High Performance Liquid Chromatography (HPLC) Analytical and Separation

By Ben Jones

A Brief History

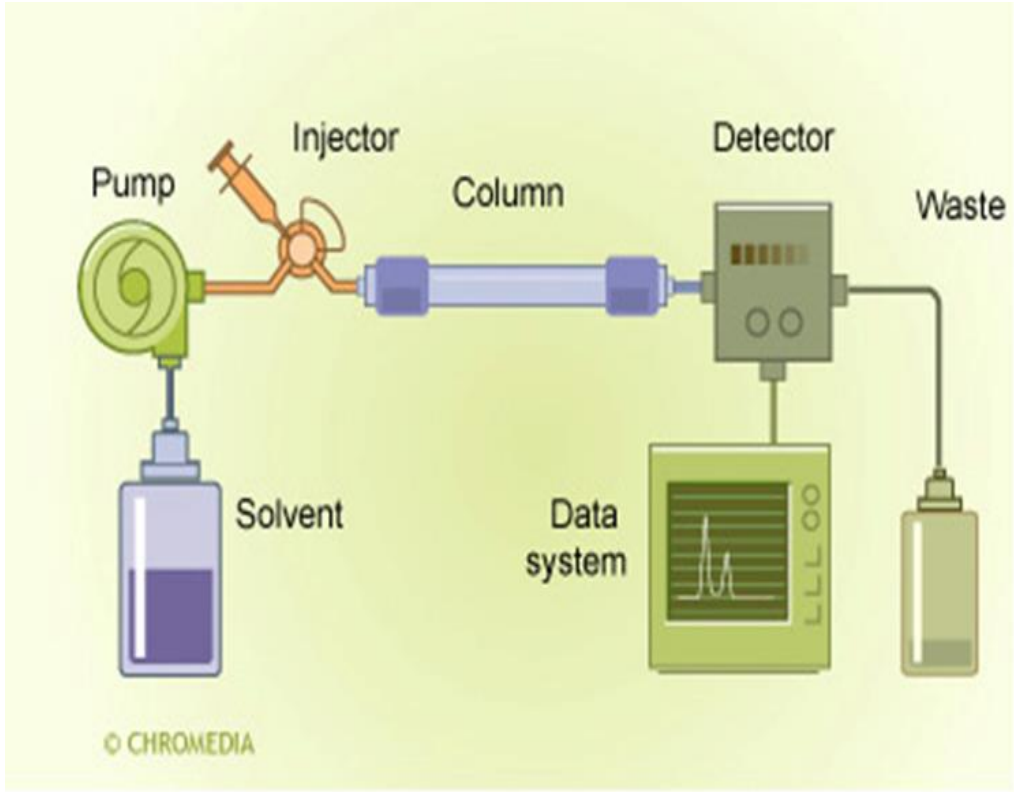
- Chromatography was discovered in 1903 and was initially used to separated plant pigments.
- However, it wasn't until the 1960s that Cal Giddings, Josef Huber, and others predicted that LC could be operated in the high-efficiency mode by reducing the packing-particle diameter substantially below the typical LC level of 150 µm and using pressure to increase the mobile phase velocity.

Why HPLC?

- Compared to other chromatographic techniques, such as TLC, HPLC is extremely quick and efficient.
- It uses a pump, rather than gravity, to force a liquid solvent through a solid adsorbent material, with different chemical components separating out as they move at different speeds.
- The process can be completed in roughly 10 to 30 minutes, and it delivers high resolution. It is accurate and highly reproducible.

Components of HPLC

- 1. Solvent: Used to elute sample through column.
- 2. Pump: Pumps the mobile phase at a specific flow rate in mL/min. The pump pressure is normally between 400-600 bar.
- 3. Injector: Introduces the sample into the column (about 5-20 µL).
- 4. Column: Provides separation through high pressure created by the small particles.
- 5. Detector: It quantifies and identify the sample components and provides information to the computer.
- 6. Computer: Takes the signals from the detector and displays the retention times and quantity of the components.



Our Machine

1. Solvent
2. Pump
3. Injector
4. Column
5. Detector
6. Computer

Types of Columns

- Normal phase: Column packing is polar (e.g silica) and the mobile phase is non polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers and chiral compounds.

