Thin Layer Chromatography and Column Chromatography: Separation of Pigments

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Introduction

Recrystallization and distillation are very effective and very useful techniques for the purification of organic compounds. Nevertheless, these methods have limitations, particularly when complex mixtures of material are encountered, and especially when the desired material is a minor component of the mixture. Only in very fortunate circumstances is it possible to find a solvent which will crystallize the desired compound while keeping all of the others in solution. Likewise, fractional distillation of many-component mixtures is very difficult to carry out efficiently - as the number of compounds in the mixture increases, so does the chance that one or more will have a boiling temperature similar to that of the desired material. Moreover, distillation is not suitable for separation of very small amounts of material, or for compounds which are not volatile, as is frequently the case for chemicals present in biological systems. Fortunately, it is just for these situations that the various techniques of *chromatography* are especially useful. The term chromatography refers to a number of very powerful separation methods, where the components of a mixture are separated on the basis of differences in their distribution coefficients between two phases, just as for extraction. With chromatography, however, one of the phases is moving (the *mobile phase*) relative to the other (the *stationary phase*). This allows for separation of the components, as described below. Different types of chromatography are defined in terms of the nature of the mobile and/or stationary phases. Thus, liquid chromatography (**LC**) refers to a system where the mobile phase is a liquid, and gas chromatography (**GC**) involves a gaseous mobile phase. In this laboratory, you will have the opportunity to experiment with two types of liquid chromatography, column chromatography, where the stationary phase is packed into a glass column, and thin layer chromatography (**TLC**), in which the stationary phase is coated on a flat surface, a plate of glass or plastic.

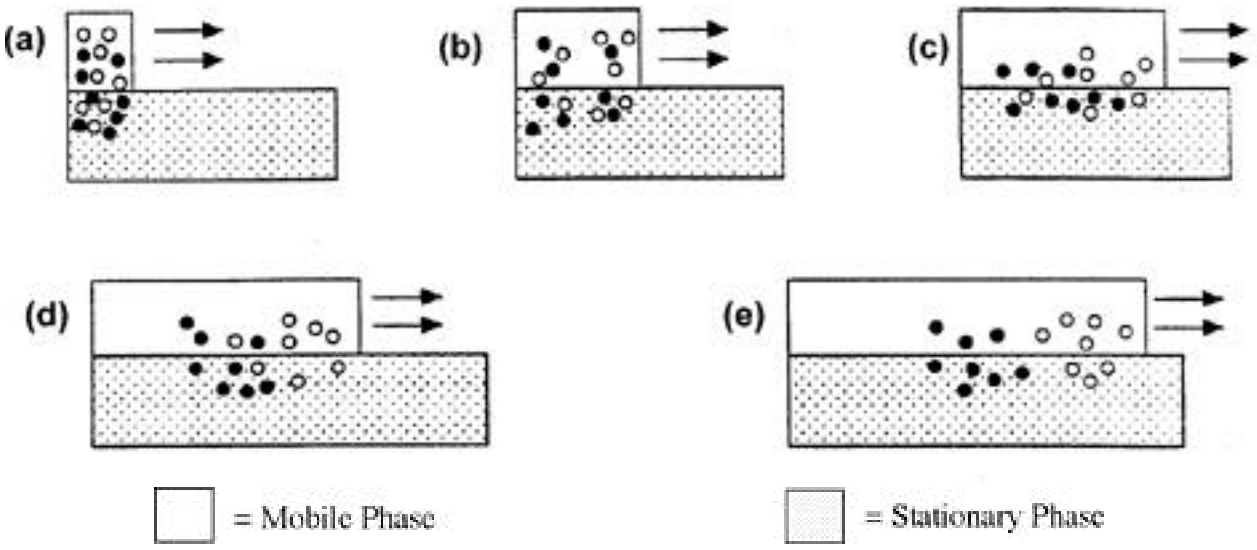


Figure 1. An illustration of chromatographic separation process. (a) A mixture of two components. Filled circles have lower K than the open circles. (b)-(e) Different rates of elution ultimately lead to separation.

How does a chromatographic separation work? As indicated above, the method depends on the partitioning of the components of the mixture between two immiscible phases. In this way, chromatography resembles liquid-liquid extraction.

We can define a partition coefficient as follows:

