Experiment 22

CHROMATOGRAPHY

Objective

The purpose of this experiment is to separate and identify mixtures using column chromatography and thin-layer chromatography (TLC).

Lab techniques

- Extraction
- Column chromatography
- Thin layer chromatography

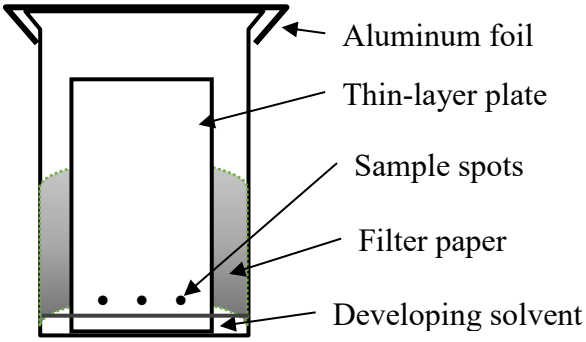
Introduction

Apart from distillation, extraction and recrystallization, chromatography is also a highly effective separation method for mixtures. Chromatography refers to a separation method based on the different distribution of different compounds between a stationary phase and a mobile phase. The stationary phase can be a solid or any liquid adhered on the surface of an inert support, while the mobile phase can be liquid or gas.

Adsorption chromatography makes use of the different adsorption forces of the stationary phase (adsorbent) toward different compounds. By continuous elution with a mobile phase, separation can be obtained. On the other hand, partition chromatography makes use of the property of solubility. Its principle is similar to that of extraction. In conclusion, there are many methods of chromatography; however, column chromatography and thin-layer chromatography are the most useful techniques in the laboratory due to their convenience.

I. Thin-layer chromatography

Thin-layer chromatography makes use of a glass, metal or plastic plate coated with a thin layer of alumina, silica gel, or any other substance containing a binding agent (calcium sulfate) as the stationary phase. Today, TLC plates are commercially available. The TLC plates bought on the market usually have a layer with a thickness of 0.2 or 0.25 mm. The mixture for separation is spotted on the bottom of the plate with a capillary tube. A smaller spot produces a higher quality of the separation. Then the plate is placed in a developing chamber filled with a shallow pool of developing solvent (eluent). Due to capillary action, the developing solvent will climb up the plate through the sample spot. Because different compounds in the mixture have different adsorption forces towards the stationary phase and the mobile phase, they will climb up the plate at different speeds. Hence, the mixture can be separated on the plate (Figure 22-1).



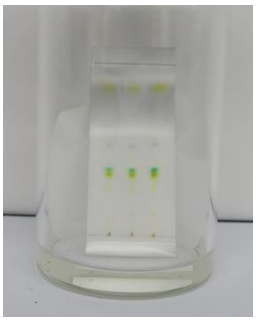


Figure 24-1 Thin-layer chromatography

(I) Retention factor

If the mixture is colored, the separated components can be identified with naked eyes. In the case of colorless compounds, physical or chemical imaging methods can be applied to identify them. By measuring the distance traveled by the compound versus the distance traveled by the developing solvent, the retention factor (R_f) can be calculated. The retention factor is a characteristic value of that compound in that particular developing solvent. This value is also dependent on the thickness of the stationary phase. Thus, it can be used for identification.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the eluent}}$$

Generally speaking, by considering the property of the components of the mixture, a suitable stationary phase and eluent can be chosen, and the best condition for separation can be found.

(II) Stationary phase and developing solvent

The stationary phase can be further divided into a normal phase system and a reverse phase system according to polarity. Silica gel is most often used in a normal phase system. The stationary phases used in a normal phase system have high polarity. A higher polarity of the compound means a stronger intermolecular force between it and the stationary phase. Thus, the distance traveled by the compound will be shorter, and the value of R_f will be smaller. In contrast, the R_f value of compounds with low polarity will be larger.

Developing solvents are those commonly used volatile solvents and have different polarities. The developing solvent with low polarity most often used in the laboratory is hexane, and that with high polarity is ethyl acetate. In a normal phase system, a solvent with high polarity will have a stronger intermolecular force with the stationary phase; hence, the compounds adhered on the stationary phase can be brought up more easily due to the subsequent replacement by the solvent. Thus, a higher polarity of the developing solvent corresponds to a higher R_f value. By tuning the polarity of the developing solvent, the traveling distance of a compound on the TLC plate can be

adjusted. This can be done by mixing hexane and ethyl acetate to obtain any desired polarity between those of the two solvents.

