methyl salicylate</th>
<th>R_f Salicylic Acid</th>
<th>R_f Acetyl Salicylic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>8</td>
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</tbody>
</table>

Label the green lids of your two 4 oz. TLC jars with mixture composition numbers assigned in the diagram and table above. Use a 10 mL graduated cylinder to prepare 10 mL of each of the two compositions assigned. Don’t try to pour out the correct amount from the supply bottles, but rather use the plastic pipet attached to the supply bottle to transfer the solvent to better control the exact volume to be measured out. Working in the hood, pour each 10 mL mixture into the corresponding wide-mouth jar. Swirl to mix and then pour about half out so that the solvent level won’t be so high that it will wash compound spots off the TLC plate. (Dispose of the excess in the non-halogenated waste bottle.) Insert a piece of 4 cm filter paper into each jar so that it wraps around the inside wall of the jar and dips into the liquid. This creates a saturated vapor atmosphere that improves spot shape and reproducibility. Cap both jars. Using a lead pencil (not a pen)
and a ruler, mark two plates as shown in Figure 7.13 in the lab guide. First, draw a light pencil line across the plate about 1 cm from the bottom of two TLC plates. Make four equally spaced vertical dashes on this line. The dashes should be about 5 mm from the edge and 5 mm apart. Then label the lanes at the top of the plate.

In a shorty vial, obtain a small amount of dichloromethane from the common shelf. Using a TLC spotting capillary tube from your desk, practice spotting on a paper towel using pure dichloromethane. After filling the capillary by dipping it in the liquid, touch it quickly to the towel so that the spot is no larger than 1 to 2 mm diameter. The smaller the spot, the better the final TLC analysis. After the solvent evaporates, you can apply more material in the same spot by again quickly touching the capillary to the surface at the same place.

Three people in your group of four or five should obtain just a few drops of one of the 1% or 2% solutions of the three standards: methyl salicylate, salicylic acid, and acetyl salicylic acid in shorty vials, properly labeled. Spot both of your plates with each of these three standards, placing the spot at the origin mark corresponding to each. Examine the plate under the UV light to see that enough of the compound has been applied by observing a visible dark purple dot, if it is not visible spot more. Using forceps, gently place one in each TLC bottle, being careful not to splash solvent up the plate (see Figure 7.9 in lab guide). Be sure that the spots are not below the solvent level or they will wash away into the solvent. Allow each plate to develop until the solvent front is approximately 1 cm from the top of the plate. Using forceps, remove the TLC plate and quickly mark the solvent front with a pencil. Allow the plates to dry in the hood.

Examine the plate under the UV lamp to see the components as dark spots against a bright orange or green-blue background. Outline the spots with a pencil. [The spots can also be visualized by putting the plate in an iodine chamber that can be found on the side shelf. After a few minutes sitting inside the closed bottle, compound spots turn brown.] Calculate the \( R_f \) value for the center of each spot as shown in Figure 7.11 in the lab guide and enter the value in Table 1 or Table 2 above. Tape the properly labeled TLC plates in your notebook using the wide sticky tape available on the side shelf. Cover the whole plate with tape.

Obtain \( R_f \) values for the other mobile phase compositions from the other students in your group and enter them in Table 1 or 2 also. As a group, decide which composition gives the best separation, in other words: (1) it does not allow any of the compounds to remain on the baseline, (2) it does not allow any of the compounds to travel with the solvent front, and (3) it provides the greatest differences in the \( R_f \) values for the compounds to be separated.

Cleaning Up. Do not dispose of the spotting capillaries; they are reusable!! They may be cleaned by dipping the ends into acetone and blotting the ends with a paper towel. Store them safely in the test tube labeled for TLC spotting capillary storage in your drawer for use in other experiments.

