HPLC

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatography

Chromatography is a **method of separation** in which the components to be separated are distributed between two phases, one of these is called **stationary phase** and the other is **mobile phase** which moves on stationary phase in a definite direction.

Chromatography Basics

It will take longer to travel through the stationary phase for a component which possesses high affinity to the stationary phase than a component which possesses lower affinity to the stationary phase.

As a result of these differences in mobilities, components of a sample can be separated as they travel through the stationary phase.

Classification of chromatography

A. On the basis of interaction of solute to the stationary phase;

- Adsorbtion Chromatography
- Partition Chromatography
- Ion Exchange Chromatography
- Size Exclusion Chromatography

Classification of chromatography

B. On the basis of physical state of mobile phase

- Liquid Chromatography (LC)

Liquid-Solid

Liquid-Liquid

- Gas Chromatography (GC)

Gas-Solid

Gas-Liquid

Classification of chromatography

C. On the basis of chromatographic bed shape;

- Two Dimensional

Paper Chromatography

Thin Layer Chromatography (TLC)

- Three Dimensional

Column Chromatgoraphy (CC)

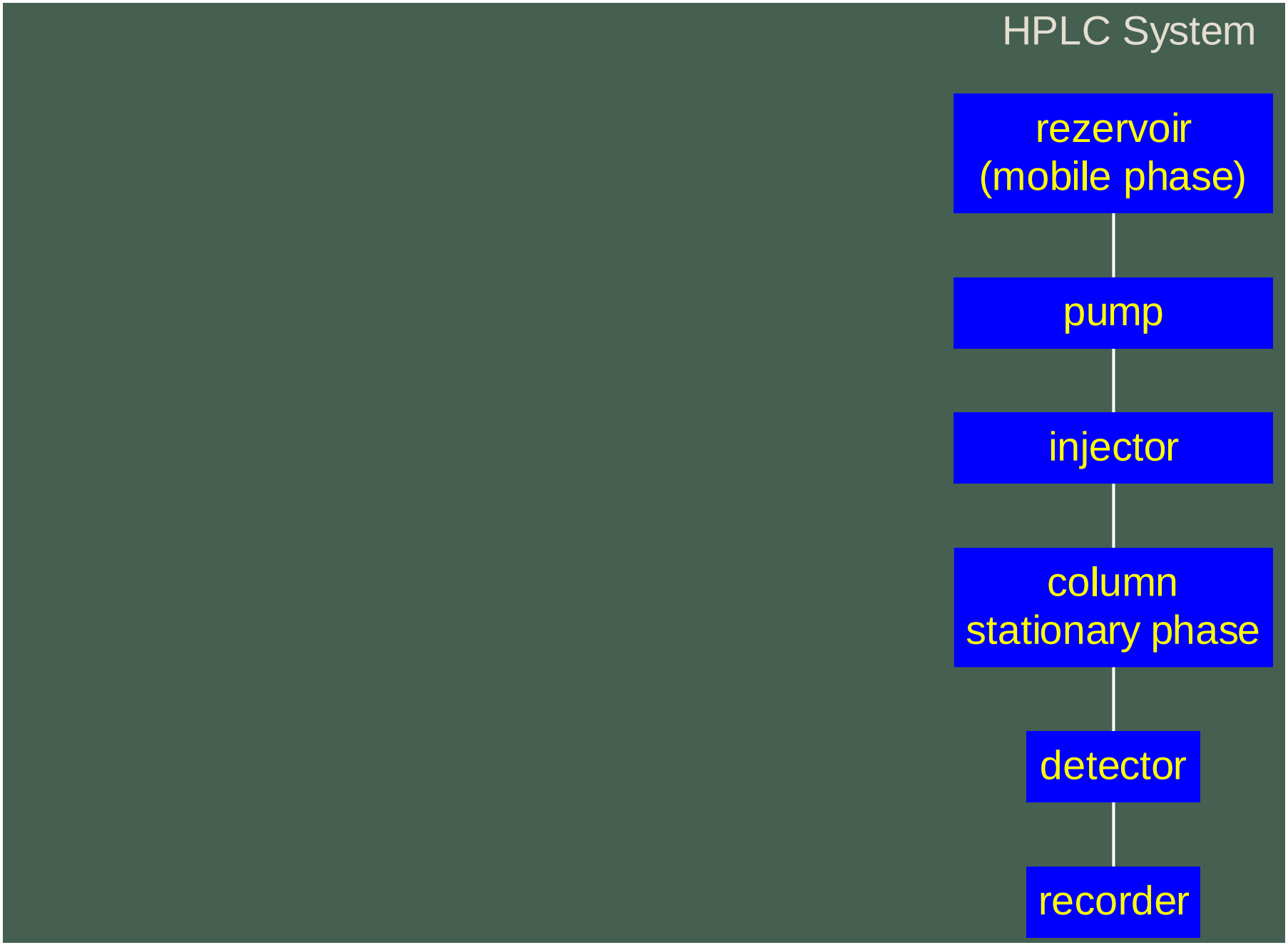
High Performance Liquid Chromatography (HPLC)