The stationary phases used in a reverse phase system have low polarity. Therefore, the underlying principle is the opposite of that of the normal phase system. The R_f values of compounds with lower polarity are smaller, and those of compounds with higher polarity are larger. Moreover, a lower polarity of the developing solvent allows the compounds to travel a longer distance.

In order to obtain the best separation results, both the stationary phase and the eluent should be chosen carefully. The quality of separation due to the eluent is not completely related to its polarity, as the interaction forces between the compound and eluents of different structures vary. Since it is not easy to determine the results of any single separation, a trial-and-error approach must be adopted.

(III) Spotting sample

The sample should be diluted with the solvent before spotting. In addition, spotting should be carried out with a capillary tube with diameter smaller than 1 mm. The concentration of the sample should not be too high, and spotting should not be repeated many times, as it may exceed the loading capacity of the stationary phase and cause tailing, which has a negative impact on the outcome of separation.

First, a horizontal line is drawn with a pencil on the TLC plate about 0.5~1 cm from one edge as the starting line. Then another very short vertical line is drawn crossing the starting line, and the sample is spotted on the crossing point. Spotting should be done with great care to control the spreading of the sample to within a diameter of 2 mm. Also note that fingers should not touch the surface of the plate at any time during operation to avoid contamination.

As the sample spot is climbing, diffusion will occur and cause the diameter of the spot to increase. Therefore, with a large sample spot to begin with, compounds with similar R_f values will overlap with each other while climbing and make the separation difficult to recognize. More than two spots can be loaded on one starting line; however, it should be noted that their separation from the edges and each other at the baseline should be more than 0.5 cm to prevent them from distortion and interfering with each other, respectively. Moreover, the solvent on the spots should be allowed to completely vaporize before elution starts.

(IV) Development

Development of samples should be done in a closed developing chamber. Developing solvent is added to the container up to a height of about 0.3 cm. Filter paper can be placed around the inner wall of the chamber and soaked in the solvent at the bottom and moistened. This can help to attain quick liquid-vapor equilibrium within the chamber and speed up the climbing of the developing solvent.

The TLC plate is placed in the center of the developing chamber with the side closer to the starting line soaked in the solvent and the other side leaning on the wall of the chamber. It should be noted that the bottom rim of the spots at the starting line should be higher than the surface level of the eluent to prevent the sample from dissolving in it. Then the opening of the chamber is covered. When the solvent has advanced to within 5 mm from the top end, take out the plate, and immediately draw a line to indicate where the solvent front is (You may draw a line before developing). After the solvent has vaporized, the position of the sample spots is observed by visualizing methods and the corresponding R_f values are calculated. The developing solvent should also be recorded at the same time.

(V) Visualization

After the development of the sample, if the sample is originally colored, then its position can be observed directly. However, most of the time, the samples are colorless. In this situation, suitable visualizing method should be used. Some commonly used visualizing methods are listed below:

1. Visualization using UV light: This method can be used to observe compounds that exhibit fluorescence. If a compound is not fluorescent but has the ability to absorb UV light, a TLC plate with the stationary phase impregnated with a fluorescent reagent that absorbs UV light with a wavelength of 254 nm can be used. Under such UV light illumination, the position on the plate with the compound will appear as a dark spot due to the adsorption of the UV light by the compound, while the other regions of the plate fluoresce due the activation of the fluorescent reagent. Thus, the corresponding positions of the components can be identified.
2. Using visualizing agents: The most commonly used visualizing agent is a 5% solution of phosphorous molybdic acid ($H_3PO_4 \bullet 12MoO_3$) in 95% ethanol. The TLC plate is first soaked in this solution for less than one second and removed immediately. The samples can then be colored by heating on a hot plate. Alternatively, this solution can be sprayed onto the plate and heated to allow visualization. Silver nitrate solution can be used as a visualizing agent for halogenated compounds. Sulfuric acid solution can also be used as a visualizing agent; dark spots can be observed after heating.
3. Iodine visualization: In this method, several iodine crystals are mixed with a 0.5 cm thick layer of silica gel in a jar. After a while, the iodine molecules will be evenly distributed on the surface of the silica gel. Then the TLC plate to be visualized is placed inside the closed jar. The jar is then lightly shaken for a few seconds to allow direct contact between the silica gel and the stationary phase of the plate. The plate is removed and the colored position is marked with a pencil. This visualizing method is based on the principle that some organic compounds

will form complexes with iodine and become colored. The color of the spot will fade away after the plate is removed due to sublimation of the iodine. Thus, the plate should be marked immediately upon removal of the TLC plate. **Note that this process should be carried out in a fume hood and iodine vapor should not be inhaled.**