Used mixed solvents should be placed in the appropriate organics waste container in your hood. Return iodine chambers to the side-shelf.
Saponification of Methyl salicylate

You will saponify this ester to produce salicylic acid, a precursor to acetylsalicylic acid. Methyl salicylate has two functional groups: an ester and a phenol. Esters are easily hydrolyzed with base to the corresponding carboxylic acid. When a strong base such as NaOH is added to methyl salicylate, three reactions occur: 1) ester hydrolysis, 2) phenoxide ion is formed, and 3) the carboxylic acid product is converted to its conjugate base. Addition of a strong acid in the reaction workup protonates both the phenolate and the carboxylate anions. Salicylic acid is insoluble in cold water so it can easily be isolated via filtration.

The hydrolysis reaction is very slow at room temperature but the reaction time can be shortened when the mixture is refluxed for a period of time. Refluxing occurs when the solvent in the reaction mixture is heated to boiling and resulting vapor is condensed and drops back into the reaction flask as a liquid after coming in contact with a condenser. You have already observed this behavior when you carried out the distillation experiment, however, in that case you were interested in transferring the vapor to a second round bottom flask. In this case, you will be returning the vapor to the reaction flask as liquid solvent.

After allowing the reaction mixture to reflux you will obtain a sample for TLC to monitor the progress of the reaction. You can stop the reaction when the spot corresponding to the starting material is only slightly visible. You will begin the workup of the reaction by cooling and acidifying the reaction mixture and isolating the resulting solid product. Salicylic acid is only slightly soluble in cold water but is soluble in hot water so you will do your filtration while the mixture is cold. After isolating the product, you can take advantage of salicylic acid’s solubility in hot water and use this solvent for recrystallization.

**Experimental Procedure:** Have one member in the group make 20 mL of a 4 M sodium hydroxide solution in a 50 mL Erlenmeyer flask. When weighing out the NaOH pellets work quickly and do not touch them with your hands. The group will share this sodium hydroxide solution for their saponifications. Weigh out 230 mg of methyl salicylate using the 100 – 1000 µL syringe into a large test tube 20 X 150 mm. Lay the test tube on the pan of the balance and have it rest on the two little dents designed to keep the tube from rolling. Add 3.5 mL of the 4 M sodium hydroxide solution to the tube containing the methyl salicylate and swirl to ensure that the two compounds mix. A white solid will quickly form. Add a 1/2 inch stir bar and clamp the tube and heat the water solution to reflux using your heating mantle. The tube is large compared to the volume of the solution so the walls of the tube will serve as the condenser. Reflux for 15 minutes, cool and check the reaction mixture by TLC. If the reaction has nearly gone to completion you can proceed to the work up section, if not reflux for an additional 5 – 10 minutes.
depending on amount of methyl salicylate remaining. Cool and check reaction mixture again by TLC.

Workup:
Place the reaction tube in a 250 mL beaker containing ice to keep the tube cool. Add 3 M sulfuric acid solution to the tube in 0.5 mL increments, (about 10 drops from a disposable pipet) until a heavy white precipitate forms and remains when the tube is stirred. Add an additional 10 drops of acid to ensure complete precipitation of the salicylic acid. Using a clean glass stirring rod test the pH of the salicylic acid solution to be sure it is acid using pH paper. Filter the cold solution using a Hirsh funnel and isolate the white solid.

The crude solid will be purified by recrystallization from water. Transfer the solid to a 10 mL Erlenmeyer flask and add 2 mL of water. Heat to boiling using a boiling stick to control bumping. Add water in small increments until all the solid is dissolved and then remove from the heat. Allow the solid to recrystallize with placing it in an ice bath. Once a significant amount of crystals have formed, cool the flask in an ice bath and then isolate the solid using vacuum filtration with a Hirsh funnel. Wash the crystals once with ice-cold water and remove the solvent using a vacuum. Transfer the crystals to a watch glass to dry until the next lab period.

Yield and Characterization:
Weigh the solid and obtain a melting point.

**Acylation of Salicylic Acid**

Now you will carry out the second step of the synthesis and produce acetyl salicylic acid.

