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Analytical Separation Techniques

Chapter 1- Chromatography

Dr Nouf Alourfi

History

- Mikhail Tswett, Russian, 1872- 1919.
- Botanist
- In 1906 Tswett used to chromatography to separate plant pigments.
- He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column.
- Chroma means "color" and graphein means to "write".



Importance

- Chromatography has application in every branch of the physical and biological sciences.
- 12 Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role.





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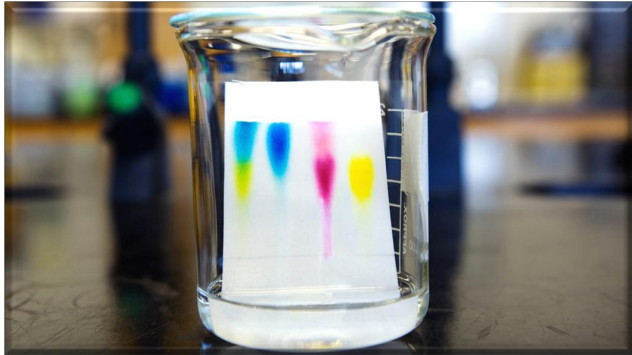
What is chromatography?

- Chromatography (from Greek word for chromos for colour) is the collective term for a family of laboratory techniques for the **separation of mixtures.**

الغاز
في
الزجاج

- It involves passing a mixture which contains the analyte through a stationary phase, which separates it from other molecules in the mixture and allows it to be isolated.

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في
الزجاج



What is chromatography?

Explanation:

- **Mixture:** Two or more substances that are mixed together, but not chemically combined.
- **Solutions:** are mixtures in which one substance is dissolved in another.
- Solutions have two parts:

✓ **solute:** is the substance that is dissolved. like salt

✓ **The solvent:** is the substance that does the dissolving.

Ex
water

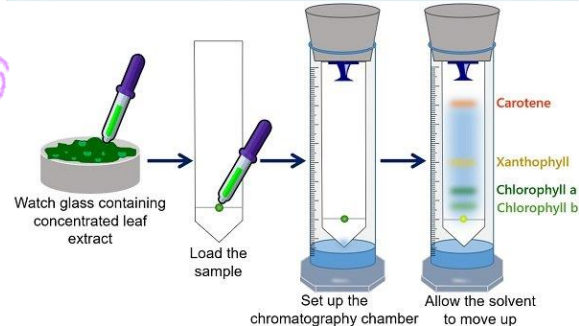
What is chromatography?

- Thus.. We can say... Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, stationary phase which is stationary and mobile phase which moves through it in a definite direction.

- Chromatography separates the components of a mixture by their distinctive attraction to the mobile phase and the stationary phase.

- Mobile phase:** gas or liquid that carries the components.
- Stationary phase:** the part of the apparatus that does not move with the sample.

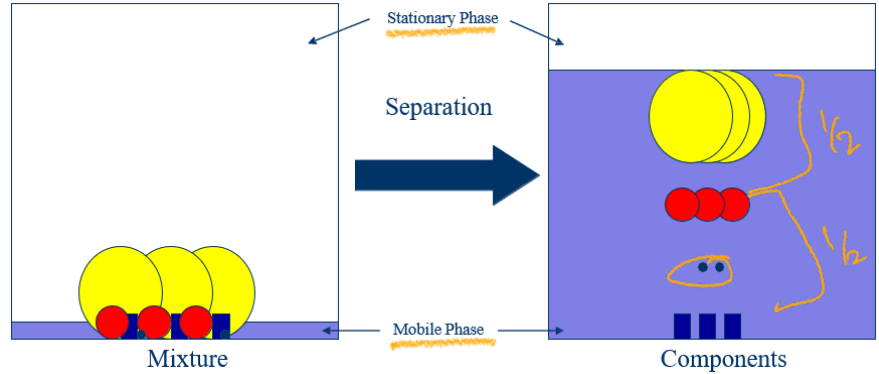
Separation of Plant Pigments through Paper Chromatography



What is chromatography?

Explanation:

- Compound is placed on stationary phase.
- Mobile phase passes through the stationary phase.
- Mobile phase solubilizes the components.
- Mobile phase carries the individual components a certain distance through the stationary phase, depending on their attraction to both of the phases.

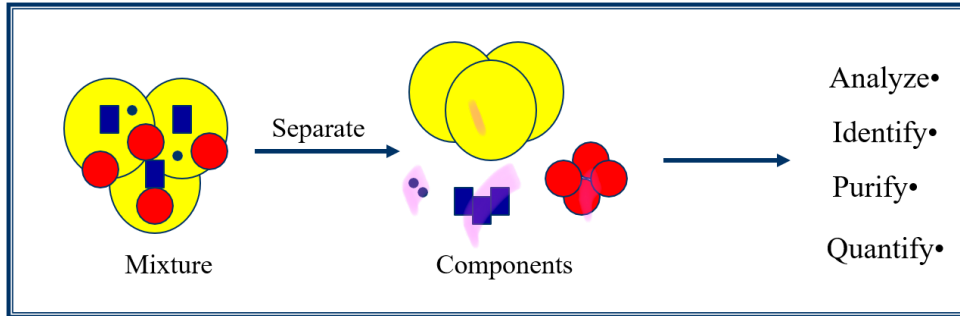


Components	Affinity to Stationary Phase	Affinity to Mobile Phase
Blue	-----	Insoluble in Mobile Phase
Black	~~~~~	√√
Red	~~~~~	~~~~~
Yellow	√√	~~~~~

Uses of Chromatography

Chromatography is a technique used for separating mixtures into their components in order to:

- 1. **Analyze** : examine a mixture, its components, and their relations to one another.
- 2. **Identify** : determine the identity of a mixture or components based on known components.
- 3. **Purify** : separate components in order to isolate one of interest for further study.
- 4. **Quantify** : determine the amount of a mixture and/or the components present in the sample.