(VI) Pros and cons

Thin-layer chromatography is quicker, cheaper, and more convenient than gas chromatography (GC) and high performance liquid chromatography (HPLC). The amount of sample required for thin-layer chromatography is extremely small. If necessary, a glass plate with a large surface area coated with a thick layer of stationary phase can also be used for preparative purpose. After development, the bands containing the separated compounds can be scraped off for extraction. However, the amount of a sample that can be separated is still limited. Therefore, column chromatography should be the first choice whenever large-scale chromatography is required.

Furthermore, only a single developing solvent can be used in thin-layer chromatography. As a result, the polarity of the developing solvent cannot change continuously throughout the process, as with gradient elution in HPLC. However, a second development can be carried out after finishing the first one, using the same plate. In this way, the quality of separation can be improved. Using the same plate, a second development can also be done by developing on another axis to attain a two-dimensional separation. Ordinary analytical thin-layer chromatography can only be used for qualitative analysis. Note that the structure of the sample cannot be identified exclusively by using this method.

(VII) Applications

Thin-layer chromatography is a very important technique for organic chemistry in many ways. It can be used for the following:

1. To estimate whether two compounds are identical. Compounds having different values of R_f must be different compounds. However, compounds having the same value of R_f may not be identical.
2. To determine the number of separable components within a mixture.
3. To choose the suitable eluting solvent for column chromatography.
4. To judge the quality of purification of different methods such as recrystallization, extraction, distillation, and column chromatography.
5. To monitor the progress of a reaction by directly analyzing the reaction mixture intermittently.

II. Column chromatography

Column chromatography is a technique carried out in a vertical wide bore glass

column (Figure 22-2). The column is filled with solid adsorbent moistened with eluting solvents. The mixture to be separated is loaded at the top of the column and then eluted downward by eluting solvents. The mixture can thus be separated. In general, this technique is used in separating a large quantity of a mixture.

The solid adsorbents in the column are the stationary phase, and the liquid eluting solvents are the mobile phase. Due to the differences in the strength of adsorption between the compounds and the stationary phase, and the intermolecular forces between the compounds and the mobile phase, the distribution coefficients of different compounds between the two phases are different. Thus, the elution rates of different compounds are different so that separation can be achieved.

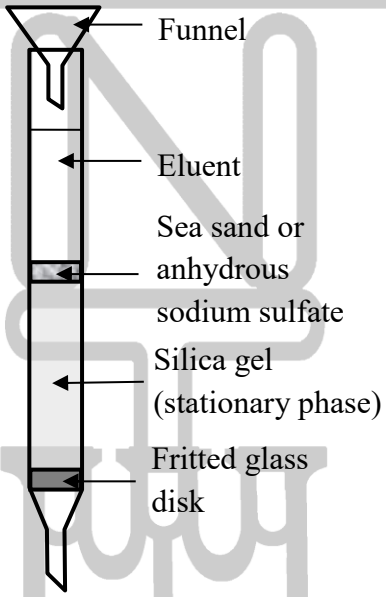


Figure 22-2 Column chromatography

(I) Factors affecting column chromatography

Several factors should be considered when carrying out column chromatography: (1) the choice of stationary phase, (2) the choice of polarity of the mobile phase (eluent), (3) the size of the column, (4) the ratio of adsorbent to the sample, (5) the packing of the column, (6) the flow rate of the eluent, (7) the loading of the sample, (8) collection of the liquid eluted out, (9) analysis of the collected fractions.