$$K_A = A_{\text{mobile phase}} / A_{\text{stationary phase}}$$

where **A** represents the amount of component **A** in the respective phases. The volumes of the phases have been left out in this expression -- we will assume that they are, constant and incorporated in K. What does K_A tell us? The partition coefficient is the ratio of the amount of **A** in the mobile phase versus the stationary phase at equilibrium. Essentially, a given molecule of **A** will move back and forth between the two phases, and so K_A represents the ratio of time, on average, that a molecule of **A** spends in the respective phases. If $K_A = 1$, a molecule of **A** will be in the mobile phase half of the time, and in the stationary phase half of the time. Larger K values indicate a higher fraction of time spent in the mobile phase, and components with smaller partition coefficients spend most of the time on the stationary phase. The main point to be made is that the partition coefficients for each of the components of the mixture will be different. Since the mobile phase is moving, and the stationary phase is not, components with higher K will move farther in a given amount of time than those with smaller K (the compounds only move during the time they are in the mobile phase). The faster moving component then becomes physically separated from the slower one. This is illustrated in Figure 1. The stippled block represents the stationary phase, and the unshaded block on top is the mobile phase, moving from left to right. The sample is applied at the left end of the system, and the individual components partition between the phases. The mobile phase is continually pushed across the stationary phase by addition of fresh mobile phase to the left-hand end of the system. As the material in the mobile phase moves along, it is exposed to new stationary phase, and a new equilibrium is established. Meanwhile, the material originally left on the stationary phase is now exposed to fresh mobile phase, and is also redistributed according to the partition coefficients. In Figure 1, the open circles represent a component of the mixture which has a higher K than the component represented by the filled circles. Initially the mixture is applied to the left-hand end of the chromatography system. As the mobile phase is pushed across the stationary phase, the components of the mixture are carried with it (a process called ***elution***). Since, however, the open circles spend a greater fraction of their time in the mobile phase than do the filled circles, they move faster, and will reach the right-hand end of the system sooner than the filled circles. The stages of the separation are represented pictorially in Figure 1. We can then collect the components as they elute separately from the right-hand end of the chromatography system.

The speed at which a compound will be eluted from beginning to end of the chromatography system will depend on its partition coefficient, which is in turn dependent on the relative affinity of the compound for the mobile phase versus the stationary phase. No compound can move faster than the mobile phase, even if the compound resides only in the mobile phase and never in the stationary phase ($K = \infty$), it will move only as fast as the mobile phase. Conversely, a compound which resides wholly in the stationary phase ($K = 0$) will not be eluted at all, not a good situation if this is a material you hope to recover! In fact, the fraction of time a compound spends, in the mobile phase is given by the ratio $K/(K + 1)$, and its elution rate is equal to this ratio multiplied by the velocity of the mobile phase. Of course, the total time required for a compound to elute through the system also depends on the length of the system. The time it takes for a compound to be eluted from start to end is called the ***retention time***, and is often symbolized by T_R. For a given chromatographic system, different compounds will each have a characteristic retention time. The retention time will be the same whether elution is begun with the pure compound, or whether that compound is a component of a complex mixture. In fact, if a component of an unknown mixture can be shown to have the same retention time as a known material on the same chromatographic system, this is often taken as strong evidence for the identity of the unknown.

Solid-Liquid Chromatography

The preceding discussion holds for all kinds of chromatography. Compounds are separated on the basis of their mobility through a chromatography system, and this can be related to the partitioning of components between a mobile phase and a stationary phase. Different types of chromatography are distinguished by the nature of the mobile and stationary phases. For now, we will focus on liquid chromatography techniques, in which the mobile phase is a liquid, usually an organic solvent or water, and the stationary phase is a solid absorbent. The partition coefficients which are so important in determining the mobility of a compound in a chromatography system will obviously depend on the compound, and also on the particular mobile and stationary phase used, so let us consider the types of phases used in liquid chromatography.

The most common stationary phases used in organic chemistry are silica gel and alumina. Both of these solids have a structure consisting of a three dimensional network of atoms (silicon or aluminum, respectively) alternating with oxygen atoms. Obviously, this arrangement must end at the outer surfaces of these materials, and the surface structure of both silica gel and alumina has a large number of polar oxygen and hydroxyl groups, as indicated in Fig. 2.

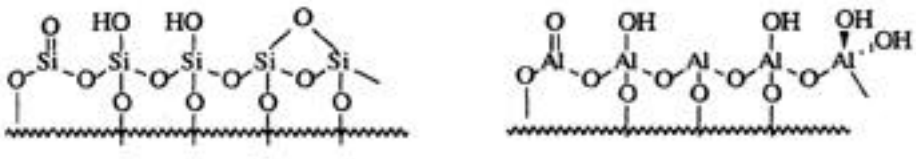


Figure 2. Stylized structure of the surface of silica gel (left) and alumina (right).

The surfaces of these materials are quite polar, capable of absorbing organic molecules by dipole attraction, hydrogen bonding, Lewis acid-base interactions, etc. It is this absorption on the surface of the particles which is involved when a molecule goes to the stationary phase. To maximize the available binding area, small particles, typically < 0. 10 mm in diameter, with a very high surface area, are used. Both silica gel and alumina are available in a variety of 'activities' (roughly speaking, polarities), and can be manufactured to tightly controlled specifications. The characteristics of each are similar, and while silica gel is used more often as a general purpose stationary phase for chromatography than is alumina, for some separations alumina is superior.