**Experimental Procedure**
Carry out this procedure in the Hood. Add 100 mg of salicylic acid produced in the TLC experiment to a clean 13 X 100 mm test tube. Add 250 µL of acetic anhydride using a disposable syringe without the needle to the test tube containing the salicylic acid. (Have everyone in the your team use the same syringe for the addition of the acetic anhydride). Add one drop of 85% phosphoric acid from a Pasteur pipet and then loosely cork the top of the test tube. Shake the tube gently to dissolve the salicylic acid and then place the tube in a beaker with hot tap water. Leave the tube in the hot water for 15 minutes. Check temperature of water and if significant cooling has occurred replace with more hot water. Monitor the reaction by TLC. When the reaction has gone almost to completion remove the test tube from the water bath and add 700 µL of water from an adjustable pipet. Allow the solution to cool to room temperature. Crystallization of the crude product should begin. Now place the sample in an ice bath and force out the remaining crystals. Vacuum filter the crude product with a Hirsh funnel and allow it to dry on a piece of filter paper until the next lab period.
**PostLab Questions:**

1) When making the measurements for the Rf values, it is conceivable to measure the distance the spot travels from the top, middle or bottom of the spot. In practice, however, it is best to measure only from the middle of the spot. Why?

2) Tell why it is important that the solvent level in the TLC developing jar be below the level of the sample spots.

3) In each pair, pick the molecule which would have the HIGHER Rf value. Explain your answer.
   
a) phenyl chloride or phenol  
b) N,N-dimethylformamide or 1,3-butadiene  
c) acetonitrile or benzene  
c) decanol or propanol

4) Assuming that 1 g of aspirin dissolves in 450 mL of water at 10 °C, how much aspirin would be lost in the 16 mL of water or 1.4 mL of water added to the reaction mixture if the mixture were at 10 °C during the filtration?

5) How much difference would the amount lost in the water make in your percent yield?

**References:**


**Final Report**

Tape Tables 1 and 2 into your In-Lab Data and Observations section. In your RESULTS AND DISCUSSION section, give the composition of the mobile phase your group chose and tell why, show explicitly how you calculated the Rf values. Describe the reproducibility of TLC Rf values. Discuss how your observed relative Rf’s corresponded to relative Rf’s predicted by you in the PreLab. Also include the % yield and weight of each purified product. You must also have an NMR spectrum for both products. Label the peaks and integrate all peaks. Include a discussion the interpretation of these spectra.
Technique of Column Chromatography

Experiment Title: Applying Column Chromatography As A Method to Isolate An Optically Pure Alcohol.

Introduction: Thus far you have utilized two techniques to isolate pure organic compounds: recrystallization and distillation. One additional isolation technique that is often employed when very small quantities of sample are available is column chromatography. This separation method is very similar in principle to thin layer chromatography, and can be used for samples of many different sizes. TLC is used to determine an effective solvent system for the separation and then column chromatography is used to separate and isolate the desired compound(s). This laboratory assignment demonstrates the utility of column chromatography to separate two enantiomerically pure compounds.

Activities
✓ In the first lab you will synthesize a racemic ester from sec phenethyl alcohol and monitor the reaction by TLC.
✓ The next lab period you will purify the racemic ester using column chromatography.
✓ On the fourth lab day you will hydrolyze the racemic ester to an alcohol with the assayed lipase and monitor the reaction by TLC.
✓ Finally you will separate the ester from the resulting ester from the alcohol using column chromatography.
✓ Then you will determine the optical purity alcohol and the ester using polarimetry.

PreLab:
Your chemical data table should include the chemicals used in the synthesis, TLC, and column chromatography activities described in detail below. Some of these chemicals may be found in your Common Shelf Data Table, and if so you will not need to include them in the Chemical Data Table. Hand in a copy of the completed Common Shelf Chemical Data Table with your PreLab.

Prelab Questions:
Why is derivatization used to isolate the R enantiomer of sec-phenethyl alcohol? Draw the chemical structure for both enantiomers of sec-phenethyl alcohol.
Why is it necessary to distill the product from the esterification of sec-phenethyl alcohol?

