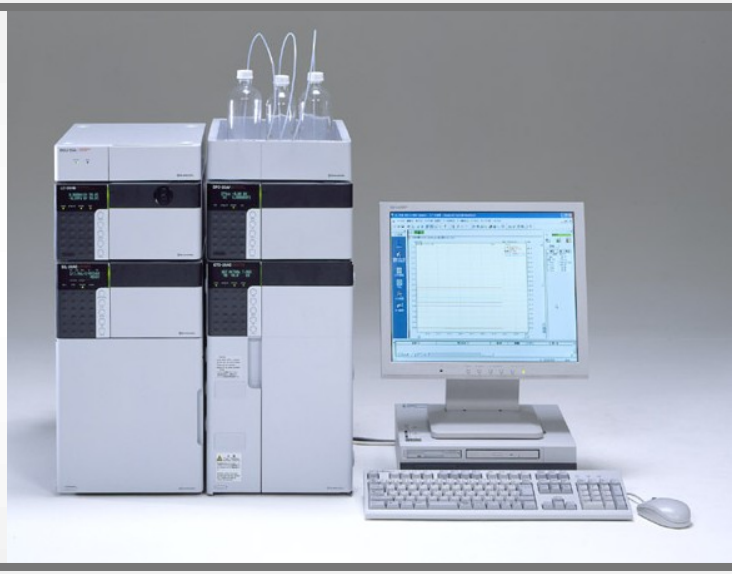
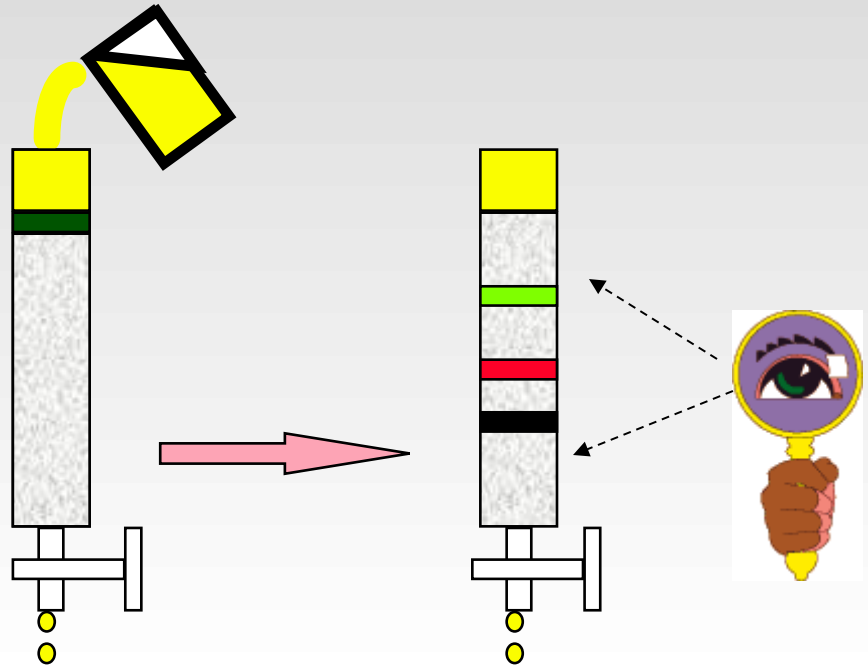
Introduction to HPLC