As a general rule, more polar compounds will have a higher affinity for the stationary phase than will non-polar materials. For the latter, such as alkanes, the absorption on the stationary phase is due mainly to weak attractions such as van der Waals forces. Alkenes are bound somewhat more strongly than are alkanes, because the polar bonds on the surface of the stationary phase can cause the _-electron density of the alkene to be distorted, creating a temporary dipole which is in turn attracted to the polar stationary phase. Molecules such as ethers and carbonyl compounds which already have polar bonds are even more strongly bound by dipole-dipole forces. Finally, alcohols and carboxylic acids, which can form hydrogen bonds to the surface groups on silica gel or alumina, have a correspondingly higher affinity for the stationary phase. Of course, the stronger the affinity for the stationary phase, the longer it will take for the compound to elute. A general order based on some common functional groups is given below**.**

If a polar stationary phase like silica gel or alumina is used, the mobile phase should be relatively non-polar to maximize the difference between the phases. Normally, an organic solvent is used as the mobile phase. The solvent of choice depends on the mixture of compounds to be separated, and may range in polarity from alkanes (for example, hexane) to alcohols (methanol) and anywhere in between. Since the selection of stationary phases is rather limited, it is the choice of mobile phase solvent which provides most of the flexibility in liquid chromatography. The goal is to select a mobile phase which will give partition coefficients for the components to be separated which are neither very large nor very small. As we have seen, if the partition coefficients are small, the compounds will spend nearly all of the time absorbed on the stationary phase, and will not elute.

On the other hand, if the partition coefficients are quite large, the components of the mixture will all stay in the mobile phase throughout, and no separation will occur. The best separation occurs if the solvent is chosen so that the compounds are more evenly distributed between the mobile and stationary phases.

As a general rule, for a compound **A**, the more polar the mobile phase, the larger the distribution coefficient K_A becomes. This is partly because the polar mobile phase can provide the same kind of attractive forces as the stationary phase, and partly because the mobile phase will compete successfully with molecules of **A** for the available binding sites on the stationary phase. Thus displaced, **A** has no place to go but the mobile phase. Therefore, an increase in the mobile phase polarity will increase the rate of elution for all compounds in the sample. Therefore, if the compounds elute too slowly, the remedy is to increase the polarity of the mobile phase. On the other hand, if the mixture doesn't separate well because everything runs as fast as the mobile phase, a switch to a less polar solvent is indicated. Often times, just the right polarity can be achieved by mixing a small amount of a polar solvent with a non-polar one. For example, petroleum ether (which is not an ether at all, but is instead a mixture of pentane and hexane isomers), is not polar enough for the separations we will need to do in this week's experiment. We will use a mixture of 20% toluene in pet ether, or 2% ethyl acetate in pet ether, to make a mobile phase with the proper polarity. Of course, the 'correct' polarity of mobile phase depends on the compounds to be separated - separation of a mixture of alcohols will require a more polar mobile phase than a separation of alkenes and alkanes. The optimum mobile phase is determined by trial and error, usually using thin-layer chromatography, as discussed below.

There are other choices of stationary phases for more specific applications. Cellulose and even sugar are occasionally useful. A variety of special stationary phases have been developed for the purification of large biomolecules such as proteins and DNA. The specifics of these are beyond the scope of this discussion, although many of the same principles apply. Of more general interest to organic chemists are the so-called reverse-phase chromatography systems. As the name implies, these involve the use of a non-polar stationary phase, in conjunction with a polar mobile phase, often water. For reverse-phase systems, it is the non-polar materials which elute most slowly, and a decrease in polarity of the mobile phase increases the rate of elution, all directly the opposite of the conventional silica gel or alumina systems. Reverse-phase systems are not used as widely as is conventional ('normal-phase') chromatography, but offers a new level of selectivity which can be very useful for separations which are difficult on silica gel.

Thin Layer Chromatography (TLC)

A variety of different liquid chromatography techniques are available. Probably the simplest and fastest is thin layer chromatography (TLC). In this method, the stationary phase is coated as a thin layer on a glass or plastic plate. A pencil line (not ink! solvents will cause ink to run) is drawn

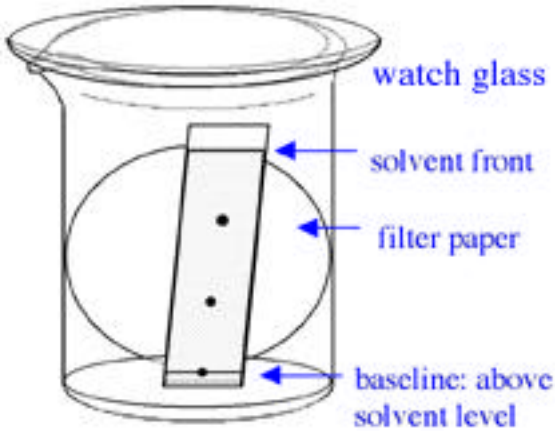


Figure 3. A beaker as a TLC developing chamber. The filter paper helps to keep the chamber saturated with solvent so the plate will no dry out.

across one end of the plate, about 5 mm from the edge. This is the ***baseline***, and marks the starting point for the mixture to be separated. The sample is applied as a small spot on this line. This is done by dipping a thin glass capillary into a solution of the sample in a volatile solvent. A small amount of solution will be drawn into the capillary. When the end of the capillary is touched to the plate , the solution will be drawn out onto the stationary phase. The object is to keep the initial spot as small as possible. This can be accomplished by a series of quick touches of the capillary to the plate, allowing the solvent to evaporate after each application. After the sample has been applied, the plate is placed in a ***developing chamber***, which contains the solvent to be used as the mobile phase.