Application of Chromatography



Real-life examples of uses for chromatography

- **Pharmaceutical Company** ^{مصانع أدوية}: determine amount of each chemical found in new product.
- **Hospital**: detect blood or alcohol levels in a patient's blood stream.
- **Law Enforcement**: to compare a sample found at a crime scene ^{1AB} to samples from suspects.
- **Environmental Agency** ^{بيئية}: determine the level of pollutants in the water supply.
- **Manufacturing Plant**: to purify a chemical needed to make a product.

Factors affect the separation in the chromatographic techniques

العوامل

Separates components in mixture can be affected by:

- ✓ polarity القطبية
- ✓ boiling point نقطة الغليان
- ✓ ionic strength قوة الأيونات
- ✓ size الحجم



معامل التوزيع

Distribution Coefficient and Retention Fraction value (Rf value)

☆ **Distribution Coefficient** =
$$\frac{\text{Concentration of component in stationary phase}}{\text{Concentration of component in mobile phase}}$$

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☆ Due to **difference in distribution coefficient** of various component of mixture, **their mobility** in two phases **differ**. Hence, **separation occur**.

■ Measure of this mobility is called **Retention Fraction value (Rf value)**.

■ **Rf** =
$$\frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

المادة المراد فصلها
المسافة التي يقطعها
المادة
المذيب

Distribution Coefficient and Retention Fraction value (Rf value)

Example 1:

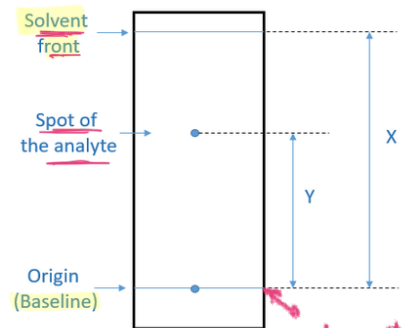
If one component of a mixture travelled 9.6 cm from the baseline while the solvent had travelled 12.0 cm, calculate the Rf value for that component.

المسافة
المسافة

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

$$R_f = \frac{9.6}{12.0} = 0.8$$

$\times 100$
 $= 80\%$



$$R_f = Y \div X$$

solvent

Distribution Coefficient and Retention Fraction value (RF value)

Example 2:

In TLC, the distance from the baseline to the centre of (component-A) spot is 6.7 cm and (component-B) spot is 7.9 cm. If the solvent front is 10.9 cm.,

1- Calculate the retention factor for the both components A and B.

2- Explain that with simple drawing.

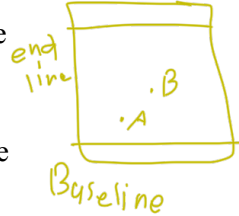
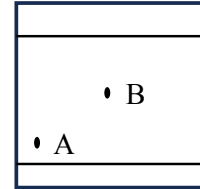
$$\text{1- } R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

$$R_f A = \frac{6.7}{10.9} = 0.6$$

$$R_f B = \frac{7.9}{10.9} = 0.72$$

کے لئے RF
مقدار
میں

mobile
Phase
↑ 20.2%



Distribution Coefficient and Retention Fraction value (Rf value)

Example 3:

A mixture of components (O + P) gave the following result in Paper Chromatography (Fig. right).

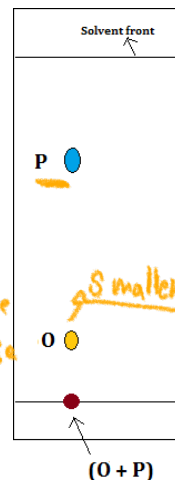
Component O is adsorbed ..

A) more strongly onto the stationary phase and has a larger Rf value than component P.

B) more strongly onto the stationary phase and has a smaller Rf value than component P.

C) less strongly onto the stationary phase and has a smaller Rf value than component P.

D) less strongly onto the stationary phase and has a larger Rf value than component P.



Classification of chromatography methods

There are three classification schemes:



- Based on physical state of the mobile phase.
- Based on interaction of solute (analyte) with the stationary phase.
- Based on chromatographic bed shape.

▪ Based on physical state of the mobile phase:

GC/LC

- 1- gas chromatography (GC).
- 2- Liquid chromatography (LC).
- 3- supercritical fluid chromatography (SCFC).

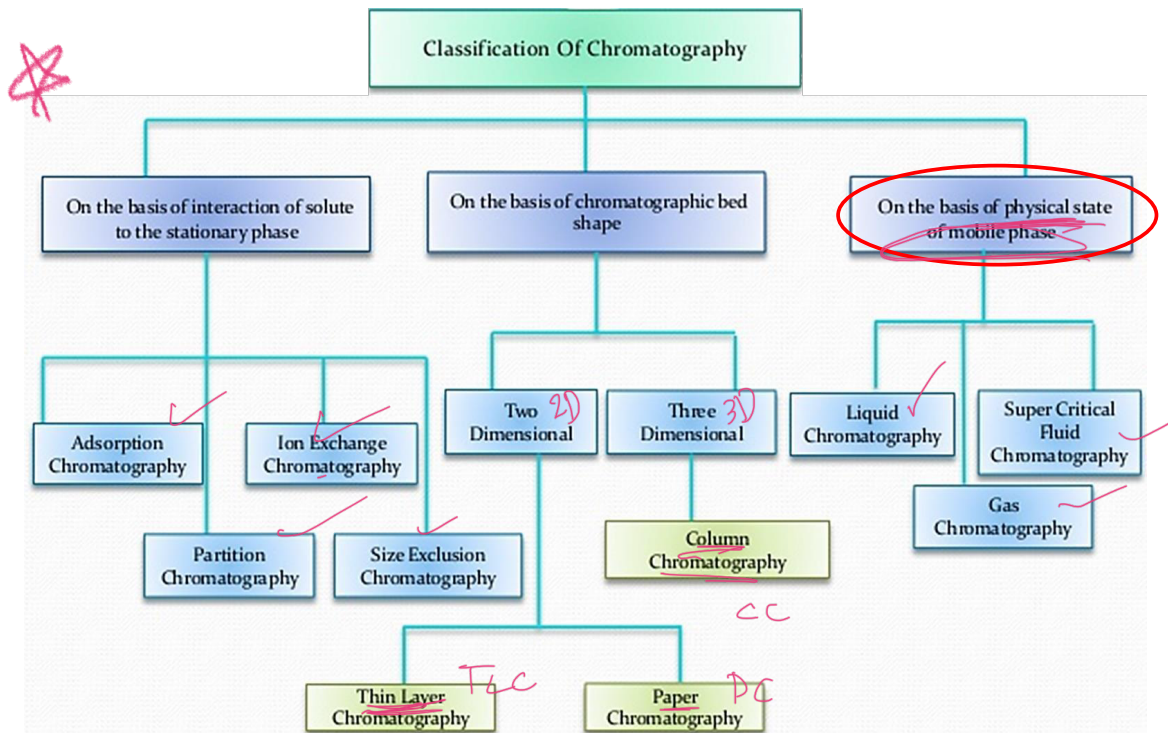
▪ Based on interaction of solute (analyte) with the stationary phase:

- 1- Adsorption الامتزاز
- 2- Ion Exchange التبادل الأيوني
- 3- Partition التقسيم
- 4- Size Exclusion

▪ Based on chromatographic bed shape:

- 1- 2D [Thin-Layer Chromatography (TLC), Paper Chromatography (PC)].
ورق التبريم
- 2- 3D (Column chromatography).

Classification of chromatography methods



Classification of chromatography methods

LC	GC	المحبة
سائل	غاز	M.P
L	غاز	

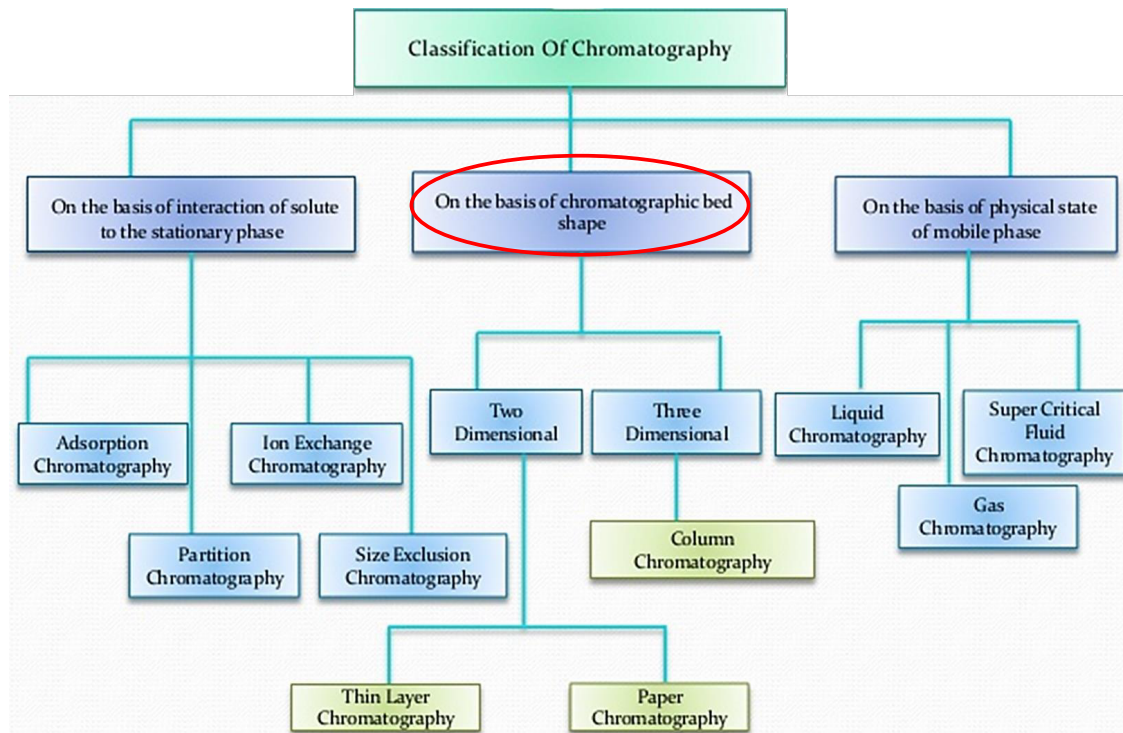


- Based on physical state of the mobile phase.

1- gas chromatography (GC): ^{يفصل العينات المتبخرة} separates vaporized samples with a carrier gas (mobile phase) and a column composed of a liquid or of solid beads (stationary phase).

2- Liquid chromatography (LC): ^{يفصل العينات السائلة} separates liquid samples with a liquid solvent (mobile phase) and a column composed of a of solid beads (stationary phase).

Classification of chromatography methods

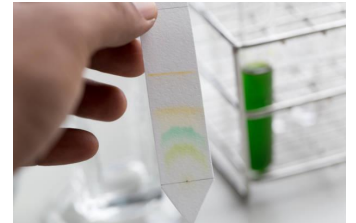
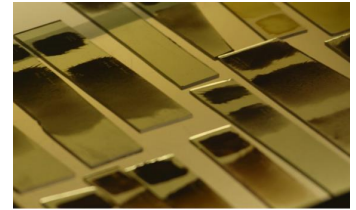


Classification of chromatography methods

- Based on chromatographic bed shape

1- 2D [Thin-Layer Chromatography (TLC), Paper Chromatography (PC)]

- ✓ **Thin-Layer Chromatography:** Uses thin plastic or glass trays to identify the composition of pigments, chemicals, and other unknown substances.
- ✓ **Paper Chromatography:** Can be used to separate the components of inks, dyes, plant compounds (chlorophyll), make-up, and many other substances. The liquid is able to move up the filter paper because its attraction to itself is stronger than the force of gravity.



