Paper Chromatography and Liquid Chromatography
Introduction

It is perhaps true that great progress in science occurs after new inventions in technique occur. In the history of science, the battery and the vacuum pump are examples of such inventions. In modern science, the invention and application of two techniques, spectroscopy and chromatography, have revolutionized chemistry and biology. Chromatography is probably the most widely used and most powerful of all the techniques of chemical analysis. Chromatography was invented in the late nineteenth century by the Russian botanist Mikhail Tswett, who used it to separate naturally occurring chlorophylls. He extracted green plant material with organic solvents and allowed the extract to percolate through glass tubes full of powdered solids (e.g., sugar and calcium carbonate). A slightly more polar solvent was then used to wash the extract through the powder. Broad bands of color separated down the length of the column of powder. Tswett claimed that the different bands were different types of chlorophyll and other pigments and that chromatography was indeed the best way to investigate the chemistry of complex natural mixtures. As with many other inventions of true genius in the history of science, Mikhail Tswett’s claims were dismissed as nonsense by the scientific establishment of the time, and the new technique was neglected for almost three decades. Chromatography was rediscovered, albeit in a different form called partition chromatography, by the English chemists Martin and Synge in the late 1930s. Martin’s research group at the Wool Industries Research Association then developed the first microanalytical chromatography method, called paper chromatography, as a means to analyzing the structure of proteins. Martin and Synge received the Nobel Prize in chemistry in 1952 for the invention of partition chromatography.

The power of chromatography as a scientific tool lies in the fact that it is a simple, gentle, inexpensive, and general way of unmixing (separating) and analyzing complex mixtures of substances. Of course, once a mixture has been separated into pure components, it becomes a relatively straightforward matter to investigate the properties of each component. An interesting historical example, briefly mentioned earlier, is the application of partition chromatography to the study of proteins. The properties and behavior of fibers (e.g., wool) are very much determined by the particular chemical structure of the fiber. Martin et al. were trying to elucidate the structure of wool by breaking down the fiber with hot acid. Unfortunately, the resulting mixture of products from the destruction of fiber was so complicated that there was little progress. The problem was resolved by the invention of the partition chromatographic method. This method enabled the horrendous reaction mixtures to be easily separated into the individual amino acid and peptide products and eventually led to a complete structure of wool protein. It is interesting to note that partition chromatographic methods are now being applied extensively to solve gene sequencing problems in molecular biology.

Chromatography is now one of the major methods for chemical analysis and purification. Advances in technology, particularly in the miniaturization of columns and detectors, have resulted in the development of extraordinarily sensitive and quantitative chromatographic instruments (chromatographs). Micro gas chromatographs have been used in outer space probes, and micro liquid chromatographs have recently been explored as in vivo implantable analytical monitors. The objective in this series of chromatography experiments is to construct several small-scale chromatographic instruments (PC, LC, and GC) and to investigate the nature of chromatographic processes in several useful applications.
Background Chemistry

Chromatography is a method of separation in which the components to be separated are distributed between two phases, one of these being a porous substance or stationary phase, the other being a fluid that flows through the porous stationary phase. A small volume of the original sample containing the components to be separated is placed at the start of the porous stationary phase. It is important in most types of separation to try to place the sample in as small a volume of the stationary phase as possible or the separation becomes more difficult. The fluid, called the mobile phase, is allowed to flow through the porous bed, and as it does, the sample components begin to migrate through the bed. Each component will have a different affinity for the stationary phase and for the mobile phase. Components that have a higher affinity for the stationary phase will be slowed down relative to the other components. Components that tend to stay in the mobile phase will move farther along the stationary phase than the other components. The greater the fraction of time a component spends in the mobile phase, the farther it will move from the start. A component that remains only on the stationary phase will not move at all!

On the molecular scale, component molecules (or ions, atoms, etc.) do not simply move directly along the stationary phase in straight lines. Even though the component molecules are being pushed along in the general direction of the mobile phase, there is a tremendous amount of molecular jostling and bumping. Component molecules, therefore, diffuse in all directions as they migrate in the general direction in which the mobile phase is going. Thus, the chromatographic process is the sum of the billions and billions of molecular events involving diffusion to and from the surface of the stationary phase, random molecular bumping, and migration along the stationary phase in the general direction of the mobile phase. Chromatography may be described graphically in terms of the concentration profiles of the various components as they move along the stationary phase. A computer modelling of the chromatographic process for the separation of four components from a sample is shown in the computer graphic sequence in Figure 18.1.

The chromatographic sequence illustrated in Figure 18.1 represents the behavior of sample components in elution chromatography. Elution chromatography, by far the most commonly used type of chromatography, is also one of the three major types of chromatography. The elution technique involves the chromatography of very small amounts of sample and results in concentration profile shapes similar to those shown in Figure 18.1.