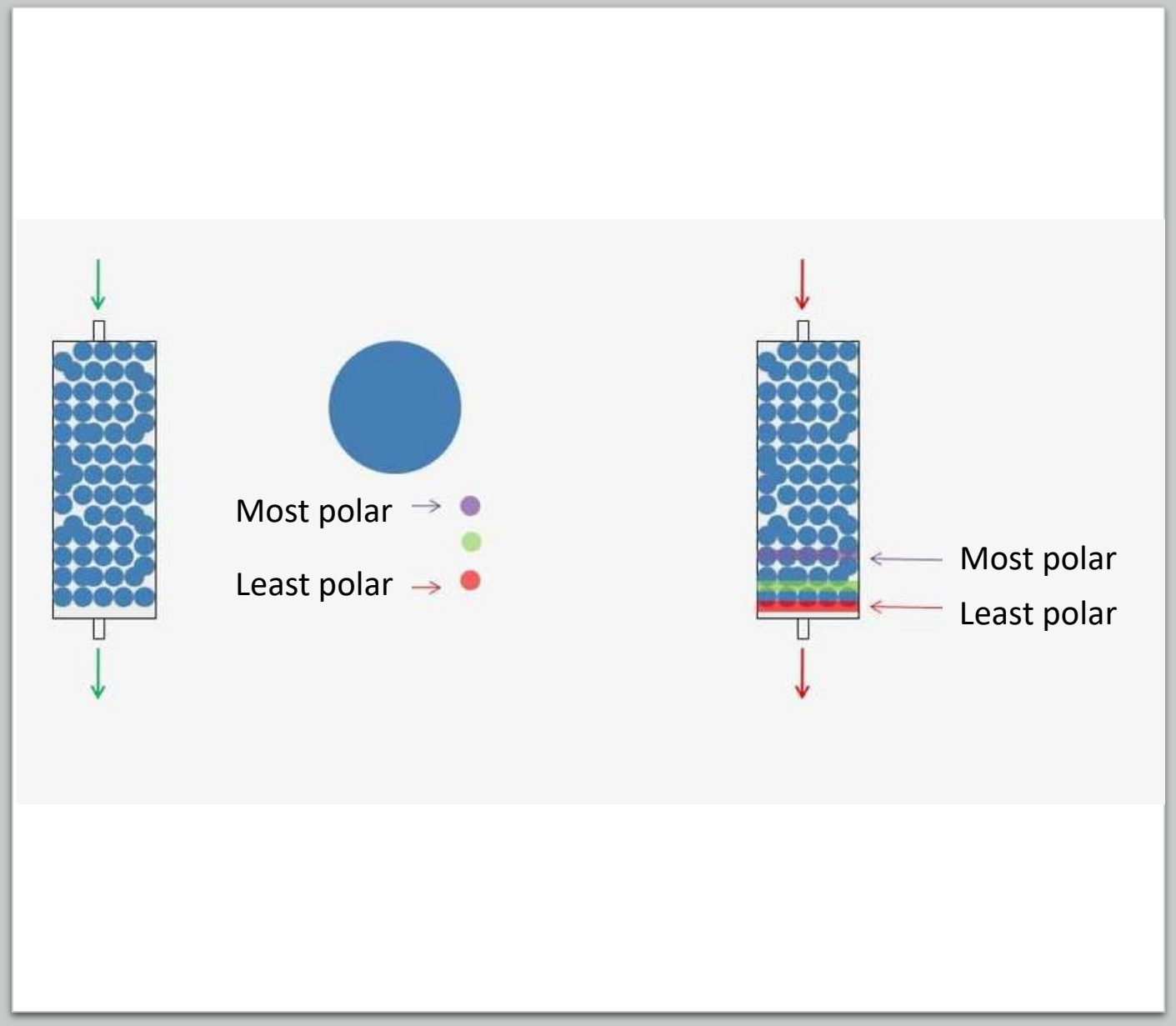
- Reverse phase: the column packing is non-polar (e.g C18) , mobile phase is water + miscible solvent (e.g methanol). It can be used for polar, non polar, ionizable and ionic samples.