How do you know by TLC that the esterification reaction is complete?

Why do you wash with aqueous 1 M HCl and then saturated aqueous NaHCO₃.
Procedure 1 Synthesis of racemic \(\alpha\)-methyl-benzyl-acetate


A low temperature esterification is carried out under basic conditions to yield \(\alpha\)-methyl-benzyl-acetate from sec-phenenthanol. Dissolve 500 mg of sec-phenenthanol in 2 mL of THF in a 10 mL 14/20 flask. Add 631 mg of acetic anhydride (d= 1.082 gm/mL, 583 uL) and 648 mg of pyridine (d= 0.978 mg/mL, 663uL) to the flask and a small magnetic stir bar. Attach a 14/20 reflux condenser and let the water run slowly through the cooling jacket. Stir the reaction mixture at room temperature for 2 hours. Monitor the reaction by TLC.

Workup: When the reaction is complete add 2 mL of 1M HCl to the flask and stir for 2 minutes. Transfer the reaction mixture to a separatory funnel and remove the water layer. Check the pH of the water layer and make sure it is acidic; if not wash with a second 2 mL of 1M HCl. Check the pH again and if acidic wash twice with 2 mL of saturated NaHCO\(_3\). Remember gas is evolved during this wash, so vent your sep funnel often. Check the pH of the water washes and make sure they are basic. If not repeat with an additional NaHCO\(_3\) wash. Dry the ether layer over MgSO\(_4\) for at least 10 minutes. After 10 minutes the ether layer should be clear with no visible signs of water. Gravity filter the solution to remove the MgSO\(_4\) into a tarred 20 mL scintillation vial. Blow off the ether with Nitrogen. Weigh the crude oily precipitate.

Purification of the Racemic \(\alpha\)-methyl-benzyl-acetate:

You will separate any remaining starting material from your product using column chromatography. Weigh your purified product and characterize it by \(^1\)H NMR before and after running your column. Determine the percent yield before proceeding to the next step.

Determining Solvent System for Column Chromatography

Before proceeding to the column chromatography separation you must determine whether or not you have produced a reaction product by examining your crude oil by TLC. Run TLC on your crude oil using a 4:1 mixture of petroleum/ether. Use the TLC standard of the sec-phenenthanol located on the side shelf to identify the spot that corresponds to the starting alcohol. Check the mixture by TLC (petroleum ether 4:1 ether). Where should the spot from your ester product appear relative to your starting material? Once you are satisfied that you have formed the product continue on with the purification of the crude oil using column chromatography.
Column Chromatography

Packing the Column. Before you assemble the column, check the small plug that fits into the bottom of the column to make sure that it has a small fritted disk inside. Next, make sure that the plug fits snugly into the glass column and is not easy to pull out. If it is loose, get a new bottom plug from the stockroom. Finish assembling the chromatography column as depicted in Figure 8.1 of the Lab Guide. Be sure to clamp the column securely and vertically.

Grasp the valve with one hand and turn it with the other. Close the valve and fill the column with ligroin to the bottom of the plastic funnel. Weigh out approximately 10 g of silica (20 mL) in a small beaker and add enough petroleum ether/ether (4:1) (150 mL) to make a slurry with the silica. Cool the slurry in cool water. Pour the slurry into the column making sure that all the silica is transferred, if necessary rinse with the ether solvent mixture. Let the silica settle tapping the sides if necessary. The column should have no visible cracks and should be packed evenly throughout. After the silica has settled carefully add a lay of sand (0.5- 1 cm). Add more solvent to the column if necessary to make sure that the entire column is below the level of the solvent.

Note: It is extremely important to never let the column run dry at any time. This will allow air to enter the column, which will result in uneven bands and poor separation.

