3.4. CHROMATOGRAPHY

Chromatography was first developed and used by Russian-Italian botanist Mikhail Tsvet in the early 1900s. Tsvet observed that the solution with petroleum ether of the plant extract had passed through the CaCO3 adsorbent in a glass column and that there was a separation with yellow, green bands on the column. The development by attracting other researchers' attention was in the 1930s because of the fact that the first publications in this area was Russian.

Chromatography is a method that allows different chemical substances forming a mixture to be separated from each other based on the dispersion balances or the different interactions between the two phases, which do not mix. Chromatography with another definition is a generic term for separating different chemical substances in a mixture based on such principles as adsorption, solubility, capillarity, ion exchange or molecular sieve between two separate phases.

The substances separated by chromatography can be identified, which is also a purification method, since they can be isolated. In other words, chromatography is a qualitative and quantitative method of identification, since it enables the identification and quantitation of separated substances.

Common to all chromatographic methods:

Stationary phase: Stable (static) phase ……...may be solid and liquid,

Mobile phase: Movable phase…….……may be liquid and gas.

Components forming the mixture (sample): They exhibit different migrations between these two phases (stationary-mobile) which do not mix and thus can be separated from each other.

3.4.1. CLASSIFICATION OF CHROMATOGRAPHIC ANALYSES

3.4.1.1. Classification of Principles (Based on Mechanism of Separation)

a- Adsorption chromatography
b- Partition chromatography
c- Ion exchange chromatography
d- Ion pair chromatography
e- Molecular sieve chromatography
f- Affinity chromatography
g- Electrochromatography

a- Adsorption Chromatography:

Adsorption is the superficial interaction between a solid substance and a liquid soluble compound. Here, the stationary phase is a high adsorption capacity (Al_2O_3, Silicagel) and the mobile phase is gas or mostly liquid.

Bonds that play role in adsorption;

- Van-der-Waals bonds
- Dipole-dipole interaction power
- Hydrogen bond
- Ionic bonds
- Chelate bonds
- Rarely irreversible covalent bonds.

The basic principles of this type of chromatography are:

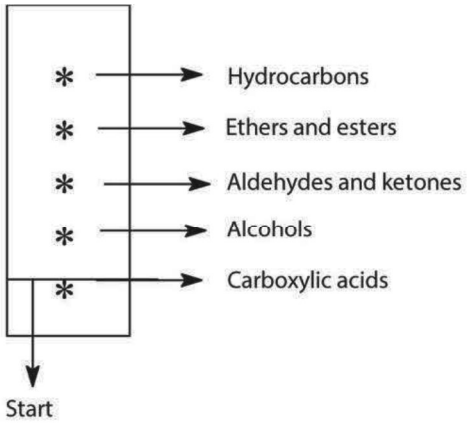
a- Saturated hydrocarbons are hardly adsorbed, so they migrate very quickly. The adsorption of unsaturated hydrocarbons increases with the number of double bonds and their conjugation numbers. As a result, a non-polar solvation is required with an active adsorbent for separation. As a result, a non-polar solvent is required with an active adsorbent for separation.

b- Generally, adsorption affinity increases with formation of functional group on a hydrocarbon. Among the functional groups, the following ranking can be made.

-CH3 ⟶ **-O-alkyl** ⟶ **C=O** ⟶ **-OH** ⟶ **-COOH**

⟶ polarity, adsorption increase

For example, if benzene is used as the solvent, in the chromatography plate the ethers and esters eluate to the upper part of the plate, the ketones and aldehydes are relatively in the middle, the alcohols are below them and the acids remain at the beginning. Therefore, the separation takes place according to the polarity of the compounds.



c- If there are many substituents in one molecule, it can be said that adsorption affinities are roughly interrelated. Especially steric effect is important for functional groups in aromatic rings

b- Partition Chromatography:

In partition chromatography, both phases are usually liquid. For this reason, liquid-liquid chromatography is also called. The stationary phase is usually more polar than the mobile phase

(mostly water) and is adsorbed as a thin layer of film on a solid support (kieselguhr, cellulose, etc.). The mobile phase is less polar than the stationary phase and another liquid that does not mix with it. In liquid-liquid chromatography it is sometimes desirable that the stationary phase is less polar. This chromatographic scheme is called “reverse phase liquid-liquid chromatography”.

This method is often used to separate homologous serials. In partition chromatography, the mobile phase can sometimes be gaseous. In this case, the stationary phase is a liquid impregnated with an inert solid which is not an adsorbent. With this method, the chromatographic separation of the readily volatile or gaseous substance is provided.