The objective of chromatography is to obtain a complete separation of the individual components of a sample mixture. In chromatographic instruments separation is obtained by trying to

- Maximize the component migration differences of all components
- Minimize the component spreading that occurs during the chromatographic process

If you look at the computer model pictures, you can see that as the chromatography proceeds, the center (or peak) of the concentration profile of each component gets
farther and farther apart, leading to a complete separation of the four components (as in the last picture of Figure 18.1). At the same time, all components spread out and occupy more and more of the space in the system. You can see that if the sample contained a large number of components, then overlapping might occur and a good separation might not be obtained.

In modern chromatography component migration differences are maximized by choosing the correct stationary phase and mobile phase based on an understanding of the chemical interactions involved. Sample components can interact with the stationary phase in a variety of ways — e.g., by partition, by adsorption, by ion exchange, by size, or by exclusion. A careful choice of the type of chemistry used in the system generally results in good separations. It must be emphasized that chromatography is now carried out on almost every conceivable type of chemical component, including proteins, gases, food dyes, etc., and on extraordinarily complex mixtures, such as oils, gasoline, urine, etc. Often, different stationary phases and different mobile phases are required in order to separate different groups of components from the same sample!

Much effort has been spent over the last 20 years to find ways of minimizing component spreading during chromatography. Narrow component bonds are particularly important if complex mixtures are to be resolved, i.e., well separated. The magnitude of the spreading process depends on several factors — e.g., the particle size of a solid stationary phase — and is usually measured in terms of the efficiency of the chromatographic system. The efficiency of a particular system is quantitatively expressed by the number of theoretical plates (N). N may be calculated from

\[ N = 16 \left( \frac{V_R}{W_b} \right)^2 \]

where \( V_R \), the retention volume, is defined as the volume of mobile phase required to carry a component through the chromatographic system. \( W_b \) is the width of the base of the component concentration profile (often called the band width). A better way of expressing the efficiency is to use the height equivalent to a theoretical plate (H), where

\[ H = \frac{L}{N} \]

and where the column is the porous stationary phase and L is its length. Modern high-performance chromatography instruments can provide many thousands of plates per meter of column (i.e., \( H < 0.1 \text{ mm} \)); consequently, the component bands are extraordinarily narrow. These high efficiencies have been achieved by technological developments in the manufacture of solid stationary phases and the tubes (columns) in which the phases are contained. In high-performance liquid chromatography (HPLC), the particles of stationary phase are often spherical, very small (diameters less than \( 5 \times 10^{-6} \text{ m} \)), and packed into the tube in a highly uniform manner. One unfortunate aspect of all this efficiency is that it has become very difficult to force the mobile
Figure 18.1  A Computer Model of the Chromatographic Process

Frame 1  A sample consisting of four components is injected into the chromatographic system. The mobile phase is allowed to flow and the various component molecules (represented by □, ○, Δ, and ×) begin to move along the column. The concentration profile of each component is shown directly below the picture of the column.

Frames 2–7  The components migrate along the column in the direction of the mobile phase. The component's migration differences increase until a complete separation (almost) of all four components is obtained (Frame 7). Notice that the molecular spreading increases as the components move farther along the column.
phase through the very small particles packed into a tube. Most modern HPLC instruments contain very high pressure pumps to force the mobile phase through the column at reasonable flow rates.

There are literally thousands of different chromatographic methods in use in a vast array of applications. The acronyms are legion — e.g., PC, IC, LC, GC, HPLC, HPTLRPC, GC/MS, GPC/EC, etc., etc. — and the techniques are used in all areas of science and technology. The ubiquity of chromatography makes it difficult to organize a simple classification of chromatographic systems. However, it is useful to classify systems on the basis of the types of phases and on the major distribution processes, shown in Table 18.1.

<table>
<thead>
<tr>
<th>Nature of the Distribution Process</th>
<th>Mobile Phase</th>
<th>Stationary Phase</th>
<th>Type of Chromatography and Acronym</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partition</td>
<td>liquid</td>
<td>liquid</td>
<td>paper and thin layer on cellulose (PC, TLC) reverse phase liquid (HPRPLC) gas liquid (GLC)</td>
</tr>
<tr>
<td>Partition</td>
<td>gas</td>
<td>liquid</td>
<td></td>
</tr>
<tr>
<td>Adsorption</td>
<td>liquid</td>
<td>solid</td>
<td>normal phase liquid, thin layer, and ion exchange (LC, TLC, IC) gas solid (GSC)</td>
</tr>
<tr>
<td>Adsorption</td>
<td>gas</td>
<td>solid</td>
<td></td>
</tr>
<tr>
<td>Size sorting</td>
<td>liquid</td>
<td>gel</td>
<td>gel permeation (GPC)</td>
</tr>
</tbody>
</table>

Table 18.1 Types of Phases and Distribution Processes in Chromatography

In this laboratory text you will have the opportunity of constructing and investigating three major types of chromatographic systems: paper chromatography (PC), liquid chromatography (LC), and gas chromatography (GC). The sections that follow provide you with some background chemistry that is pertinent to paper and liquid chromatography. Gas chromatography is discussed in Chapter 19, although you should note that the introduction given earlier pertains to all elution chromatography, including GC.

