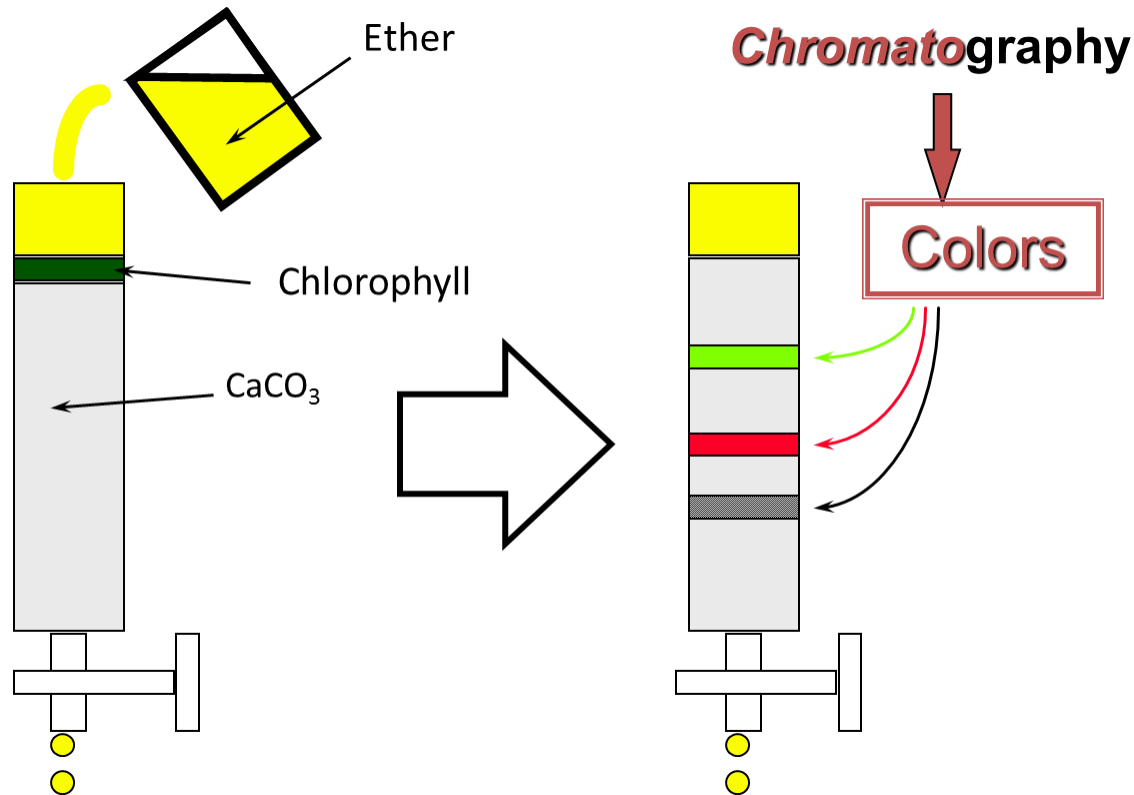


# High-Performance Liquid Chromatography (HPLC)

Assist. Prof. RANJEET RANJAN



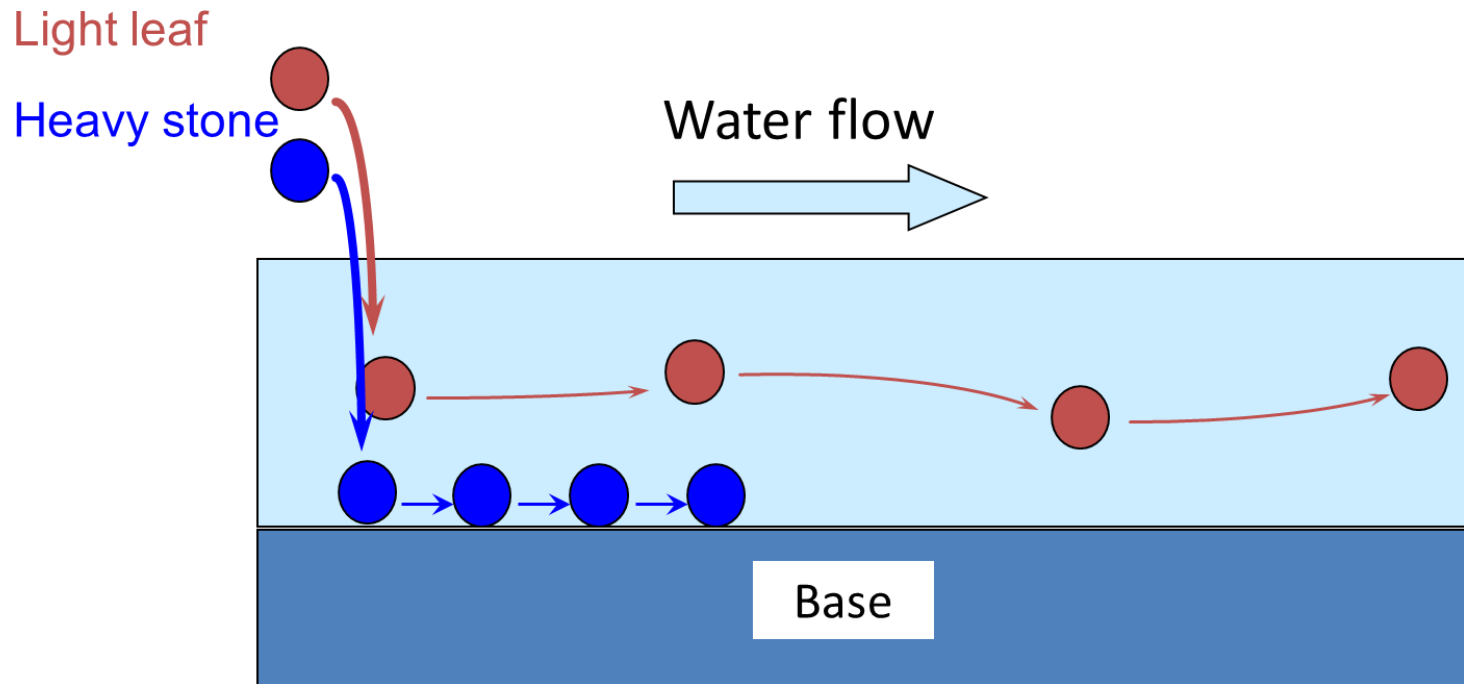
# Invention of Chromatography by M. Tswett



The **Russian-Polish botanist M. Tswett** is generally recognized as the first person to establish the principles of chromatography.

In **1906**, **Tswett** described how he filled a glass tube with chalk powder ( $\text{CaCO}_3$ ) and, by allowing an ether solution of chlorophyll to flow through the chalk, separated the chlorophyll into layers of different colors. He called this technique “chromatography”.

# Comparing Chromatography to the Flow of a River



❖ Chromatography can be often compared to the flow of a river.