1. Choosing the stationary phase:

   Silica gel and alumina are widely used as the stationary phase. Commercially available alumina can be further divided into three grades: neutral, acidic, and basic. The activity of alumina can be adjusted by adding a fixed amount of water, where the adsorption force decreases with increasing amounts of water added. Both silica gel and alumina are materials with high polarity; hence, they have a stronger adsorption force with polar compounds. Thus, during elution, compounds

with high polarity will stay in the column for a longer time, while those with low polarity will stay for a shorter time. If the compounds to be separated are acidic, an acidic adsorbent should be chosen as the stationary phase. If the compounds to be separated are basic, a basic adsorbent should be chosen as the stationary phase. This can help to avoid very strong intermolecular force between acid and base, which would cause tailing and loss of compounds. Moreover, some compounds are unstable on the adsorbent. For example, lipids are easily hydrolyzed by alumina of low activity with high water content. Thus, the stationary phase should be chosen carefully in order not to destroy the compounds to be separated.

2. Polarity of the mobile phase:

   When an adsorbent of high polarity, such as silica gel and alumina, is used, the compounds will stay in the column for a shorter time when using an eluent with higher polarity. In contrast, the compounds will stay longer on the column when using an eluent with lower polarity. Choose an eluent with suitable polarity, the mixture can be eluted out at a desired rate. If the composition of the mixture to be separated is very complicated, a single solvent may not be enough for its separation. In this situation, gradient elution can be carried out with solvents of different polarities, starting with the one with the lowest polarity. The most commonly used eluting solvent of low polarity is hexane and the most common one of high polarity is ethyl acetate. These two solvents can be mixed to obtain an eluting solvent with the desired polarity. However, the best condition for separation is not easy to determine in most cases. Hence, thin-layer chromatography should be done in advance to find a suitable separation condition. For a silica gel system, developing solvents in which the desired sample having an R_f value of around 0.25 in thin-layer chromatography are usually used as eluent in column chromatography.

3. Size of the column:

   The most suitable ratios of the length of the column to the diameter range from around 4:1 to 8:1. Enough space should be reserved at the top of the column to hold the eluting solvent.

4. The ratio of adsorbent to the sample:

   Since separation is dependent on the exposed surface of the adsorbent, a higher ratio of adsorbent/sample will allow better separation. The average granular size of the adsorbent will also affect the separation. With the same amount, the smaller the granular size the better the separation. The type and amount of adsorbent used should be based on the ease of separation. Generally, a ratio in the range of 20:1 to 50:1 by weight is used.

5. Packing of the column:

   The adsorbent and a suitable amount of eluent are added into a beaker. Then the

mixture is stirred with a glass rod until slurry is formed and no gas bubbles can be observed. The amount of eluent added should be controlled so that the slurry is not too thick, and the slurry should be poured quickly into the column before the adsorbent settles. The column can then be tapped gently from the side to make the packing tighter and to flatten the top of the column at the same time. Note that the quality of column packing will directly affect the results of the separation. If the top of the column is not flat, or gas bubbles and cracking are present in the column, the flatness of the separation band will be damaged, causing a lower quality of separation. Moreover, the tighter the column is, the better the quality of separation will be.

6. Flow rate of the eluent:
   When the rate of elution is too fast, equilibrium between the adsorbent and different compounds cannot be attained. Thus, the quality of separation will be affected. It is true that time should be allowed for the equilibrium to be attained by lowering the rate of elution. However, slow diffusion of the sample bands will occur at the same time, widening the separation band and causing the bands to overlap. Thus, too slow a rate will not only lengthen the separation process but also lower the quality of the separation. Studies have shown that the best rate of elution is about 2.5 cm per minute (i.e., the descending height of the eluent). The eluting speed can be increased by increasing the pressure at the top of the column or by applying suction at the bottom. Safety measures should be taken while applying pressure.
7. Loading of sample:
   Undiluted samples should **not** be loaded on the column directly; if this is done, the column will be overloaded, causing widening of the separation band with detrimental effect. In general, the sample is diluted with the eluting solvent to give a 5-10% solution before being loaded on the column. Moreover, elution should be started after the sample solution has been totally submerged into the column top. Sample loading will invariably affect the flatness of the column top to some degree. In order to protect the top of the column, a layer of sea sand or solid anhydrous sodium sulfate is usually added as an inert buffer.
8. Collection of the liquid eluted out:
   The eluent is generally collected in small fractions with test tubes. If the amount of each fraction is too large, overlapping is more likely to occur in the separation region. On the other hand, if the amount of each fraction is too small, a large number of sample fractions will need to be analyzed, and the process will be time consuming. In general, half of the column volume should be taken as a standard value for each fraction, and adjustments should be made according to the ease of

separation. With a tough separation, the probability of overlapping will increase. In this case, smaller amounts should be collected for each fraction.

