Experiment 27

CHROMATOGRAPHY

Objective

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

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 (TLC) makes use of a glass, metal or plastic plate coated with a mixture 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 as shown in Figure 27-1.

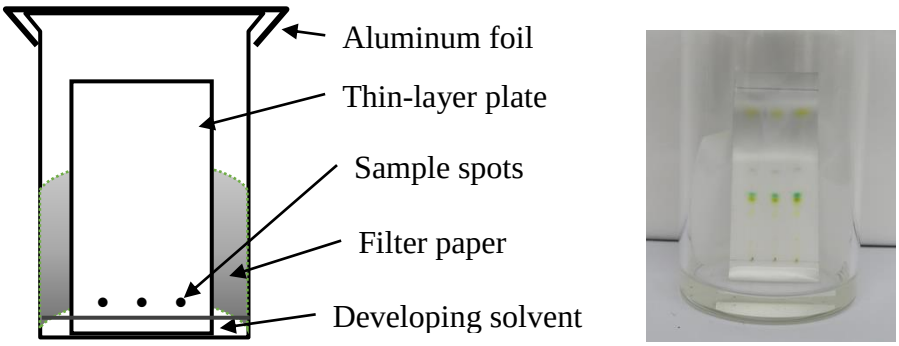

Figure 27-1 Thin-layer chromatography

(I) Retention factor

If the mixture is colored, the separated components can be identified with the naked eye. 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 of thin-layer chromatography

The stationary phase can be further divided into a normal phase system and a reverse phase system according to polarity. Silica gel is always 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 easily due to the subsequent replacement by the solvent. Thus, a higher the polarity of the developing solvent corresponds to a higher R_f value. If a developing solvent with a suitable polarity is chosen, 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. Thus, 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 on the TLC plate about 0.5~1 cm from one edge of it 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. It should be noted that the line drawing and the spotting should be done gently to avoid damaging the plate.

As the spot is climbing, diffusion will occur and cause the diameter of the sample to increase. Therefore, if the sample is spotted too large, compounds with similar R_f values will overlap with each other 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 at the baseline should be more than 0.5 cm to prevent them from interfering with each other. 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 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 starting line should be higher than the surface level of the eluent to prevent the sample from dissolving in it. Then the chamber is covered. When the solvent has advanced to within 5 mm from the end, take out the plate, and draw a line to indicate where the solvent front is immediately (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. Using visualizing agents: The most commonly used visualizing agent is phosphorous molybdic acid ($H_3PO_4 \bullet 12MoO_3$) dissolved 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 can also be used as a visualizing agent; dark spots can be observed after heating.
2. 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 mixed with a fluorescent reagent that absorbs UV light with a wavelength of 254 nm in the stationary phase 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 presence of the fluorescent reagent. Thus, the corresponding positions of the components can be identified.

(VI) Pros and cons of using thin-layer chromatography

Thin-layer chromatography is quicker, cheaper, and more convenient than gas chromatography (GC) and high performance liquid chromatography (HPLC).

To begin with, the amount of a 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 chromatography. 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. A second development can also be done by developing on another axis, using the same plate 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.

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 components within a mixture.
3. To determine the suitable eluting solvent for column chromatography.
4. To determine the quality of purification of different methods such as recrystallization, extraction, distillation, and column chromatography.
5. To examine the process of a reaction.

II. Column chromatography

Column chromatography is a technique carried out in a vertical wide bore glass column (Figure 27-2). 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, and the differences allow separation of the compounds.

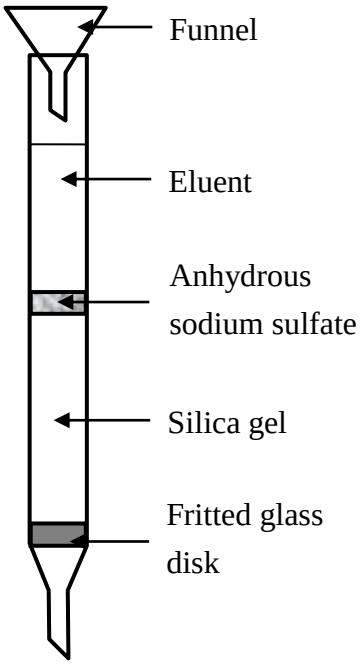

Figure 27-2 Column chromatography

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.

Apparatus

Mortar and pestle, TLC plate (Merck, TLC Silica gel 60 F_{254}), capillary tube, test tubes (20), droppers, funnel, glass rod, beaker (30, 100, 250 mL), aluminum foil, tweezers, UV light

Chemicals

Hexane (C_6H_{14})
Ethyl acetate ($CH_3CO_2C_2H_5$)
Acetone (CH_3COCH_3)
Anhydrous sodium sulfate (Na_2SO_4)
Silica gel (230-400 mesh)

Procedures

I. Prepare the sample solution

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, it may need more solvent to extract.
2. Use a dropper withdraw the extract into a graduated cylinder as the sample solution; the amount of solution is about 2 mL.

II. Column chromatography

3. 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.
4. 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.
5. Packing the column:
   (1) Clamp the column vertically on an iron stand and place a funnel on top of it.
   (2) Add 5 mL of eluent to the column through the funnel to force out the entrapped air bubbles.
   (3) Stir and pour the slurry prepared in the previous step into the column as quickly as possible.
   (4) 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.)
   (5) 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 gas bubbles and cracking form which may influence the results of separation.
6. Applying the sample:
   When the surface of the eluent reaches the top of the stationary phase, apply the sample solution (with a dropper) to the top of stationary phase gently to form a small layer. As soon as the sample solution is submerged into the surface of the silica gel completely, add small layer of eluent to drain into the column until the column just dries.
   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 draining it circularly so as to allow the sample to spread into a thin film which slowly descends to cover the entire adsorbent surface.
7. Continue adding eluent to start the chromatography. When the first colored band is about to come out, collect the fraction in the test tube for one milliliter per tube. Then 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.

III. Thin-layer chromatography

8. Get a thin-layer plate supplied by the laboratory.
9. Draw a starting line and three spotting positions at about 0.5 cm above the bottom of the TLC plate. Use a capillary tube to place 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.
10. Add developing solvent (hexane/acetone = 7:3) to a 30 mL beaker to a height of about 0.3 cm. Then attach a cut filter paper to the inside wall of the beaker as shown in Figure 27-1 and cover the beaker with aluminum foil.
11. 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.
12. Observe the climbing of the developing solvent. Take the plate out when the solvent front is 0.5 cm from the top of the plate. Record the position of the solvent front and the distance it has traveled with a pencil.
13. 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).
14. Discard the organic waste, TLC plates, and capillary tubes to the waste bins.

References

1. National Taiwan University, Department of Chemistry; *Experiments of Organic Chemistry*, 8th ed.; NTU Press: Taipei, 2006.
2. Pasto, D. J.; Johnson, C. R.; Miller, M. J. *Experiments and Techniques in Organic Chemistry*; Englewood Cliffs: N.J., 1992.
3. Pavia, D. L. *Introduction to Organic Laboratory Techniques: A Microscale Approach*, 1st ed.; Saunders College Pub.: Philadelphia, 1990.