- This is a technique which is used for the separation of the components in a mixture, identification of the components and quantification of these components.
- It relies on the pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with an adsorbent material.
- Each component in the sample shows different interactions with the stationary phase, causing different flow rates for different components and leading to the separation of the components as they flow out the column.

HPLC system is a mandatory tool in most of the labs involved in research.

The fields of research include

- Medical,
- Biological,
- Chemical,
- Biochemical,
- Phytochemical (plant chemical) researches


HPLC System
rezervoir
(mobile phase)
pump
injector
column
stationary phase
detector
recorder

Parts of HPLC - Pump

- The main function of the HPLC pump is to force a liquid (which is also known as the mobile phase) via a liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).
- Operating pressure limits for regular HPLC systems are often in the range of 6000 – 10000 psi. An ideal pump should have solvent compatibility and corrosion resistance characteristics. For this reason, they are made of stainless steel.
- For analytical purposes, pumps with a flow rate of 0-10 mL/min are used and for preparative purposes, pumps over a flow rate of 100 mL/min may be used.
- There are two types of pump operation:
  - ✓ isocratic pump
    delivers constant mobile phase composition
  - ✓ gradient pump
    delivers variable mobile phase composition

Parts of HPLC – Injector System

The test sample is loaded into the stationary phase via an injector system.

- Manuel injection
- Automatic injection (Autosampler)

Parts of HPLC - Column

- The separation of the compound mixture takes place in the column. HPLC columns is made up of **stainless steel**.
- Most HPLC columns are resistant to the usual HPLC pressure and also relatively inert to chemical corrosion.
- Glass tubes, tantalum tubes, and flexible polyethylene tubes are also rarely used as column tubing.
- The stationary phase is packed in the column and it is usually a solid adsorbent (silicagel, alumina, polimers).
- The mobile phase moves through the stationary phase with the test sample. And the compounds in the test sample are separated in here. Mobile phase serves only as a carrier of the test sample. According to the polarity differences, the mixed compounds are separated.

Column diameter

Columns, of i.d. 2-5 mm are generally used for analytical purposes. Wider columns of i.d. between 10 mm and 24.5 mm may be used for preparative work.

Column lenght

Columns 5, 10, 15 or 25 cm long are common if microparticulate stationary phases of 10 µm or less are used.

A longer column increases the retention volume, thus decreasing the concentration of the peak in the eluate and impairing the detection limit.

Yet for preparative purposes columns up to 1 m in length are used.

Parts of HPLC - Detectors

- Detector records the relative concentrations of different components in the test sample with respect to their retention time. **Retention time** is the time taken by the compound to be eluted through the column. Retention time is calculated from the time of injection until the compound is eluted. Detection is based on several different physical and chemical principals.