9. Analysis of the collected fractions:
   The fractions collected should be analyzed with thin-layer chromatography or other methods. Then the decision on which portions to collect can be made.

Apparatus

Fresh green leaves (ca. 1 g), mortar and pestle, graduated cylinder (10 mL), TLC plates (Merck, Silica gel 60 F254), capillary tube, test tubes (20), dropper (229 mm), funnel, glass rod, beaker (30, 100, 250 mL), tweezers, three prong clamp, Erlenmeyer flask, aluminum foil.

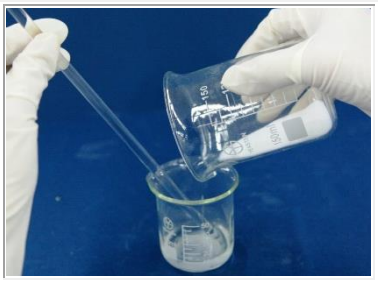
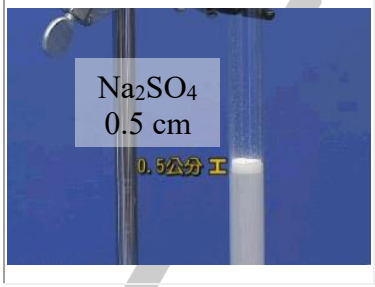
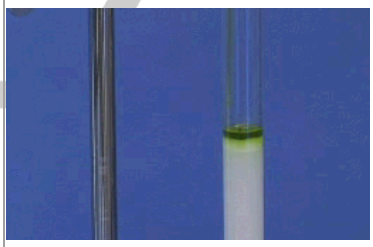
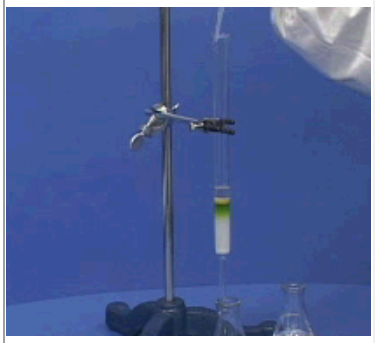
Shared: Scissors, UV light source, rubber squeeze bulb.

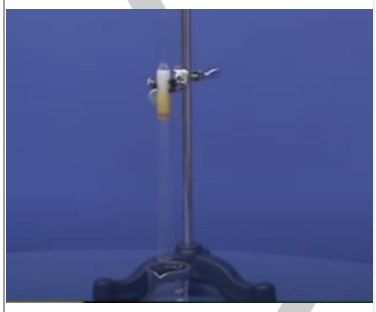
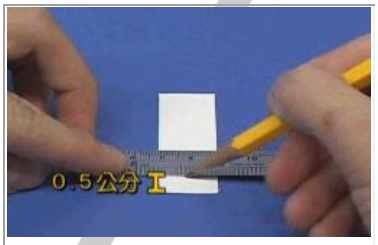
Chemicals

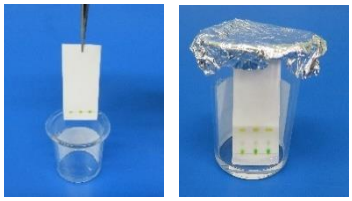
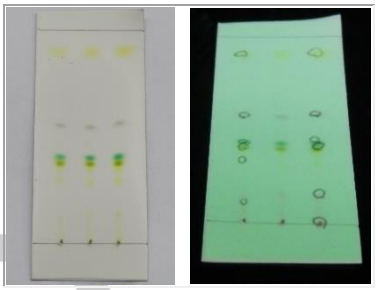
Hexane (C_6H_{14}), ethyl acetate ($CH_3COOC_2H_5$), acetone (CH_3COCH_3), anhydrous sodium sulfate (Na_2SO_4), silica gel (230-400 mesh).

Procedures

	Procedure	Illustration
I.	Prepare the sample solution	
1.	(1) Cut ca. 1 g of fresh green leaves to pieces and grind with 10 mL hexane/ethyl acetate (8:2, v/v) solvent to extract the chlorophylls. Because of evaporation, more solvent may be added during the process. (2) Use a dropper to withdraw about 2 mL of the extract into a graduated cylinder as the sample solution.	
II.	Column chromatography	
2.	(1) Clamp the column vertically on an iron stand and place a funnel on top of it. (2) Prepare ca. 60 mL hexane/acetone (7:3, v/v) solution as the eluent for column chromatography, and 20 test tubes to collect the fractions.	

