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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OFENGINEERING & TECHNOLOGY

LT.6. Column Chromatography & Thin layer Chromatography

Content outline

1. Column Chromatography: Definition & Types
2. Elution condition
3. Performance measurement of column chromatography
4. Thin layer chromatography

Column Chromatography

In column chromatography the stationary phase is packed into a glass or metal column. The mixture of analytes is then applied and the mobile phase, commonly referred to as the eluent, is passed through the column either by use of a pumping system or applied gas pressure. As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the eluate as it leaves the column.

Examples of column chromatography

•liquid chromatography (LC),

•High-performance liquid chromatography (HPLC),

•and gas chromatography (GC).

Column chromatographic techniques can be subdivided on the basis of the development and elution modes.

a. Zonal development

b. Affinity or Displacement development

In zonal development: The analytes in the sample are separated on the basis of their distribution coefficients between the stationary and mobile phases. The sample is dissolved in a suitable solvent and applied to the stationary phase as a narrow, discrete band. The mobile phase is then allowed to flow continuously over the stationary phase, resulting in the progressive separation and elution of the sample analytes.

Displacement or affinity development: The sample of analytes dissolved in a suitable solvent is applied to the stationary phase as a discrete band. The analytes bind to the stationary phase with a strength determined by their affinity constant for the phase. The analytes are then selectively eluted by using a mobile phase containing a specific solute that has a higher affinity for the stationary phase than have the analytes in the sample. Thus, as the mobile phase is added, this agent displaces the analytes from the stationary phase in a competitive fashion, resulting in their repetitive binding and displacement along the stationary phase and eventual elution from the column in the order of their affinity for the stationary phase, the one with the lowest affinity being eluted first.

Elution

- **To effect elution, following modes can be applied**
- If the composition of the mobile phase is constant as in GC and some forms of HPLC, the process is said to be isocratic elution.
- To facilitate separation however, the composition of the mobile phase may be gradually changed, for example with respect to pH, salt concentration or polarity. This is referred to as gradient elution. The composition of the mobile phase may be changed continuously or in a stepwise manner.

Performance measurement of Column Chromatography

➢A chromatogram is a pictorial record of the detector response as a function of elution volume or retention time. It consists of a series of peaks or bands, ideally symmetrical in shape, representing the elution of individual analytes.