The developing chamber can be as simple as a covered beaker (see Figure 3), and the depth of the solvent is kept below the level of the baseline. Since it is important that the developing chamber be saturated with solvent vapor, a piece of filter paper is placed along the inner wall of the beaker - this serves as a wick to ensure solvent saturation. The plate is set in the chamber on the baseline edge. The solvent will be drawn up through the stationary phase by capillary action, and the ***solvent front*** can be seen to move up the plate. As the solvent passes the spot where the sample was applied, the sample will begin partitioning between stationary and mobile phases, and separation will occur (see Figure 4). Note that each component appears as a separate spot. Each spot moves proportionately to the distance traveled by the solvent front - if the solvent is allowed to rise twice as far up the plate, the spots will all move twice as far up the plate. Since the separation increases as well, the plate should be left in the chamber until the solvent front reaches within a few mm of the top of the plate, at which time it is removed from the chamber and allowed to dry. The ratio of the distance traveled by a particular spot, compared to the distance traveled by the solvent front (both measured from the baseline) is called the R_F value. An R_F of 1.0 indicates that the component spends all of its time in the mobile phase ($K = \infty$), while a spot that remains at the baseline ($K = 0$) has an $R_F = 0$. In fact, it can be easily seen that $R_F = K/(K + 1)$, and corresponds to the fraction of time the component spends in the mobile phase. Like the retention time, the R_F is characteristic of a compound under a defined set of stationary and mobile phases, and can be used to help identify unknowns.

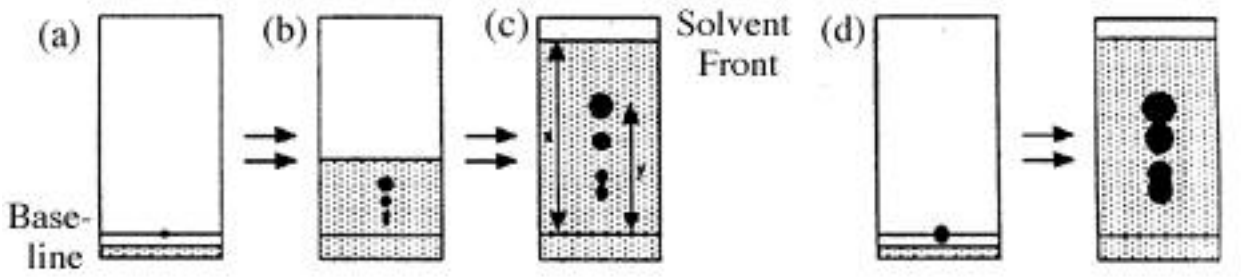


Figure 4. TLC separation. (a) before development; (b), (c) the components move proportionately to the solvent front. The R_F of the faster moving spot is given by y/x. (d) See what happens when your spots are to big?!

The main advantage of TLC is its simplicity. Development of each plate takes only a few minutes, and so it is possible to screen several mobile phases to determine the optimum solvent system for a separation. Usually, the solvent polarity is adjusted so that the compounds of interest have R_F's in the range 0.2 - 0.6; this provides the best separation of components. TLC is often used to monitor the progress of a reaction - as the reaction proceeds, the spot due to the starting material will diminish, and that of the product will dominate. Side products or impurities which form will likewise be evident as additional spots.

For TLC, a critical factor in determining how completely two components are separated on the plate is the size of the original sample spot. In fact, the component spots increase in size somewhat during the chromatography, since the components can diffuse in all directions when in the mobile 1 phase. If the R_F's for two components do not differ by very much, as is often the case,

the centers' of the corresponding spots will be separated by only a small distance on the plate. If the spots are small, clear separation may be observed, but if the original spot is too large (Figure 4d), the components will be run together even after the chromatography. It is very important to take care during sample application to keep the sample spot small.

In this experiment, we will be separating pigments which are colored, and so the spots will be visible to the eye. Many organic compounds, of course, are colorless, and the spots on the TLC plate must be visualized differently. One method is to place the developed plate in a jar containing a few crystals of iodine. Iodine vapor has a very high solubility in organic material, and tends to concentrate in those areas of the plate where the organic compounds are, and the spots turn a brown color. Other staining agents, which react with organic materials, work similarly. Some compound are colorless but will fluoresce and can be visualized under an ultraviolet (UV) lamp. A different technique involves the use of TLC plates which have a fluorescent material bound to the stationary phase. Many organic materials will prevent (quench) the fluorescence. When observed under an ultraviolet lamp, the plate glows, except for dark spots which mark the location of the organic components.

