6. Gas Chromatography

A. Introduction

Chromatography is the general name for several extremely powerful methods for separating mixtures of many types of compounds. The word **chromatography** implies color, and dates back to the earliest separations of mixtures of colored materials, such as plant pigments, into the individual components. While most separations no longer involve colored compounds, the name has been retained for all of these related methods which are then subclassified according to the details of the separation method.

Some of the terms used to describe these methods are column chromatography, paper chromatography, gas chromatography, thin-layer chromatography, high-performance liquid chromatography, gel permeation chromatography, ion-exchange chromatography, size-exclusion chromatography, and electrophoretic chromatography.

All chromatographic methods are based upon a common principle. A **moving phase,** which may be either a liquid or a gas, moves through a **stationary phase** consisting of a finely-divided solid, which is often coated with a non-volatile, or non-soluble, liquid phase. In many forms of chromatography, the stationary phase is contained in a **column** constructed of metal or glass tubing. The moving phase is continuously passed through this column. A mixture of compounds is injected at the front of the column and swept along through the column by the moving phase. **Separation of the mixture is achieved because the components of the mixture travel through the column at different rates** and emerge from the column at different times. The times for the emergence of the components are monitored by a detector and the components may be collected individually if desired.

In **gas chromatography** the mobile or moving phase is a non-reactive gas, usually **helium.** If the stationary phase is an uncoated solid, the technique is termed **gas-solid chromatography** and it is used mainly for separating mixtures consisting of low molecular weight compounds which are normally gases at room temperature. If the stationary phase is a solid coated with a nonvolatile liquid, then the method is termed **gas-liquid chromatography.**

Gas-liquid chromatography (GLC), often referred to simply as gas chromatography (GC), is a powerful analytical method for both qualitative and quantitative analysis of mixtures of volatile components. By this method the components of the mixture can be identified and their concentrations measured, even at very low concentrations. In this experiment gas-liquid chromatography is used to analyze the various fractions obtained in the fractional distillation of the mixture of three esters. Sample sizes are of the order of 1–10 microliters (1–10 mg), but equipment is available commercially for either much smaller or much larger samples.

B. Gas-Liquid Chromatography

1. The Stationary Phase

In gas-liquid chromatography the **stationary phase** consists of a very fine granular **solid support,** which has been coated with a layer of a **nonvolatile liquid.** The solid support is comprised of an inert material (originally ground firebrick) which has been carefully "sieved" to a uniform particle size. These particles are very rough and thus have

a large surface area. The solid support is coated with 5 to 20% by weight of a nonvolatile liquid, which forms a very thin film on each particle, perhaps 0.1 micron or less in thickness (1 micron = 10^{-6} m) (see Figure 1).

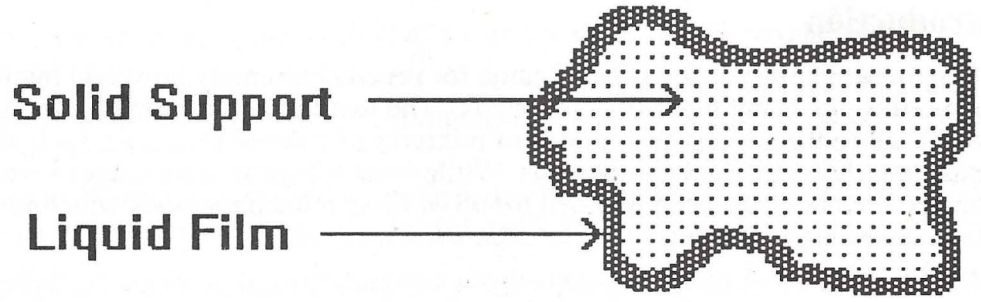

Figure 1. Magnified particle of solid support.

Many **nonvolatile** liquids are available for use as liquid phases, ranging from quite nonpolar substances to relatively polar materials. Thus the solvent properties of the liquid phase can be varied considerably. Even though the solid support is coated with a liquid, it appears to be a dry powder. When this powder is packed uniformly into a metal tube, typically 1/8- or 1/4-inch, one obtains, in effect, a highly-dispersed nonvolatile liquid anchored in the tubing. This **column** is coiled to fit into an instrument called a **gas chromatograph.**

2. The Gas Chromatograph

Figure 2 is a block diagram of a gas chromatograph. The mobile phase, **the carrier gas,** is helium and the gas is supplied at constant pressure to ensure a steady flow rate, usually about 60-100 mL/min. The helium first passes through an **injection port,** where samples are injected with a syringe through a septum into the helium stream. Since the injection port is heated, liquid samples are "flash vaporized." The components of the sample are then swept through the column by the carrier gas and then through the **detector.** The detector generates a signal when components from the sample pass through it and this signal is fed to a strip-chart recorder which continuously plots the magnitude of the signal as a function of time, as indicated by a distance measured on the chart paper. This chart is called a **chromatogram** (Figure 3).