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أفوكو فسي

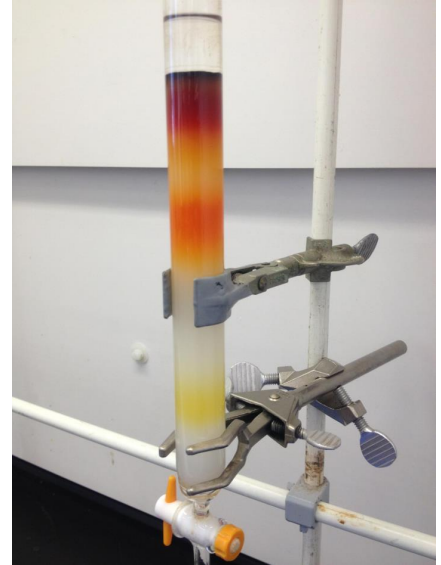
Classification of chromatography methods



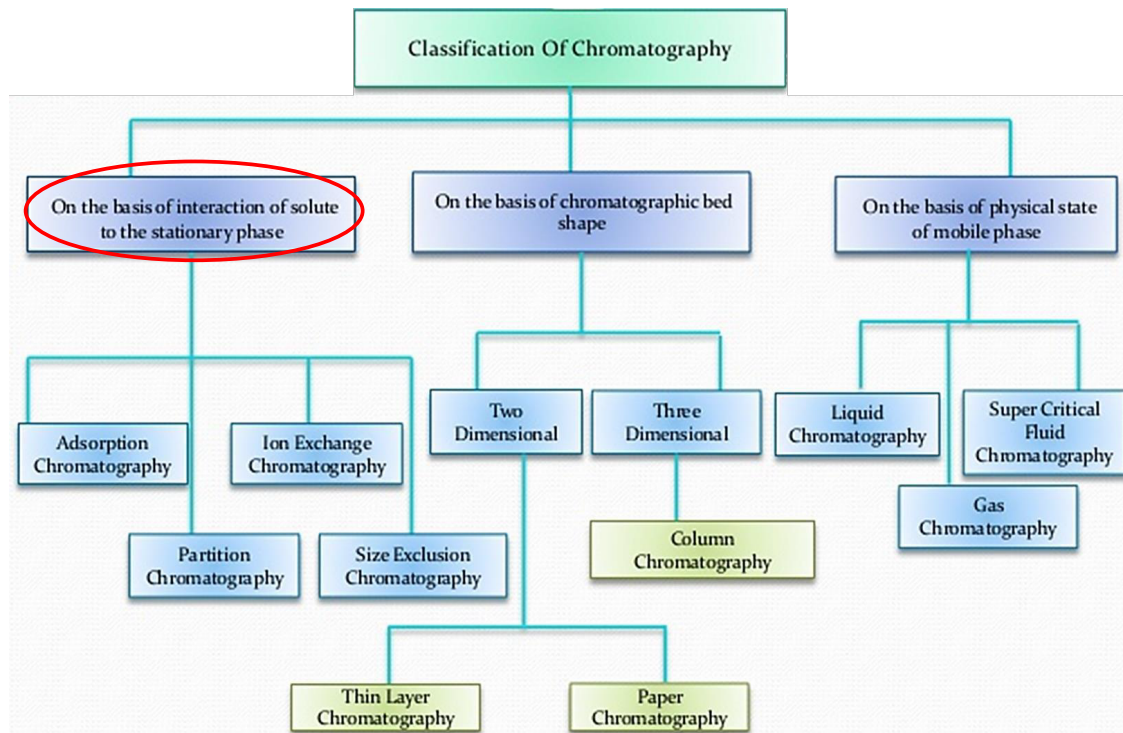
- Based on chromatographic bed shape

2- 3D (Column chromatography).

- The main vessel used for this chromatographic process is a column.
- The stationary phase is set up inside the tube and filled with solid stationary components.
- The mobile phase is passed along the stationary solid phase, which adsorbs the solute particles from the mixture.



Classification of chromatography methods



Classification of chromatography methods

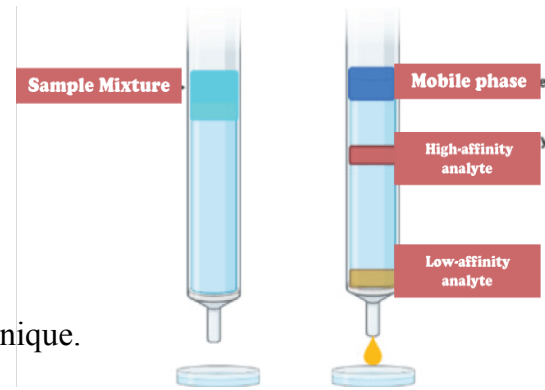
- Based on interaction of solute (analyte) with the stationary phase.

1- Adsorption:

- Adsorption chromatography is the oldest type of chromatography technique.

* The principle of adsorption chromatography is that different molecules or ions can be adsorbed (stuck) onto the surface stationary phase to different degrees, according to how strongly they are adsorbed (their different affinity to stationary phase).

- Analytes that have a strong affinity to stationary phase will stay on the column longer.
- The stationary phase is usually a porous material such as activated carbon, alumina, or silica gel.
- The sample is then allowed to migrate through the column by the force of gravity.



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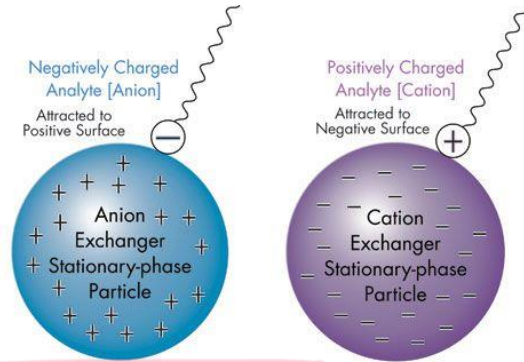
قوة الجاذبية

Classification of chromatography methods

- Based on interaction of solute (analyte) with the stationary phase.

2- Ion Exchange.

- It helps in separating ions and polar molecules based on their difference in charge properties.
- Ion-exchange chromatography is a form of chromatography that separates ions and polar molecules based on their affinity to the ion exchanger.
- ① The cationic exchanger consists of negatively charged groups and attracts positively charged cations in the mobile phase.
- The anionic exchanger consists of positively charged groups and attracts negatively charged anions.



Classification of chromatography methods

- Based on interaction of solute (analyte) with the stationary phase.

3- Partition

The chromatography technique which is based on the partitioning of components of a mixture between stationary and mobile phases.