- Ion exchange: Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

- Size exclusion: molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

Normal Phase

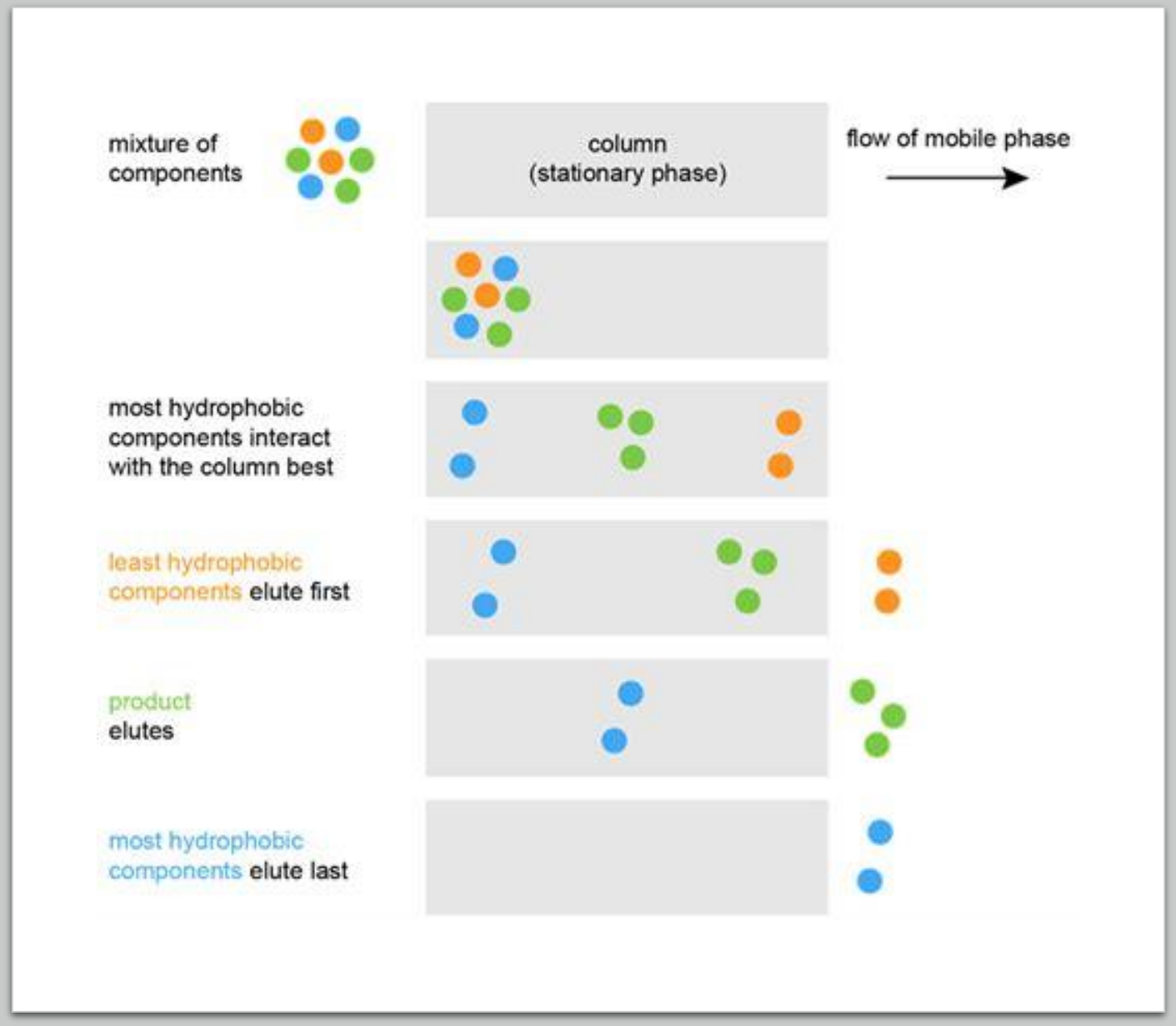
- Polar stationary phase and non-polar solvent.
- Polar compounds in the mixture being passed through the column will stick longer to the polar silica while the non-polar compounds will pass through.



Reverse Phase

- Non-polar stationary phase and polar solvent.
- Non-polar compounds (hydrophobic) in the mixture being passed through will stick longer to the column while the polar compounds will pass through first.