Open the stopcock and continue to tap the column as you allow the solvent to drain slowly until the solvent just barely covers the surface of the sand, collecting the solvent in an Erlenmeyer flask. Remember the solvent system is the one you used when you tested the product mixture by TLC experiment.

Adding the sample: Add just enough petroleum ether/ether to dissolve the oil you isolated from ether extractions (add drops at a time) Note: It is important to use a minimum of solvent to dissolve the oil, if too much solvent is used the mixture will elute too rapidly and poor separation will result.

The solvent is drained just to the surface of the sand, which should be perfectly flat. Add the dissolve oil with a Pasteur pipette to the surface of the sand. Be sure to add the sample as a solution and should any sample crystallize, add a drop more (to be determined). (This is done so that the sample to be added to the column is in the most concentrated solution possible.) Drain some liquid from the column until the product solution just barely covers the surface of the sand. Add more solvent to the top of column being very careful not to disturb the surface. Drain some liquid from the column until it barely covers the surface of the sand and repeat again. Now the sample should be well within the silica. Elute the first fraction with approximately 20 mL of the ether solvent mixture and label it fraction A. Check this fraction periodically by TLC to make sure it contains only the ester component. Four or five samples can be applied and analyzed at the same time using one TLC plate. When you do not see anymore of the ester component you will be ready to elute the alcohol component. Elute the second fraction with approximately 30 mL of 1:1 petroleum ether/ether and label it fraction B.
Again check the progress using TLC. When you no longer see any more alcohol in the solvent you can stop draining the column. Evaporate the solvent from both fractions using the nitrogen in your hood.

Cleaning up. When you are done with the column, pour out the excess solvent into the proper waste container, pull out the bottom, and leave the “wet” column out in the beaker in your desk. The column will dry out by the next lab, and the dry used silica can then be easily emptied out into the waste bin.

**Preparation for the Lipase Reaction**

You will be using a lipase enzyme to selectively hydrolyze one enantiomer of your racemic ester. You will need to make the buffer before can prepare the enzyme stock solution. Below is a short introduction to buffers and a tutorial to walk you through the process of making the buffer. Follow the directions given in the tutorial to prepare 500 mL a 0.1 M pH 7 phosphate buffer solution per group. The tutorial will assist you in calculating the amount of acid and base component to add to the buffer.

**Introduction**

Maintenance of pH is vital to all cells. Biological processes such as metabolism are dependent on enzymes, and enzymes, in turn, are effective only within a narrow pH range. Organisms have a variety of mechanisms to keep the pH of their intra- and extracellular fluids essentially constant, but the primary protection against detrimental pH changes is provided by buffer systems.

**Buffer Theory**

A buffer is a mixture of a weak acid (HA) and its conjugate base (A⁻) or a weak base (B) and its conjugate acid (BH⁺). The pH of a buffer does not change significantly when a limited amount of strong acid or base is added. This is because the strong acid is consumed by A⁻ (Figure 1A) or B (Figure 1B) and the strong base is consumed by HA (Figure 1C) or BH⁺ (Figure 1D).

\[
\begin{align*}
\text{A} & \quad \text{H}^+ + \text{A}^- \rightarrow \text{HA} \\
\text{B} & \quad \text{H}^+ + \text{B} \rightarrow \text{BH}^+ \\
\text{C} & \quad \text{OH}^- + \text{HA} \rightarrow \text{A}^- + \text{H}_2\text{O} \\
\text{D} & \quad \text{OH}^- + \text{BH}^+ \rightarrow \text{B} + \text{H}_2\text{O}
\end{align*}
\]

Figure 1.

A specific example of a buffer is a solution of ammonia (NH₃), a weak base, and ammonium ion (NH₄⁺), NH₃’s conjugate acid. When HCl is added to this buffer, NH₃ reacts with H⁺ to become NH₄⁺. Since this H⁺ is locked up in NH₄⁺, the pH of the solution
does not decrease significantly. When NaOH is added to the same buffer, \( \text{NH}_4^+ \) reacts with \( \text{OH}^- \) to form \( \text{NH}_3 \) and water. Again, since this \( \text{OH}^- \) is locked up in water, the pH of the solution does not increase significantly.