This analogy represents the components of chromatography in the following way:

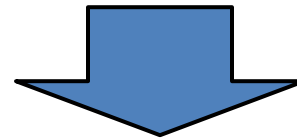
- River: Separation field
- Leaf and stone: Target components of sample (analytes)
- Standing to watch at the river mouth: Detector

# Fields in which HPLC is used

Field	Typical Mixtures
Pharmaceuticals	Antibiotics, sedatives, steroids, analgesics
Biochemical	Amino acids, proteins, carbohydrates, lipids
Food products	Artificial sweeteners, antioxidants, aflatoxins, additives
Industrial chemicals	Condensed aromatics, surfactants, propellants, dyes
Pollutants	Pesticides, herbicides, phenols, polychlorinated biphenyls
Forensic science	Drugs, poisons, blood alcohol, narcotics
Clinical chemistry	Bile acids, drug metabolites, urine extracts, estrogens

# From Open Column Chromatography to HPLC

- Higher degree of separation.
  - Refinement of packing material (3 to 10  $\mu\text{m}$ ).
- **Reduction of analysis time.**
  - Delivery of eluent by pump
  - Demand for special equipment that can withstand high pressures



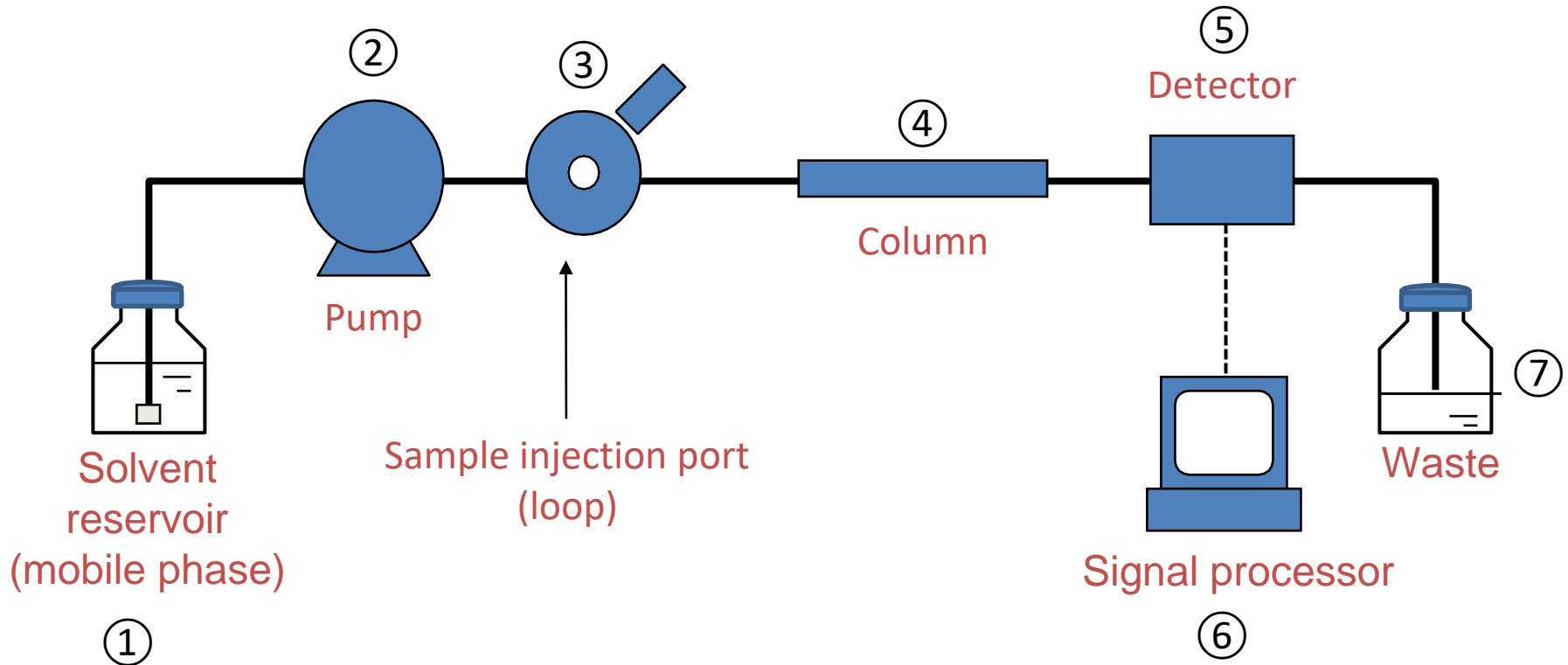
The arrival of **High-Performance  
Liquid Chromatography (HPLC)**



# Advantages of HPLC

- High separation capacity, enabling the batch analysis of multiple components,
- Superior quantitative capability and reproducibility,
- Moderate analytical conditions,
  - Unlike GC, the sample does not need to be vaporized.
- Generally high sensitivity,
- Low sample consumption,
- Easy preparative separation and purification of samples.

# HPLC Block Diagram



- Other accessories may be added as necessary.



# ① Solvent Reservoirs

➤ The requirements for a solvent reservoir are:

1. The reservoir and its attachment to the pump should be made of materials that will **not react with or contaminate the mobile phase**: Teflon, glass, or stainless steel.
  2. The vessel should **have a cap** to prevent particulate matter from contaminating the mobile phase.
  3. Caps have **another hole** to allow air to enter the reservoir otherwise removal of mobile phase by the pump will create a vacuum. This prevents mobile phase from flowing the pump, creating a "vapor lock" within the pump.
- Besides providing extra protection against particulates entering the pump, the inlet filter serves to hold the inlet line at the bottom of the reservoir.



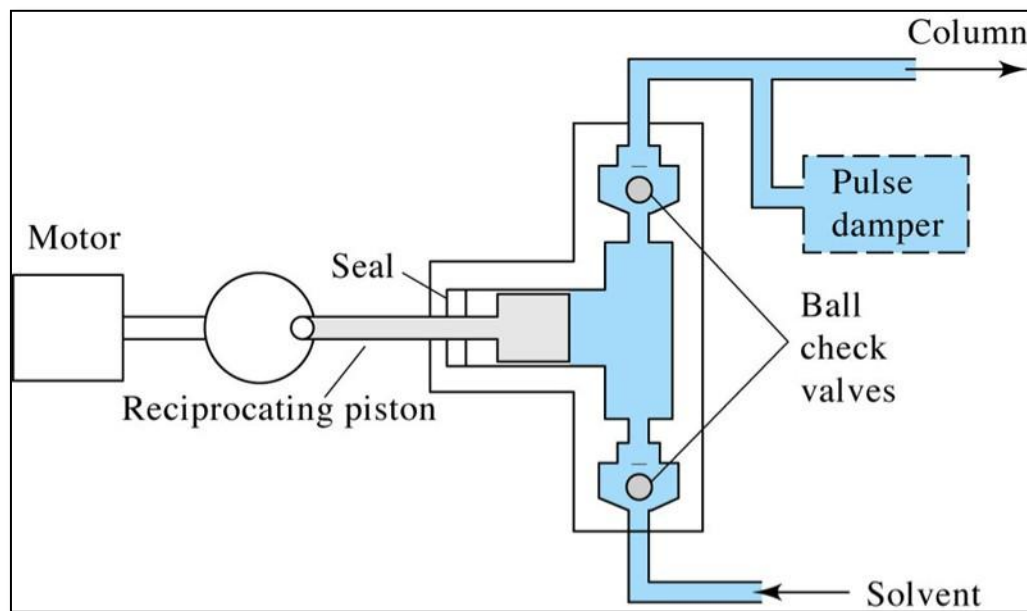
## ② Solvent Delivery Pump(s)

### ❖ Performance Requirements:

1. Constructed of materials inert toward solvents to be used,
2. Deliver high volumes (flow rates) of solvent (up to 5 mL/min),
3. Deliver precise and accurate flow (<0.5% variation),
4. Deliver high pressure (up to 400 atm),
5. Deliver pulse-free flow,
6. Have low pump-head volume,
7. Be reliable.