**PAPER CHROMATOGRAPHY**

*Paper chromatography* (PC) is a technique developed in England in the 1940s by Martin, Synge, and Consden that uses paper for separating the complex mixtures of amino acids obtained from the breakdown of wool. The separation and subsequent identification of individual amino acids and peptides led to the elucidation of the structure of wool protein. Paper chromatography is the name that is used to describe chromatography carried out on a stationary phase consisting of specially-prepared, porous paper. The sample, usually dissolved in a solvent, is directly applied to one end of a sheet of paper. The sheet is then placed in a large glass or plastic chromatography tank and mobile phase is allowed to contact the paper. The mobile phase is "pulled through" the paper by capillary action. PC has a somewhat limited use and is not used extensively now, although it is interesting to note that there have been several recent applications to the separation of complex enzyme mixtures with modified celluloses.
Paper is cellulose and has the structure shown in Figure 18.2. The hydroxyl groups (–OH) in the cellulose are responsible for the hydrogen bonding of water, which makes up about 6% of the weight of the paper. It is this water layer, along with more water that is sometimes adsorbed during the chromatographic process, that forms the stationary phase in PC. Chromatography paper is unsized and is carefully manufactured to produce a highly porous paper with relatively uniform fiber structure. If the edge of a sheet of chromatography paper is placed in a liquid, the liquid will be pulled through the paper by capillary action. The liquid moving through the pores in the paper constitutes a mobile phase, and chromatography can occur. The mobile phase may be pulled along, up, or down, depending on where the liquid is initially applied.

Let us now look at the factors that control the way in which components move in a paper chromatographic separation. Consider a single nonelectrolyte component applied to the paper at the start (see Figure 18.3).
The component molecules are distributed by partition between the aqueous stationary phase and the nonpolar mobile phase. The partition coefficient $k$ is defined as the ratio of the concentration of the component in the mobile phase to that in the stationary phase. In the above diagram, $k = 2$ because 1 volume of the mobile phase contains 2 molecules, and an equal volume of stationary phase contains 1 molecule. In practice, however, the volumes of the two liquid phases are not equal. For many chromatography papers the mobile phase volume is 3 times the stationary phase volume, and the phase ratio $r$ is

$$ r = \frac{\text{volume of mobile phase}}{\text{volume of stationary phase}} = \frac{3}{1} $$

The distribution ratio is defined as $kr$ — i.e., the number of molecules in the mobile phase per solute molecule in the stationary phase. In our example, $kr = 6$. In PC the measure of retention is the $R_f$. $R_f$ is defined as the fraction of time spent by an "average" molecule in the mobile phase and is equal to the fraction of molecules present in the mobile phase. Since, in general, the total number of molecules is $kr + 1$ (kr molecules in the mobile phase and 1 in the stationary phase) then,

$$ R_f = \frac{kr}{kr + 1} $$

Rearranging,

$$ kr = \frac{R_f}{1 - R_f} $$

In an actual PC separation, the $R_f$ value is defined experimentally by

$$ R_f = \frac{\text{distance moved by the component}}{\text{distance moved by the mobile phase front}} $$

As you can see, the relative migration distance $R_f$ depends on the partition coefficient $k$, the value of which depends on the chemical nature of the component and mobile phase. In a chromatographic experiment in which a sample mixture containing several components is applied to the paper at the start, the $R_f$ value of each component will depend on the partition coefficient for each component in the system employed.

The discussion thus far has been restricted to the chromatography of a single nonelectrolyte component. Paper chromatography was really introduced to separate polar substances, such as amino acids, sugars, drugs, metabolites, etc. Most of these compounds are weak acids or bases. We must therefore blend together acid-base and distribution principles in order to explain how various components behave in a PC system.

The migration of a weak electrolyte in chromatography is very dependent on the state of the acid-base equilibria that can occur. Consider a weak organic base B (e.g., nicotine) that may accept a proton to give the conjugate acid BH⁺:
\[ B + H_2O \rightleftharpoons BH^+ + OH^- \]

\[ BH^+ \rightleftharpoons B + H^+ \]

In a paper chromatography system in which the mobile phase is a nonpolar solvent and the stationary phase is an aqueous buffer of known pH, we can picture the system as shown in Figure 18.4.