The central equation for buffers is the **Henderson-Hasselbalch equation**, which is merely a rearranged form of the \( K_a \) equilibrium expression. For the purpose of the derivation, imagine a buffer composed of an acid, HA, and its conjugate base, A⁻:

\[
\text{HA} \leftrightarrow \text{H}^+ + \text{A}^-
\]

\[
K_a = [\text{H}^+][\text{A}^-]/[\text{HA}]
\]  \(1\)

\[
\log K_a = \log ([\text{H}^+][\text{A}^-]/[\text{HA}]) = \log[\text{H}^+] + \log([\text{A}^-]/[\text{HA}])
\]  \(2\)

\[
-\log [\text{H}^+] = -\log K_a + \log([\text{A}^-]/[\text{HA}])
\]  \(3\)

\[
pH = pK_a + \log([\text{A}^-]/[\text{HA}])
\]  \(4\)

The Henderson-Hasselbalch equation can be rearranged further:

\[
pH - pK_a = \log([\text{A}^-]/[\text{HA}])
\]  \(5\)

\[
10^{pH-pK_a} = 10^{\log([\text{A}^-]/[\text{HA}])}
\]  \(6\)

\[
10^{pH-pK_a} = [\text{A}^-]/[\text{HA}]
\]  \(7\)

\[
10^{pH-pK_a} [\text{HA}] = [\text{A}^-]
\]  \(8\)

In the laboratory, the desired pH of a buffer determines which buffering compound will be selected. In general, buffers are effective within a pH range equal to the pKₐ of the buffering compound ± one pH unit. After choosing a buffer, the next step is to decide its concentration. The buffer concentration must be sufficient to maintain the pH within acceptable limits with the changes in [\( \text{H}^+ \)] expected to occur. For biological systems, this generally means that the total buffer concentration is within a range from 1 mM to 200 mM. The total buffer concentration is defined as the sum of the concentrations of the acid and conjugate base forms.

\[
\text{Total buffer concentration} = [\text{HA}] + [\text{A}^-]
\]  \(9\)

Equation 8 can be modified to account for the total buffer concentration:

\[
\text{Total buffer concentration} = 10^{pH-pK_a} [\text{HA}] + [\text{HA}]
\]  \(10\)

\[
\text{Total buffer concentration} = (10^{pH-pK_a} + 1) [\text{HA}]
\]  \(11\)
How to prepare a buffer

There are several ways to prepare a phosphate buffer. The following method is conceptually easy and will help you become comfortable with buffer preparation.

1. Choose a pH. (In this case it is pH 7)

2. Choose a phosphate buffering compound such that the pKₐ of its weak acid (HA) form is within approximately one pH unit of the desired pH.

3. Choose a total buffer concentration. (In this case it is 0.1M)

4. Use equations 9 and 11 to determine the concentrations of [A⁻] and [HA] at the desired pH.

5. Convert these concentrations to masses.

   NOTE: You will use the salt form of A⁻ to introduce the appropriate amount of A⁻ into solution. Therefore, you will use the formula weight of the salt form of A⁻ when you convert the concentration of A⁻ to a mass (see sample problem below).

6. Dissolve the appropriate forms of the buffering compound in a volume of distilled/deionized water (this water does not come out of a tap in the organic lab it will be found in a carboy labeled deionized water) approximately equal to 60 % of the total volume of the buffer.

7. Adjust the pH (if necessary with 1M NaOH or 1 N HCl).

8. Dilute to the correct total volume with distilled/deionized water.

Sample problem

How would you prepare 100 mL (or 0.100 L) of 100 mM sodium acetate buffer, pH 4.0?