### ❖ Types of HPLC pumps:

1. Reciprocating pumps
2. Syringe pumps
3. Constant pressure pumps

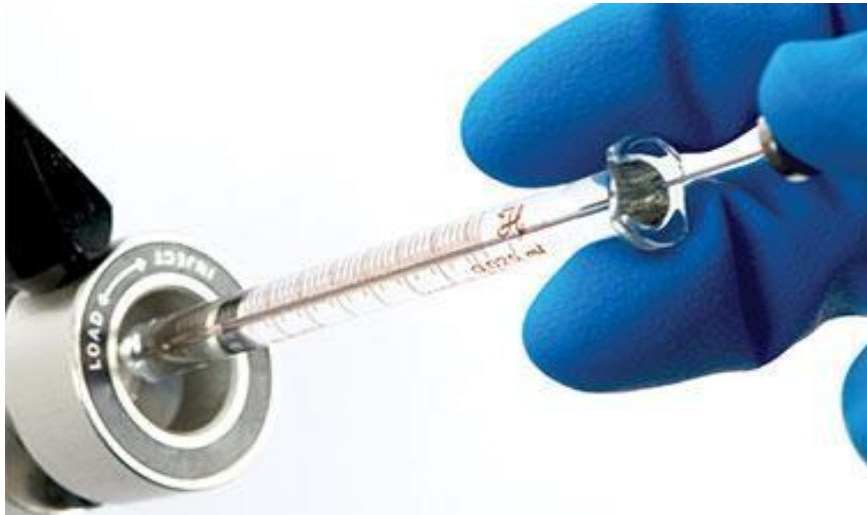


A reciprocating HPLC pump

### ③ Sample Injection Port (Loop)

#### Sample Injectors

Manual



Rheodyne/Valco injectors

Automatic

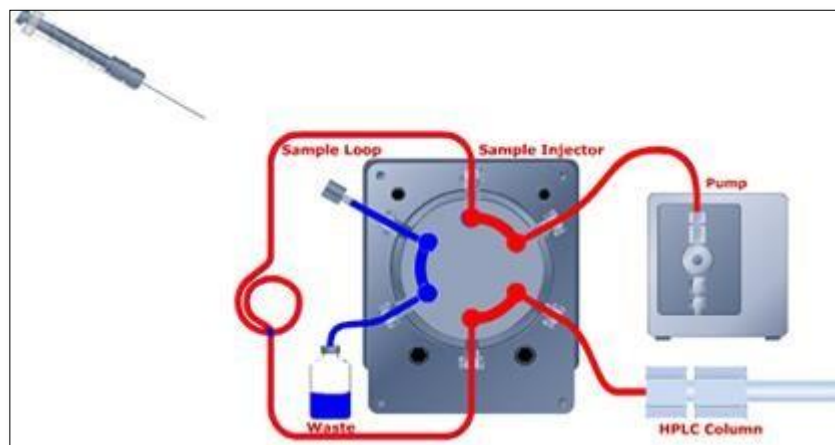


Autosamplers

# Manual injectors

- Injection is done through specially designed 6-port rotary injection valve.
- The sample is introduced at atmospheric pressure by a syringe into a constant volume loop.
- In the **LOAD** position the loop is not in the path of the mobile phase. By rotating to the **INJECT** position the sample in the loop is moved by the mobile phase stream into the column.
- It is important to allow some sample to flow into waste from the loop so as to ensure there are no air bubbles in the loop and previously used sample is completely washed out to prevent memory effects.

HPLC injection  
port



# Automatic injections

- ✓ Advanced HPLC systems are equipped with an auto injector along with an auto sampler.
- ✓ Automatic injection improves laboratory productivity and also eliminates personal errors.
- ✓ The software programs filling of the loop (generally from 0 to 100  $\mu\text{L}$ ) and delivery of the sample to the column.
- ✓ The computer also controls the sequence of samples for injection from vials kept in numbered positions of the auto sampler.
  - However, feeding the vial number correctly on auto sampler rack and listing out the sequence correctly in the computer is very important.



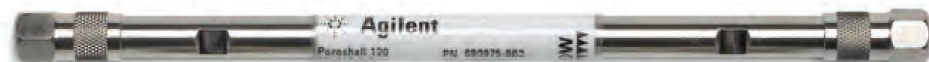
HPLC vials and septa

# ④ HPLC Columns

## HPLC Columns

Guard

Analytical

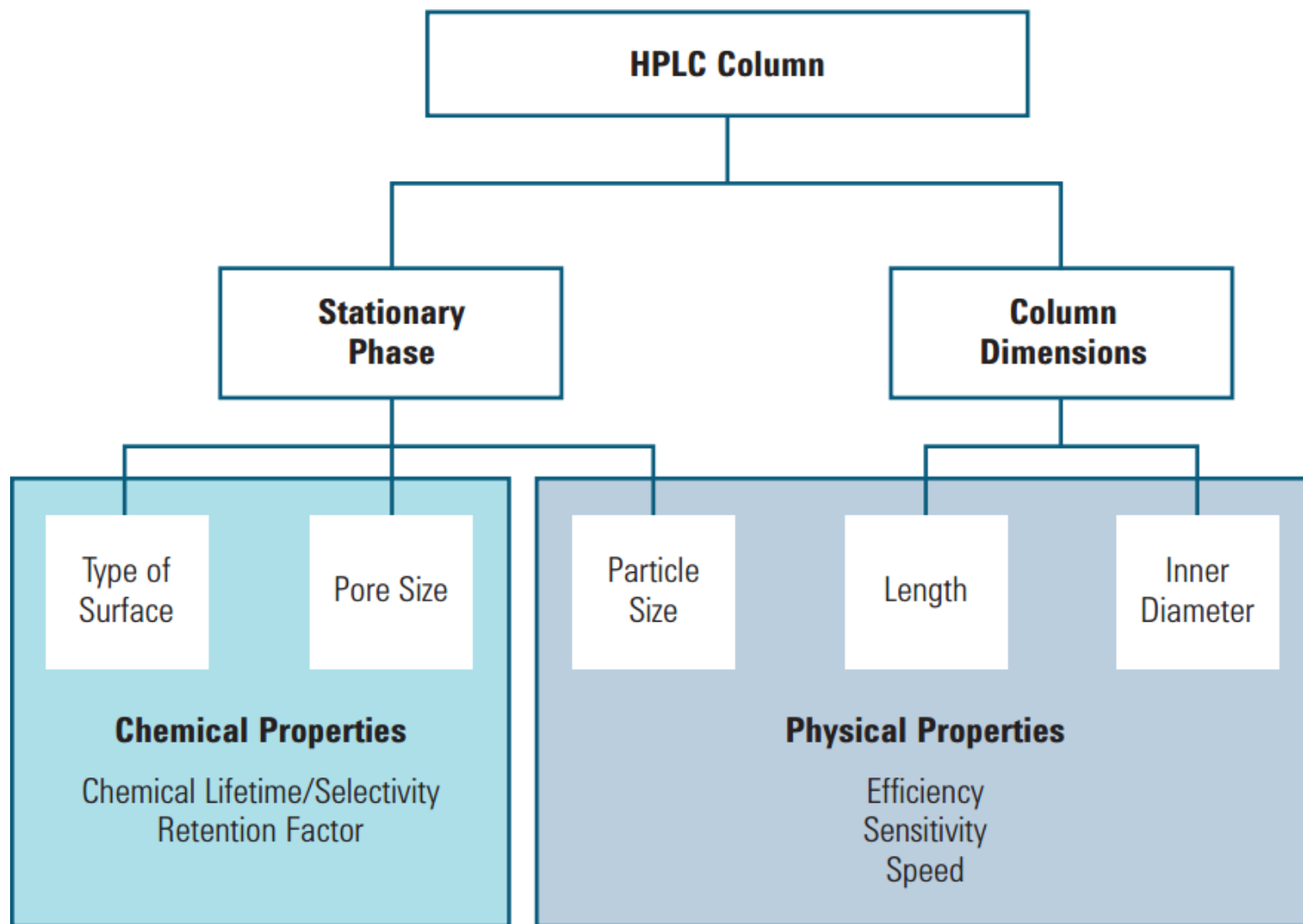


## Guard Column

❑ A guard column is introduced before the analytical column to:

1. **increase the life of the analytical column** by removing not only particulate matter and contaminants from the solvents but also sample components that bind irreversibly to the stationary phase.
  2. The guard column **serves to saturate the mobile phase with the stationary phase** so that losses of this solvent from the analytical column are minimized.
- The composition of the guard-column packing is **similar** to that of the analytical column; the particle size is usually larger.
  - When the guard column has become contaminated, it is **repacked** or **discarded** and **replaced** with a new one.