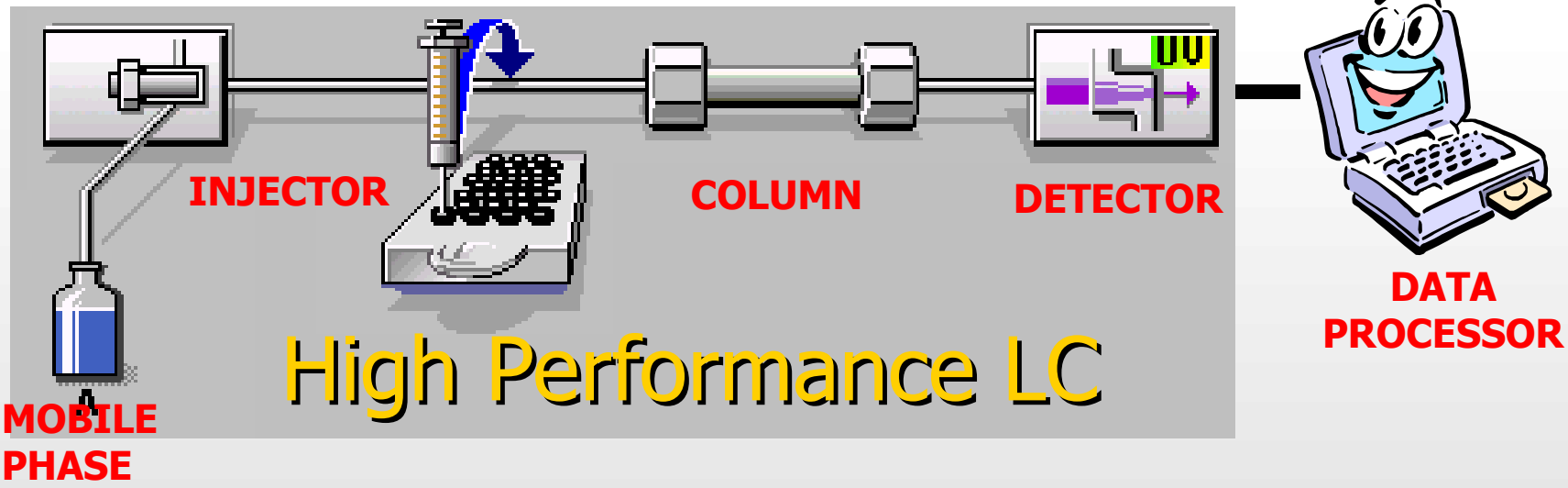


SPC-CSC

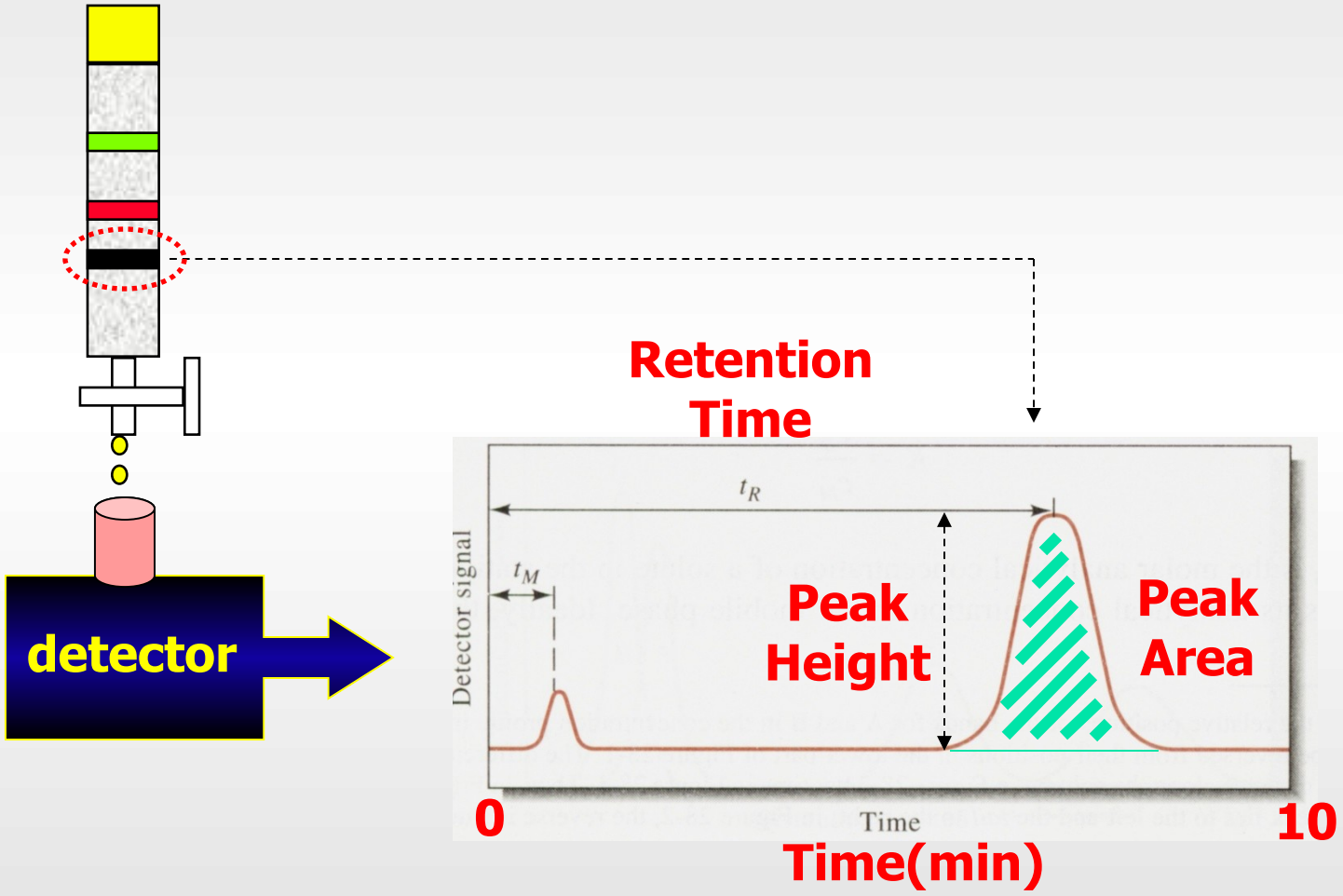
Applications for HPLC

- Pharmaceuticals
  - Antibiotics
  - Vitamins
  - Antipyretic & Analgesic drugs
- Environmental
  - Inorganic ions
  - Pesticides
- Polymers
  - Antioxidants
  - Plasticizers
- Food
  - Preservatives
  - Vitamins
  - Sugars
  - Organic acids
- Medical
  - Amino acids
  - Drugs
  - Metabolites