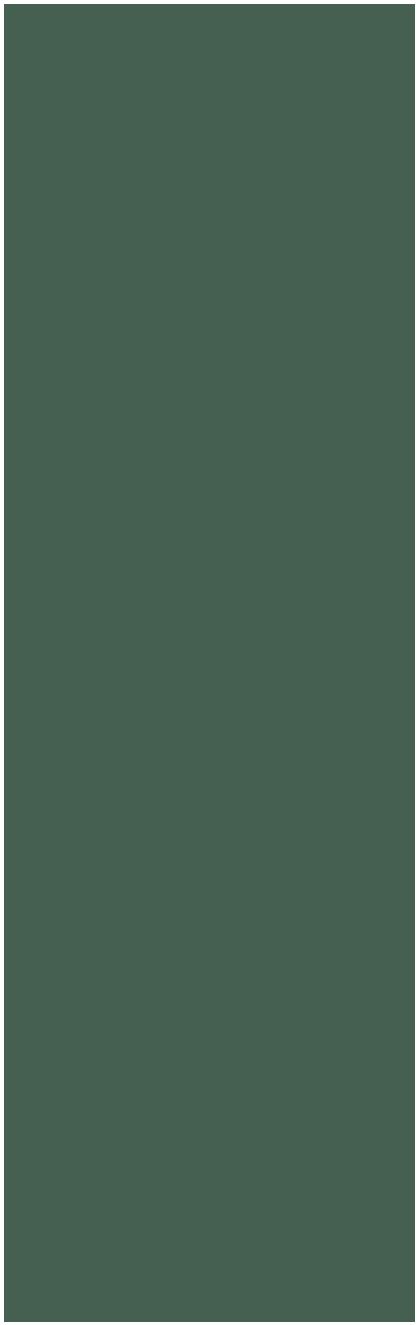
- The detector is connected to the recorder.

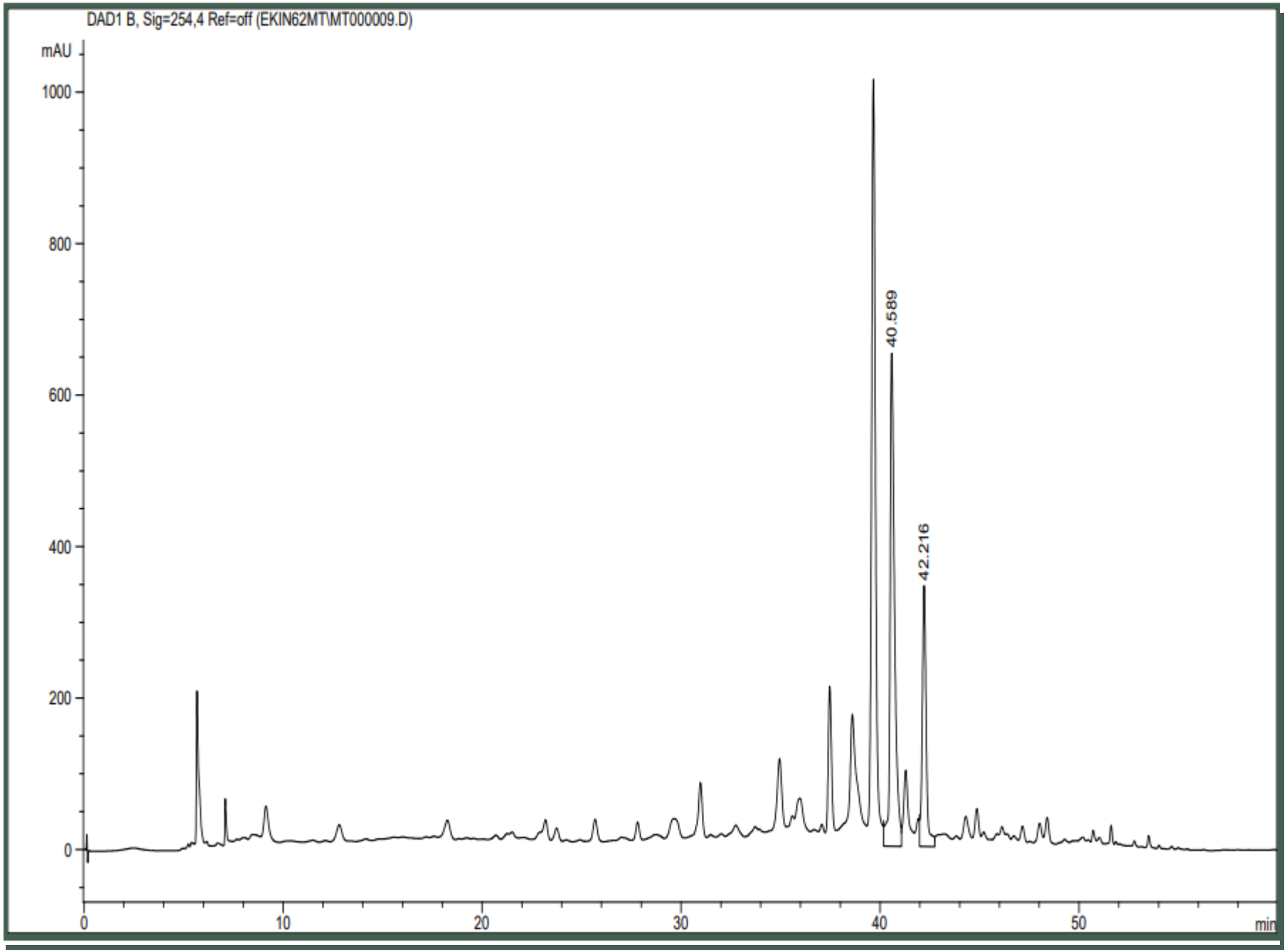
- Recorder reads the output given from the detector and it creates peaks indicating relevant detected compounds.