![Figure 18.4 Distribution of a Weak Base in PC](image)

The only species that will distribute into the nonpolar mobile phase is B. The charged species BH⁺ remains in the aqueous stationary phase. The extraction coefficient D is equal to the ratio of the total concentration of component in the mobile phase to that in the stationary phase:

\[ D = \frac{[B_{org}]}{[B] + [BH^+]} \]

NOTE: an absence of subscript refers to the aqueous phase.
Dividing by \([B]\) and substituting,

\[ k = \frac{[B_{org}]}{[B]} \quad \text{and} \quad \frac{[BH^+]}{[B]} = \frac{[H^+]}{K_a} \]
Then

\[ D = \frac{k}{1 + \frac{[H^+]}{K_a}} \]

Now for weak electrolytes,

\[ R_f = \frac{D_r}{D_r + 1} \]

Substituting the expression for $D$ and rearranging gives

\[ R_f = \frac{kr}{kr + 1 + \frac{[H^+]}{K_a}} \]

and

\[ K_a = \frac{[H^+]}{\frac{kr}{R_f} - 1 - kr} \]

The $R_f$ expression shows how the migration of a weak base depends on $k$, $r$, $K_a$, and on the $[H^+]$ of the aqueous stationary phase. Analogous expressions can be derived for weak acids, i.e.,

\[ R_f = \frac{kr}{kr + 1 + \frac{K_a}{[H^+]}} \]

All these expressions (and many others for different types of equilibria) were originally derived by Soczewinski, Waksmundski, et al., and a group of chemists, biochemists, and pharmacologists working in Lublin, Poland. Graphs of $R_f$ versus pH of the stationary phase in paper chromatography for various weak electrolytes are shown in the diagrams of Figure 18.5.

The most important results that can be obtained from the mathematical analysis of $R_f$ versus pH data are the following:

- The chromatographic separation of weak acids and bases can be obtained simply by choosing an optimal pH of the stationary phase.
- Chromatography can be used to measure the $K_a$, $K_b$, and $k$ values of any weak electrolyte.

The technique is fast, easy, inexpensive, and will work with extraordinarily small amounts of material.
LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is the name that is now universally used to describe chromatographic separations carried out in small tubular columns packed with small particles of stationary phase. As the name implies, the mobile phase is liquid and may be a pure solvent or a variable composition mixture of solvents. The LC apparatus may be a simple plastic tube with gravity flow of liquid or a very complex instrument with stainless steel tubes, high-pressure mobile phase pumps, and sophisticated, sensitive analytical detectors. Much of the chemical analysis that is done in clinical, industrial, and research laboratories is carried out on commercial high-performance liquid chromatography (HPLC) instruments costing thousands of dollars. A quantitative analysis is obtained by injecting a known volume of sample into the instrument and by quantitatively measuring the concentration of each separated component with a detector placed at the end of the column. HPLC is often performed in the reverse phase mode (HPRPLC). A chromatographic process in which the liquid mobile phase is more polar than the stationary phase is referred to as a reverse phase process. One of the most common HPRPLC stationary phases consists of 5 μm particles of silica gel that has been derivatized (chemically reacted) with octadecyl hydrocarbon groups, as shown in Figure 18.6.
The mobile phase is often aqueous buffer or a water/alcohol mixture. HPLC columns are extremely efficient, producing very narrow component bands. The efficiency is mostly due to the extremely small uniform particle size of the stationary phase and homogeneous packing in the column.
Pre-Laboratory Quiz

1. Who first used chromatography for the separation of natural products?

2. What type of chromatography was invented to help discover the structure of proteins?

3. Give a definition of chromatography.

4. In order to obtain good separations in chromatography, what factor must be maximized and what factor must be minimized?

5. What is one measure of the efficiency of a chromatographic system?
6. What is partition?

7. What does HPRPLC stand for?

8. In the partition chromatography of a nonelectrolyte, give the expression that relates \( R_f \) to partition coefficient and distribution ratio.

9. Draw a simple diagram showing how the \( R_f \) of a weak base varies as the pH of the stationary phase is changed.

10. Describe 1 reverse phase-stationary phase used in HPLC.
Laboratory Experiments

Flowchart of the Experiments

Section A. Paper Chromatography of Dyes

Section B. Moist Buffered Phase Chromatography of Nicotine

Section C. Calculation of the $K_b$ of Nicotine from Chromatographic Data

Section D. Preparation of a Liquid Chromatography Column

Section E. Investigations of Column Parameters and Processes

Section F. Derivatization of the Silica Gel Stationary Phase

Section G. Chromatography of Selected Synthetic Dyes

Section H. LC of Beet Pigments

Requires one three-hour class period to complete
Section A. Paper Chromatography of Dyes

Goal: To carry out a series of ascending paper chromatographic separations of inks and food dyes in a small-scale tank system.