PUMP
INJECTOR
COLUMN
DETECTOR
DATA PROCESSOR
MOBILE PHASE
High Performance LC

Chromatographic Data



Modes of HPLC

- Normal Phase mode
- Reverse Phase mode
- Reverse Phase Ion Pairing mode
- Ion Exchange mode
- SEC mode (GPC / GFC)
- Chiral separation mode

Normal Phase Mode

- First technique used
  - $CaCO_3$ in Separation Column
  - Petroleum ether as Eluting Solvent
- We define this combination as

Normal Phase mode

Column : polar property

Solvent : non-polar property

Reverse Phase Mode

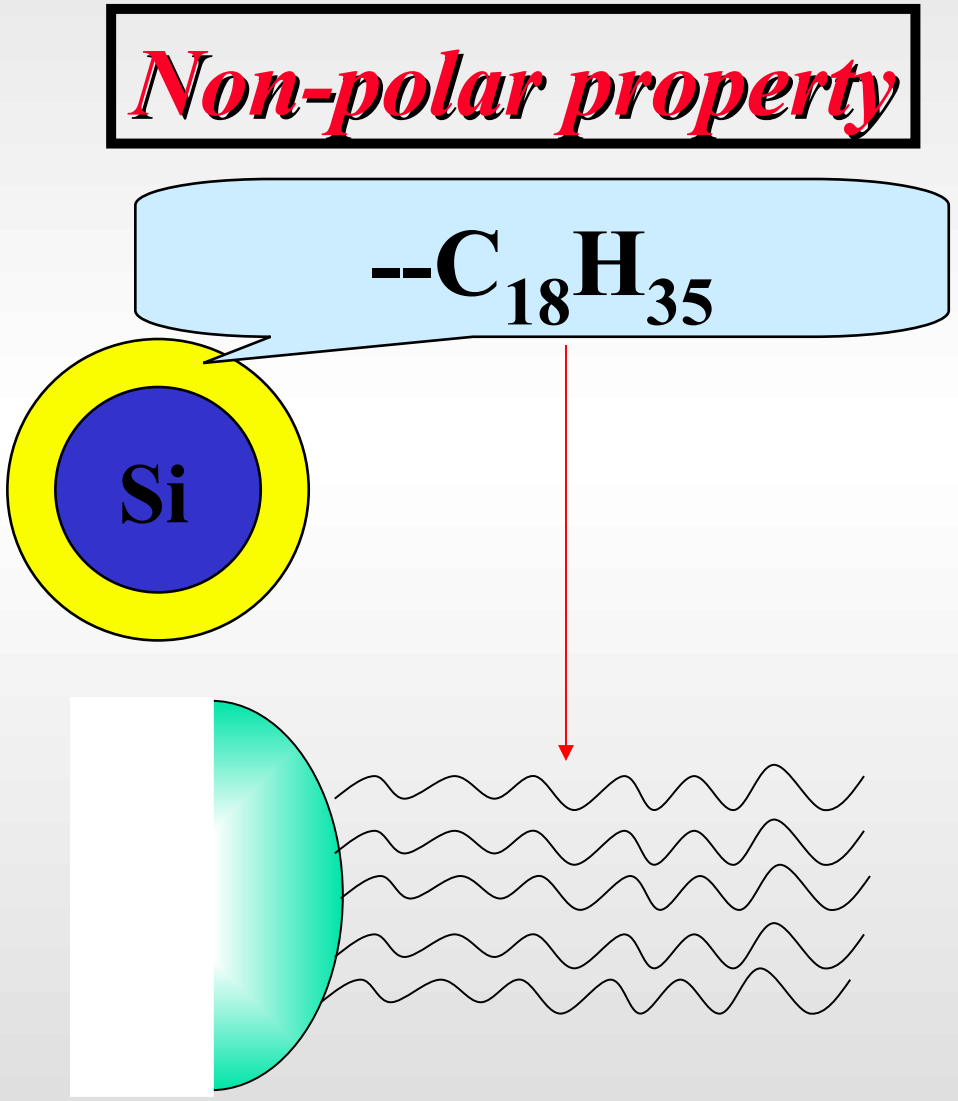
Column : Non-polar property

Solvent : Polar property

water /methanol / acetonitrile

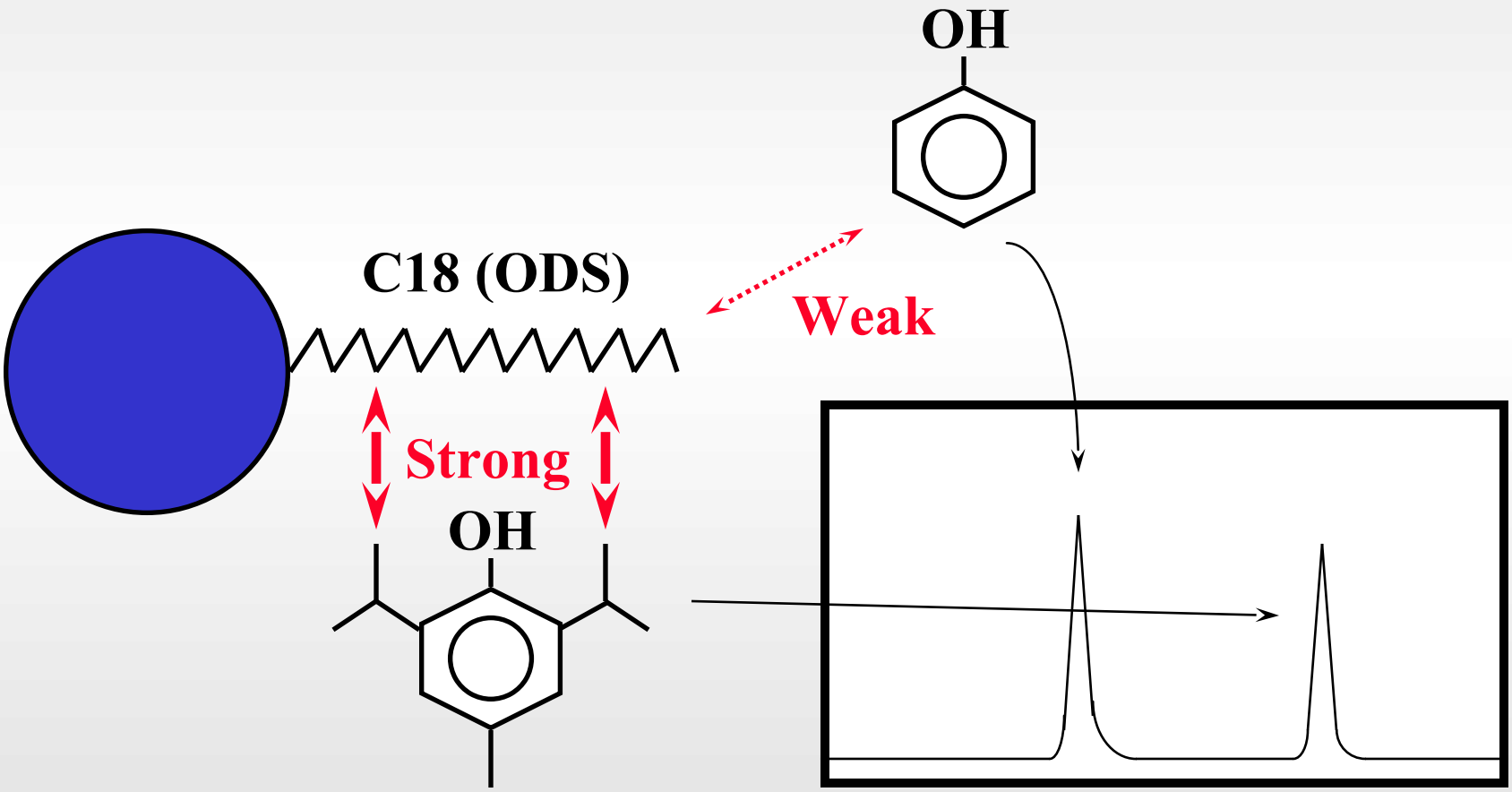
Reverse Phase HPLC Columns

- C18 type
- C8 (octyl) type
- C4 (butyl) type
- Phenyl type
- TMS type
- Cyano type