Detectors

- UV Detectors
  - -Fixed-wavelength
  - -Variable-wavelength
  - -Diode-Array Detectors (DAD)*
- Refractive Index Detectors
- Florescence Detectors
- Electrochemical Detectors
- Light-scattering Detectors
- Photoconductivitiy Detectors

The eluted compounds are transported by the mobile phase to the detector and recorded as Gaussian (bell-shaped) curves. The signals known as «peaks» and whole entitiy is the **«chromatogram»**.
The peaks give qualitative and quantitative information about the mixture.

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mAU
1000
800
600
400
200
0
40.589
42.216
0
10
20
30
40
50
min

Normal Phase (NP) HPLC

- Stationary Phase: Polar (Silica)
- Mobile Phase: Non-polar (hexane, isopropyl ether)

Reverse Phase (RP) HPLC

- Stationary Phase: Non-polar (octadecylsilane [ODS], C-18)

Reverse Phase HPLC

- Mobile Phase: Polar (Aqueous)
- Methanol
- Acetonitrile
- Ethanol
- Isopropanol
- Dimethylformamide
- Propan-1-ol
- Dioxane
- Tetrahydrofuran

Most of the HPLC analysis are reverse phase.

HPLC and Spectroscopy

- HPLC can be combined with the following spectroscopic methods:

- UV spectrometer (HPLC-UV/DAD)
- HPLC-NMR (Nuclear Magnetic Resonance)
- LC-MS (Mass Spectrometry)

Applications of HPLC

- Clinical diagnosis of diseases.
- In scientific research for discovery.
- In pharmaceutical labs for analysis.
- In food industry for quality control.
- For standard controls by government.
- For separation of similar molecules

Use of HPLC in Pharmacognosy

- Preparation of standardized extracts
- Qualitative and quantitative phytochemical analysis
- Determination of the purity of an isolated compound
- Stability testing of herbal products

It is given prominent importance due to some advantages of HPLC;

- High sensitivity i.e. ability to evaluate samples at **very small concentrations** like in nano-gram and picogram
- **Precise detection of** similar molecules
- **High accuracy** in the identification of components of complex mixtures.