In partition chromatography, the partition coefficient of Nerst is valid. According to Nerst; "The ratio each other of the concentration in two seperate liquid phases of a third substance dissolved in the two liquids mixture, which do not mix with one another, is fixed."

$K = C_s / C_m$

K : Partition coefficient

C_s : Concentration at the stationary phase

C_m : Concentration at the mobile phase

If the K value is large, the concentration at the stationary phase is higher than at the mobile phase. It means that the molecule stays at the stationary phase longer.

c- Ion Exchange Chromatography:

Some solid substances may be ion exchanged between solute (mixture to be separated into components) and solid when they are exposed to ionizable substance solutions. These solid things are called “ion exchangers”. The ion exchangers may be inorganic or organic. Examples of inorganics include clay and zeolite. Organics are frequently used in the analysis process and are called "ion-exchange resins". Ion exchange resins are polymer compounds containing ionizable groups and are usually present in the form of small spheres or granules. Cation-exchange resins and anion-exchange resins are divided into two. In the cation exchange resin, the resin part of the polymer has anionic character and the polymeric cation is replaced by another cation in the solution. In the anion-exchange resin, the resin part has cationic character. The ion-exchange resins are generally styrene and divinyl benzene copolymers. In ion-exchange chromatography, the stationary phase forms the resin. The mobile phase is only liquid. In this method, the analyzed solution is distributed according to the ionic equilibrium rules between the mobile and the stationary phase.

d- Molecular Sieve Chromatography:

This method is used to separate macromolecules. The stationary phase and the mobile phase are the same structure and composition. In this method, a porous structure which supports the stationary phase is needed. For this purpose, hydrophilic or hydrophobic gels are used.

Hydrophilic gels: They are used with aqueous solvent and swell in aqueous media. This type of molecular sieve is called “gel filtration chromatography”. It is often used in biochemical applications to especially remove the salt of the protein solution.

Hydrophobic gels: They are used with organic solvent. This chromatographic application is called “Gel permeation chromatography”.

e- Affinity Chromatography:

Polyamide is used as the stationary phase in affinity chromatography.

Hydrogen bonds are formed between the phenol or nitro compounds present in the mixture to be analyzed and the polyamide stationary phase, thus adsorbing. Here the mobile phase is often liquid, and rarely gas.

3.4.1.2. Classification by Application Technique (Based on Shape of Chromatographic Bed)

Chromatographic methods can be seen as a rich area of analysis with different application techniques.

A - Planar Chromatography	A_1 - Thin layer chromatography
	A_2 - Paper chromatography
	A_3 - Preparative thick layer chromatography
	A_4 - Electrochromatography (electrophoretic chromatography)
B - Column Chromatography	B_1 - Column chromatography
	B_2 - Gas chromatography
	B_3 - High pressure liquid chromatography (HPLC)
	B_4 - Capillary electrochromatography

A_1 - Thin Layer Chromatography (TLC)

The TLC is a physicochemical separation method. It has an important place with fast results, good resoluion and economic application advantages. The most commonly used adsorbents for the stationary phase are silicagel, aluminum oxide, kieselguhr, cellulose and its derivatives and polyamides. The adsorbent material used as stationary phase in İTK is coated on glass, plastic or aluminum plates as a thin layer and in homogeneous thickness. For coating the plates, the adsorbent is mixed well with a 1: 1.5 ratio distilled water until a homogeneous mixture is obtained, taking care not to form air bubbles in a wide-necked ball. About 0.5%, more distilled water is added and mixed again. This mixing time should not exceed 90 seconds. The suspension prepared in this way is applied onto the plate with the help of the spreader. The prepared plates are used after being activated by holding the body 30 ' at 110 ° C. Activation is important. This is because the adsorbent has a significant effect on the separation.

The mobile phase used in the TLC may consist of one or several solvents. Solvent elution in adsorption chromatography is grouped under the so-called eluotropic series according to their effect, i.e. their dragging power, and is increased by the polarity of the elution effect of a solvent. Polarity is proportional to the "dielectric constant" of a substance. In this case, the substance, which has bigger dielectric constant, has greater polarity, and accordingly the effect of elution is higher.

Solvent	**ε (dielectric constant) at 20 °C**
n-Hexane	1.890
Heptane	1.924
Cyclohexane	2.023
CCl_4	2.238
Benzene	2.284
$CHCl_3$	4.806
Ether	4.34
Ethyl acetate	6.02 (25 °C)
Pyridin	12.30 (25 °C)
Acetone	20.70 (25 °C)
Ethanol	24.30 (25 °C)
Methanol	33.62
Water	80.35

Benzene or chloroform can be initially selected for an unknown substance. If the substance remains at the start, a second solvent is added to the used solvent. On the contrary, if the substance is rapidly migrating and drifting near the front, a weaker should be selected.