The amount of material which can be separated by TLC is quite small, typically 10^{-6} g or less, and this technique is normally used for analysis of mixtures, but not for purification. Although it is possible to use TLC on larger scale, preparative-scale purifications are more typically accomplished by column chromatography. Even then, chromatography is limited to the separation of relatively small quantities of material - most laboratories are not equipped to handle more than a few grams.

Column Chromatography

In column chromatography, the stationary phase is packed into a vertical tube. The sample is applied to the top of the column and the mobile phase is allowed to percolate down through the stationary phase. The various components are separated as they move down the column, and are collected individually as they are eluted from the bottom of the column. Normally, the column will have a stopcock at the bottom so that the flow of mobile phase can be controlled.

It is very important that the stationary phase be packed evenly and efficiently for column chromatography. Air spaces or gaps in the packing will cause the elution of different parts of the sample to proceed at different rates, and this will adversely affect the separation of components. The column is normally a glass tube with a stopcock at one end. A small wad of glass wool is stuffed into the bottom end of the column, to serve as a support for the stationary phase. A relatively simple way to pack a column efficiently is the slurry method. The stationary phase is mixed with enough of the mobile phase to form a thin slurry, which is then poured into the column. The stationary phase settles evenly, and air bubbles are eliminated. At this point, a very small amount of clean sand is dusted through the solvent, and allowed to settle on top of the stationary phase. The layer of sand should be < 5mm deep. It's purpose is to help protect the top of the column from getting stirred up when the sample is applied (see next paragraph). The packing is completed by drawing the excess mobile phase through the column, until the solvent level is just even with the top of the sand layer. The flow of solvent through the column helps to compress the packing. A modification of the slurry method is to fill the column about half full of mobile phase, and slowly add the dry stationary phase. The packing will sink through the solvent - as it settles, it packs efficiently. The sand layer is then added, and packing continued as above.

Application of the sample to the top of the column must be done with great care. The idea is to place the mixture as a thin, even band at the top of the column. The profile of the sample at the start is maintained during elution of the column. If the sample is applied carelessly so that it appears as a jagged zig-zag, it will not be possible to separate closely eluting components (see Figure 5); the problem is exactly analogous to making the sample spot too large in TLC. Likewise, if too polar a solvent is used to apply the sample, it will spread down the column, making a very wide band, again hurting the separation efficiency. The proper way to apply the sample is as

follows: With the mobile phase even with the top of the sand layer, the sample, dissolved in a minimum amount of solvent (the mobile phase or, better, a less polar solvent can be used), is slowly and carefully run down the wall of the column with a dropper, so as not to disturb the top of the column. The sample solution will collect as a layer above the sand. Enough mobile phase is then

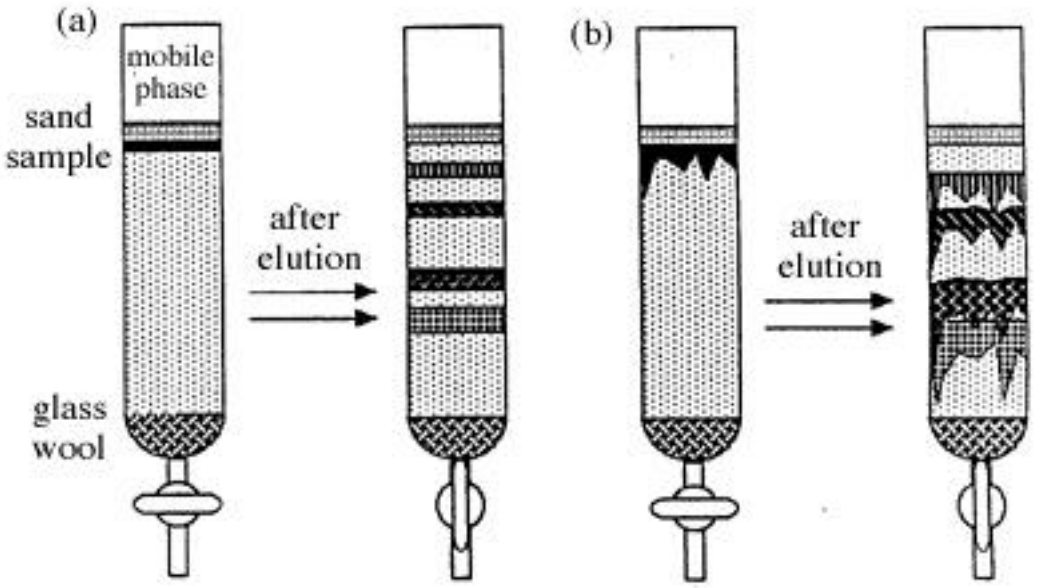


Figure 5. The effect of initial sample band shape on chromatographic resolution. (a) Good sample band shape leads to good separation of components. (b) Careless application of the sample will cause erosion of the column.