3.	Preparation of the adsorbent: Take 20 mL of eluent in a 100 mL beaker. Slowly add 4 g of silica gel to it and stir the mixture with a glass rod thoroughly until no gas bubbles can be observed.	
4.	Packing the column: (1) Add 5 mL of eluent to the column through the funnel to force out the entrapped air bubbles in the glass frit. (2) Stir and pour the slurry prepared in the previous step into the column as quickly as possible. (3) After the adsorbent has settled, tap the wall of the column gently to flatten the top of the column and pack the column more tightly. Collect the eluted solvent in a beaker. (It is clean and could be reused.) (4) As soon as the solvent submerges into the top of the silica gel, add a layer of anhydrous sodium sulfate of 0.5 cm thickness at the top and flatten it by tapping the column lightly. Add eluent whenever necessary to prevent the surface of the eluent from dropping below the column top to avoid formation of gas bubbles and cracking which may influence the results of separation.	  
5.	Applying the sample and collecting the fractions: (1) When the surface of the eluent reaches the top of the stationary phase, gently apply the sample solution (with a dropper) to the top of stationary phase to form a small layer. As soon as the sample solution is completely submerged into the surface of the silica gel, apply a small amount of eluent to rinse the residual sample attached to the wall into the column. (2) Continue adding eluent to fill the column and start the chromatography. When the first colored band is about to come out, collect the fraction with test tubes in one milliliter per tube.	 

	(3) Continue the process to collect the following colored bands. Observe and record the results of the chromatography (the eluent collected is reusable if it is colorless), and check each tube with TLC. Note: The top of the column should not be damaged when the sample is loaded. This is done best by touching the dropper tip to the inside of the column and slowly apply the sample circularly so as to allow the sample to spread into a thin film that slowly descends to cover the entire adsorbent surface.	 
6.	Disposal of solid adsorbent: (1) Clamp the glass column upside down above a beaker, let the solvent drip dry and solid packing drop out. (2) Alternatively, a rubber squeeze ball can be used to connect the inverted glass column, apply pressure to accelerate the discharge of the solid packing. Discard solid adsorbent in a waste bin.	
III. Thin-layer chromatography		
7.	Get a thin-layer plate supplied by the laboratory. Draw a starting line and three spotting positions at about 0.5 cm above the bottom of the TLC plate (Figure 22-3). Use a capillary tube to apply spots of the fractionations and one spot of the original extract. Note: Rinse the same capillary tube with clean solvent and drain on tissue towel 2~3 times to reuse it between different samples.	 
8.	Add developing solvent (hexane/acetone = 7:3) to a 30 mL beaker to a height of about 0.3 cm. Then attach a cut strip of filter paper to the inside wall of the beaker with the bottom immersed in the eluent, and cover the beaker with aluminum foil.	

9.	Place the plate into the developing chamber with the tweezers carefully. Then cover the beaker with aluminum foil (the height of the spots should be higher than the surface level of the developing solvent) and allow the solvent to advance up.	
10.	Observe the climbing of the developing solvent. Take the plate out when the solvent front is near 0.5 cm from the top of the plate. Mark the position of the solvent front with a pencil and record the distance it has traveled.	
11.	Locate the center of each spot and measure the subsequent distances they traveled. Then calculate their values of R_f. You may also detect the sample spots under the UV light (254 nm).	
12.	Discard the organic waste, TLC plates, and capillary tubes to the waste bins.	

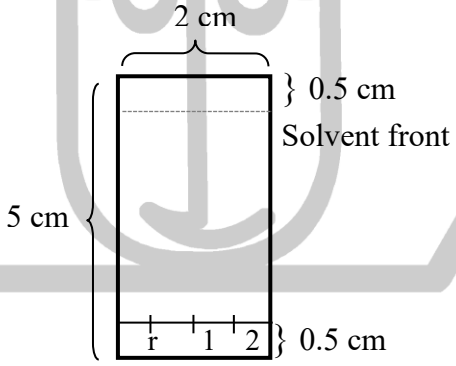


Figure 22-3 Thin layer chromatography plate

References

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2. Pasto, D. J.; Johnson, C. R.; Miller, M. J. *Experiments and Techniques in Organic Chemistry*; Englewood Cliffs: N.J., 1992.
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