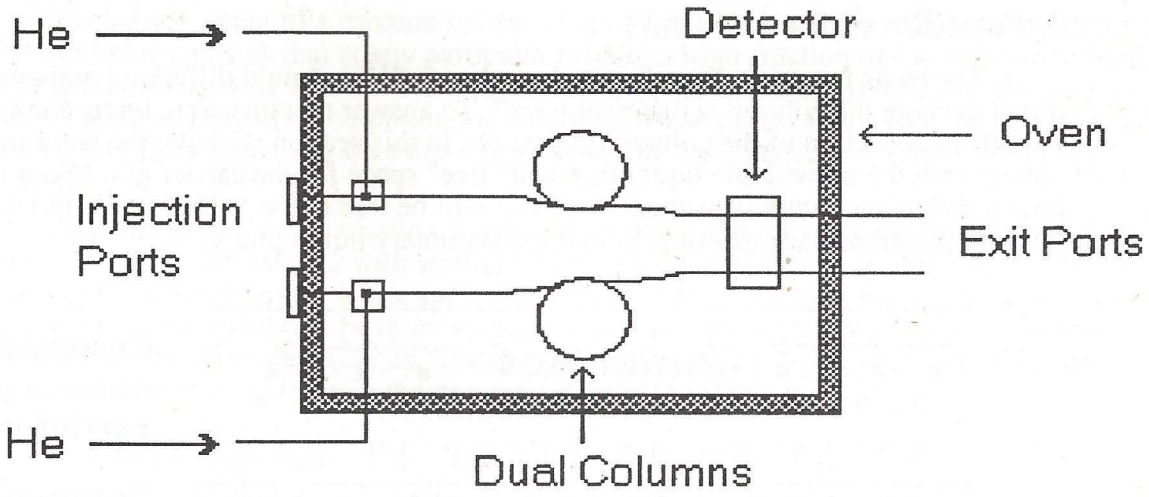

Figure 2. Gas chromatograph.

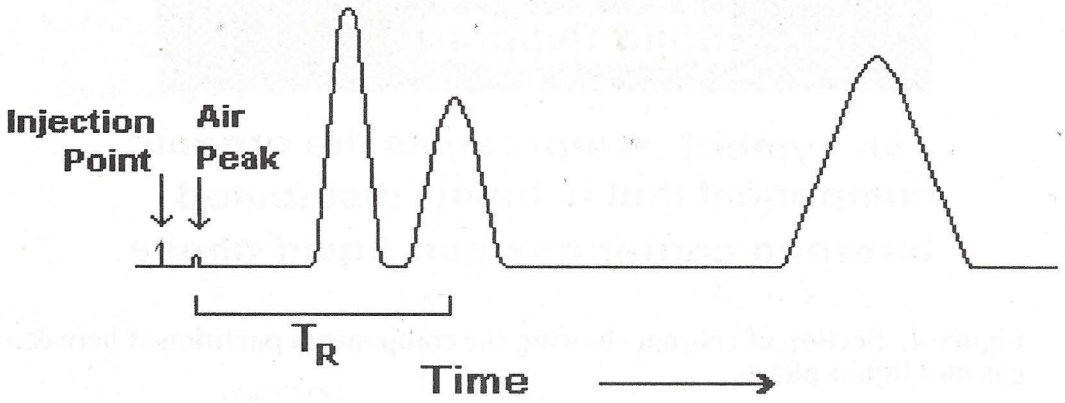

Figure 3. Appearance of a chromatogram.

Ideally, each component produces a symmetrical peak and, if the separation is good, the chromatogram will consist of a series of separate peaks. The time for a component to travel through the column, measured from the injection point to the peak maximum, is called the **retention time,** T_R. For a given column operating at a specific temperature and flow rate, **the retention time, T_R, is a characteristic property of each compound**; i.e., each compound will show the same retention time upon repeated injections under the same conditions. Thus, retention times can be used to identify components of a mixture.

Since the area under the peak is proportional to the **amount** of the substance passing through the detector, quantitative information can be obtained as well. We can find out what compounds are present in a mixture and how much of each is present.

3. Function of the Column

The basis for separations lies in the column. Why should different components travel through the column at different rates? To answer that question, let us consider a hypothetical section of the column (Figure 4). In this section we have the solid support coated with the nonvolatile liquid film and "free" space for the carrier gas. (No matter how tightly the column is packed, there still will be free space.) The carrier gas moves through this free space, passing around the stationary liquid phase.

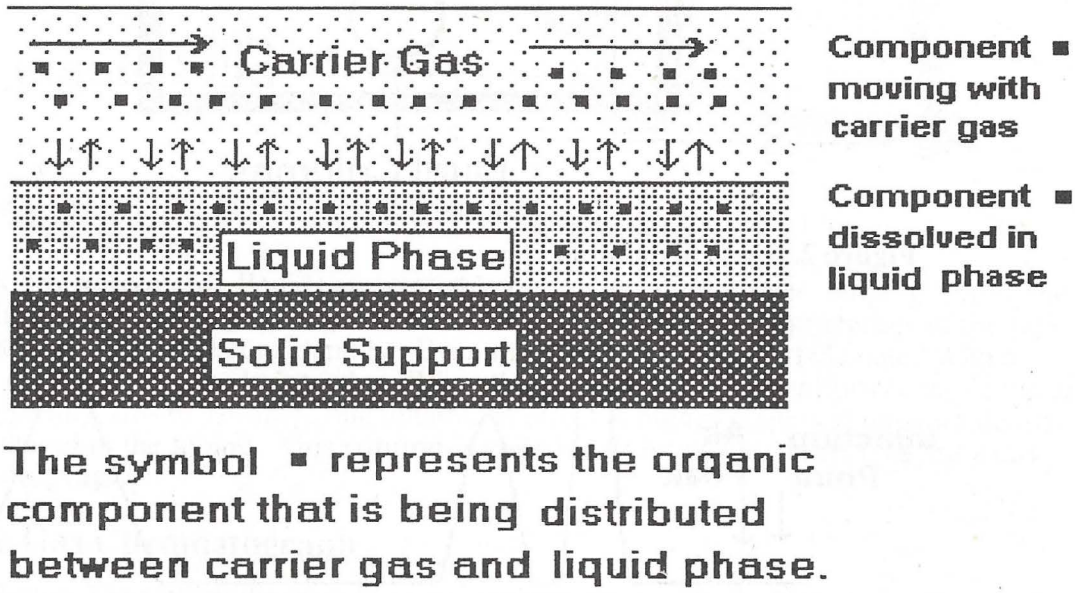

Figure 4. Section of column showing the components partitioned between carrier gas and liquid phase.