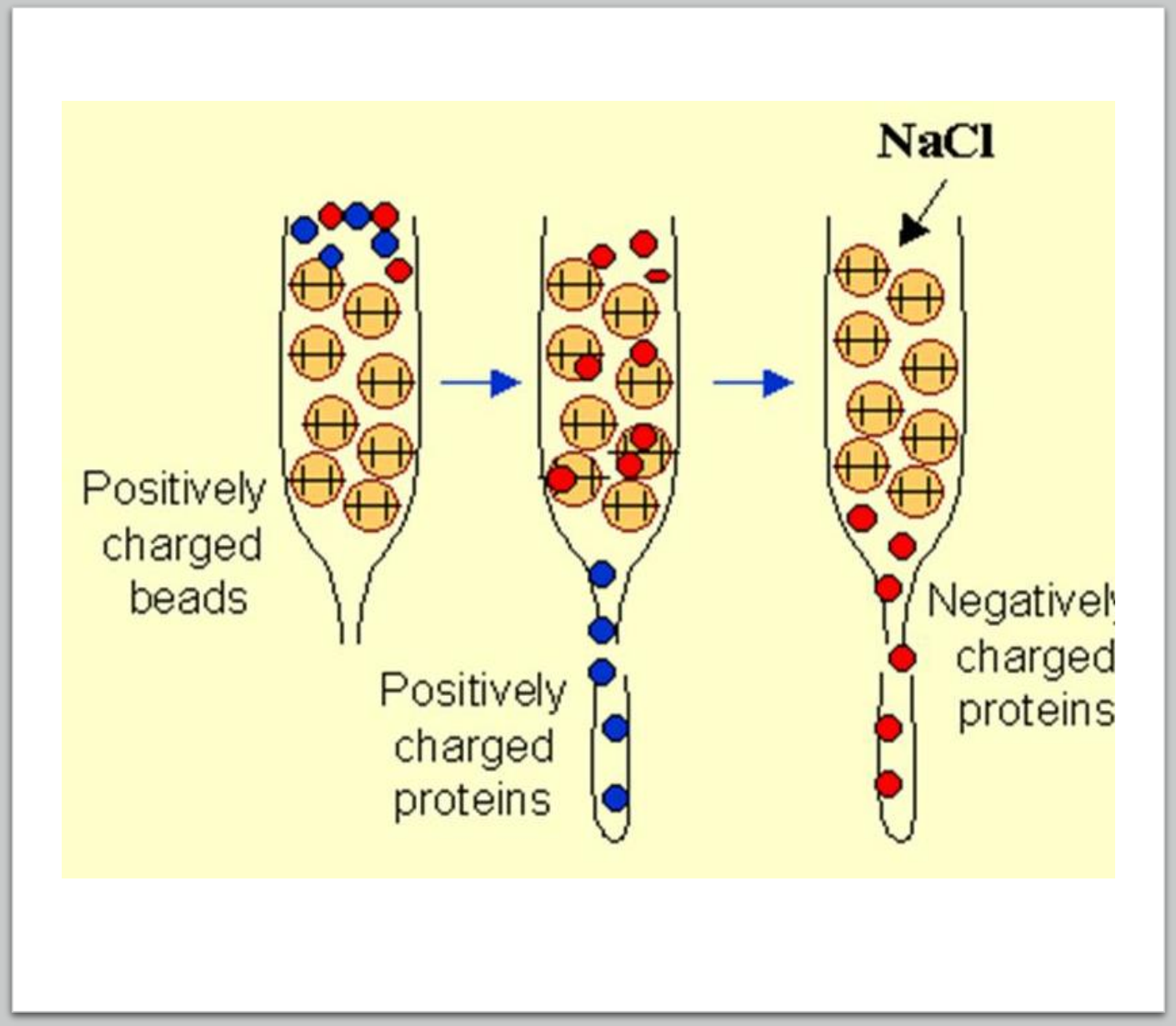
- Applications: Most of the applications in HPLC require the evaluation of drugs, biochemical molecules and other substances used by humans and they are polar (water soluble) in nature. So, reverse phase HPLC is widely used.



Ion Exchange

- Positively charged column/beads.
- Negatively charged compounds in the mixture being passed through will interact with the beads while the positively charged compounds will pass through.