# Analytical Column



Properties of an HPLC column



# Analytical Column

## HPLC Columns (or LC)

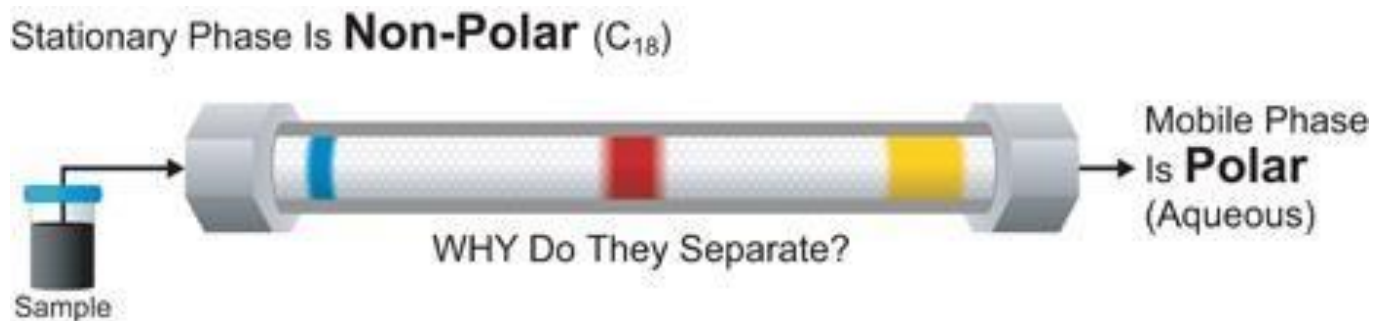
**Normal-phase**

**Reversed-phase**

	<b>Stationary phase</b>	<b>Mobile phase</b>
<b>Normal-phase</b>	High polarity (hydrophilic)	Low polarity (hydrophobic)
<b>Reversed-phase</b>	Low polarity (hydrophobic)	High polarity (hydrophilic)

# Reversed-phase HPLC (RP-HPLC)

- ❖ Stationary phase: Low polarity
  - Octadecyl ( $C_{18}$ ) group-bonded silical gel (ODS), etc.
- ❖ Mobile phase: High polarity
  - Water, methanol, acetonitrile, acetone, tetrahydrofuran etc.
  - Salt (or buffer) is sometimes added to adjust the pH or to form ion pairs.



# Analytical Columns for RP-HPLC

## ➤ C<sub>18</sub> (ODS) type

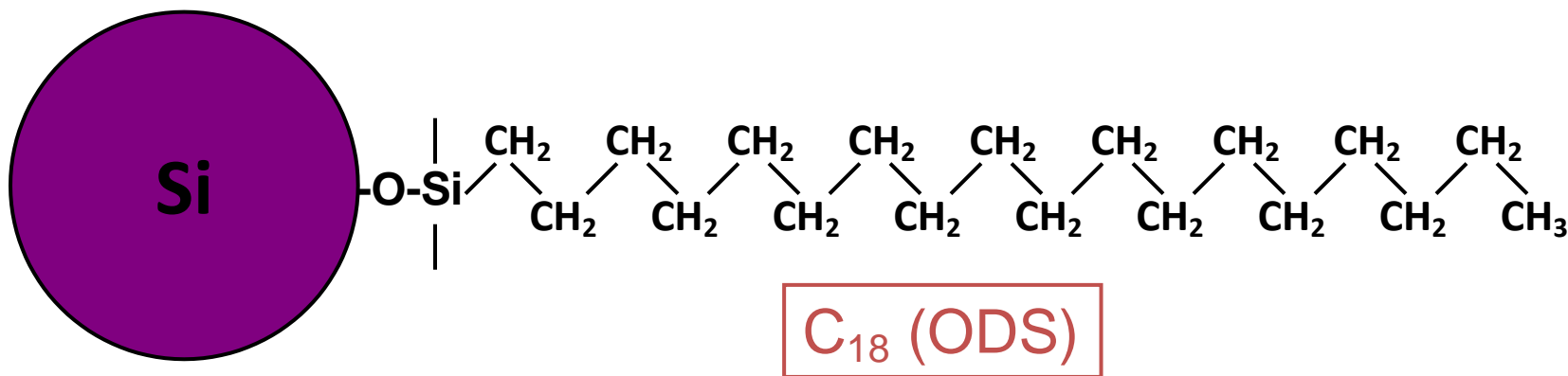
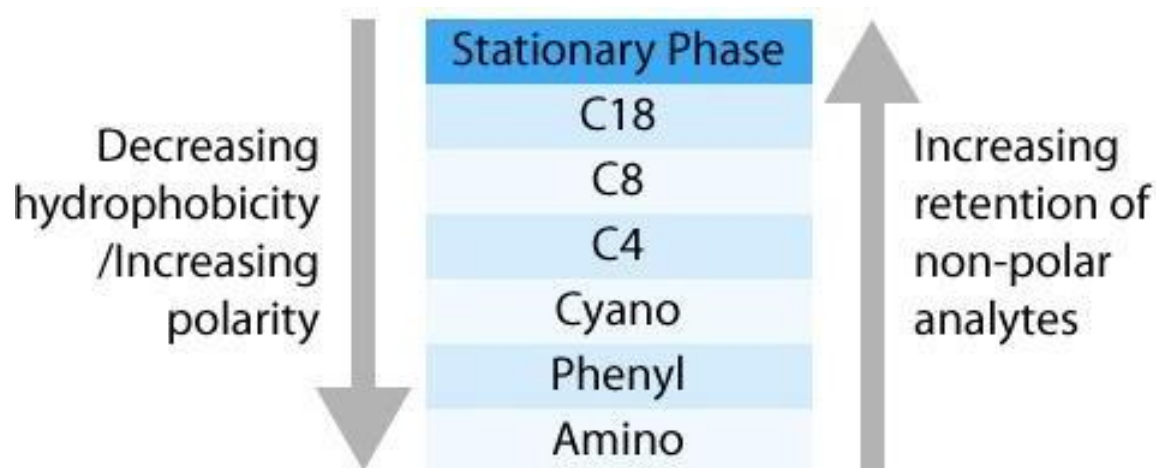
➤ C<sub>8</sub> (octyl) type