Necessary information:

pKₐ of acetic acid = 4.76
FW acetic acid = 60.05
FW sodium acetate = 82.03

Using equation 9:

100 mM = [acetic acid] + [sodium acetate]

Using equation 11:
100 mM = \(10^{4.0 - 4.76} + 1\) [acetic acid]

Solve for [acetic acid]:

[acetic acid] = 85 mM (2 significant figures...keep extra digits for later calculations)

\((85.19483458533 \text{ mM})(0.100 \text{ L})(1 \text{ mol/10}^3 \text{ mmol})(60.05 \text{ g/mol}) = 0.51 \text{ g} (2 \text{ significant figures})\)

Solve for [sodium acetate]:

[sodium acetate] = 100 mM – 85.19483458533 mM = 15 mM (2 significant figures...keep extra digits for later calculations)

\((14.80516541 \text{ mM})(0.100 \text{ L})(1 \text{ mol/10}^3 \text{ mmol})(82.03 \text{ g/mol}) = 0.12 \text{ g} (2 \text{ significant figures})\)

To prepare this buffer, dissolve 0.51 g acetic acid and 0.12 g sodium acetate in approximately 60 mL of water. Adjust the pH to 4.0 (if necessary). Dilute the buffer to a total volume of 100 mL.

**Phosphate buffers**

You may recall that phosphoric acid is a polyprotic acid with the following titration curve

![Titration Curve for Phosphoric Acid](image-url)
Figure 2.

The following equilibria and pKₐ values can be obtained from the titration curve:

\[
\begin{align*}
H₃PO₄ & \leftrightarrow H₂PO₄^- + H^+ \quad \text{pK}_{a1} = 2.15 \\
H₂PO₄^- & \leftrightarrow HPO₄^{2-} + H^+ \quad \text{pK}_{a2} = 6.82 \\
HPO₄^{2-} & \leftrightarrow PO₄^{3-} + H^+ \quad \text{pK}_{a3} = 12.15
\end{align*}
\]

Phosphate buffers are typically used when performing enzyme-catalyzed reactions because pKₐ₂ is close to physiological pH. Since you will be using phosphate buffers with pH values near 7, you will use the H₂PO₄⁻ and HPO₄^{2-} forms of phosphoric acid to prepare your buffers. The stockroom should have the sodium salt forms of these species.

Reference: Buffer Tutorial by Andrea Cerrone, Chemistry Graduate Student in Dr. Philip Bevilacqua Group.

**Procedure 2. Lipase catalyzed enantioselective hydrolysis of racemic α-methyl-benzyl-acetate**

**Introduction**

“The kinetic resolution of an ester catalyzed by an enantioselective enzyme is a common method for the synthesis of enantiopure compounds. Lipases, that is to say enzymes which in nature catalyze the hydrolysis of fats, are particularly versatile in this reaction (why?). In an extensive screening program it was shown, that Candida antartica Lipase B (CAL-B) is particularly suitable for the enantioselective hydrolysis of one of the racemates of α-methyl-benzyl-acetate. The enzyme works best at pH 7.0 therefore the reaction is preformed in aqueous buffer at pH = 7. The water of the buffer participates in the reaction; after all it is a hydrolysis. Under extremely acidic or alkaline conditions the CAL-B, like any enzyme, might denature. During the reaction acetic acid is released, therefore the acid has to be neutralized with sodium hydroxide to maintain the correct pH. The reaction can give up to 95% yield of both (s)-ester and the (R)-alcohol and both can be obtained with excellent optical purity (ee > 98%).”  Stetca, D.; Arends, I.W. C. E.; and Hanefeld, U. J Chem Ed. 79, 1351, 2002.

**Experimental Procedure:**

Each person in the group will carry out the enzymatic hydrolysis of the ester. Everyone will follow these instructions:

Add 100 mg of racemic α-methyl-benzyl-acetate to a 10 mL Erlenmeyer flask. Note racemic α-methyl-benzyl-acetate is an irritant and any eye and skin contact should be avoided. Add 1 mL of buffer to the flask and gently stir this two-phase mixture. Slowly
add 20 mg of lipase to the flask and continue stirring. Make sure the lipase is room temperature before adding. Monitor the pH of the reaction by touching the reaction mixture with the tip of a Pasteur pipette then transferring this small drop of solution to a piece of pH paper. If the pH drops below 7.0 slowly (dropwise from a 1 mL syringe) add some 1M NaOH to maintain a pH of 7.0. Continue to read and adjust the pH of the solution. The reaction is complete when the pH remains stable.