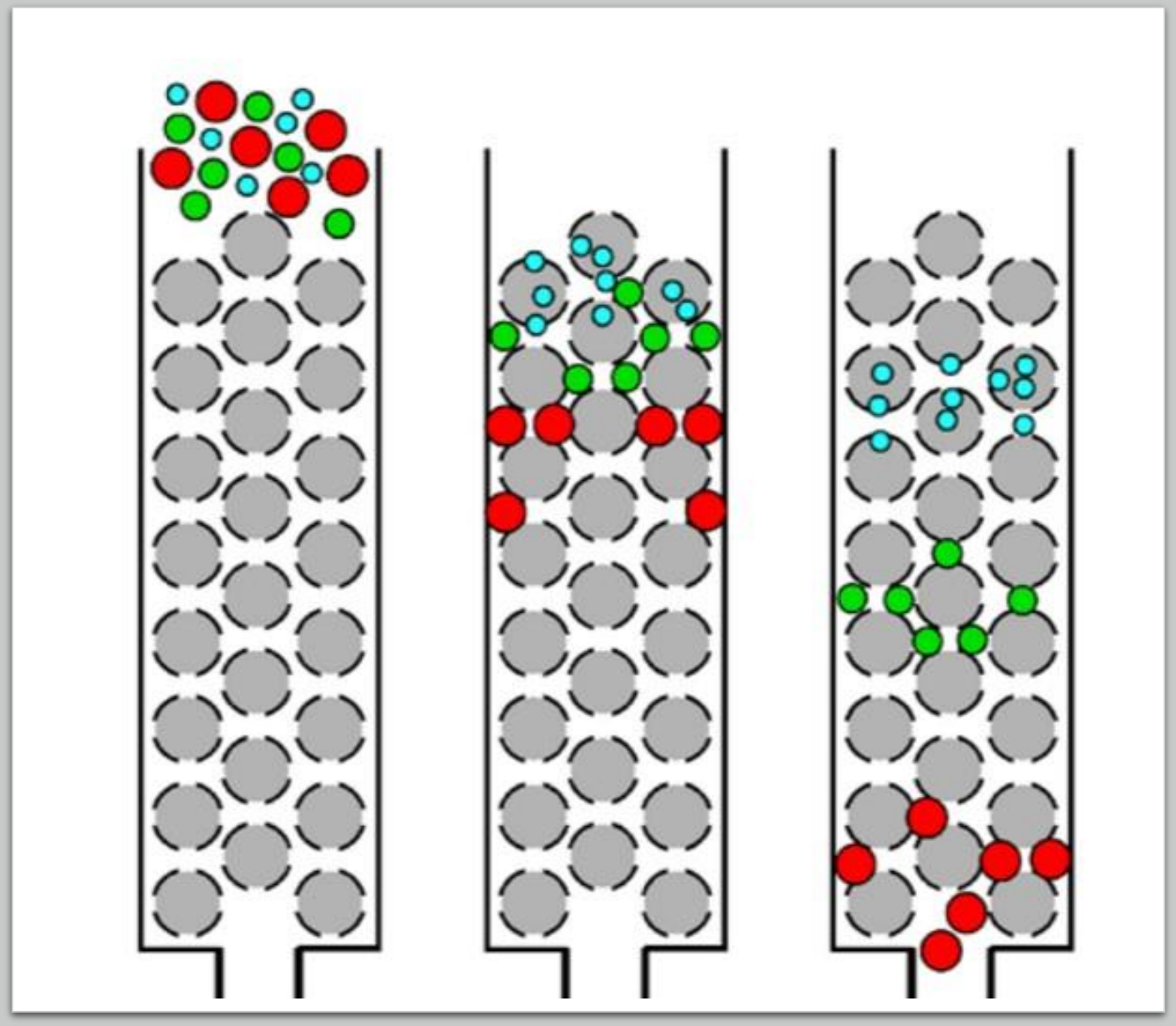
- Applications: It is used specifically for separation and estimation of acidic and basic compounds.



Size Exclusion

- Porous beads as stationary phase.
- Smaller compounds are captured in pores and retained.
- Larger compounds do not interact and pass through.

- Applications: Size-exclusion chromatography is applied for separation of macromolecules (proteins, polys accharides) by molecular weights.



Techniques of HPLC

Analytical

Analytical chromatography is used to determine the existence and possibly also the concentration of analyte(s) in a sample.