There are a number of factors that affect the development. These:

a- Polarity of the substance: The more polar substance, the more held by the adsorbent.

b- The polarity of the solvate forming the mobile phase: The more polar the mobile phase, the weaker the bond between the substances and the stationary phase. So the substance drifts so much over the plaque.

c- Activity of Adsorbent: The more active adsorbent, the greater interaction with the substance.

TLC cuvettes or tanks: It is important to ensure good atmospheric saturation when working with high precision. This can be achieved by using filter paper. Small tanks are preferred due to the minimum volume and the advantages associated with it.

Some of the terms used in chromatographic analyzes are:

Solute: Mixture of substances (sample) to be separated into their components.

Start: The point where the soluton is applied on the stationary phase.

Front: The distance the mobile phase reached on the stationary phase.

Development: The solution of the mixture of the substances to be separated in a suitable solvent is applied on the plate in small droplets and this plate is placed in a tightly sealed tank containing a suitable solvent system (mobile phase). On the mobile phase adsorbent, while the capillaries move up, the substances in the mixture are separated from each other. This is called developing the plaque.

In a normal development, the distance between start and front is 10 cm. The mobile phase is also utilized in a gradual, bi-directional and circular development which can provide better discrimination than simple development with a 10 cm rise.

TLC Application

After the mobile phase has been filled in the tank at a height of 5-8 mm, a clean filter paper is placed so as to surround the tank inner wall all around. This ensures that the tank is saturated with solvent vapor.

The solution of the sample in the appropriate solvent is spotting on the plate that it is 1 cm from the side edge of the adsorbed-coated plate and 1.5 cm from the lower edge. The distance between the spots should not be less than 10 mm and the diameter of the spots should be 3-5 mm. In this way, spotting on a line 1.5 cm from the bottom edge of the plate with glass tubes.

The prepared plate is placed in the saturated tank and the plate is removed from the tank after the end of the mobile phase rise (separation of the substance mixture from each other) (development) is over. The front is marked with a sharp-pointed pen.

What is important here is that the sample solution and the reference mixture are always spotted side by side on the plate. Otherwise, if the reference solution is not spotted, comments can not be made or if they are not side by side, faulty interpretations can be made.

When any synthetic environment is examined chromatographically, the following information can be accessed.

a- The reaction is not progressing (only if there is staining of the starting materials in the chromatogram)

b- The reaction proceeds over time (if the chromatogram contains both starting materials and product stains)

c- The starting substances have been depleted or have been completely converted into products,

d- Transition to the final product on an intermediate product, etc.

In order to identify the substances that are discriminated in the manner described above, in other words, to reveal the spots;

a- Physical methods

b- Chemical methods

c- Biological and enzymatic methods are applied.

It is simple to identify spots on the chromatogram if the excluded substance absorbs itself in the UV region or if it shows fluorescence when irradiated with UV rays at 254 nm or 366 nm. Otherwise, a chromatographic marker is sputtered and substance spots become apparent by utilizing the process of forming colored derivatives of a substance by a chemical reaction. In some cases, spots can also be detected with biological methods.

For the identification of the substance; the distance between the midpoint of the visible spots and the start is precisely measured with a millimetric ruler (A). This distance is proportional to the development distance (B), the distance between start and front.

R_f (Resolusion factor) is a constant in certain conditions that determine the position of a substance in an TLC plate.

$$R_f = \frac{\text{The distance between the midpoint of the spot and start (A)}}{\text{The distance between start and front (Development distance) (B)}}$$ Always $R_f < 1$

Under certain conditions, the R_f value of a compound is a physical constant and helps identify the compound by determining other properties of the compound. It is not only true to speak of an absolute R_f value. Because it may change depending on the circumstances.

Factors Affecting R_f Value:

Adsorbent quality: Adsorbent particle size is important. However, this has no effect on the R_f value when affecting the development time.

Layer thickness: The standard thickness of the coating technique with aqueous method is 0.25 mm. However, for a reproducible R_f according to Stahl, the thickness should not be less than 0.15, although it falls below this value as a result of drying. It can rise up to 5mm thick in preparative works. The layer thickness does not change R_f but it changes the speed of development.