The carrier gas is moving through the column at a velocity represented by the long arrows. The sample component is represented by the small black squares and is being carried along by the carrier gas. What happens to a component of a mixture when it enters this section of the column? Once in this section (and in all sections of the column), the component will **partition** itself between the **gas phase** and the **stationary liquid** phase. Partitioning means that **some** of the component will **dissolve** in the liquid phase and **some** of it will be in the gaseous state being carried along with the carrier gas. (Some of the black squares of Figure 4 are now situated in the liquid phase as well in the gas phase.) According to Henry's Law, the partial pressure (and thus the concentration) of the component in the gas phase is directly proportional to its concentration in the liquid phase (and ***vice versa***). There is a **dynamic equilibrium** in which the molecules of the component rapidly transfer back and forth between the gas phase and the liquid phase. This partitioning of the component (small black squares) is represented by the vertical arrows of Figure 4. At any given instant, only a **fraction** of the component will be in the gas phase and it is only when the component is in the gas phase that it can move through the column. If this fraction is, for example 1/4, then this component will move along the column only 1/4 as fast as the carrier gas.

Each component of a mixture behaves similarly. A **low** affinity of a compound for the liquid phase means that at any particular instant, a **high** fraction of the component will be in the gas phase and thus the component will move relatively rapidly along the column. A **high** affinity of a compound for the liquid phase means that a **low** fraction of it will be in the gas phase and thus this component will move relatively slowly along the column.

In general, the more volatile a compound, the faster it will move through the column. However, compounds with similar boiling points may have very different retention times if they differ in other ways, such as polarity. If the stationary liquid phase is **polar,** then polar components will be more soluble in the polar phase and move relatively more slowly than nonpolar components. If the liquid phase is **non-polar,** then non-polar compounds will be more soluble in the non-polar phase and move more slowly.

4. The Chromatogram

The components of the mixture emerge from the column in the form of symmetrical peaks and the greater the retention times, the broader the peaks (Figure 3). With large differences in T_R the peaks are well separated. On the other, hand if the T_R values are close, the peaks move toward each other and may overlap (Figure 5). The width of a peak at half of the peak height, $W_{1/2}$, is directly proportional to the retention time. (This behavior is "ideal," but is closely followed provided that we don't "overload" the column with too much sample.)

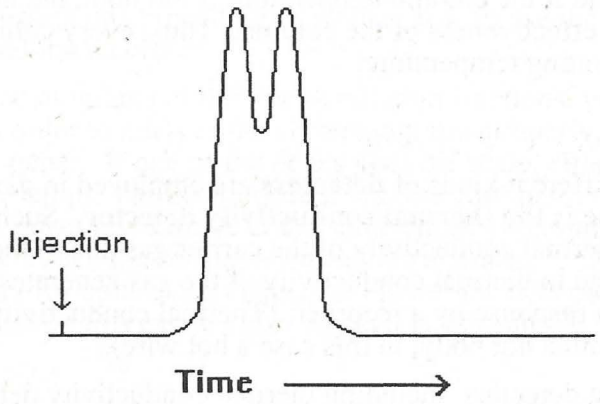

Figure 5. A chromatogram showing poor resolution.

The degree of separation of peaks is a measure of the **resolution** of the column (Figure 5). **High** resolution means sharp peaks and better separations, whereas **poor** resolution means broader, overlapping peaks, and poor separation. Many factors determine the degree of resolution of a column, but the most critical factors are set when the column is packed with stationary phase. Proper packing is essential for a column to give good resolution and the technique involved includes a high degree of art as well as science.

The operating temperature of a gas chromatography column is an important factor in controlling the separation of components of a mixture. As the temperature is increased, the fraction of sample in the gas phase increases, lowering T_R. The relationship is that log T_R is proportional to the reciprocal of the temperature given in degrees Kelvin (Figure 6). Normally the oven is maintained at constant temperature (isothermal

operation), but sometimes it is advantageous to program a steady increase in temperature as separation proceeds. Programmed temperature gas chromatography is particularly useful in analyzing mixtures where the components have widely different boiling points.

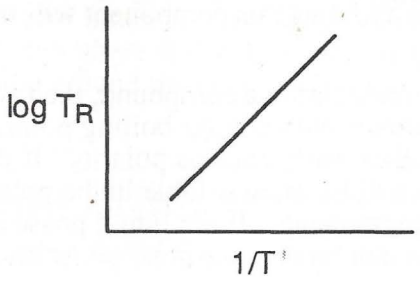

Figure 6. Relation between retention time (TR) and temperature. T in Kelvin.

Higher column temperatures give shorter retention times and result in faster analyses. But increasing the column temperature usually reduces the resolution of compounds with similar retention times and leads to overlapping peaks, as in Figure 5. The idea is to adjust the temperature to give fast analysis times, while still maintaining clean separation of the individual peaks.