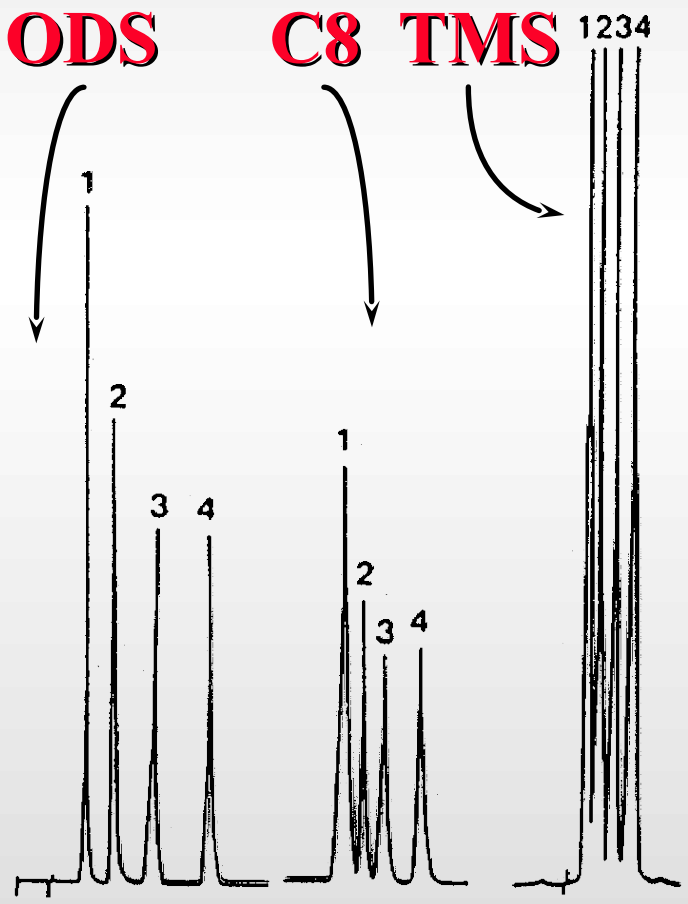


Principle of separation

"like dissolves like"

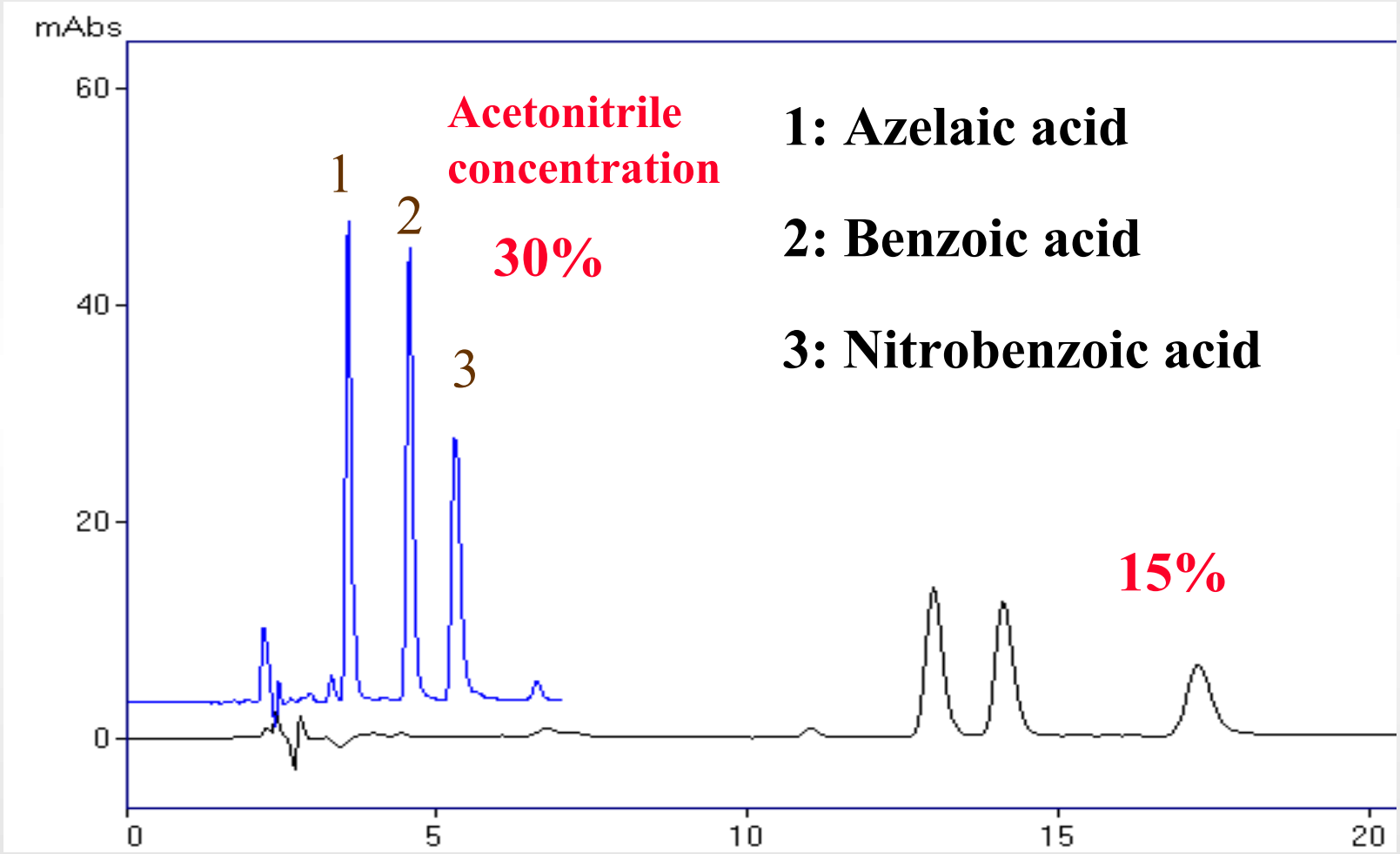


Effect of stationary phase



- **Analytical Conditions**
  - **Column : Shim-pack CLC-ODS**
  - **Mobile phase : MeOH : H2O = 7 :3**
  - **Flow rate : 1.0 mL/min**
  - **Temperature : 40 C**
  - **Injection volume : 10 uL**
  - **Detection : UV-254 nm**
- **Peaks**
  1. **Methyl benzoate**
  2. **Ethyl benzoate**
  3. **n-Propyl benzoate**
  4. **n- Butyl benzoate**