➤ C<sub>4</sub> (butyl) type

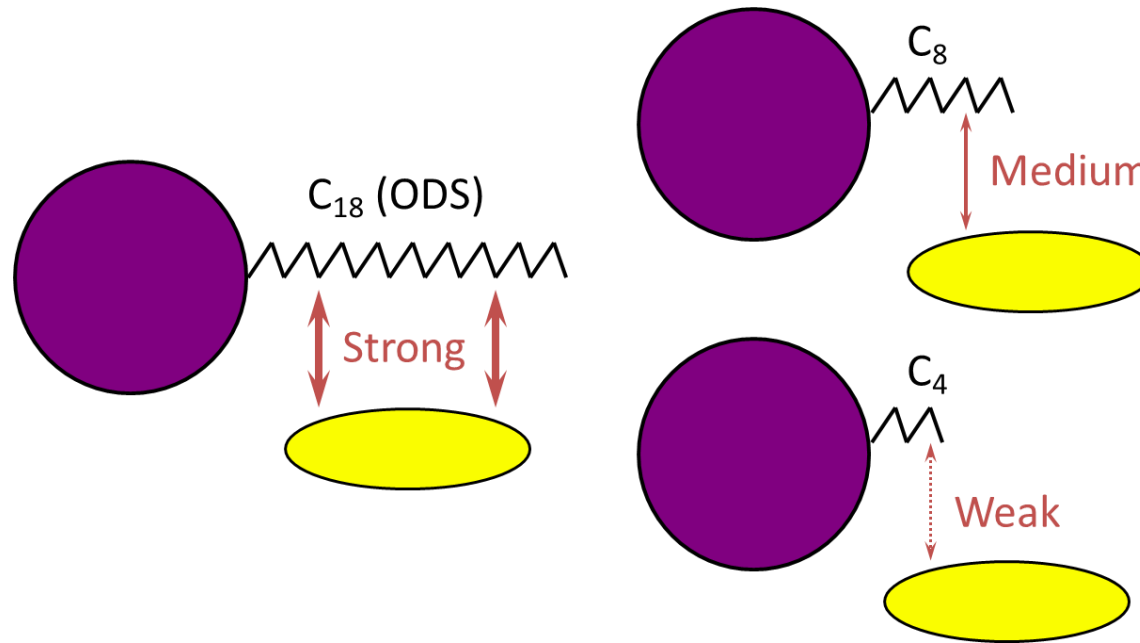
➤ Phenyl type

➤ Cyano type

➤ Amino type

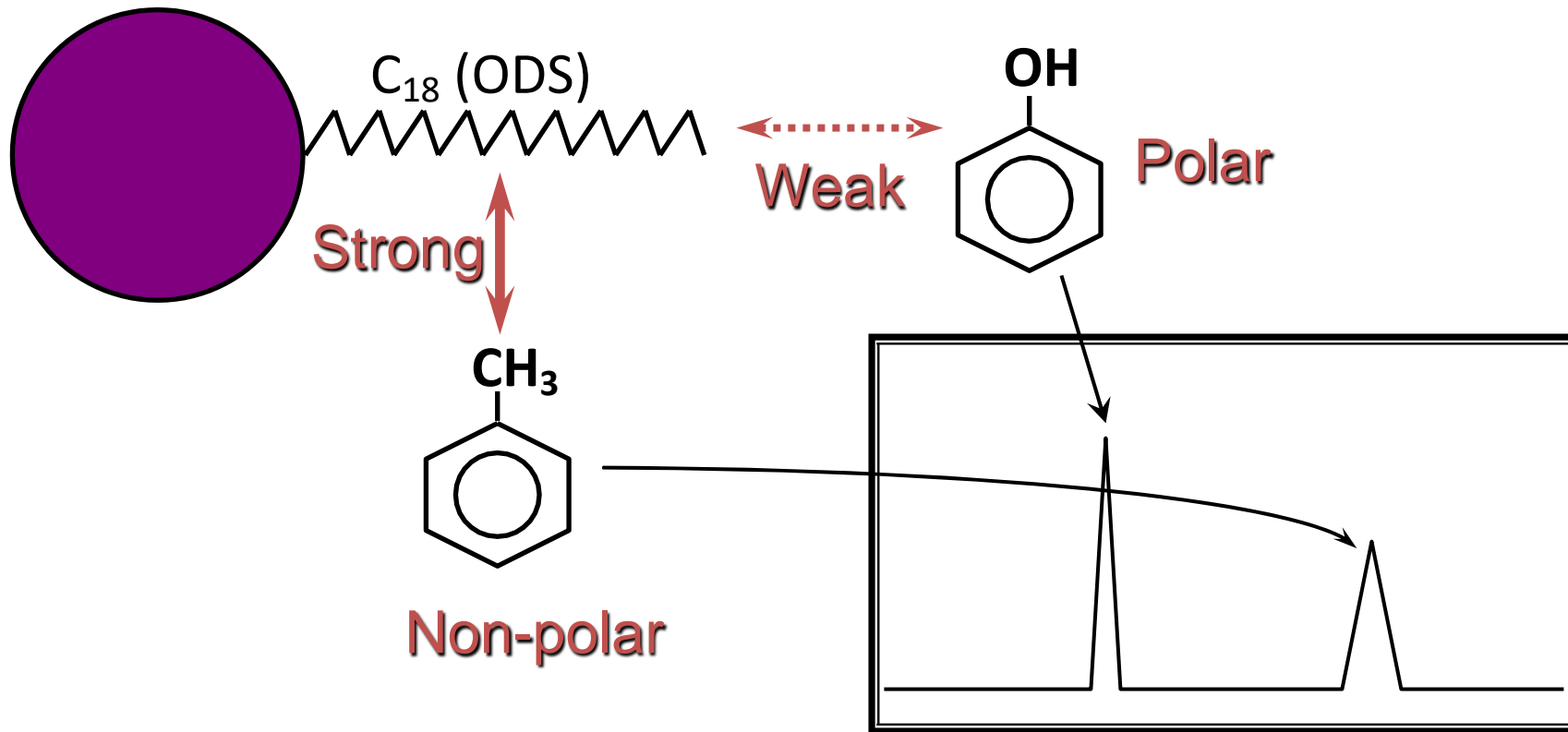


# Effect of Chain Length of Stationary Phase



- If a stationary phase produced by chemically bonding an aliphatic chain to silica gel is used, the length of the aliphatic chain influences the retention strength for the solute.
- It is said that, in general, **longer chains have a greater retention strength**. Beyond a certain length, however, the retention strength does not change significantly.

## Relationship Between Retention Time and Polarity in RP-HPLC



- In RP-HPLC, strongly **hydrophobic** substances (i.e., substances with a relatively low polarity) are **strongly retained** by the stationary phase, and thus have relatively **long retention** times. Therefore, in a chromatogram containing multiple peaks, the substances are generally eluted in **decreasing order of polarity**.

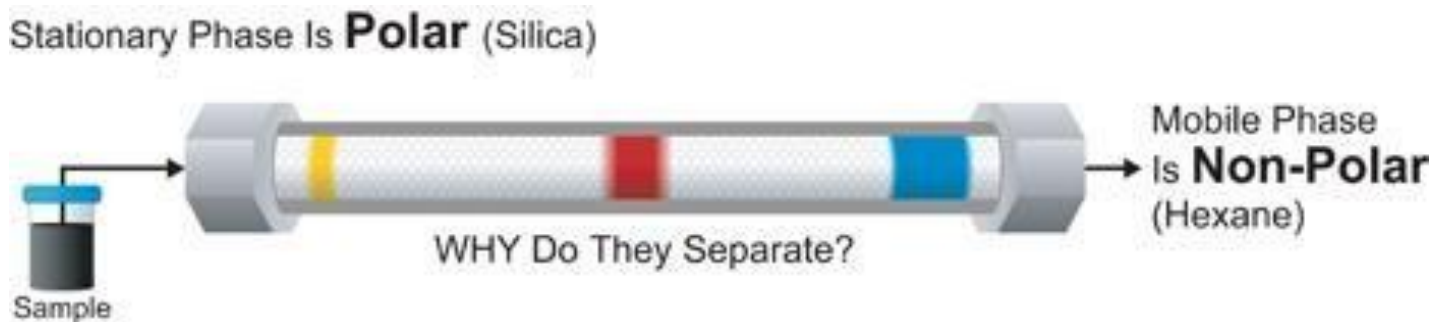
# Normal-phase HPLC (NP-HPLC)