Semi-preparative

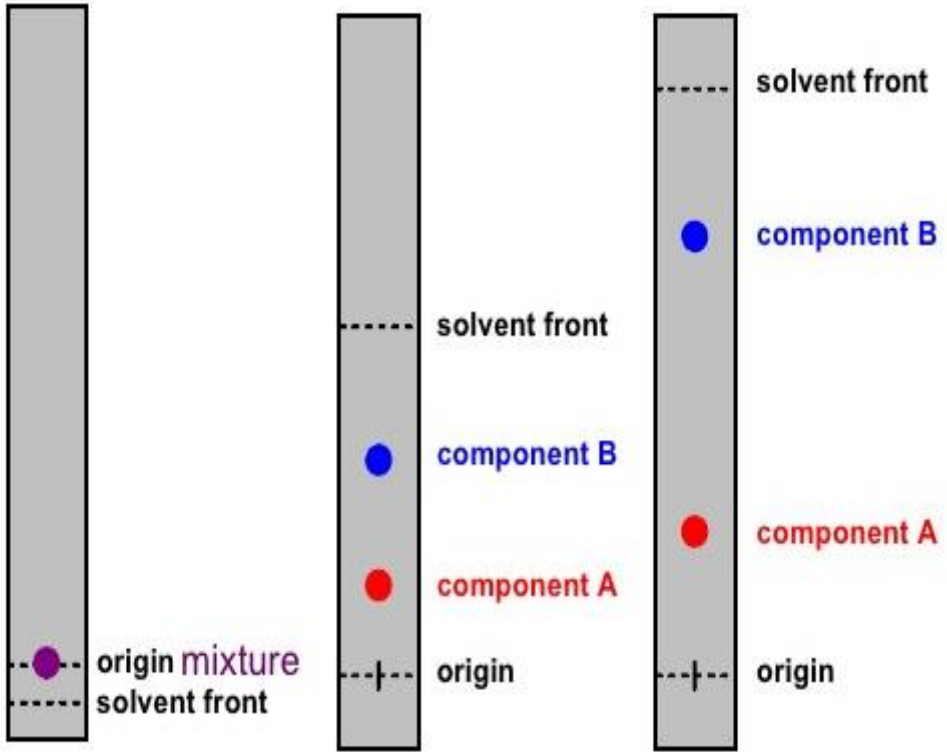
Semi-preparative refers to preparative LC performed on analytical (4–5 mm i.d.) or slightly larger (6–10 mm i.d.) columns. Normal injection size would be milligram- to low-gram size samples.

Preparative

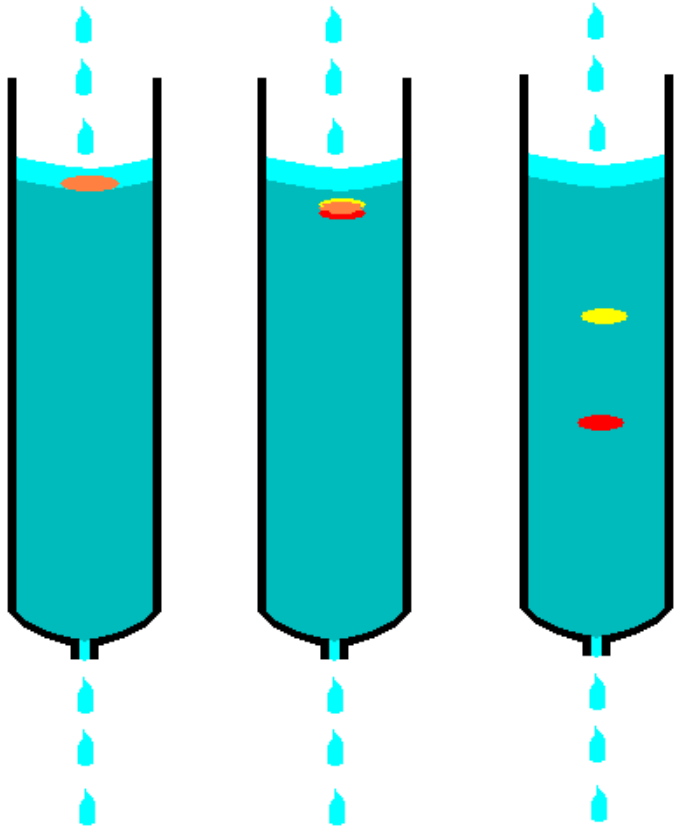
Preparative chromatography is used to purify sufficient quantities of a substance for further use, rather than analysis.