Discussion: You will also examine the effect of mobile phase composition on separation and use Rf parameters to characterize individual dye components in commercially available dye mixtures.

Before You Begin: Paper chromatography is a microanalytical technique. Extraordinarily small sample volumes are used (microliters), and the separation often yields very small amounts (often less than nanomoles) of product. It is particularly important to maintain good technique and to try to avoid contamination of the chromatography paper. Work on a clean piece of paper or paper towel when you are drawing lines and spotting samples.

Experimental Steps: 1. Obtain 2 pieces of chromatography paper from Reagent Central. On one piece, draw in the lines and points with a pencil, as shown below.

2. At your lab place will be a plastic chromatography tank and a solvent tray. Take the tray to Reagent Central and deliver 3 pipets of the mobile phase solvent, 2:1 1-propanol/water, to the tray.

3. Take the tray to your bench and place the tank over it.

4. Take a clean 1 x 12 well strip to Reagent Central and deliver 2 drops each of standard FD and C food dye solution to different wells. In the remaining wells deliver 2 drops of commercial food dye mixtures (you may select these from the variety available).

   - Note in your laboratory record the location of each dye.
5. Obtain a piece of cutoff thin stem (cut at an angle) as an applicator. Dip the applicator into the dye solution in the first well and then transfer the dye solution to the first application mark on the paper. Keep the applicator vertical.

NOTE: Apply enough dye to see the color easily, but keep the spot small!

6. Spot the rest of the food dye samples, cleaning the applicator after you spot each dye.
   - Record where each dye is spotted.

7. Obtain the pens (which contain water-soluble inks) and touch each pen tip to a sample application mark.

NOTE: Keep the pen vertical and try to make the spots small by touching and removing the tip quickly.

8. Roll the paper into a cylinder and hold it while you staple it as shown below. Do not overlap the 2 edges of the cylinder.

9. Lift the tank from the solvent tray and place the paper cylinder into the solvent as evenly and centrally as possible.

10. Place the tank over the cylinder.

11. Watch the mobile phase for a few moments so that you can see the mobile phase front as it climbs up the paper by capillary action.

   The chromatography will take about 20-25 minutes for the front to reach the solvent front line. From time to time check the progress of the front. Organize your time so that you do other things — e.g., keeping up your notes and preparing for the next section — while the chromatography is occurring.

12. When the front reaches the solvent front line, lift the tank and quickly remove the cylinder. Place it onto a clean paper towel. Replace the tank.
13. Pull the cylinder apart and hang it up to dry on the straw drying system (tray + straw + clothespin). The drying time is about 20 minutes.

14. While the paper is drying, start marking out the second piece of chromatography paper, as shown in Step 1 of Section B.

15. When the paper is dry (or when you are ready), place it flat on a towel on the table and locate the component zones or spots. This is done by drawing a circle around the leading edge of the oval color area.

- Measure the distance that each component migrated from the original sample application mark.
- Calculate the $R_f$ value for each component, where

$$R_f = \frac{\text{component migration distance}}{\text{mobile phase migration distance}}$$

- Use $R_f$ values and color to identify the unknown food dyes in the commercial mixtures.

16. Attach the chromatogram to your notebook.

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### Section B. Moist Buffered Phase Chromatography of Nicotine

**Goal:**

To obtain an $R_f$-pH profile for a cigarette extract and for a pure nicotine standard by moist buffered phase paper chromatography.

**Experimental Steps:**

1. Mark out a second piece of chromatography paper as shown.
2. Clean one 24-well tray and take it to Reagent Central. Fill 1 well about 1/2 full with pH 7.0 buffer solution, another with pH 8.0 buffer solution, and so on. (You will have pHs 7.0, 8.0, 9.0, 10.0, and 11.0.)

3. Fold a clean piece of paper towel in half and place it on the table.

4. Lay the marked piece of chromatography paper on the towel.

5. With a clean thin-stem pipet, transfer some pH 11.0 buffer and wet the paper by moving the pipet down the pencil lines, as shown.

   NOTE: You want to completely wet the rectangle marked pH 11.0 with the buffer without letting the pH 11.0 solution soak into the area marked pH 10.0.

6. Wash the thin-stem pipet twice with water.

7. Transfer some pH 10.0 buffer from the well and wet the area marked pH 10.0 in the same manner as in Step 5.

8. Carry on with each buffer until you have finished with pH 7.0.

9. Wash the pipet and 1/2 fill it with water.

10. Wet the dry ends with water so that the whole piece of paper now appears uniformly wet.

11. Blot evenly and firmly with a folded paper towel.

12. Hang the paper on the drying rack (in still air!) and note the time on a watch or lab clock. Let the paper semidry for 3 minutes.