Activation of the layer: 30-60 minutes - 105-110 °C to dry, desiccator is required to keep. (Cellulose is 10 minutes at 105 ˚C)

Quantity of substance: 10-20 µg for many substances. Excessive substance may cause increased or decreased R_f value.

Solvent quality: For solubility analysis, pure solvent should be used. Due to evaporation, the rate of the solvate system will change, so it should be renewed frequently.

Temperature: Adsorption chromatography is less affected by temperature than dispersion chromatography. Generally, an increase in R_f values with temperature is observed. (Not important between 18-38 ˚C)

Tank atmosphere: If the atmosphere of the tank or bath is saturated with the solvent system, the development time is shortened. The R_f value in the unsaturated atmosphere rises.

Application technique: Chromatographic plate placed in the tank can change R_f even if the plate gradient is small. However, different R_f's are achieved with the descent, descent, or horizontal technique.

Adsorbent pre-adsorption of solvated vapors: Important for dispersion chromatographic technique. R_f values.

Development size: R_f increases slightly as the distance increases.

Second substances: Especially in the adsorption technique, it changes R_f.

Advantages of the TLC:

1- The basic tools used are quite simple and economical,

2. The distinctions are quite rapid (better than the column and paper)

3- Corrosive reagents may be used to identify the spots,

4- It gives definite and repetitive results for many applications,

5- Provides the possibility of using a wide variety of adsorbents,

6- With High Performance Thin Layer Chromatography (HPTLC) system

a- Densitometric chromatogram scanning,

b- Quantitative calculation and printing of results is possible.

A_2 - Paper Chromatography

Although it is a very important method of analysis in the beginning (especially for polar-hydrophilic compounds), it has largely left its place TLC today.

It is generally a separation method based on the principle that the substances adsorbed on the filter paper, move differently on paper with the help of a suitable solvent. The filter paper naturally contains some water. For this reason, this method is a chromatograph where the liquid-liquid diffusing principle is valid. The separation of the substances applied to paper depends on the difference in dispersion between the solvent that moves on the paper and the water that the paper contains. Here, the stationary phase is water molecules, the paper serves only as support. The mobile phase consists of a solvent or solvent mixture.

The paper chromatography and TLC applications are similar. Three separate solutions are prepared for the identification of the unknown substance in both TLC and paper chromatography.

- the solution containing the substance to be analyzed,
- the solution containing the standard (reference)
- Solution containing both sample and reference substance in equal concentration.

$$R_f = \frac{R_f\ (\ddot{o})}{R_f\ (c)} = 1 \longrightarrow \text{The sample is the same as the reference.}$$

The Rm value is also calculated using the TLC and Paper chromatographs.

Indicated by the formula:

$$Rm = \log\left(\frac{1}{R_f} - 1\right)$$

Different development methods are applied in paper chromatography.

* Descending method: Solvent system is given from top to bottom.
* Ascending method: Solvent system is given from bottom to top.
* One-way and two-way chromatography
* Circular chromatography

In circular paper chromatography, the center of the special circle chromatographic paper is first marked. Approximately 1 cm away from the center, a solution of the mixture of substances to be separated in circular form is applied. A ring with a diameter of about 2 mm is then opened in the center. At the same time, a petri dish containing the appropriate solvent system is saturated with solvent vapor. Subsequently, a sheet of paper or cotton wool rolled so as to be able to bridge between the solvent and the chromatography paper is placed in the ring opened to the center of the chromatography paper and closed in the mouth of the chromatography paper petri dish. The solvent proceed in the stationary phase by drawing circles originating from a center. Meanwhile, the substances in the mixture migrate differently, forming R_f's different spot circles.

The ascending method is applied according to the tube technique. Here again, after being cut into special chromatography paper strips, the substance solution is applied approximately 1 cm above the lower edge. Once the appropriate solvation system has been placed in a given glass tube, the paper ribbon is placed in the tube in such a way that it contacts the mobile phase, and the mouth is tightly closed. In this way, the substances in the mixture are separated.

The value of R_f is again used to evaluate the results in paper chromatography. The R_f value varies depending on various factors. These:

1- The type of paper used

2- Method used

3- Used solvent

4- Concentration of substance and application area

5- Direction of development

6- Temperature

R_f values are more reliable in paper chromatography if certain conditions are provided.

A_3 - Preparative Thick Layer Chromatography

It is another chromatographic method based on the principle of TLC. The difference from the TLC is that the adsorbent layer thickness and the amount of applied substances are much. For this reason, it is possible to isolate and separate substances at the milligram or gram level. In this method, substance solution in strip form is applied to an adsorbent plate conveyed thick, and after the end of development, the strips formed are removed by scraping with a spatula, extracted with a suitable solvent, filtered. After evaporation of the liquid portion, the residue is crystallized by dissolving in a clean and suitable solvent.