Techniques Explained

- Analytical = TLC



- Preparative = Column

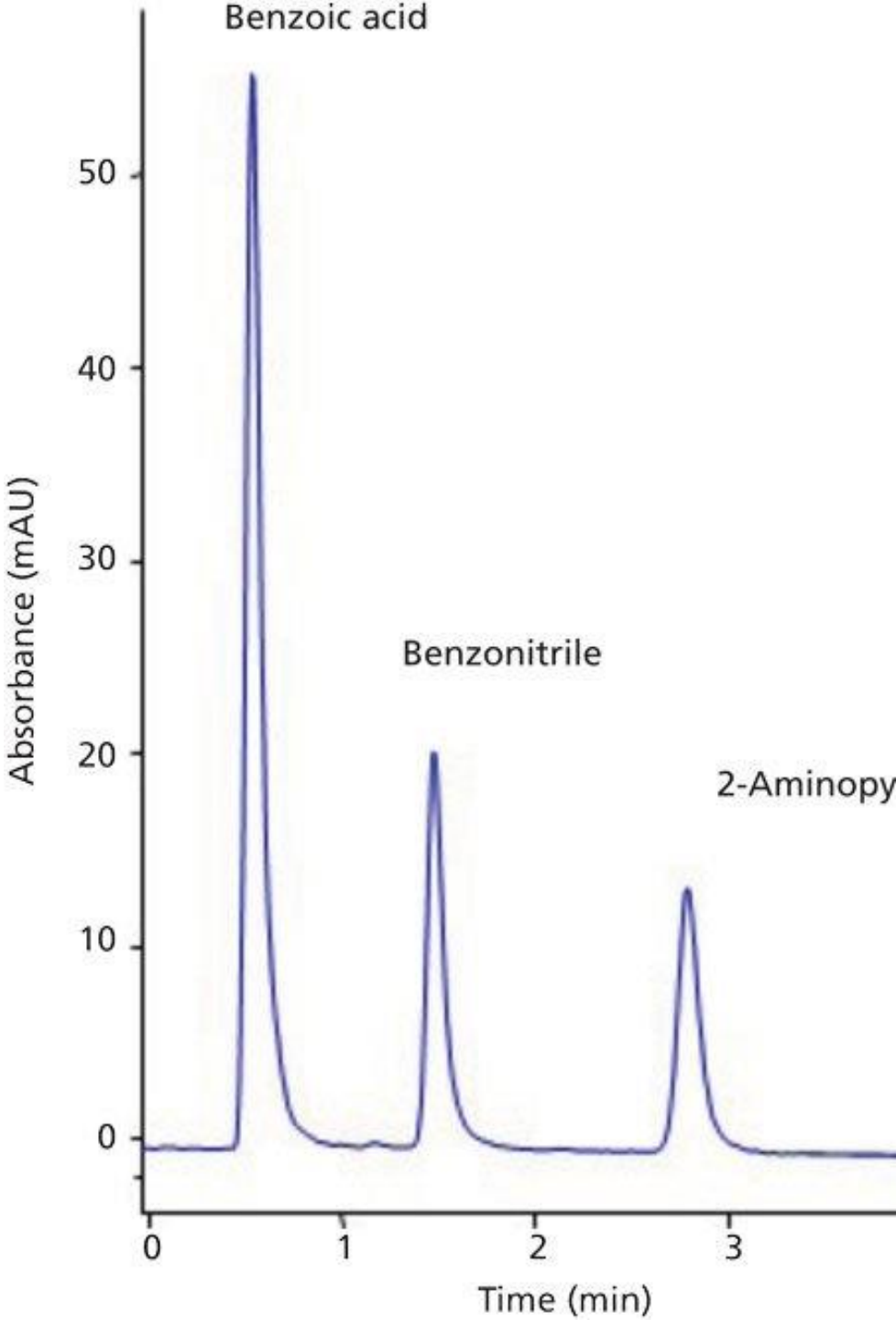


Scale Definition

Parameter	Analytical	Semi-preparative	Preparative
Column Size (mm)	120 - 250 x $2^{-4.6}$	120 - 250 x 8^{-16}	120 - 250 x 20^{-62}
Particle Size (µm)	up to 5	5-10	>10
Stationary Phase (g)	up to 5	5-30	5-450
Flow Rates (mL/min)	0.1 - 2	5 - 50	100 - 1000
Sample Size (mg)	0.01 - 2	0.1 - 50	1 - 700
Flow Cell (mm)	10	3	0.5 - 2