13. While the paper is semidrying, pour out the n-propanol/water mobile phase from the solvent tray into the special waste container. Dry the tray with a paper towel.
14. Put 2 squirts of hexane ($C_6H_{14}$) solvent into the tray. Cover the tray with the tank.

15. Obtain the 2 pens, one that contains a standard pure nicotine solution and the other that contains a cigarette extract.

We have found that this is one of the best ways to store and apply very dilute nicotine solutions. The solutions in these pens also contain a dye that will enable you to see the precise location and size of a nicotine or a cigarette sample when it is applied to the chromatography paper.

16. At 3 minutes elapsed time, take the paper down and place it on a folded towel. Apply the standard pure nicotine solution to the left sample application mark of each pH area by holding the pen almost vertical and touching the paper. Keep the size of the applied sample small.

17. Now apply the cigarette extract to the right sample application mark of each pH area in the same manner.

18. Roll the paper into a cylinder and staple as before (Section A). Do not overlap the edges!

19. At 6 minutes elapsed time from Step 11, put the cylinder, evenly and centrally, into the hexane in the solvent tray and replace the tank.

20. Watch the chromatography proceed. It will take a short time (only about 2 minutes!) As soon as the mobile phase reaches the solvent front line, remove the cylinder and place it on a dry paper towel. The hexane will evaporate quickly.

21. Open the cylinder and place the chromatogram on a paper towel.

22. Locate the nicotine spots by applying Dragendorff's reagent to each buffered area.

NOTE: The nicotine spots will appear as orange colored spots.

CAUTION: Do not get Dragendorff’s reagent on your hands. If you do, wash with cold water and check with your instructor.

23. Hang the paper to dry. It will take about 30 minutes.

Organize your time and begin to set up Sections C and D during the waiting period.

24. Lay the dried paper down and draw a circle around the leading edge of each orange nicotine spot.

- Measure and record the $R_f$ values for each nicotine spot. You may average the $R_f$ values for the standard pure nicotine and the nicotine from cigarette extract spots for each pH.

NOTE: If the moist buffered phase chromatograph did not give you a reasonable $R_f$ versus pH profile (see Section C), you may repeat the experiment.
Section C. Calculation of the $K_b$ of Nicotine from Chromatographic Data

Goal:
To use data obtained from a plot of $R_f$ versus pH to calculate the base dissociation constant $K_b$ for nicotine.

Discussion:
In order to carry out this calculation, it is important that you understand the derivation of the $R_f$-pH relationship for weak bases. The derivation is presented in the Background Chemistry section at the beginning of this laboratory module.

Experimental Steps:
1. Plot a graph of $R_f$ versus pH of the stationary phase.
2. Draw a smooth curve through the points.
   The chromatograms at various pH values and the $R_f$ versus pH plot should look similar to the following.
3. Find the threshold $R_f$ value at which the $R_f$ is pH independent. This is easily obtained by drawing a horizontal line as a tangent to the $R_f$ versus pH curve at the flat part of the curve (at about pHs 10 and 11).

At the threshold $R_f$, the nicotine is virtually all in the free base form (B) and, therefore, is acting as a nonelectrolyte. The chromatographic relationship for a nonelectrolyte is simply

$$R_f = \frac{kr}{kr + 1}$$

which, on rearrangement, gives

$$kr = \frac{R_f}{1 - R_f}$$

where $R_f$ is the threshold $R_f$, $k$ is the partition coefficient for nicotine between hexane and aqueous buffered stationary phase, and $r$ is the phase ratio.

- Calculate $kr$ from the expression.

4. Look at the graph and select a pH value at which the $R_f$ value is in the middle of the curved part of the curve. Draw a vertical line through the selected pH until it cuts the curve. Draw a horizontal line to the $R_f$ axis.

- Note the pH and $R_f$.
- Calculate the $[H^+]$ for the selected pH.
- Now use the expression derived earlier to calculate the $K_a$ for BH+, the conjugate acid of the free base B of nicotine:

$$K_a = \frac{[H^+]}{kr - 1 - kr}$$

where $R_f$ is the $R_f$ value at the selected pH (and therefore $[H^+]$), and $kr$ is the constant you calculated in Step 3.

- Now for conjugate acids and bases, in aqueous solutions,

$$K_w = K_a \cdot K_b$$

where $K_w$ is the ion product of water, $1.0 \times 10^{-14}$ (at $25^\circ C$). Calculate $K_b$ for nicotine.

- At what pH of the stationary phase would you expect the nicotine to have an $R_f$ value of zero?
- Which form of nicotine, B or BH+, would you expect to pass most easily through a lung cell wall into the blood stream?
- How could the concentration of nicotine in tobacco be determined by using moist, buffered phase paper chromatography?
Section D. Preparation of a Liquid Chromatography Column

Goals:

(1) To construct a liquid chromatography system from straws. (2) To prepare a silica gel chromatography column that can be used to test chromatographic processes and separations.