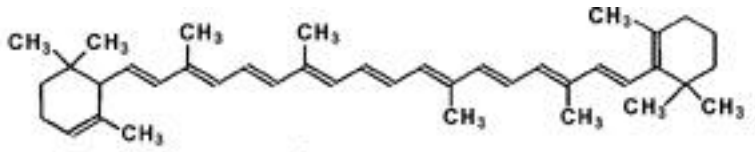
run out of the bottom of the column to bring the liquid back to the level of the sand. The walls of the column can then be rinsed with a small amount of fresh solvent, and enough mobile phase run out so that this liquid is drawn to the top of the column. This process can be repeated once more. By this time, all of the sample should collect as a thin, even band absorbed near the top of the stationary phase. At this point, fresh mobile phase is run down the walls of the column, once again with care to prevent stirring up the top of the packing. Enough of the mobile phase is added to fill the column, and the rate of elution is controlled by adjusting the flow through the stopcock. Once the column is packed, it is absolutely critical to avoid letting the top of the column dry out. If the level of solvent drops below the top of the column, air pockets and cracks will develop in the packing. This will lead to uneven elution. Therefore, during the sample application procedure it is essential that the solvent level is carefully controlled just to the top of the packing. During elution, the level of mobile phase should always be kept at least 2 inches above the top of the packing, and may need to be replenished one or more times.

The separation efficiency is also effected by the elution rate. The walls of the column provide significant drag on the flow of the solvent , and at rapid flow rates, the velocity of the mobile phase is substantially higher than near the walls of the column. This will distort the band shape of the sample from a disk to a conical profile, as the material in the center of the column moves faster than that near the edge. Of course, this is to be avoided, since it will degrade the separation ability of the column. The problem is most severe with small columns such as those we will be using, and it is particularly important to control the elution rate between 0.5 and 1 ml/min. This is no place to rush - your chromatography will suffer from your haste. With proper care, you will be rewarded with a nice separation of bands, corresponding to the different components of the mixture, as the elution proceeds down the column. Each band can be collected as it elutes from the end of the column in a separate test tube and voilà, you have separated the mixture! When the compounds are

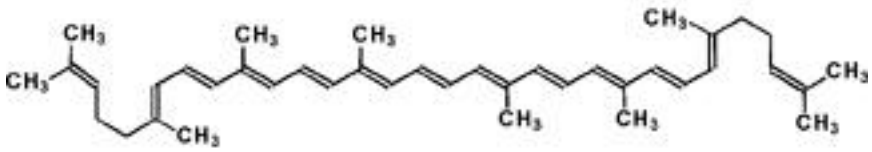
visible, as in this experiment, your eye will serve as a detector to determine when a compound is coming off the column. For colorless organic materials, small fractions are collected blindly - these are then analyzed separately, often by TLC, to determine which fractions contain which components.

Pigments in Tomatoes

The red color of tomatoes is due to the presence of a large number of colored compounds called pigments. The pigment of red tomato is a very complex mixture of many different kinds of structures, most of which are present in very small amount . In this experiment, we will be interested in two of these, β-carotene (λ_{max} = 451 nm) and lycopene (λ_{max} = 475 nm):



β-Carotene ($C_{40}H_{56}$)



Lycopene ($C_{40}H_{56}$)

These compounds absorb visible light due to the extended conjugated pi-system. β-Carotene, which is also found as a pigment in carrots, is yellow, while lycopene is a more orangish, almost golden color. β-Carotene is slightly less polar than lycopene, and so will elute faster. Both of these compounds are present in very small amounts in tomatoes, but because their color is so intense, a little bit makes a lot of color.

We will isolate these pigments from tomato paste. The crude pigment mixture must first be extracted from the tomato paste, and is then separated and purified by chromatography. Most of the water can be removed from the tomato by extraction with ethanol, and then the pigments can be extracted from the insoluble solids using petroleum ether (bp 35-60 °C), a mixture of low boiling alkanes (mostly pentanes and hexanes). This solution of pigments is then concentrated by evaporation, and subjected to chromatography.

The amount of material which is isolated is much too small for us to determine accurately by weighing. Instead, we will determine the quantity of purified lycopene by measuring its absorbance using a spectrophotometer. The absorbance of the lycopene solution will depend on the concentration of lycopene present according to the Lambert-Beer relation: $\mathbf{A} = \varepsilon \boldsymbol{l}\mathbf{c}$, where **A** is the measured absorbance (at 475 nm), ε is the molar absorptivity (=1.85 x 10^5 $M^{-1}cm^{-1}$ for lycopene at 475 nm), ***l*** is the cell path length in cm (we will use 1 cm cells), and **c** is the concentration of lycopene. The concentration of lycopene can therefore be determined from the absorbance, and, knowing the volume of the lycopene fraction, you will be able to calculate the number of moles and the number of grams of purified lycopene obtained.