- ✓ **Normal**: analyte is nonpolar organic; stationary phase MORE polar than the mobile phase.
- ✓ **Reverse**: analyte is polar organic; stationary phase LESS polar than the mobile phase.

	Stationary phase (st)	Mobile phase (m.p)	
✓ Normal	Polar	non polar	↑
✓ Rev	non polar	Polar	↓

Reverse

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المحلول
المحلول

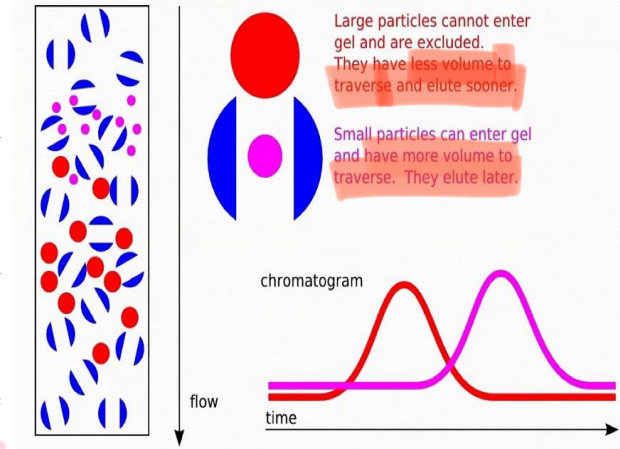
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Classification of chromatography methods

- Based on interaction of solute (analyte) with the stationary phase.

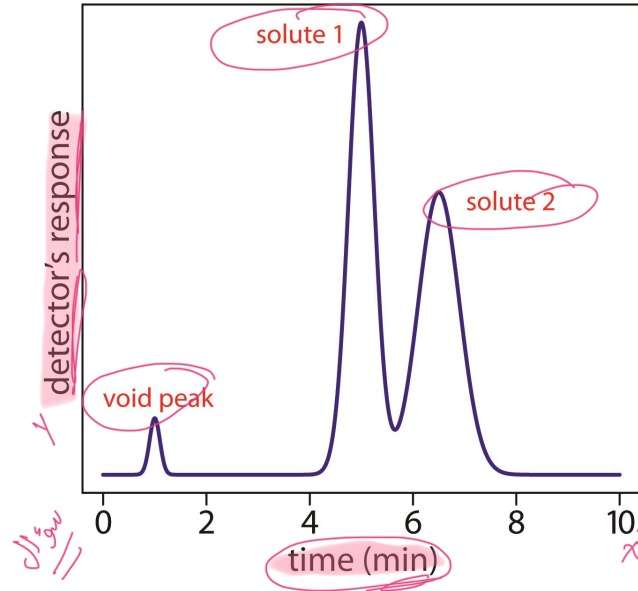
4- Size Exclusion

- It separates the molecules **based on their size** by filtration through a gel. It is also known as gel-filtration chromatography.
- The chromatography column is packed with porous beads such as polymers. The pore size of the beads is varied and different.
- The mobile phase is passed through the column where the smaller molecules are trapped in gel pores, whereas larger molecules are released out. Thus, the small molecules are later eluted out through an elution process.



Chromatogram

- A chromatogram is essentially the output of a chromatography run.
- It is a two-dimensional plot with the y-axis representing detector response for concentration, and the x-axis representing time.
- The height of the peak in the detector response depends on concentration of the particular component.





Analytical Separation Techniques

Chapter 2- Paper Chromatography

Dr Nouf Alourfi

Paper Chromatography- definition

يستخدمون رقاقة لا صلبة
ليتم تشييع عثبات تكون مركزة في نقطة واحدة

Simple chromatography

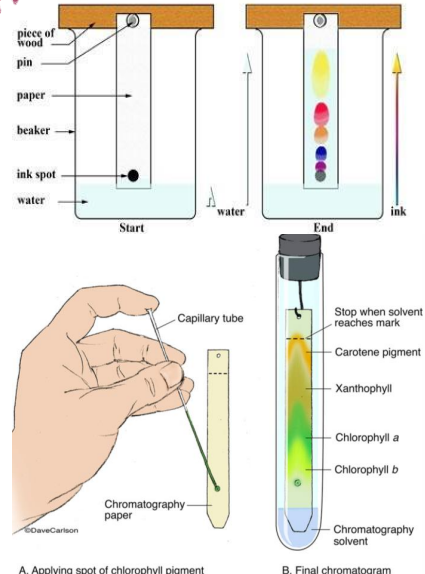
- A method of partition chromatography using **filter paper strips** as carrier or inert support (Stationary Phase) and liquid solvent as **mobile phase** to separate **dried liquid samples**.

- **Stationary Phase**: **Filter paper sheet**. The filter paper sheet consists of **cellulose (solid)** and **water (liquid)**.

- **Mobile phase**: **liquid** (usually mixture of solvents).

يكون
Water only

Water

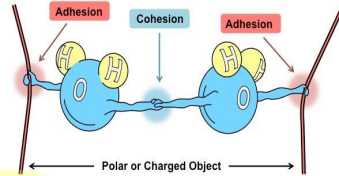


A. Applying spot of chlorophyll pigment

B. Final chromatogram

Principles of Paper Chromatography

- **Capillary Action:** the movement of liquid within the spaces of a porous material due to the forces of adhesion, cohesion, and surface tension. The liquid is able to move up the filter paper because its attraction to itself is stronger than the force of gravity.



- **Solubility:** the degree to which a material (solute) dissolves into a solvent.

Solutes dissolve into solvents that have similar properties. (Like dissolves like) This allows different solutes to be separated by different combinations of solvents.

- **Separation of components depends on** both their solubility in the mobile phase and their differential affinity to the mobile phase and the stationary phase.

Factors affect The mechanism of separation in paper chromatography

- The mechanism of separation in paper chromatography depend on **two forces**:

1- Retarding Force قوة التثبيط

2- Propelling Force قوة الدفع



Factors affect The mechanism of separation in paper chromatography

1-Retarding Force

→ stationary phase

■ Adsorption

Related to the cellulose exist in the filter paper as stationary phase. The substance which is more adsorbed on the cellulose will be retarded so, it will be near the baseline.