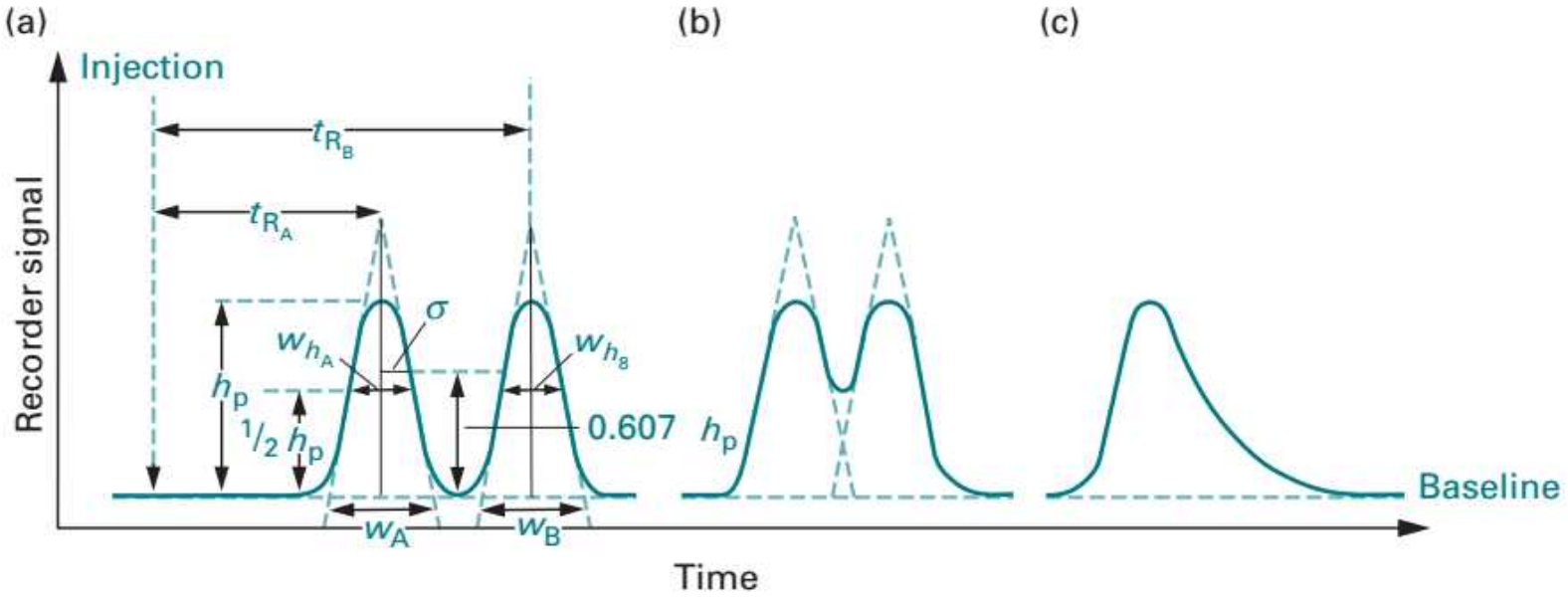


Fig. 11.1 (a) Chromatogram of two analytes showing complete resolution and the calculation of retention times; (b) chromatogram of two analytes showing incomplete resolution (fused peaks); (c) chromatogram of an analyte showing excessive tailing.

Image source: Wilson & Walker

- Broadening and tailing in chromatograms results due to diffusion in colum which tend to oppose the separation and which result in non-ideal behaviour of each analyte.
- **Retention time:** Simply a time spent by an analyte in the column. The retention time tR for each analyte has two components. The first is the time it takes the analyte molecules to pass through the free spaces between the particles of the matrix coated with the stationary phase. This time is referred to as the dead time, tM.The second component is the time the stationary phase retains the analyte, referred to as the adjusted retention time, tR. This time is characteristic of the analyte and is the difference between the observed retention time and the dead time:

$$t'_R = t_R - t_M$$

Relative retention time: It is simply the retention time for the analyte divided by that for the standard.

Retention factor: It is simply the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which, by definition, has a k value of 0.

$$k = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M}$$

Resolution: The ability of a chromatography column to separate two analyte peak from another is known as resolution. Resolution (RS) is defined as the ratio of the difference in retention time (tR) between the two peaks (tRA and tRB) to the mean (wav) of their base widths (wA and wB):

$$R_S = \frac{\Delta t_R}{w_{av}} = \frac{2(t_{R_A} - t_{R_B})}{w_A + w_B}$$

➢When Rs=1, the separation of two peaks is 97.7% and a column with Rs more than 1.5 considered good. The number of distribution events govern the ability of a column to separate the two analytes.

➢In column chromatography, each thin plain of column matrix participate in distribution of molecule. Assume height of a distribution plain is **H** and length of a column is **L**, hence number (**N**) of distribution plain in a column is given by:

$$N = \frac{L}{H}$$

$$N=16\ (t_R/W)^2$$

$$N=5.54\ (t_R/W_{1/2})^2$$

Thin Layer Chromatography

Thin Layer Chromatography (**TLC**) is a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase) used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_f) expressed as:

$$R_f = \text{dist. travelled by sample / dist. travelled by solvent}$$

The *Rf* value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions, the compound with the larger *Rf* value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower *Rf* value.

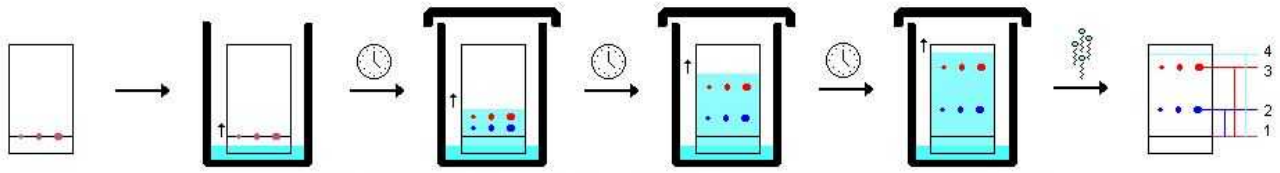
If it is desired to express positions relative to the position of another substance, x, the *Rx* (relative retention value) can be calculated:

$$R_x = \frac{\text{distance of compound from origin}}{\text{distance of compound x from origin}}$$

➢The factors affecting retardation factor are the solvent system, amount of material spotted, absorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatography techniques.

Principle

TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots. A complex mixture applied to the plate, in a suitable solvent as a spot or as a band, is dried and the chromatogram developed by allowing solvent to spread along the plate as a sharp front from one edge toward the opposite edge of the plate. A selection of solvents can be used in any order so long as the previous solvent is dried off before the next one is applied. Components of the sample usually have different affinities for the separating medium.



The sequence involved in TLC. Image used with permission (CC BY-SA 3.0; Wikipedia).