Chances are, the lycopene solution directly from the column will be too concentrated to get an accurate absorbance reading. An absorbance of 2.0 means that only 1% of the light at that wavelength is transmitted, and this is too little to be accurately determined. Measurement on a

solution with absorbance of 0.3 - 0.7 is much more reliable. How, then, can we get a solution with this absorbance? The answer, of course, is to dilute the mixture with solvent until the absorbance is in this range. Of course, you must keep track of the dilutions you make, so that you can back-calculate the concentration of the original solution from that after dilution. Now, it is not necessary (and is, in fact, wasteful) to dilute the entire sample. If you decide that you need to dilute 10-fold, take 1.0 ml of the solution and dilute to 10.0 ml with fresh solvent. If you need to dilute 100-fold, do not take 1.0 ml and dilute to 100 ml. Instead, do two successive 10-fold dilutions - you will end up with the same dilution, but will consume only 18 ml of solvent instead of 99 ml. In addition, you will have the chance to check the solution after the first 10-fold dilution, in case that is sufficient.

This part of the experiment seems to cause the most confusion. If the solution you made the absorption measurement was derived by a 10-fold dilution, then the original solution was ten times as concentrated. If the measured solution was the result of successive 10-fold and 5-fold dilutions, then the original solution must be 10 x 5 = 50 times as concentrated. From the volume of the original eluent (before removing some to dilute!), the total amount of lycopene can be determined.

The amount of dilution required for a reading of 0.3 - 0.7 will vary for each of you. Take a reading on the original solution. From the (approximate) absorbance value, you can judge the next action. If you are well above 2, try a 10-fold dilution. We will use the small graduated cylinder for this purpose: place 1.0 ml of the solution in the graduated cylinder, and carefully add solvent to the 10.0 ml mark (or 5.0 ml for a 5-fold dilution). Note carefully that this is not the same as adding 10 ml of solvent to 1.0 ml of sample, and is not strictly equivalent to mixing 1.0 ml of sample with 9 ml solvent.

Suppose, then, that 4.6 ml of eluent containing lycopene is obtained. When 1.0 ml of this solution is diluted to 10.0 ml, and the new solution has absorbance at 475 nm of ≈ 3 (too high). If 1.0 ml of this solution is diluted to 10.0 ml, an absorbance reading of 0.28 is obtained (below the recommended range, but close enough). How much lycopene was obtained? First, we determine the concentration of the diluted solution:

$$c = A/(\varepsilon l) = (0.28) / (1.85 \cdot 10^{5}\ M^{-1}\ cm^{-1} \cdot 1.0\ cm) = 1.51 \cdot 10^{-6}\ M$$

The concentration of the original solution (symbolized c') is therefore one hundred times this value (two successive 10-fold dilutions = 10 x 10 = 100-fold dilution), and the amount of lycopene is given by:

$$\text{moles of lycopene} = V_{lyc} \times c' = 4.6 \cdot 10^{-3}\ l \cdot 1.51 \cdot 10^{-4}\ M = 6.95 \cdot 10^{-7}\ \text{mole}$$

$$\text{mass of lycopene} = 6.95 \times 10^{-7}\ \text{mole} \cdot 536.90\ \text{g/mole} = 0.37\ \text{mg}$$

The mass of lycopene can then be expressed as a weight percent of the original mass of tomato paste used. It will be a small number!

Experiment 4

The crude pigment extract from tomato paste will be analyzed by thin layer chromatography, and the lycopene and β-carotene will be purified by column chromatography. The amount of lycopene present will be determined spectrophotometrically.

Safety: Methylene chloride, ethyl acetate, toluene, petroleum ether, all of these chemicals (solvents) are extremely flammable! No flames will be permitted after the first 10 minutes of lab. All solvents, etc. must be discarded in the containers provided.

At the beginning of lab, flames will be permitted so that you may prepare capillaries for spotting TLC plates. These are made from melting point tubes. The tube is held at each end and rotated rapidly in a burner flame. When the glass has softened, the tube is removed from the flame and smoothly stretched to a fine capillary. One pull should give you enough for two or three spotters, enough for this laboratory.

Now, TURN OFF THE FLAMES.

Weigh about 3 g of tomato paste (this is approximately 3/4 tsp, anything less than 5 g is OK) in a small beaker. Add 10 ml of 95% ethanol, and mix thoroughly with a spatula. The alcohol will carry away water and some soluble salts; very little of the pigment dissolves. The liquid is decanted from the beaker, and the paste is squeezed against the side of the beaker to remove all of the ethanol. The ethanol can be discarded down the drain - after all, we eat this stuff. The dehydrated paste is transferred to a clean beaker, and treated with 10 ml of methylene chloride. Again, this is thoroughly mixed with a spatula to dissolve as much of the pigment as possible (Some of the red pigments are different materials, and will not dissolve). The reddish-orange liquid is then filtered through a conical funnel with filter paper into a 6 in. test tube**.** The residual paste is squeezed with a spatula to release as much of the pigment solution as possible. Add one boiling chip to the test tube, and in the hood, warm the tube gently in a boiling water bath (prepared in a beaker). Heat only to a gentle boil - you should be able to easily hold the end of the tube with your fingers. Too-rapid heating will cause your hard-earned pigment mixture to bump out of the tube, so be careful! Just in case, be sure to point the tube away from everyone. Boil the solution down to dryness. This should only take a minute or so. Remove from the heat and dissolve the pigment residue in 1 mL of pet ether (bp 35-60 °C).