■ Partition

Related to the water exist in the filter paper as stationary phase. The substance which is more soluble in the water of paper will be retarded so, it will be near the baseline.

Factors affect The mechanism of separation in paper chromatography

2- Propelling Force

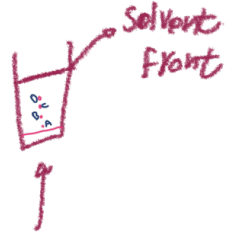
mobile phase

■ Solubility

The component which is more soluble in the mobile phase will be close to the solvent front.

■ Solvent flow

The flow of solvent running on the filter paper stationary phase depend on many factors such as viscosity, density, temperature,----etc where as the solvent flow increase as the components become more closer to the solvent front.



المواصل

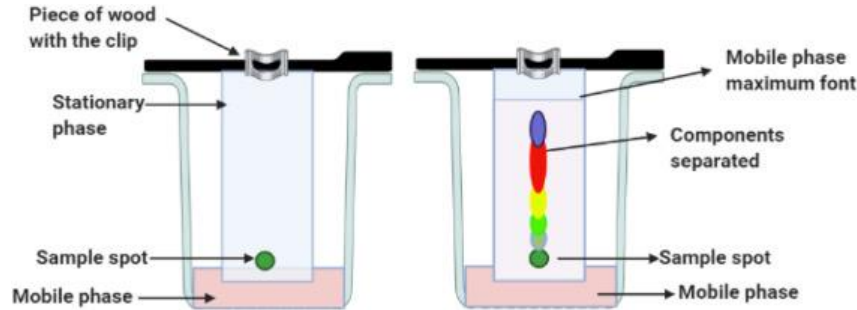
Factors affect The mechanism of separation in paper chromatography

عوامل

The factor governing the separation of mixtures of solutes on filter paper is mainly the partition between two phases.

Where:

- ✓ One is usually water adsorbed on cellulose fibres in the paper (stationary phase).
- ✓ The second is the organic solvent (mobile phase) flows past the sample on the paper.



Factors affect The mechanism of separation in paper chromatography

Partition occurs between the mobile phase and the stationary aqueous phase bound by the cellulose.

The isolation depends on partition coefficient of the solute.

- $K = \frac{\text{Concentration of component in stationary phase}}{\text{Concentration of component in mobile phase}}$

Types or modes of paper chromatography

Based on the way the development of chromatogram on the paper is done in the procedures, we have broadly 5 types of chromatography.

1- Ascending chromatography:

As the name indicates, the chromatogram ascends. Here the development of paper occurs due the solvent movement or travel in upward direction on the paper. The solvent reservoir is at the bottom of beaker. The paper tip with sample spots just dips into the solvent at the bottom such that spots remain well above the solvent.

Ascending upward
Descending downward

Types or modes of paper chromatography

2- Descending chromatography:

Here the development of paper occurs due to the solvent travel downwards on the paper.

The solvent reservoir is at the top. The movement of solvent is assisted by gravity besides capillary action.

3- Ascending- descending mode: 1 on 2

Here the solvent first travels upwards and then down wards on the paper.



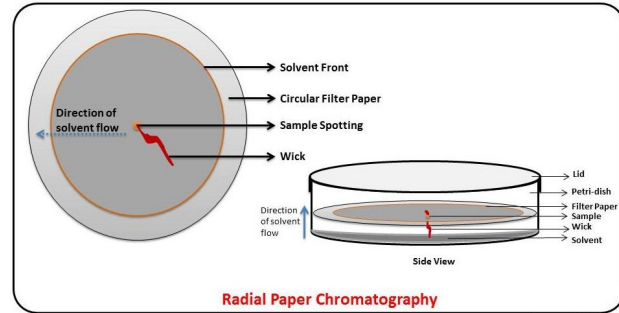
Types or modes of paper chromatography

4. Radial mode:



Here the solvent travels from centre (mid-point) towards periphery of Circular chromatography paper. The entire system is kept in a covered petridish for development of chromatogram.

The wick at the centre of paper dips into mobile phase in a petridish by which the solvent drains on to the paper and moves the sample radially to form the sample spots of different compounds as concentric rings.



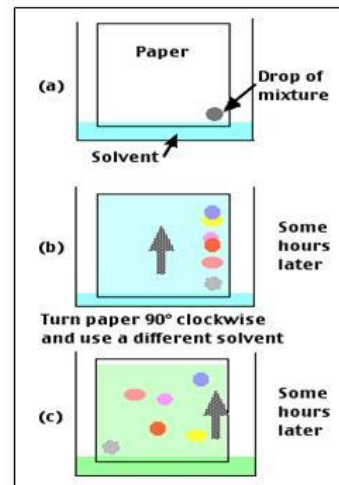
Namrata Heda

Types or modes of paper chromatography

5- Two-dimensional chromatography:

When large numbers of substances are to be separated on a single chromatogram two-dimensional chromatography is used. Development in a direction perpendicular to the first, and with a solvent system different from that used initially is often necessary. The sample is applied on one corner of a square piece of paper and after development with the first solvent, the paper is dried, rotated 90° and developed in the second direction.

Usually, different types of solvents systems are used in each direction. It is essential that the first solvent be completely volatile.



General Procedure

- 1- Choice of paper and solvent to be used.
- 2- Desalting of sample.
- 3- Application of the sample.
- 4- Equilibration of paper.
- 5- Development.
- 6- Detection.
- 7- Identification of substances.

General Procedure

1- Choice of paper and solvent to be used.

2- Desalting of sample.

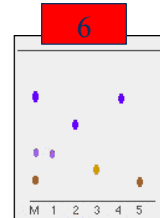
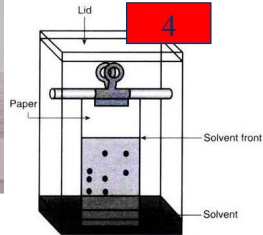
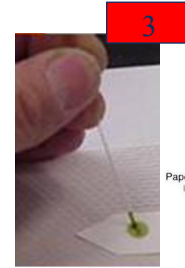
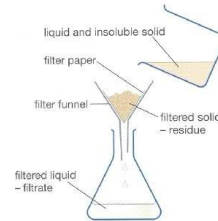
3- Application of the sample.