NOTE: Please do not discard the various plastic materials used in making the chromatography apparatus. We will recycle them wherever possible.

Experimental Steps:

1. First build a stand for the chromatography column. This is easily done by using 2 straws and a 96-well tray (round-bottomed wells). Use a 1/4" office punch to punch a hole about 2" from the end of one straw and a hole in the middle of the other straw.

2. Push the first straw into well H–12 of the tray.

3. Push the second straw through the hole in the vertical straw. If necessary, cut the end of the straw at an angle to facilitate pushing it through the hole.

4. Obtain a column tip (the small plastic piece with a narrow hole). Place a small wad of polyester fiber into the tip.

   NOTE: Do not pack it too tightly or it will impede the flow of mobile phase.

5. Obtain a third straw. Push the column tip firmly into the end of the third straw.

6. Push the third straw through the hole in the horizontal straw.

7. Place a small cup under the column straw and squirt a stream of water into the straw to wash the walls and wet the polyester.

8. Use the plastic scoop (provided for you) to put 1 scoop of 100–200 mesh silica gel into a small cup.

9. Add about 15 mL of water to the cup and stir thoroughly with the slurry pipet. Suck up some slurry. Transfer the slurry to the column quickly!

   NOTE: The idea is to do this quickly to avoid settling of the large particles.

   NOTE: If you get an air block in the column, insert a slim straw and the block will disappear.

10. Transfer more slurry while the first batch is settling. Use the slim straw to work the packing in the column. Try to make a column silica gel slurry packing about 5–6 cm long.

11. Add water to the column until it is full (to the top).

12. Obtain a pump syringe and pull the plunger almost to the end, in order to push air not pull air!

13. Push the plastic tubing into the top of the column and hold it there firmly. Gently push the syringe plunger in and push water through the column.
CAUTION: Do not push the liquid level below the top of the packed column or you will have to repack the column because severe channeling and cracking will occur.

The liquid level will not go below the top of the packing if you don't push it. The pressure you used in Step 13 helps to pack the silica gel tightly, and this pressure reduces the void volume considerably. Reducing the void volume increases the efficiency of the column.

- Why?

14. Tap the straw at the top of the packing and rotate the straw so that the top of the silica gel stays flat.

15. Add 2-3 cm of water to the column and tap to flatten.

16. Place a punched circle of no. 3 paper (obtain from Reagent Central) into the top of the column. Push the circle down the column with a slim straw.

NOTE: As it enters the water, the air bubble will disappear.

17. Push the paper until it is flat against the silica gel packing. Gently smooth with the slim straw while rotating the column.

18. Fill the column with water from your wash bottle and push water through with the syringe pump as in Step 13. Do not push water below the paper circle.

19. Allow the column to drain naturally, and the liquid level will stay at the level of the paper.

You have just carried out a sophisticated slurry-packing procedure for the preparation of a liquid chromatography (LC) column of good efficiency. Note that the procedure appears to be a little difficult the first time, but once you are familiar with the technique, it takes about 2 minutes to pack a column. If you do make a mistake while making or running any of the columns, it's OK. Simply pump the mobile phase and any sample off the column, pull off the tip, wash the silica gel out, and repack the column.

Section E. Investigations of Column Parameters and Processes

Goal:
To examine the chromatographic characteristics of the LC column prepared in Section D.

Discussion:
You will use an unretained dye component to quantitatively measure band spreading as a function of band migration distance, and you will also measure the void volume of the column.

Experimental Steps:
1. Obtain 3 drops of 0.05 %wt bromocresol purple solution in well A-1 of your tray.

2. Cut off the top 2 cm of the column with scissors so that the thin-stem pipet will reach the paper circle.
3. Mark a point (with a fine permanent marker) close to the top of the straw. Call this point 1.

4. With a thin-stem pipet, suck up a small volume of bromocresol purple dye, following the techniques shown below. Very carefully, keeping the pipet vertical, lower the tip of the pipet until it just touches the paper circle in the middle.

5. Very, very gently squeeze the pipet bulb until the dye is just soaked into the paper and not on the packing. Remove the pipet. Have a ruler ready.

6. Quickly fill the column with water to point 1 (use a wash bottle), and the chromatography will start.

7. Rotate the column 90° so that it is horizontal. The flow will stop (the water will stay in the column).
   - Measure the width of the band of color (W_b in mm) and measure the band migration distance — i.e., the distance from the paper circle to the middle of the band of color. Estimate both lengths to a mm.