A strip of compound solution is applied to a thick adsorbent plaque, and after the end of development, the strips formed are removed by scraping with a spatula, extracted with a suitable solvent, filtered. After evaporation of the liquid portion, the residue is crystallized by dissolving in a clean and suitable solvent.

B1 - Column Chromatography

The compounds to be separated from the mixture have different adsorption constants against an small particles filled in a glass tube as small particules. The adsorbent in the glass tube is called the stationary phase, while the solution containing mixture of the compounds poured into the column for separation is called the mobile phase, which is the fresh solvent that allows adsorption and desorption to proceed in the column. Solut brings adsorbed and desorbed compounds by the influence of the mobile phase as different bands. If the compounds contained in the solute are colored, they appear in colored bands; if they have the fluorescence properties, they can be rendered visible with UV light.

In order to have a good discrimination for the adsorbent in column chromatography,

* Having a high but selective adsorption power,
* The surface area should be large (small particle fraction, particle diameter too small).

In column chromatography, cellulose, silicagel, active magnesium silicate, active aluminum oxide are used as the stationary phase.

Column is prepared in two forms, dry and wet.

Preparing column by wet method:

Approximately 25 g of the adsorbent is thoroughly mixed with 75 ml of organic solvent to form a slurry, cotton and round cut filter paper placed into the bottom of cleaned column and the mixture poured slowly and carefully. A long glass bag is inserted into the colon to prevent air bubbles. When the column is filled with the adsorbent in the slurry, wait for the adsorbent to precipitate. The adsorbent particles adhering to the inner wall of the glass tube are washed with the same organic solvent and sent to the column. After the column is homogeneously filled, a round cut filter paper is placed on the adsorbent. The solution in the mobile phase of the sample is carefully added from the side of the column with the help of a baguette, taking care that the level of organic solvent is at least 4-5 cm higher than the filter paper. The tap speed is adjusted. With the addition of fresh solvent continuously from above, the solution is allowed to proceed in the column. By observing with TLC, different fractions of substances are obtained. If necessary, the elution can be continued with solvent mixtures according to the structures of the substances to be separated. The process is completed at the point where the final fraction does not contain any spots on the TLC.

3.4.1.3. CLASSIFICATION OF PHASE TYPES (Based on Phases)

1- Liquid Chromatography	a- **L**iquid / **S**olid **C**hromatography (LSC)
	b- **L**iquid / **L**iquid **C**hromatography (LLC)
2- Gas Chromatography	a- **G**as / **S**olid **C**hromatography (GSC)
	b- **G**as / **L**iquid **C**hromatography (GLC)

Table 3.1. Sumary of Chromatographic Methods

Stationary Phase	**Mobile Phase**	**Method of Application**	**Based on Physical Priciple**
Solid	Liquid	TLC, Column Chrom.	Adsorption (if solid phase is ion exchange resin, ion exchange)
Liquid	Liquid	TLC, Column Chrom., Paper Chrom., HPLC	Partition
Solid	Gas	Gas / Solid Chrom..	Adsorption
Liquid	Gas	Gas / Liquid Chrom.	Partition

Enantiomer Separation in Chiral Compounds:

Equal proportion of the (+) and (-) enantiomers is called racemate. The whole physicochemical properties of the enantiomers are the same except the angle of rotation of polarize light. Since the solubilities of the enantiomers are the same, the resolution of the racemic compounds (enantiomeric separation) is not as simple as the separation of the other mixtures. In general, the conversion of enantiomers to diastereomeric salts facilitates their separation by differences in their physicochemical properties. Some methods applied for this purpose in chromatographic studies are listed below. Also in these methods, the main purpose is to form diastereomeric salts.

a- Separation by using Chiral Derivatizing Agents (CDA):

In the achiral stationary phase, the reaction of the mixture (racemate) with CDA provides separation of diastereoisomeric derivatives.

b- Separation by using Chiral Mobile Phase Additives (CMA):

In the achiral stationary phase, the reaction of the mixture (racemate) with CMA provides separation of diastereoisomeric derivatives. Here, addition of Chiral-Counter-Ions (CCI) to the mobile phase is required.

c- Separation by using Chiral Stationary Phase (CSP):

In the chiral stationary phase, the separation is provided with the diastereomeric complexes are formed by the mixture enantiomers