A problem sometimes encountered is that stationary liquid phases are often somewhat volatile, and if the column temperature is too high, the liquid phase will "bleed-off" and destroy the effectiveness of the column. Thus, every column has a specified upper limit for its operating temperature.

5. Detectors

Several different kinds of **detectors** are employed in gas chromatographs. One of the simplest type is **the thermal conductivity detector.** Such a detector responds to a change in the thermal conductivity of the carrier gas due to the presence of another substance. A change in thermal conductivity of the gas generates a signal which is then translated into a response by a recorder. (Thermal conductivity refers to how fast heat is carried away from a hot body, in this case a hot wire).

With most detectors, including thermal conductivity detectors, the **area under a peak is proportional to the amount of component passing through the detector.** In very accurate work, we determine a **response factor** for each compound by determining the signal area produced by a fixed amount, e.g., one milligram, of each component. With thermal conductivity detectors, **equal weights of different organic compounds produce nearly equal peak areas** irrespective of the retention times of the compounds. As an example, for a mixture containing compounds A and B components, if the areas of peaks A and B of the chromatogram are in the ratio of 3:1, then the original sample was 75% A and 25% B.

C. Experimental Procedure

1. Running the Chromatograph to Obtain a Chromatogram

Most gas chromatographs have two columns, each consisting of a 1/4-inch or 1/8-inch copper or steel tube filled with the column packing and coiled to fit in the oven of the instrument. For a good pair of columns, one may contain a relatively nonpolar

silicone oil liquid phase while the other has a more polar "Carbowax" (polyethylene oxide) liquid phase. Either column can be used, but the one selected will depend on the type of compounds to be separated. The instructor will provide operating procedures for your particular gas chromatograph.

Special microliter syringes are used to inject the small samples required for gas chromatography. **Handle the syringes with extreme care, as they are easily damaged.** The 25-microliter syringe has a glass barrel with a carefully fitted stainless steel plunger. The syringe should be cleaned several times with acetone before use to ensure that the sample does not become contaminated. The house vacuum can be used to evaporate the acetone. The instructor will demonstrate the proper handling of a syringe.

To load a syringe, place the needle below the surface of the liquid and pump the plunger up and down three or four times to remove air from the syringe. Then fill to the desired sample size. Wipe off the needle and back the plunger out a little (perhaps 1-2 microliter) to trap a slug of air in the end of the needle. The air provides an air peak on the chromatogram and also clears sample from the end of the needle to avoid premature volatilization of the sample as it is injected into the chromatograph.

To inject a sample, hold the syringe with both hands and carefully and gently push the needle through center of the rubber septum and continue to push it until the needle is all the way in. **If it hits something, do not force the needle further in because the needles are easily damaged.** Push the plunger in gently, but firmly, to eject the sample, **being especially careful not to bend the plunger.** Quickly withdraw the syringe from the injection system. A mark should be made on the chart at the time of injection. The instructor will demonstrate this procedure as well as how to use the other controls on the gas chromatograph and the recorder.

Record the chromatograms of the five distillation fractions, vials 1–5 from the previous experiment. In order to analyze the chromatograms properly, it is necessary that all peaks be on the chart paper. If one of the peaks goes off scale, either the attenuator is set too high, or too much sample has been injected into the chromatograph. To bring all peaks on scale, either inject less sample at the same chromatograph settings, or use the same sample size and reduce the sensitivity using the attenuator. On the other hand, very small peaks that do not fill up the chart paper will lower the accuracy of the quantitative analysis measurements. In this event, either inject a larger sample, or set the attenuator to higher sensitivity.

2. Analysis of the Chromatogram

Analyze the chromatogram according to the example shown in Figure 7.

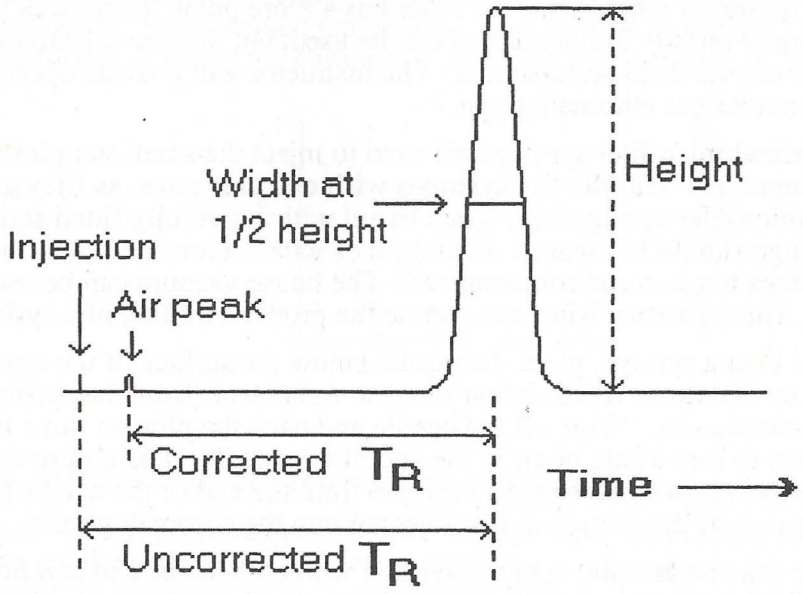

Figure 7. Analysis of the chromatogram.