Stationary phases

Stationary Phase	Chromatographic Mechanism	Typical Application
Silica Gel	adsorption	steroids, amino acids, alcohols, hydrocarbons, lipids, aflaxtoxin, bile, acids, vitamins, alkaloids
Silica Gel RP	reversed phase	fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	partition	carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids
Aluminum oxide	adsorption	amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins, alkaloids
PEI cellulose	ion exchange	nucleic acids, nucleotides, nucelosides, purines, pyrimidines
Magnesium silicate	adsorption	steroids, pesticides, lipids, alkaloids

Useful solvent mixture for TLC

•A solvent that can be used for separating mixtures of strongly polar compounds is ethyl acetate : butanol : acetic acid : water, 80:10:5:5.

•To separate strongly basic components, make a mixture of 10% NH_4OH in methanol, and then make a 1 to 10% mixture of this in dichlormethane.

•Mixtures of 10% methanol or less in DCM can be useful for separating polar compounds

Analysis

The components, visible as separated spots, are identified by comparing the distances they have traveled with those of the known reference materials. Measure the distance of the start line to the solvent front (=d). Then measure the distance of center of the spot to the start line (=a). Divide the distance the solvent moved by the distance the individual spot moved. The resulting ratio is called R_f-value. The value should be between 0.0 (spot did not moved from starting line) and 1.0 (spot moved with solvent front) and is unitless.

Visualization of spot on TLC

Reagent	Works well for	Colors	Notes
Iodine	Unsaturated and aromatic compounds	Brown spots	Not permanent
Sulfuric acid	General stain	Brown or black spots	
Chromic acid	For difficult to stain compounds	Black spots	
UV light	Compounds with extended conjugation like aromatic compounds	Pink on light green background	Only visible under UV light
Cerium sulfate	Good general stain, very well for alkaloids		
Ferric chloride	Phenols	Purple	
Ninhydrin	Amino acids, amines	Purple	
2,4-Dinitrophenylhydrazine	Aldehydes, ketones	Yellow/orange	also called "DNP"
Vanillin	Good general stain, very well for hydroxyl or carbonyl compounds	Colors vary	
Potassium permanganate	Works well for all compounds that can be oxidized	Yellow on purple Yellow or light brown on purple	at r.t. for alkenes and alkynes upon heating for alcohols, amines, sulfides
Bromocresol Green	Carboxylic acids (pK_a<5)	Yellow spot on blue background	
Cerium molybdate (CAM, 'Hanessian's Stain', Ceric staining)	Good general stain, very well with polyhydroxylated and carbonyl compounds	Blue or green spot	Upon heating, very sensitive!
p-Anisaldehyde	Good general stain, particularly sensitive towards nucleophiles	Varying colors on light pink plate upon heating	Does not work with alkenes, alkynes or aromatic system unless functional groups are present
Phosphomolybdic acid (PMA)	Very sensitive	Dark green spot on light green plate	Sensitivity can be enhanced by use of cobalt(II) chloride
Ehrlich's Reagent (Dimethylaminobenzaldehyde)	Indoles, amines	Pink or red-violet	
Dragendorff-Munier Stain	Amines even the ones that are low in reactivity	Various colors	

Thin Layer Chromatography Applications

•The qualitative testing of various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.

•TLC is extremely useful in biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.

•Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc

•It is widely used in separating multicomponent pharmaceutical formulations.

•It is used to purify of any sample and direct comparison is done between the sample and the authentic sample

•It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives

•It is used in the cosmetic industry.

•It is used to study if a reaction is complete.

Disadvantages of Thin Layer Chromatography:

•Thin Layer Chromatography plates do not have longer stationary phase.

•When compared to other chromatographic techniques the length of separation is limited.

•The results generated from TLC are difficult to reproduce.

•Since TLC operates as an open system, some factors such as humidity and temperature can be consequences to the final outcome of the chromatogram.

•The detection limit is high and therefore if you want a lower detection limit, you cannot use TLC.

•It is only a qualitative analysis technique and not quantitative.

Test your understanding

Thin layer chromatography is

a. partition chromatography
b. electrical mobility of ionic species
c. adsorption chromatography
d. none of the above

Thin layer chromatography can be used to distinguish between different amino acids. If a particular amino acid has low solubility in the mobile phase used, then the amino acid

a. will move at a speed close to that of the solvent
b. must have a high molecular mass.
c. will have a low Rf value.
d. will spend more time dissolved in the mobile phase than attached to the stationary phase.

Isocratic elution refers to

a. Elution of sample from column by keeping composition of mobile phase constant
b. Elution of sample from column by varying composition of mobile phase over time
c. Introduction of sample into column
d. None of these

References & Further reading

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