## ❖ Stationary Phase

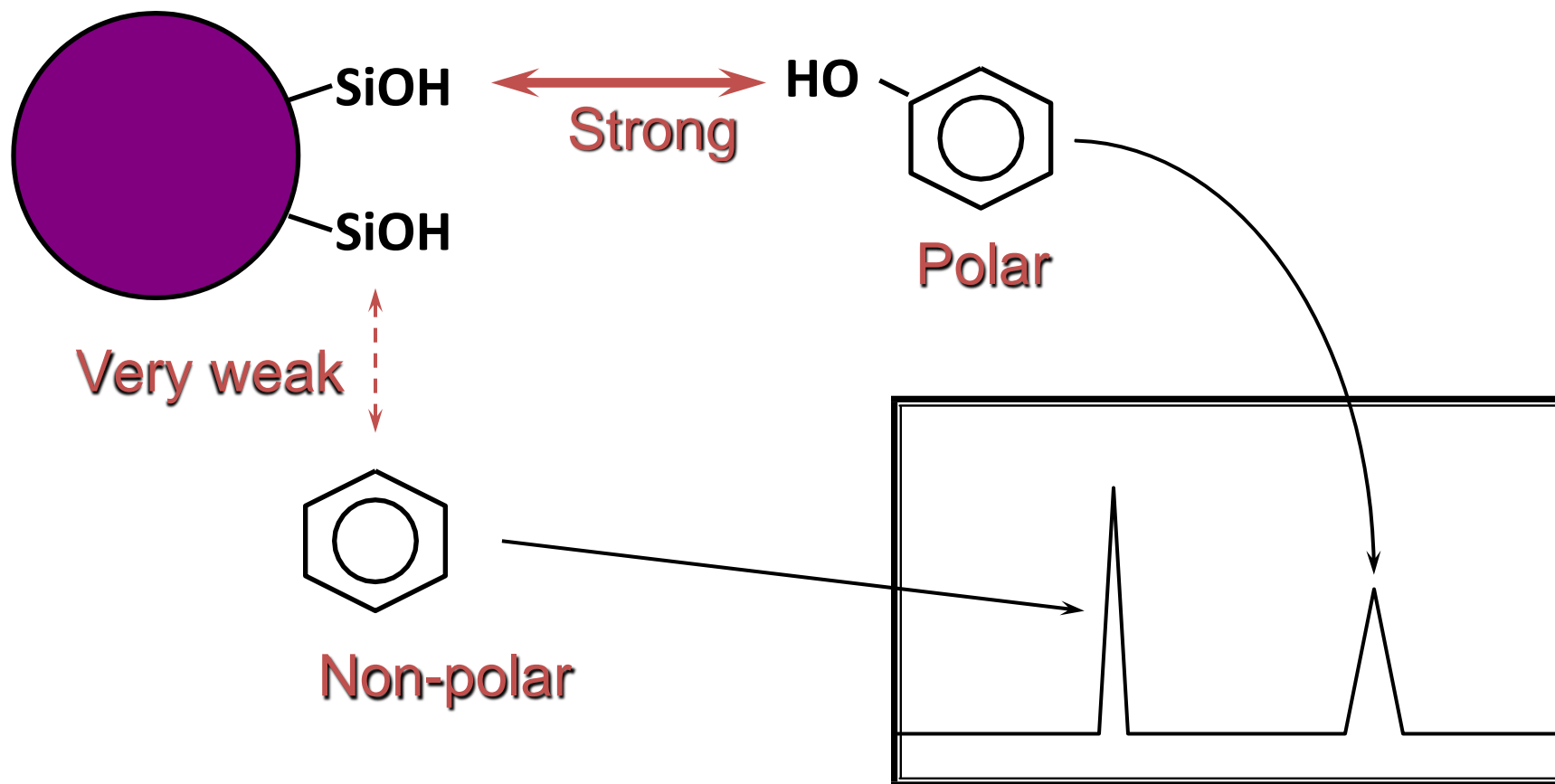
- Silica gel:  $-\text{Si}-\text{OH}$
- Cyano type:  $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CN}$
- Amino type:  $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$
- Diol type:  $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}(\text{OH})-\text{CH}_2\text{OH}$

## ❖ Mobile Phase

- Main solvents: Aliphatic hydrocarbons (e.g., hexane, cyclohexane), aromatic hydrocarbons (e.g., toluene), etc.
- Modifier solvents: Alcohols, ethers, etc.

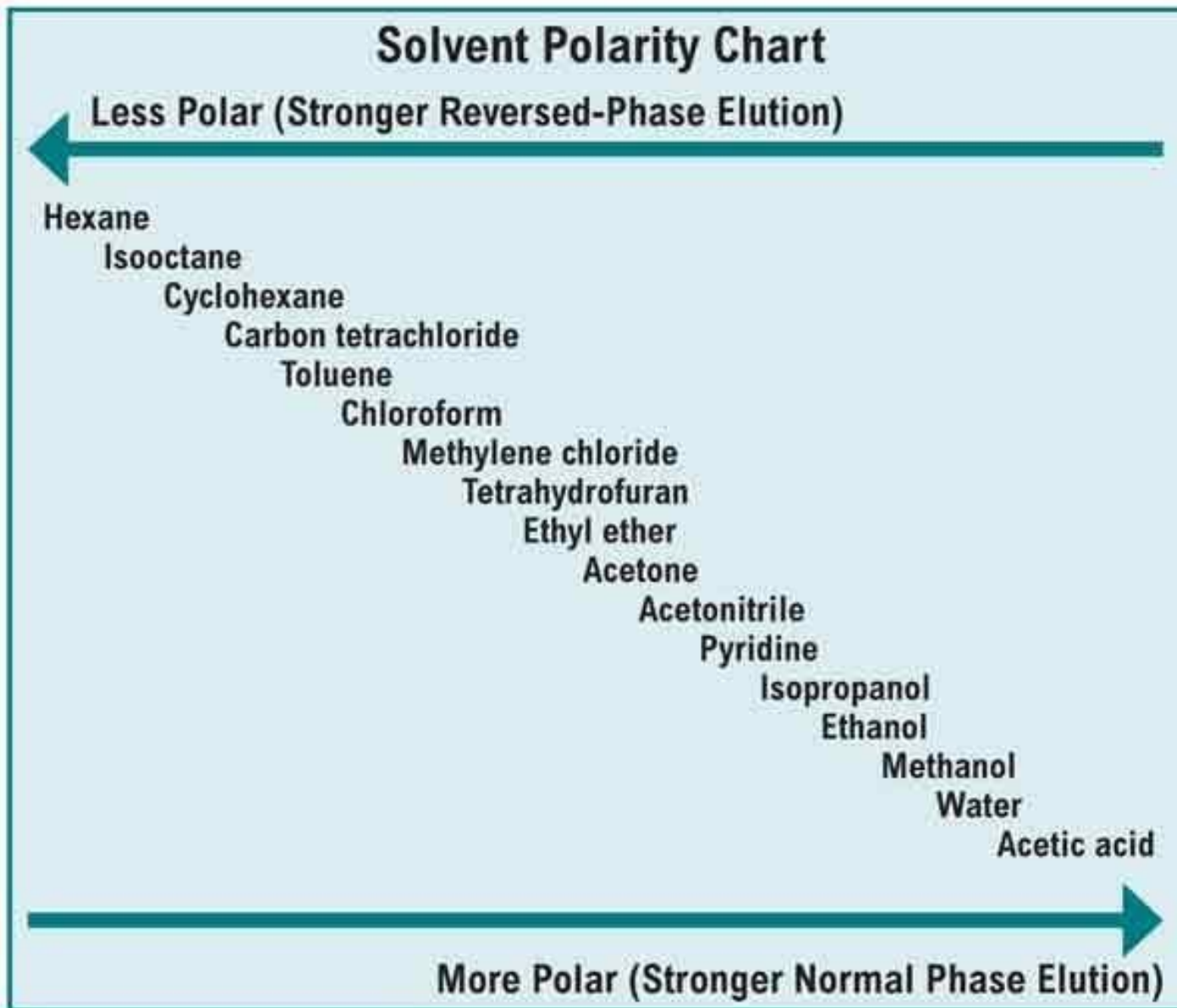


# Relationship Between Retention Time and Polarity in NP-HPLC



- In NP-HPLC, **hydrophilic** substances (i.e., substances with a relatively high polarity) are **strongly retained** by the stationary phase, and thus have relatively **long retention** times. Therefore, in a chromatogram containing multiple peaks, the substances are generally eluted in **increasing order of polarity**.