Effect of Mobile Phase Composition



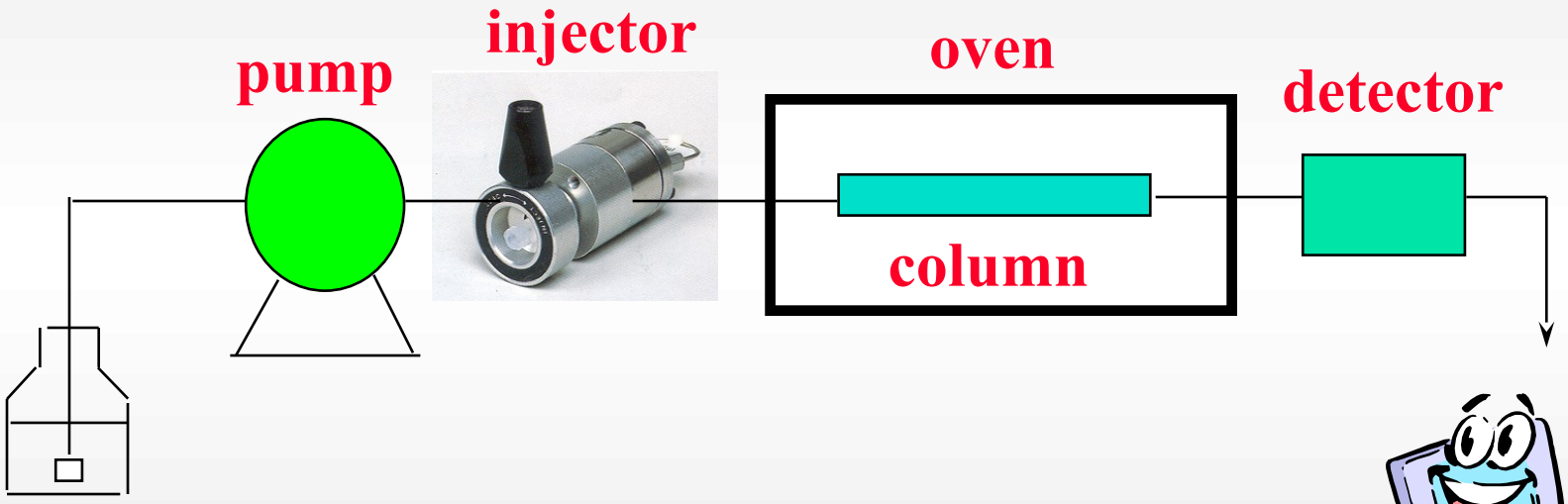
Normal vs Reverse Phase

- Normal Phase
  - good separation for stereo isomer (Vitamin E etc..)
  - variable retention time
- Reverse Phase
  - good repeatable retention time
  - rugged stationary phase

HPLC System

- Isocratic elution system
  - Single solvent of constant composition
- Gradient elution system
  - Multiple solvents of variable composition
    - High pressure gradient system
    - Low pressure gradient system