Retention times measured from the time of injection are called "uncorrected." As noted earlier, usually a little air is injected along with the sample. This air moves through the column at virtually the same speed as the carrier gas. When the air emerges it generates an "air peak." Retention times are often measured from the air peak (taken as zero) and these are termed "corrected" (Figure 7).

Measure retention times with a ruler, or use the scale on the chart paper. If an air peak is present, measure retention times from the air peak; if not, measure the retention times from the moment of injection. Retention times depend upon the carrier gas flow rate, the column temperature, the amount and nature of the column liquid phase, and the length of the column. For a given column at a constant temperature, the relative retention times of different components (the ratios of the retention times) are constant, i.e., they are independent of flow rates, chart speeds, etc., and in fact do not vary much with moderate changes in temperature.

The area under a peak on a chromatogram is directly proportional to the amount of the component which produces the peak. On research instruments, peak areas are usually measured using electronic integrators. Without an integrator the area under each peak can still be estimated by one of the following methods.

1. Measure the peak width at one-half of the peak height, $W_{1/2}$, and multiply by the peak height, h (Figure 3).

$$\text{Area} \propto h \times W_{1/2}.$$

While this method can give good results, it requires reasonably wide peaks in order to measure $W_{1/2}$ accurately. This means that chromatograms have to be spread out by running at higher chart speeds than would otherwise be needed.

2. The area of a peak is proportional to the product of the peak height times the retention time (Figure 3).

$$\text{Area} \propto h \times T_R \quad \text{or} \quad \text{Area} = k \times h \times T_R$$

This relationship arises because the peak width increases linearly with retention time. If **all** areas are measured using the product h x T_R, you do not need to determine the proportionality factor k. In most cases, approximating each area by the product of the peak height times the retention time provides adequate accuracy and is the method of choice.

3. Refractive Index

The refractive index (R.I.) is a useful property for characterizing a liquid. The refractive index measures the ratio of the velocity of light in a vacuum to the velocity of light in the liquid (or solid). All light slows down as it passes through matter. Thus, we find refractive indices are all greater than one. Typical organic liquids have refractive indices in the range of about 1.3 – 1.7.

The instrument used to measure a refractive index is called a refractometer. There are many different types of refractometers and your instructor will explain the operation of the type of refractometer avaiiable. Only a drop or two of liquid is required.

Perform the operation carefully. It is particularly important to avoid scratching the prism. Also, be sure to clean the prism with a tissue soaked with ETHANOL when you have finished. DO NOT USE ACETONE, OR THE CEMENT HOLDING THE PRISM MAY BE DAMAGED.

Look through the eyepiece of the refractometer and adjust the prism so that a section of light resembling a spectrum appears. You should also see a dark band as well. Adjust the **dispersion control** so that the dividing line between light and dark is as sharp as possible. Then adjust the prism so that the sharp line of light intersects the cross-hairs precisely. At this point the view should appear as in Figure 8, left. Then switch to the scale to read the refractive index. The scale should appear as in Figure 8, right.

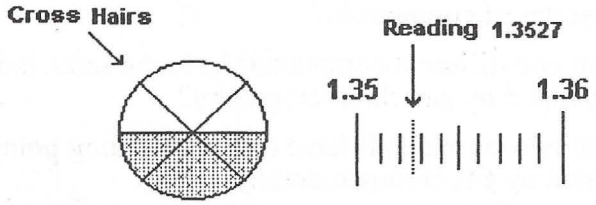

Figure 8. Determining the refractive index.

The dotted line in the Figure is the marker indicating the refractive index and it is now necessary to read the position of the marker. The numbers at the top will give the first two decimal places and immediately we see that the R.I. is between 1.35 and 1.36. Count the number of small divisions starting from the number on the left and proceeding up to the R.I. marker. In the example of Figure 8, the number of divisions is 2. Thus, the third decimal is 2. We now know that the R.I. lies between 1.352 and 1.353. Finally, estimate the distance from 1–10 that the R.I. marker is past the small division. In the example, this distance is estimated to be 7.

The reactive index is now known to 4 decimal places (5 significant figures) and is reported as $n_D^{25} = 1.3527$. This is accurate to ± 2 in the 4th decimal place.

The "D" refers to the D-line of the sodium light. Although white light was used to measure the refractive index, the instrument is calibrated to give readings as if the orange sodium-D light had been used. The 25 refers to the **temperature** of the instrument in degrees Celsius. When you measure the refractive index, note the actual temperature of the instrument, usually the ambient temperature, and record this as indicated above. Recording the temperature is necessary because the R.I. changes with temperature. Actually the R.I. **decreases as the temperature increases** because the **density** of the liquid decreases.

For a mixture, the refractive index is generally closely related to the **weight fractions** of the components as shown in the following relation:

$$n = \Sigma n_i W_i$$

where n is the refractive index of the mixture and n_i is the refractive index of a component which has a weight fraction of W_i. Thus the R.I. can be used to measure the composition of a binary mixture when the R.I. of the two individual components are known.

Determine the refractive index of the contents of vial #3 and vial #5. Referring to the refractive indices of the pure components, estimate the composition of each fraction. What do these data suggest concerning the purity of the samples? Compare these results with the compositions of the samples as determined by gas chromatography. Explain your results.

Using the composition of each of these two fractions as determined by gas chromatography, together with the refractive indices of the pure components, calculate the R.I. expected for each fraction. Compare these data with the observed R.I. values.