# Relative Polarities of Common HPLC Solvents





# ⑤ HPLC Detectors

❖ **Types of Detectors:** HPLC detectors are of two basic types:

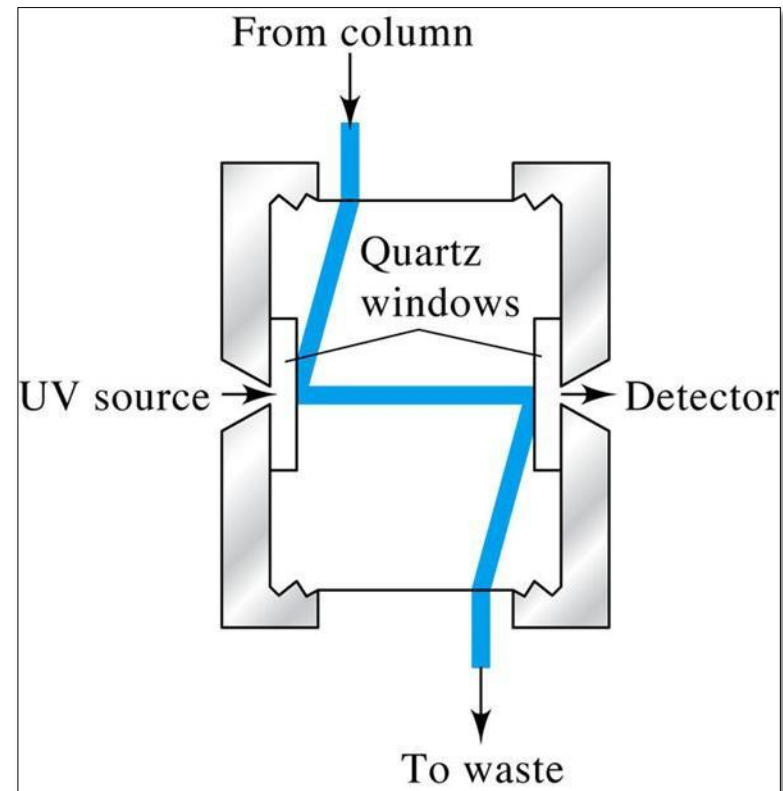
1. **Bulk property** detectors which respond to a **mobile phase** bulk property, such as refractive index, dielectric constant, or density.
2. **Solute property** detectors which respond to some property of **solutes**, such as UV absorbance or fluorescence, that is not possessed by the mobile phase.

Performance of HPLC detectors

HPLC Detector	Commercially Available	Mass LOD* (typical)	Linear Range † (decades)
Absorbance	Yes	10 pg	3–4
Fluorescence	Yes	10 fg	5
Electrochemical	Yes	100 pg	4–5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg–1 ng	5
Mass spectrometry	Yes	<1 pg	5
FTIR	Yes	1 µg	3
Light scattering	Yes	1 µg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4–5
Photoionization	No	<1 pg	4

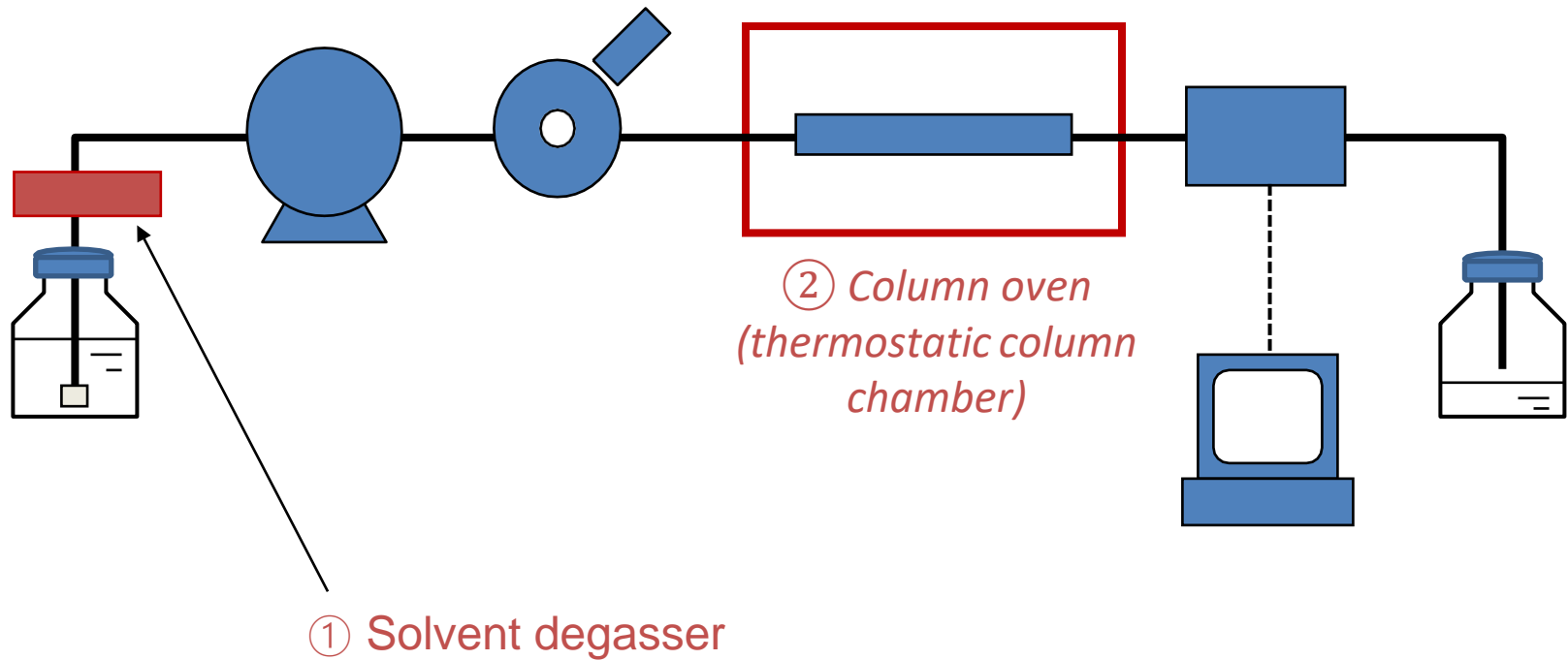
- **Absorbance Detector:** Is a Z-shaped, flow-through cell for absorbance measurements on eluents from a chromatographic column.
- Many absorbance detectors are **double-beam** devices in which one beam passes through the eluent cell and the other through a filter to reduce its intensity.

➤ **UV Absorbance Detector with Monochromator:** There are detectors that consist of a scanning spectrophotometer with grating optics. Some are limited to UV radiation; others encompass both UV and visible radiation. The most powerful uv spectrophotometric detectors are diode-array (**DAD**) instruments.



UV detector cell for HPLC

## Other HPLC Accessories



- Other necessary HPLC accessories may include:
  1. Solvent degasser,
  2. Column oven (thermostatic column chamber)

## ❖ Why is degassing of HPLC mobile phase necessary?

Bubble formation on mixing of solvents can lead to a number of problems in HPLC analysis which can be prevented by degassing of mobile phase. These include:

1. Unstable and noisy baselines,
2. Air bubbles passing through detectors lead to spurious (fake) peaks,
3. Excessive pressure can develop which can lead to eventual pump failure.