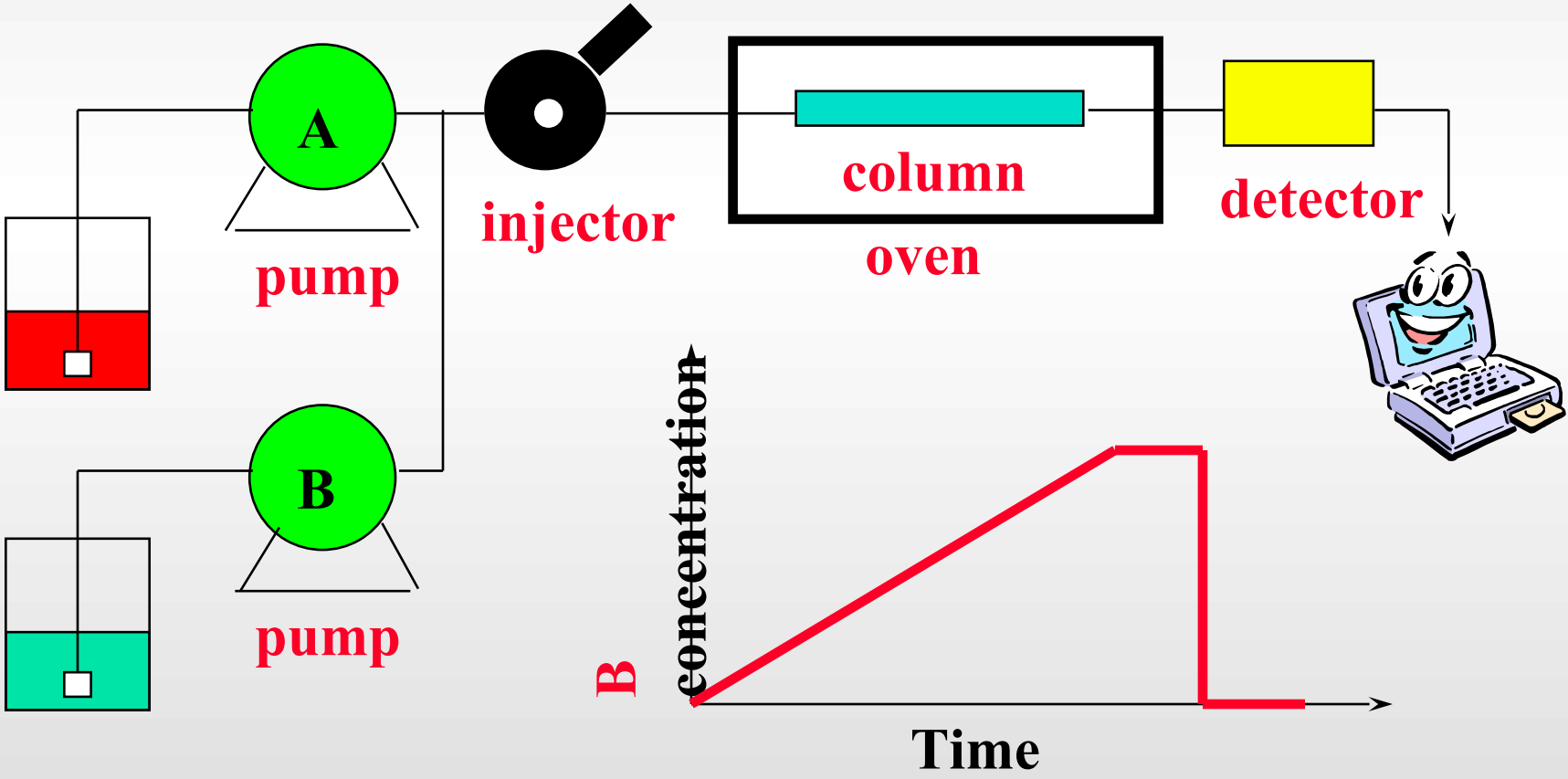
Isocratic Elution System



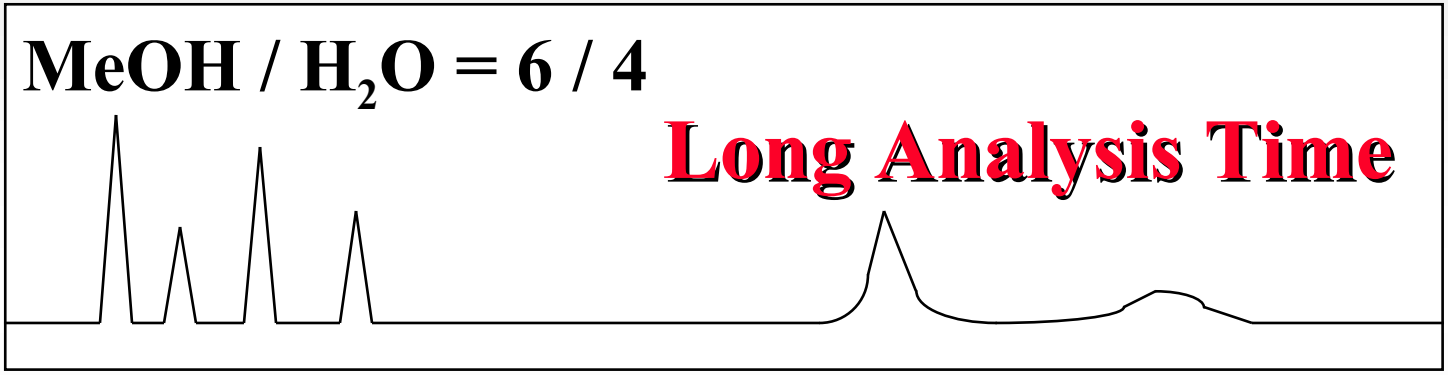
data processor

Single Solvent

Gradient Elution System