Note: When all analyses have been completed, return the distillation fractions to the bottle provided in the laboratory.

D. Prelab Questions

1. Predict qualitatively how the retention times in gas chromatography will vary for a homologous series of compounds?
2. Does the fact that two different compounds have the same boiling point mean that they cannot be separated by gas chromatography?
3. Does the fact that two compounds have different boiling points mean that they definitely can be separated by gas chromatography?
4. Suppose that you find that two compounds have identical retention times. What can you do to (try) to get a separation?
5. The "air-peak" is assumed to represent the time it takes the carrier gas to move through the column. Why does air move as fast as the carrier gas while organic substrates move much more slowly?

E. Postlab Questions

1. Based on the analysis of your samples by gas chromatography, which of the three samples is most nearly pure? Which is least pure? Explain the results.

2. For otherwise identical conditions, how will the retention time of a substance vary with the **amount** of liquid phase used in the packing? How will **relative** retention times vary with the amount of liquid phase?

3. Suppose you do a GC analysis on a sample consisting of 15% A, 60% B and 25% C by weight. A and B are volatile and have different retention times but C is nonvolatile under the conditions used and therefore does not generate a peak on the chromatogram. Assuming that you do not know that there is nonvolatile material in the sample, what will you conclude from the GC analysis, i.e., what percentages of A and B would you calculate?

4. To get around the problems presented in problem 2, the **internal standard** technique is used. A volatile component which does not interfere with the GC analysis (i.e., it gives a **separate** peak) is added to the sample which is then analyzed. For example, 10.0 mg of D was added to 50.0 mg of the mixture of A, B and C (problem 2). The sample is then analyzed by GC (note that you need to inject only 1 or 2 μL) giving a chromatogram with peaks for only A, B, and D. The relative peak areas (i.e. relative to D) are found to be: A, 0.75; B, 3.00; D, 1.00. For 10.0 mg of D, what weights of A and B are indicated by this chromatogram; i.e. what weights of A and B are in the original 50 mg sample? Thus, what are the weight percentages of A and B in the original sample?

5. Now apply the concepts developed in problem 3 to analyze a different mixture containing A and B as well as unidentified nonvolatile materials. An 8.0 mg sample of D was added to 40.0 mg of an unknown. Analysis of this mixture gave the following peaks, listing the component with the retention time and the peak height (in parentheses), both given in chart paper units: A, 20.0 (90.0); B, 30.0 (70.0); D, 50.0 (24.0). Calculate the weight percentage of A and B in the original 40 mg sample. Show all work.

7. Thin-Layer Chromatography

A. Introduction

Thin-layer chromatography (TLC) evolved from paper chromatography and column chromatography.

In paper chromatography, a mixture of compounds is "spotted" on a section of filter paper along a pencil line called the origin. The filter paper is placed in a developing solvent with the origin above the liquid level. The solvent rises through the paper by capillary action, sweeping the components along with it, each component travelling at a different rate. When the solvent has risen some distance, the paper is removed, the line of the solvent front is marked with a pencil, and the paper is dried. If the components are colored, they can be seen as spots on the paper. Generally the components are colorless and some means of visualization is performed. Sometimes a reagent is applied to the chromatogram to give colored spots. Ultraviolet light may cause the compounds to fluoresce. Paper chromatography gives good results with certain hydrophilic compounds, such as amino acids, but most organic compounds do not work well on a paper substrate.

In column chromatography, a finely-divided solid adsorbent, usually alumina (Al_2O_3) or silica gel (SiO_2), is packed into a glass tube similar to a buret to form a column. A solvent is then run through the column to displace the air. A short layer of a concentrated solution of the mixture to be separated is then placed on the top of the column and allowed to run into the column of packing material. A solvent "the eluant," is then passed through the column and carries the components of the mixture along with it. The components move down the column, generally at different rates. The eluant is continually run through the column until the components emerge from bottom. Ideally, each component is eluted from the column separately in a series of fractions which can be collected in separate vessels.

In thin-layer chromatography (TLC), the same adsorbents (alumina, silica gel) that are used in column chromatography are supplied coated on thin sheets of plastic (or glass). The procedure is similar to paper chromatography in that the mixture to be chromatographed is spotted along a line drawn on the TLC plate. A developing solution rises by capillary action into the coating carrying the components with it.

TLC, as well as column and paper chromatography, is based on a partitioning of the compound between the moving phase (the developing solution) and adsorption on a polar stationary phase substrate (alumina, silica gel, or paper). Some compounds bind tightly to the adsorbent and others do not. This difference in adsorptivity means that different compounds spend a greater or lesser fraction of time in the moving liquid phase. The smaller the adsorptivity, the greater the time spent in the moving phase, and the greater will be the rate of travel through the substrate. Since different compounds have different absorptivities for the substrate, they move at different rates and are thus separated. After the chromatogram is completed, each of the separated components must be "visualized" by one or more of several techniques available.

B. Experimental Procedure

1. General Techniques for Thin-Layer Chromatography

TLC plates coated with silica gel and containing an ultraviolet fluorescent indicator, are used in this experiment. The silica coating must not be touched, because finger-

prints can interfere with the analysis of the chromatogram. Always hold the plates by the edges. With a **pencil** draw a light line about 1 cm from the end of the plate, being careful not to break through the surface of the silica coating.