8. Turn the column until it is vertical again, and the flow of mobile phase will resume.

9. Stop the flow by 90° rotation about every cm or so of band migration.
   - Measure and record the width of the band of color and the band migration distance.
10. When the band of color begins to enter the column tip, try to estimate the point in time when 1/2 of the band has come off the column and 1/2 of the band is still on. At this time make a mark on the straw at the water level. Call this point 2. Also estimate the width of the band of color as it leaves the column.

The chromatographic progress of an unretained component through the column is shown in the following:

- Measure and record the distance d — i.e., the distance between point 1 and point 2 (in cm, estimate to a mm).
- Measure the length L of the actual column packing.

**CALCULATIONS ON BAND SPREADING**

- Plot a graph of band width \( W_b \) (vertical axis), versus band migration distance (horizontal axis).
- Compare your graph with the graph shown in Figure 18.7 which shows some typical data for the same type of experiment.

These plots show several significant trends. The *increase* in band spreading \( W_b \) gets smaller as the particle size of the silica gel decreases. Columns packed with very small particles are generally considered to be very efficient chromatographic systems.
CALCULATION OF VOID VOLUME ($V_0$), THE NUMBER OF THEORETICAL PLATES ($N$), AND THE HEIGHT EQUIVALENT TO A THEORETICAL PLATE ($H$).

$V_0$, $N$, and $H$ are calculated from the experimental data obtained in Step 10 and the following discussion. The void volume can be calculated by assuming that the straw column is a cylinder of constant radius 0.3 cm and therefore

$$V_0 = d \times \pi \times (0.3)^2$$

• Calculate and report the void volume (in mL) for your column.

The number of theoretical plates $N$ is given by

$$N = 16 \left( \frac{V_0}{W_b} \right)^2$$
where \( V_b \) is the void volume and \( W_b \) is the width of the band of color as it leaves the column. \( V_b \) and \( W_b \) must be in the same units (i.e., mL), and, therefore, you need to calculate \( W_b \) in mL using the formula for the volume of a cylinder:

\[
W_b \text{ (in mL)} = V_b \text{ (in cm)} \times \pi \times (0.3)^2
\]

Calculate \( N \). You can now calculate the height equivalent to a theoretical plate \( H \), which is one of the most important efficiency factors in a chromatographic system:

\[
H = \frac{L}{N}
\]

where \( L \) is the length (in mm) of the silica gel column packing.

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Section F. Derivatization of the Silica Gel Stationary Phase

**Goal:**
To carry out a derivatization of the silica gel in which the pH of the surface of the gel is changed from 6.8 to 4.5.

**Discussion:**
The derivatization is achieved by allowing a dilute acetic acid solution to flow through the column.

**Experimental Steps:**
1. Use your column from Section E. Use a 24-well tray to obtain 0.1 M CH\(_3\)COOH from Reagent Central.
2. Transfer the 0.1 M CH\(_3\)COOH to the column with a thin-stem pipet. Make sure you have at least 5 cm of CH\(_3\)COOH in the column.
3. Allow the CH\(_3\)COOH to flow under gravity until all the solution has gone through.
4. Pump 2 column lengths of water through the column to remove excess CH\(_3\)COOH. Do not pump the water level below the paper circle.

The surface of the silica gel has now been protonated, and the surface pH is about 4.5.

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Section G. Chromatography of Selected Synthetic Dyes

**Goal:**
To chromatographically separate a synthetic dye mixture into individual components using a derivatized silica gel column packing.

**Experimental Steps:**
1. Use good transfer technique and a thin-stem pipet to apply a small volume of the synthetic dye mixture to the paper circle on the column (as shown in the diagram in Section E, Step 4). Do not overload the column.
2. Clean the thin-stem pipet and use it to add a 30% v/v ethanol/water solution (the mobile phase) to the column (point 1). The chromatography will proceed.
3. Watch the chromatographic process.
- Draw a picture of the separation.

4. As the middle of each band of color comes off the column, make a mark on the straw at the mobile phase level.

5. Collect the effluent from each band of color in a separate well of a 1 x 12 well strip. This will allow you to collect pure fractions of each dye that may be used for spectroscopic examination.

6. Calculate the retention volume $V_R$ for each of the dye components. The calculation of $V_R$ may be carried out in the same manner as void volume in Section E.

**Section H. LC of Beet Pigments**

**Goal:** To obtain a separation of the two major betacyanin pigments in natural beet (Beta Vulgaris) juice.

**Experimental Steps:**

1. Use the LC column that you used for the separation of the synthetic dye mixture. You can clean the column by pushing 3 column volumes of water through the column. Be careful not to push the liquid level below the paper.

2. Obtain a beet extract. This may be obtained easily by squeezing a small section of a fresh red beet or by using the juice from canned beets.

3. Carry out an LC separation on the juice and present samples of the 2 major pigments to your instructor.