**Workup**

The enzyme is filtered off with a Büchner funnel (do not forget the filter paper. It works better if it is moistened by water). Wash the enzyme three times with ether (3X 1 mL) to isolate the product. Extract the filtrate with ether (3X 1 mL) and combine the layers. Wash the ether with brine and dry over MgSO₄. Gravity filter the dried ether solution and wash the MgSO₄ with ether. Check the mixture by TLC (petroleum ether 4:1 ether). Both products should be present. Evaporate the ether extract and you should be left with an oily residue containing both products.

**Purification of the Enantiomerically Pure Products**

You will isolate the enantiomerically pure alcohol and remaining enantiomerically pure ester using column chromatography. Before you begin the purification step run a ¹H NMR on the crude oil. Since you will be separating the same compounds you separated with the first column using the same procedure and the same solvent system. Check your fractions by TLC and isolate the pure alcohol and pure ester by evaporating off the chromatographic solvents using nitrogen. Weigh each of the fractions and run a ¹H NMR on each product.

**Polarimetry**

Pure enantiomers have a specific optical rotation. The values for image and mirror image are the same but for one of them it is positive while the other is negative. If the optical rotations of the pure compounds are known, the values are determined for samples of unknown purity can be used to determine optical purity. The specific optical rotation at room temperature in methanol of (R)-1-phenyl-ethanol is [α]₀ = 45° and of (S)-α-methyl-benzyl acetate is [α]₀ = -114.05°. (Roberts, S.M. *Preparative Biotransformations*; John Wiley and Sons; Chichester, New York, Brisbane, Toronto, Singapore, 1993, 1:6.41-1:6.48).

Dissolve each product in approximately 5 mL of methanol and transfer to a 10 mL volumetric flask. Make sure you know the weight of your sample before you dilute it in methanol. Fill the volumetric flask to the mark with methanol. Shake well. Fill the polarimeter cell with the clear solution and make sure that no air bubbles remain in the cell. Measure the optical rotation and use the following equation to determine the optical rotation.

\[ [\alpha] = \alpha / c \times 1 \]
Where:

\[ \alpha = \text{measured optical rotation} \]
\[ c = \text{concentration [g product/mL MeOH]} \]
\[ l = \text{pathlength of the cell [dm]} \]

The optical purity of your compounds can now be calculated with the formula given below:

\[
\text{Optical purity} = \frac{\text{measured optical rotation of your compound}}{\text{specific optical rotation of the pure enantiomer}} \times 100\% 
\]

**PostLab Questions:**

1) How much base (NaOH) needs to be added to the enzyme hydrolysis before the reaction is complete? Why is it important to add the base? What will happen to the reaction product mixture if you do not monitor the pH during this reaction?

2) Why is a brine wash added in the workup of the enzyme hydrolysis reaction?

3) How do you know which component in the reaction mixture will elute first from the column in the workup of the enzyme hydrolysis?

4) Why is the eluent changed after the first component is removed from the column?

**References:**


A document created by Andrea Cerrone for Chem 36 B,


**Final Report**
Include the weight and percent yield of the racemic ester. Include a $^1$H NMR Spectral analysis of the racemic ester prior to column separation and a $^1$H NMR of the two major fractions following the chromatographic separation. A similar set of spectra should be run following the reaction with the lipase and following your final column separation. Run the NMR spectra on the 400 MHz instrument. The weight, percent yield and optical purity of the final ester and alcohol should also in the discussion. Attach all TLC plates and calculate Rf s for all spots. The Rf s must be tabulated for both products.