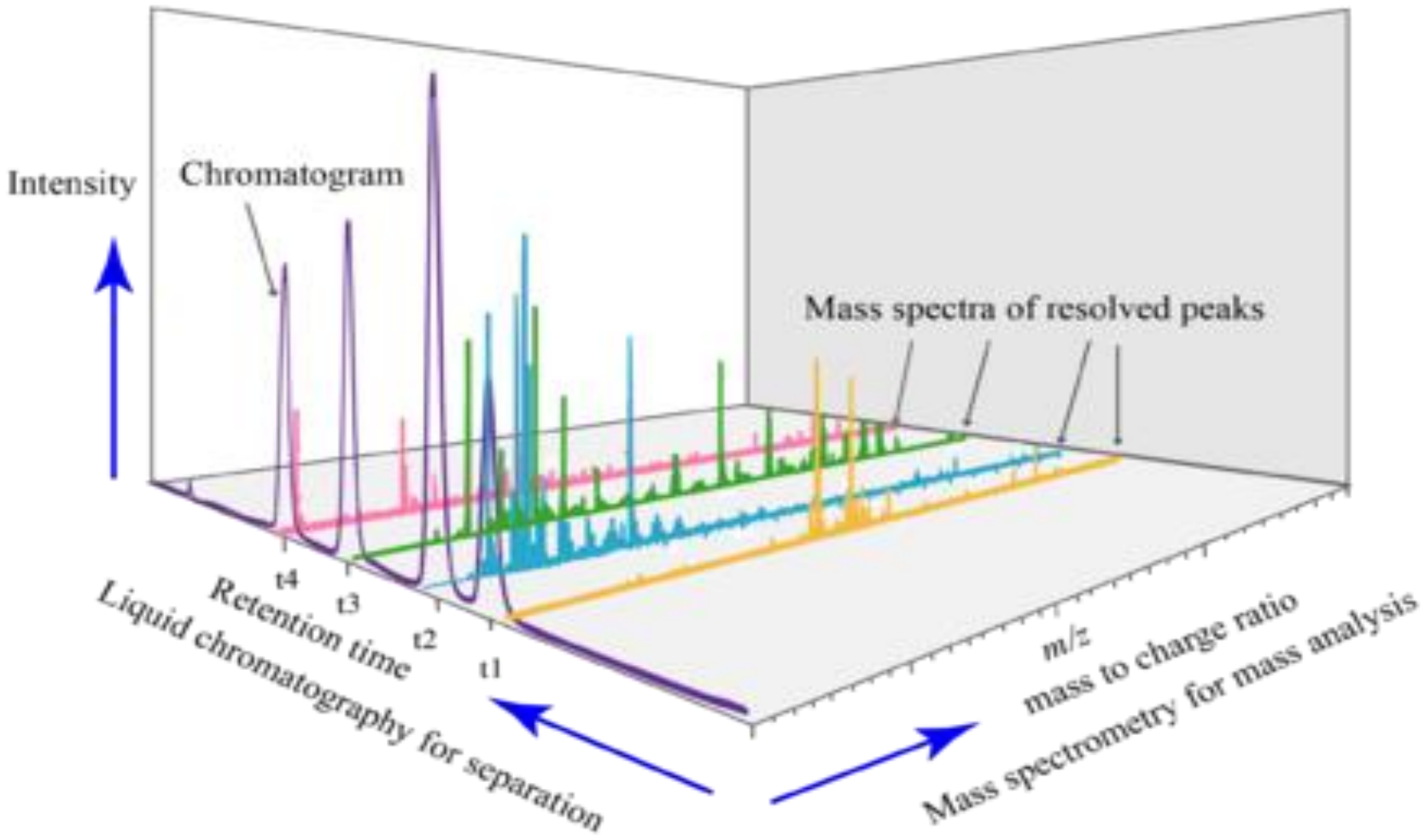
What can we expect?

- Mixture of benzoic acid, benzonitrile and 2-aminopyridine.
- Compound must absorb light from 250-800 nm.
- Peaks show good separation.



Additional Components

- HPLC-MS




THANK
YOU!