4- Equilibration of paper.

5- Development.

6- Detection.

7- Identification of substances.



7- Identification of substances

after spraying with ninhydrin

Paper chromatography experiment method

The experimental method involves:

a) **Selection of suitable type of development:** This depends on complexity of the mixture, solvent, paper etc. But in general ascending type or radial type of chromatography are used as they are easy to perform, handle, less time consuming and give chromatogram faster.

b) **Selection of suitable filter paper:** Filter paper is selected based on pore size, quality of the sample to be separated and also mode of development.

Paper chromatography experiment method

c) **Preparation of sample:** Preparation of sample involves dissolution of sample in suitable solvent used in making mobile phase. The solvent used should be inert with the sample under analysis.

d) **Spotting of sample on the paper.** Samples are to be spotted at proper position on the paper using preferably a capillary tube

e) **Development of chromatogram:** Sample spotted paper is subjected to development by immersing it in the mobile phase. The mobile phase moves over the sample on the paper under the capillary action of paper.

Paper chromatography experiment method

f) Drying of the paper and detection of the compounds:

Once the development of chromatogram is over, The paper is held carefully at the borders so as to avoid touching the sample spots and dried using an air drier. Sometimes the detecting solution (locating agent) is sprayed in the developed paper and dried **to identify the sample chromatogram spots.**

بالمين العنبرية

✱ Coloured compounds: Identified by naked eyes.

✱ Colourless compounds: Identified by UV or Chemical methods:

Uses and applications of paper chromatography

تعداد

- Paper chromatography is specially used for separation of mixture having polar and non-polar compounds.
- For separation of amino acids.
- It is used to determine organic compounds, biochemicals in urine etc.
- In pharma sector for determination of hormones, drugs.
- Sometimes used for evaluation of inorganic compounds.
- like salts and complexes.



Analytical Separation Techniques

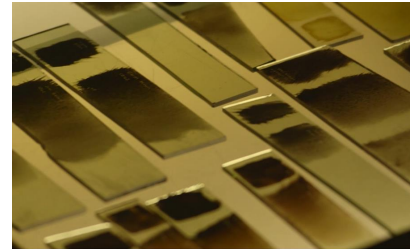
Chapter 3- Thin layer chromatography (TLC)

Dr Nouf Alourfi

Thin layer chromatography (TLC)- definition

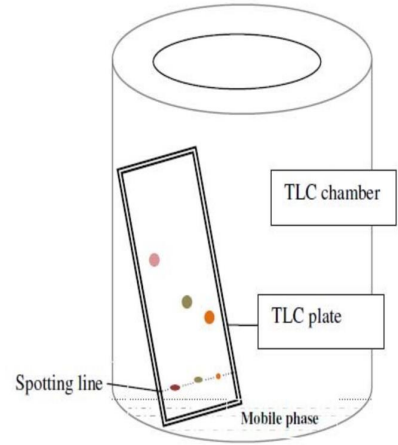
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- TLC is a method for identifying substances and testing the purity of compounds.
- TLC is a useful technique because it is relatively quick and requires small quantities of material.
- ^{عمل} Separations in TLC involve ^{توزيع الخليط} distributing a mixture of two or more substances between a stationary phase and a mobile phase.



Thin layer chromatography (TLC)- definition

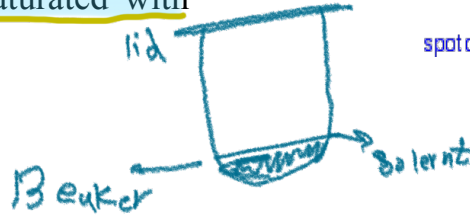
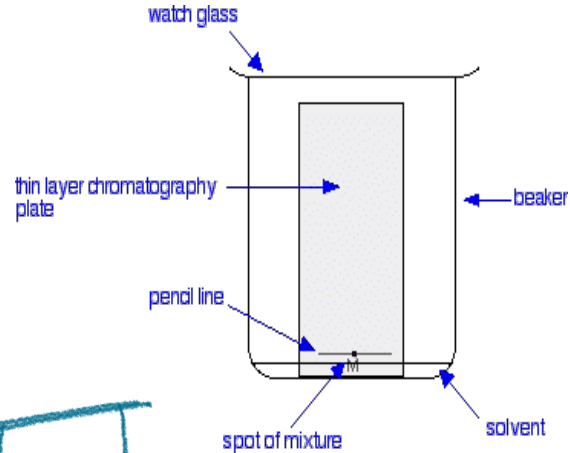
- **Stationary Phase:** is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate made of plastic or glass.
- **Mobile phase:** is a developing liquid which travels up the stationary phase, carrying the samples with it.
- Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase **versus** how much they dissolve in the mobile phase.



Thin layer chromatography (TLC)- Preparing the Chamber

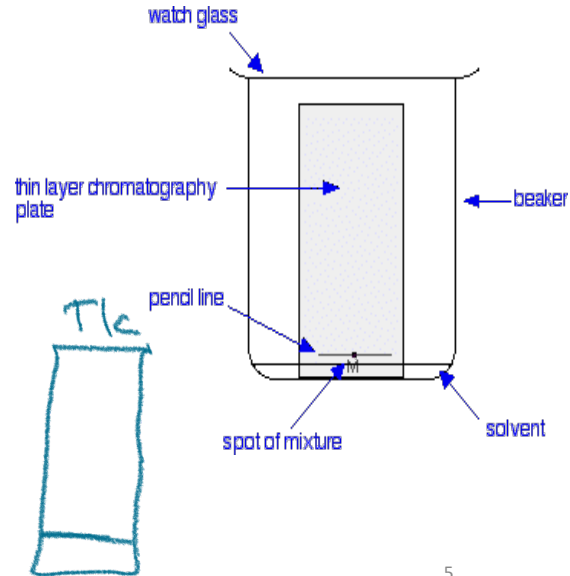
- To a jar with a tight-fitting lid add enough of the appropriate developing liquid so that it is 0.5 to 1 cm deep in the bottom of the jar.