Set up for TLC (see Figure 3). Curl a small (5.5 cm) circle of filter paper around the inside wall of a clean, dry 100 ml beaker. Add a mixture of 20% toluene : 80% pet ether (v/v) to a depth of ≈ 3 mm in the beaker, swirl to facilitate saturation of the chamber with solvent vapor, cover with a watch glass, and let stand for a few minutes. You will get two (silica gel) thin-layer chromatography plates, approximately 1" x 3". Draw a pencil line across the width of each, about 5 mm from the edge. Take a TLC spotting capillary, and dip it into the pigment concentrate. Check to see that pigment solution has been drawn into the capillary. Briefly touch the end of the capillary to one of the TLC plates on the pencil line, and blow gently on the resulting spot to evaporate the solvent. Touch again in the same spot to apply more sample. Try to keep the spot smaller than 1mm in diameter. You can try several spots along the baseline to get some practice, but keep the individual spots separated by 3-4 mm. Try to get spots with 5, 10, and 20 drops (touches) from the capillary - the heavier applications might permit more minor components to be observed. Stand the plate with the baseline edge down in the developing chamber, replace the watch glass, and watch the solvent front rise up the plate. When the solvent front has reached within a few mm of the top of the plate, remove the plate, and mark the position of the solvent front (it will dry quickly). With a pencil, circle the visible spots - they tend to fade rapidly. You should be able to see the red-orange

lycopene, perhaps the yellow β-carotene, and maybe others (baseline?). Determine the R_F values for these, make an accurate sketch of the TLC plate in your notebook, and record the R_F values.

Set up for column chromatography. This can be done ahead of time -- you may want to get this done early and avoid standing in line. Check out a chromatography column from the storeroom, and make sure that it is clean and dry. Tamp a small wad of glass wool into the column with a long glass tube. Clamp the column as close to vertical as possible. Close the stopcock, and fill the column with 20 ml of 2% ethyl acetate in pet ether. Weigh out 15 g of alumina (≈ 15 ml volume) in a beaker, and add this in small portions to the liquid in the column Add slowly to avoid clumping of the stationary phase at the top of the column. After the solid has settled, gently shake the column to wash down alumina which has stuck to the walls of the tube. Add a very small amount of sand to the column, so that an even layer of 2 - 5 mm of sand settles on top of the alumina. If you are preparing the column early, stop at this point until your pigment concentrate is ready.

To continue, open the stopcock and drain the mobile phase into a clean flask. This solvent can be used for elution of the column. When the solvent drops to the level of the sand layer, close the stopcock. Take the pigment up in the Pasteur pipet (dropper) and very carefully let it run down the inner wall of the column. Drain enough mobile phase from the column so that the solvent layer is once again even with the sand. Rinse the walls of the column twice with 1 ml portions of pet ether, each time drawing the appropriate amount of mobile phase out of the bottom of the column. At this point, all of the sample should be absorbed as a relatively narrow band on the top of the alumina column. Very carefully rinse small portions of the mobile phase (2% ethyl acetate in pet ether) down the wall of the column, until the column is filled near the top. Begin the flow of solvent by opening the stopcock to maintain a flow of 0.5 - 1.0 ml/min (you can watch how rapidly the solvent level drops). Look carefully for the β-carotene fraction. The yellow band is very difficult to see on the column, but you will be able to see the yellow solution dripping out the end of the column. Collect the δ-carotene fraction in a clean dry test tube. The lycopene fraction should be visible as a red-orange band which moves down the column. This should be collected in a clean test tube as it comes off the column. Measure the volume of the lycopene-containing fraction. Determine the concentration of lycopene spectrophotometrically, after appropriate dilution. Your TA will demonstrate the operation of the spectrometers. Calculate the amount (moles, mg) of lycopene isolated from the sample, and determine the weight per cent of lycopene obtained from the sample of tomato paste.

Finally, run a TLC of the purified β-carotene and lycopene samples. These can be done simultaneously on the same TLC plate. Simply spot the lycopene sample on one side of the plate, and the β-carotene sample on the other. You may also want to 'co-spot' both pigments on the center of the baseline, to see how the mixture would separate. Check to see that you still have TLC solvent left, and develop the plate. How does the TLC of the individual pigments compare with the mixture before column chromatography? Determine the R_F's of the lycopene and the carotene, if you can see it.

Clean-up: When finished, the extra mobile phase should be drained from the column. All of the used solvents, pigment solutions, etc. should be poured into the solvent waste container. Remove the stopcock assembly from the column, and blow the used packing material into the solid waste box using the air hose which is set up for this purpose. The glass wool, TLC plates, and the tomato paste solids also go in this box.

Post-laboratory Questions

1. What is the relationship between R_F in TLC and T_R in column chromatography?

2. What would be the effect of using 20% ethyl acetate in pet ether for the column chromatographic separation of the tomato pigments?

3. Compare the R_F's of the components in the crude pigment mixture with those of the isolated lycopene and ß-carotene.

4. What happens to the lycopene band as it moves down the column. Why?