The developing chamber (Figure 1) consists of a 250-mL beaker having a height a little greater than the length of the TLC plate and covered with a watch glass or aluminum foil. Enough developing solution is added to the beaker to bring the level to about 0.5 cm. Place a circle of filter paper in the beaker so that one edge is in the developing solution and the rest of the paper is pressed against the wall of the beaker. Swirl the solution around so that the paper is saturated with the developing solution. The purpose of the paper is to help maintain an atmosphere that is saturated with the vapors of the developing solution. This prevents premature evaporation from the TLC plate which will cause distortion of the chromatogram.

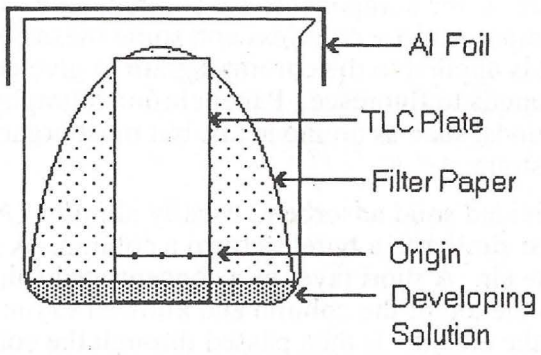

Figure 1. TLC chamber.

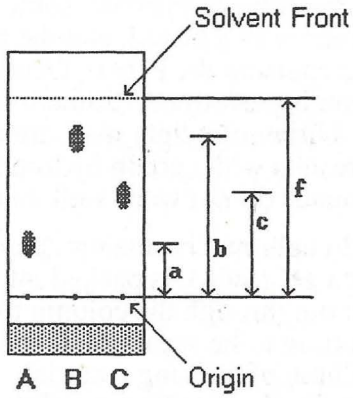

Figure 2. TLC chromatogram. $(R_f)_A$=a/f, $(R_f)_B$=b/f, $(R_f)_C$=c/f

The sample is dissolved in an appropriate solvent and spotted on the pencil line of the TLC plate using a capillary pipet. The pipet is made by drawing out a melting point tube in a flame to a very fine capillary. (Alternatively the capillary pipets may be supplied to you.) The pipet need only be dipped into the sample solution and the solution will run up into the pipet by capillary action. (Your instructor will demonstrate how to make and use a capillary pipet). The spot on the TLC plate should be **as small as possible,** perhaps 1 mm in diameter. A small identifying code number (**using pencil**) should be placed on the plate below the spot to identify the material spotted at that point. More than one sample, spaced about 1 cm apart, can be spotted on a plate. After spotting, allow the spot to dry, place the plate in the developing chamber, and cover the chamber with a watch glass or aluminum foil.

Make sure that the level of the developing solution in the chamber does not cover the spots. If this happens, your chromatogram is ruined, and you must start over. If the level of the solvent in the developing chamber is above the level of the spots, the solvent merely dissolves out the components of the spot and there will be no separation.

The solvent rises up the plate by capillary action, a process that takes several minutes. As the solvent moves through a spot, the components making up the spot move also, but at different rates. Thus the components are separated into individual spots on the plate (Figure 2).

When the solvent front nears the top of the plate, the plate is removed from the chamber and the solvent front is **quickly marked with a pencil before the solvent evaporates.** If the solvent front is not marked quickly, the solvent will evaporate and the position of the solvent front will be lost, and then it will be impossible to determine the R_f values for the compounds. After marking the front, allow the solvent to evaporate. The plate is now ready for "visualization" of the spots.

If the spots are colored they can usually be seen without aid. However, there may be spots that are colorless. Thus, special techniques must be employed to visualize the colorless spots. The TLC plates have a small amount of a fluorescent indicator bound to the silica. The short wavelength ultraviolet light (254 nm) of a UV-lamp causes the indicator to fluoresce, emitting an orange glow best seen in semi-darkness. Because many compounds quench the fluorescence, the components on the TLC plate appear as dark spots under the UV-lamp. Examine the plate with the UV-lamp to visualize the spots. Then with a pencil, draw a small circle around each spot that you have observed.

Using a millimeter ruler, determine the R_f value for each component. The R_f value is the distance a component (spot) has moved from the origin divided by the distance that the solvent front has moved from the origin (see Figure 2). With a given solvent system, the R_f value is a characteristic of a compound and can be used to identify the compound in an unknown mixture.

The UV lamp may also provide long wavelength UV light (366 nm) which does **not** trigger fluorescence by the indicator. This wave length may cause one or more of the spots to fluoresce, providing another way to make the spots visible. You should try both wave lengths and **record** the way the spots respond to UV light.

If a UV lamp is not available, an iodine chamber may be used to visualize the spots. Using this technique the dried plate is placed in a closed chamber containing crystals of solid iodine. The vapor of the iodine reacts with most compounds to give dark spots on the plate that are readily visible. When the spots are clearly visible, remove the plate and draw circles around the spots as they may disappear in time. Tape the plate into your notebook with Scotch tape with the tape completely covering the TLC plate. The tape will prevent the spots from disappearing.

TLC has the great advantage that very simple equipment is employed, yet remarkable separations can be achieved. The degree of separation depends upon the stationary phase (the silica on the TLC plate), the nature of the mobile phase (the developing solvent), and the care exercised in performing the procedure. It is essential that a sample be applied to a TLC plate as a **tiny, concentrated spot.** The spot **must be as small as possible** to reduce excessive spreading when the plate is developed. One must not use too much material, even with a very small spot, or the spot will spread too much and may overlap with other spots. Other conditions such as spreading, streaking, tailing, fronting, may also be observed. To achieve optimum results in TLC, it is often necessary to try a variety of solvents and solvent mixtures in order to achieve acceptable separations.