- ✱ Close the jar tightly, and let it stand for about 30 minutes so that the atmosphere in the jar becomes saturated with solvent.



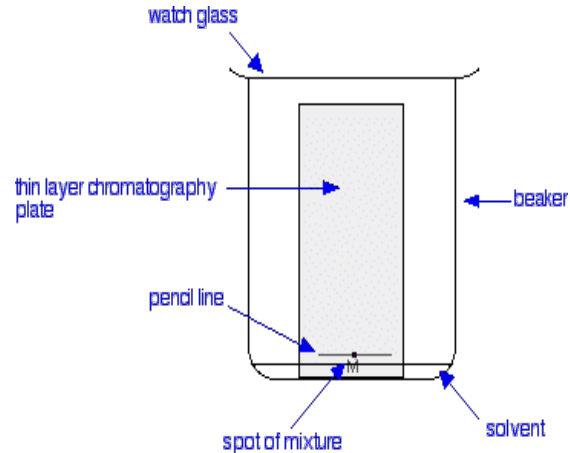
Thin layer chromatography (TLC)- Preparing the Chamber

- With a pencil, draw a line about 2 cm from the bottom of the plate.
- The line must be farther from the bottom of the plate than the depth of the solvent in the jar.
- Using a drawn-out capillary tube, spot the samples on the plate so that they line up with the line you have drawn.



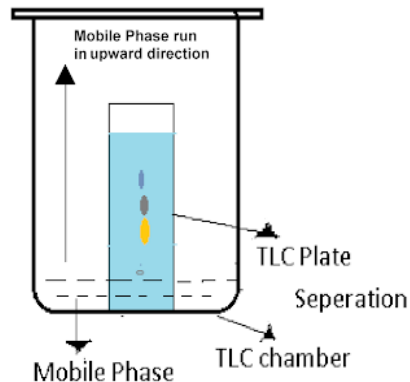
Thin layer chromatography (TLC)- Preparing the Chamber

- After preparing the development chamber and spotting the samples, the plates are ready for development.
- Be careful to handle the plates only by their edges, and try to leave the development chamber uncovered for as little time as possible.
- When the plates are removed from the chamber, quickly trace the **solvent front** (the highest solvent level on the plate) with a pencil.



Thin layer chromatography (TLC)- Identifying the Spots (visualization)

- If the spots **can be seen**, outline them with a **pencil**.
- If no spots are obvious, the most common visualization technique is to hold the plate under a **UV lamp**.
- Many organic compounds can be seen using this technique, and many commercially made plates often contain a substance which aids in the visualization of compounds (Visualizing Agents).



Thin layer chromatography (TLC)- Visualizing Agents

بعض

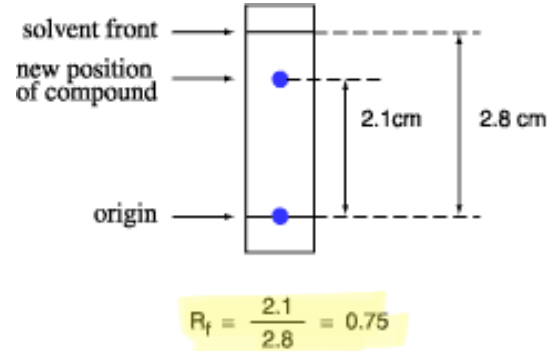
An examples of Visualizing Agents:

- **For Alkaloids:** The agent is Dragendorff's reagent, give a **Orang** or **red** colour appears.
- **For Sugar:** The agent is Aniline phthalate, give a **bright red** colour.
- **For Amino acids:** The agent is Ninhydrin, give a **violet** colour appears



Thin layer chromatography (TLC)- Interpreting the Data

- The **R_f** (retention factor) value for each spot should be calculated.
- It is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates.
- Hence, known R_f values can be **compared** to those of unknown substances to aid in their identifications.



Thin layer chromatography (TLC)- Interpreting the Data



A hand-drawn diagram of a TLC plate. A horizontal line represents the solvent front. A spot is marked with a blue 'X' at a distance from the bottom. The distance from the bottom to the spot is labeled 'Distance travelled by substance'. The distance from the bottom to the solvent front is labeled 'Distance travelled by solvent'.

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

- (Note: R_f values often depend on the temperature and the solvent used in the TLC experiment).
- the most effective way to identify a compound is to spot known substances – authentic - next to unknown substances on the same plate.
- In addition, the purity of a sample may be estimated from the chromatogram.
- An impure sample will often develop as two or more spots, while a **pure sample will show only one spot**

Thin layer chromatography (TLC)- Summary

- A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). *sta*
- A small amount of the mixture to be analyzed is spotted near the bottom of this plate.
- The TLC plate is then placed in a chamber containing a very small amount of liquid at the bottom of the plate.
- This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. *again so is*
- As the solvent moves past the spot that was applied, an equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are dissolve in the mobile phase.

Thin layer chromatography (TLC)- Summary

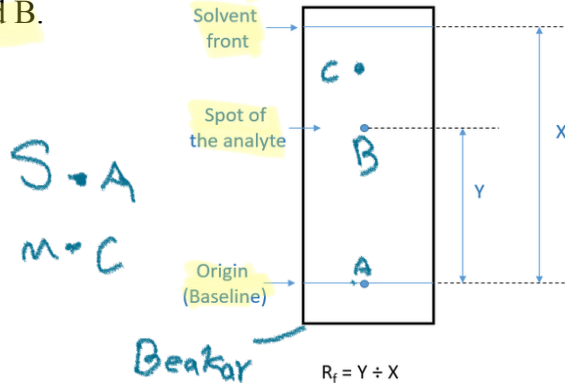
- In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others.
- When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized.
- If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp or chemical method is used to visualize the plates.

Thin layer chromatography (TLC)- Problem



In TLC, the distance from the baseline to the center of component-A spot is 6.7 cm and component-B spot is 7.9 cm. If the solvent front is 10.9 cm.

Explain with the help of a simple drawing and calculate the retention factor for the both components A and B.



Handwritten notes in yellow: 'sub b', 'm = C', and 'Beakar'.