### ☐ Degassing techniques

Commonly used degassing practices for HPLC mobile phase are:

1. Vacuum filtration,
2. Sonication
3. Helium purging.



Vacuum filtration

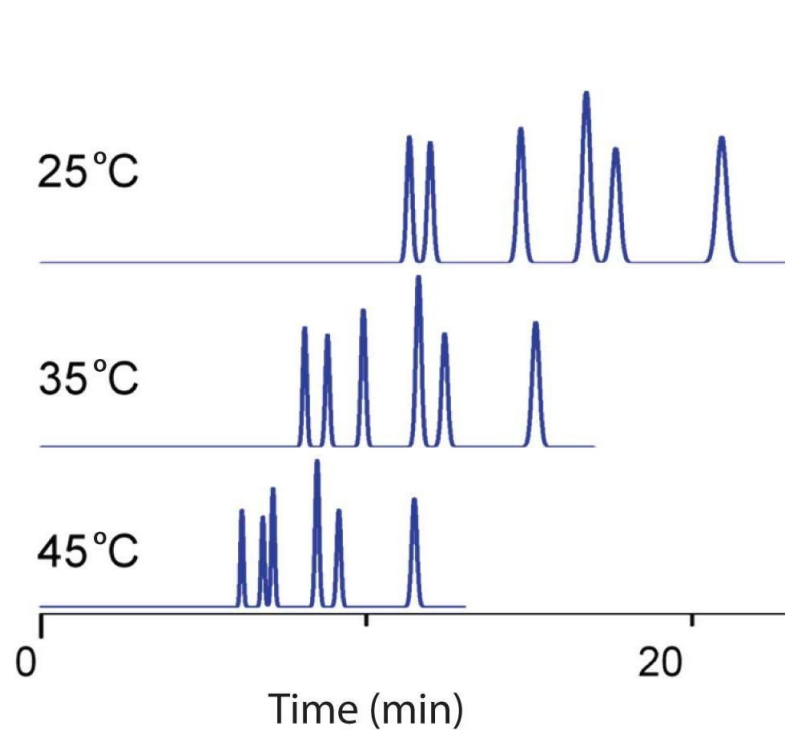


Ultrasonic bath

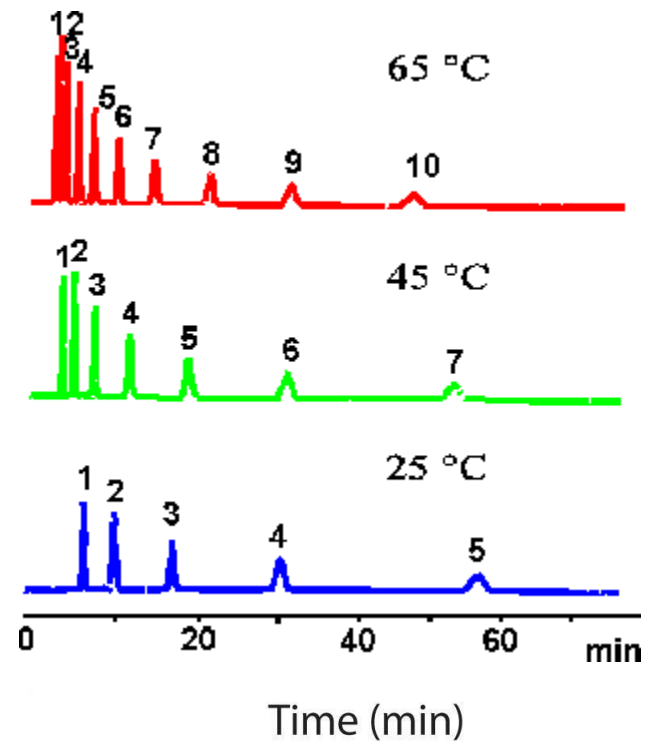
## Why is it necessary to thermostat the column?

Temperature is an important factor in HPLC that may change:

- a) Retention time,
- b) Resolution,



(a)



(b)

# Gradient System

## ❖ Isocratic system

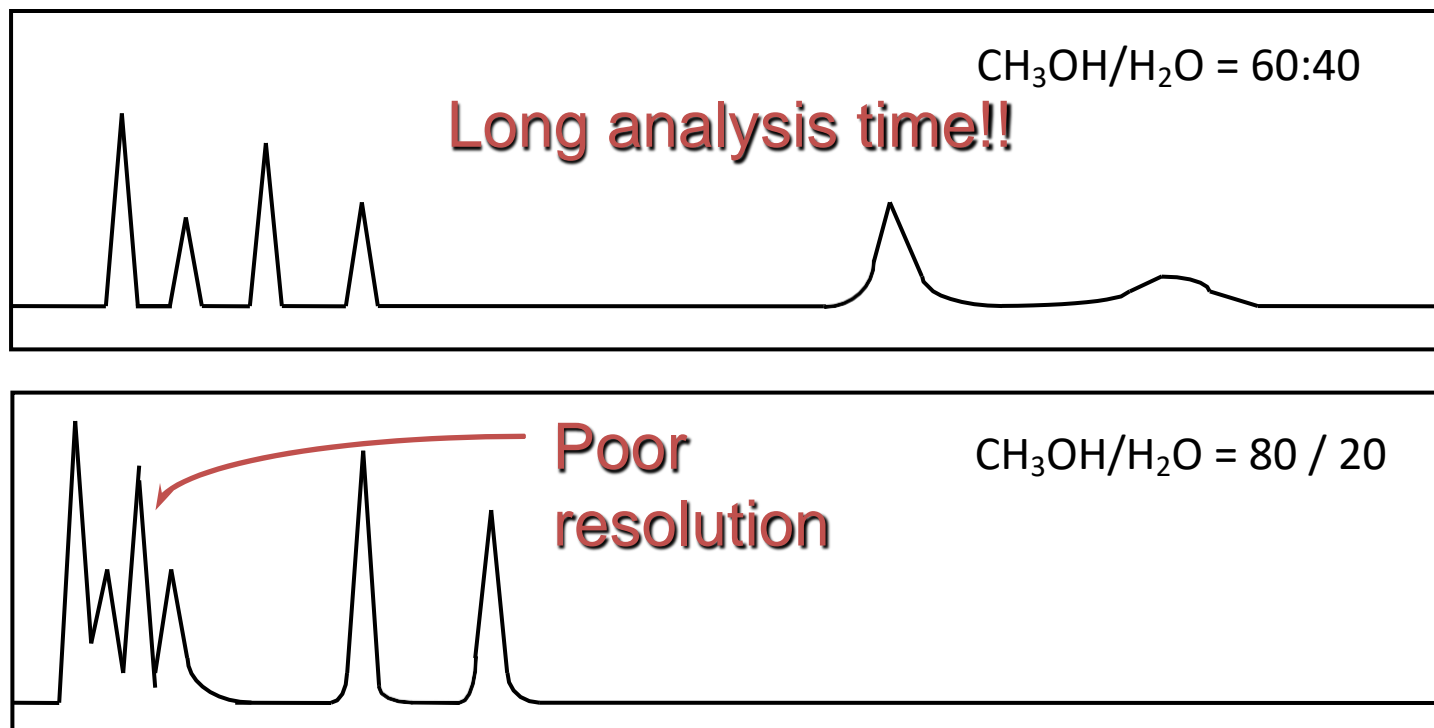
Constant eluent composition.

## ❖ Gradient system

Varying eluent composition.

- The technique of delivering solution with a constant composition as the eluent is called “isocratic elution”.
- The technique of varying the eluent composition during a single analysis is called “gradient elution”.

## In isocratic mode:



In the analysis of multiple components using HPLC, attempting to clearly separate every single component results in an extremely long analysis time.

On the other hand, attempting to reduce the analysis time by changing the eluent composition has an adverse effect on separation among components with relatively short retention times.

## In gradient mode:

If the eluent composition is changed gradually during analysis...