2. Separation of Acetaminophen, Acetysalicylic Acid, and Ascorbic acid.

Note: TLC plates from different manufacturers often give different results, even though the substrates may appear identical. Eastman Chromatography Plates with Fluorescent Indicator, Number 13181, work well for the experiments described here.

In this experiment, an unknown mixture of colorless compounds is to be analyzed by thin-layer chromatography and by examination of the chromatogram the components of the mixture are to be identified. The compounds in the mixture (all readily available at the drug store) are as follows: acetaminophen (Tylenol), acetyl salicylic acid (aspirin), and ascorbic acid (vitamin C). These compounds will be available in the laboratory as alcohol solutions or as solids and will serve as comparison samples to aid in the identification of the components of the unknown mixture. If the compounds are available as solids, obtain small samples and add a few drops of ethanol to each. It is not necessary that all of the compound dissolve.

Prepare several capillary pipets from melting point tubes as described above. Prepare a 4 x 8 cm TLC plate by drawing a light pencil line 1 cm from the end of the plate. Obtain a sample of the unknown. If the unknown is a tablet, crush it into a powder and add enough ethanol to cover the solid material. If the unknown is a solution, then obtain about 0.5 mL in a small test tube or vial. Using the capillary pipets, one for each sample, spot the three known standards and the unknown sample spaced out along the pencil line of the 4 x 8 cm TLC plate. The spots should be spaced about 1 cm apart and no closer than 0.5 cm to an edge. Mark each spot with a code number **in pencil** just below the spot.

To develop the chromatogram, a solution of 10% water in acetone will be used. Add the developing solution to the 250-mL beaker, making sure that the level of developing solution will be below the pencil line of the TLC plate when it is placed in the beaker. Add the sheet of filter paper, cover the beaker and swirl it to saturate the free space with solvent vapors. Stand the TLC plate in the beaker and cover the beaker with a watch glass or aluminum foil. Allow the plate to develop until the solvent nears the top of the plate. Remove the plate and quickly mark the solvent front with a pencil. Allow the plate to dry and observe the spots using the short wavelength tube of the UV lamp. Circle each spot with **a pencil** and determine the R_f values. Place the plate in an iodine developing chamber. When the spots are well developed, remove the plate, circle any additional spots and measure the R_f values. Cover the plate with transparent tape to preserve the iodine spots. Identify the components in the unknown sample.

3. Separation of the Pigments of Paprika

Place about 100 mg of paprika in a 4-dram vial or a conical vial and add about 0.5 ml of ethanol. Heat the mixture on the hot-plate and swirl for about 5 min. The ethanol solution is then suitable for TLC analysis directly.

Place two spots of the ethanol solution of the pigments along the pencil line of a 2.5 x 8 cm TLC plate. Allow the spots to dry and spot again at the same places with additional sample. Be careful when you do this and make sure to place each additional spot exactly on top of the original spot. Keep the spots as small as possible. Repeat the spotting about 8–10 times to build up sufficiently high concentrations of the pigment at the two spots. This repeat spotting is necessary, as otherwise the pigment spots of the developed plate will be faint and very difficult to see.

The developing solution in this case is pure methylene chloride. Develop the plates as before. To get a good separation, the developing solution should be allowed to travel almost to the top of the plate. Remove the plate and mark the solvent front quickly because methylene chloride evaporates rapidly. Observe the spots before the plate is quite dry because the spots tend to fade on drying. Mark as many spots as you can with a pencil. Number the spots and determine their Rf values. Record the colors of the spots. If a UV lamp is available, observe the spots under the short wavelength lamp and mark any additional spots that appear.

Dispose of waste chemicals in labeled waste containers provided in the laboratory.

C. Prelab Questions

1. Define the R_f value in TLC (use a sketch).
2. In TLC on alumina or silica, there is no stationary liquid phase as there is in GLC. What sort of property causes components to be "retained" by the substrate; i.e., what is the physical basis of the separations which are observed?
3. Consider two compounds of similar molecular weight: Compound A (nonpolar) and compound **B** (polar, with a carbonyl group and an OH group). Which one would be expected to have the **smaller** R_f value considering the fact that the alumina and silica gel are strongly polar? Explain.
4. Although we put a sample on a TLC plate as a **small** spot, we always find that the farther the component moves during development, the **larger** the spot gets. Suggest why this spot broadening occurs. What analogous behavior is found in gas chromatography?

D. Postlab Questions

1. Why does spotting too much sample on a TLC plate (overloading) lead to broad and/or irregular spots such as "streaking"?
2. In general, we assume that in a mixture, each component will show the same R_f value it shows when it is run alone. Sometimes the presence of a large amount of a second component **will affect** the R_f value of a component of interest. Suggest why this behavior is found.
3. **Thick-layer chromatography** is performed on a **preparative** scale in the same general fashion as TLC but the plates have much thicker layers of silica or alumina. Relatively large amount of a mixture are spotted in a virtual band along the origin (many spots are placed side by side). The plates are then developed in the usual fashion. Suppose a 75:25 mixture of A ($R_f = 0.80$) and B ($R_f = 0.40$) is run. Draw the developed chromatogram you should expect to see. How can A and B be **recovered** from the plate?
4. How could you use TLC as an aid in finding out how to separate a **large** quantity of material (5 g) by column chromatography?