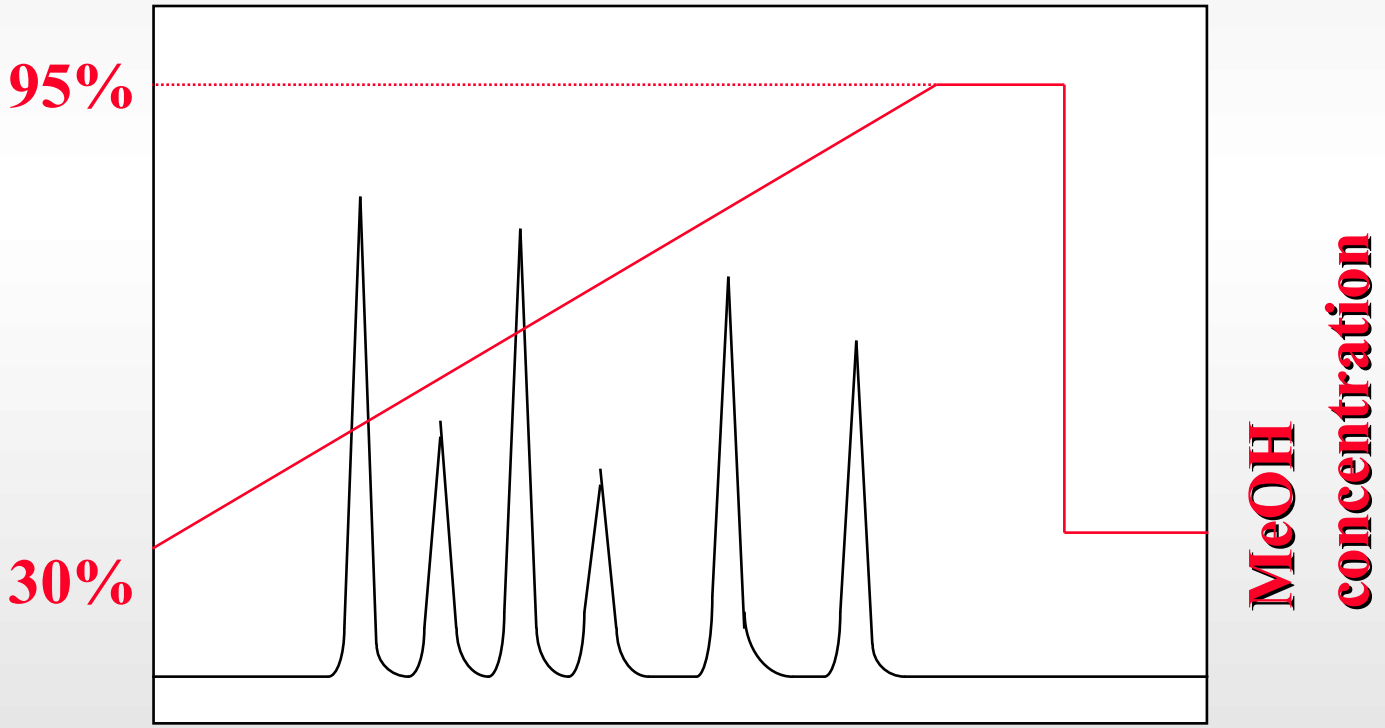


Isocratic Elution Mode



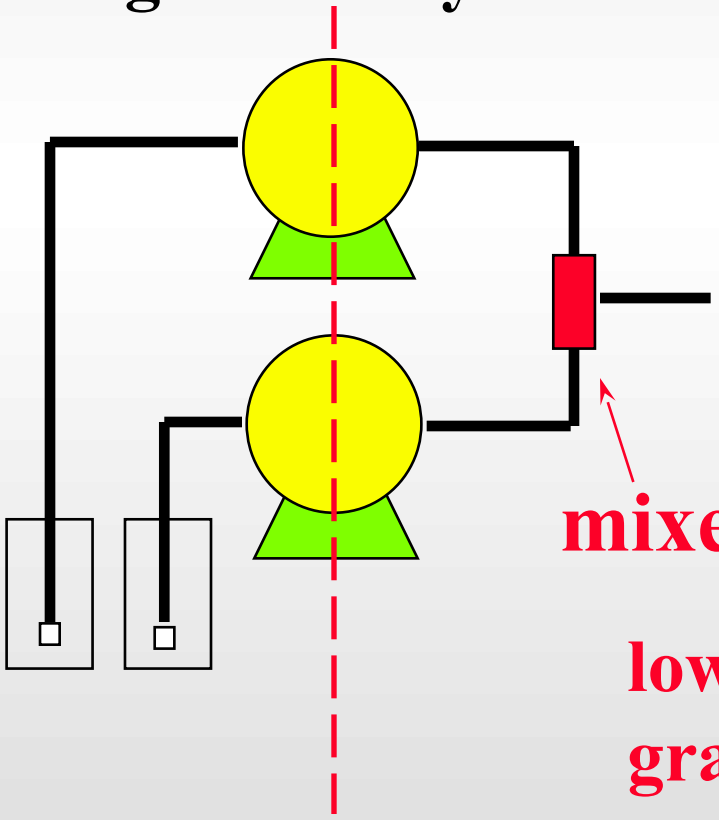
(column : ODS type)

Gradient Elution Mode



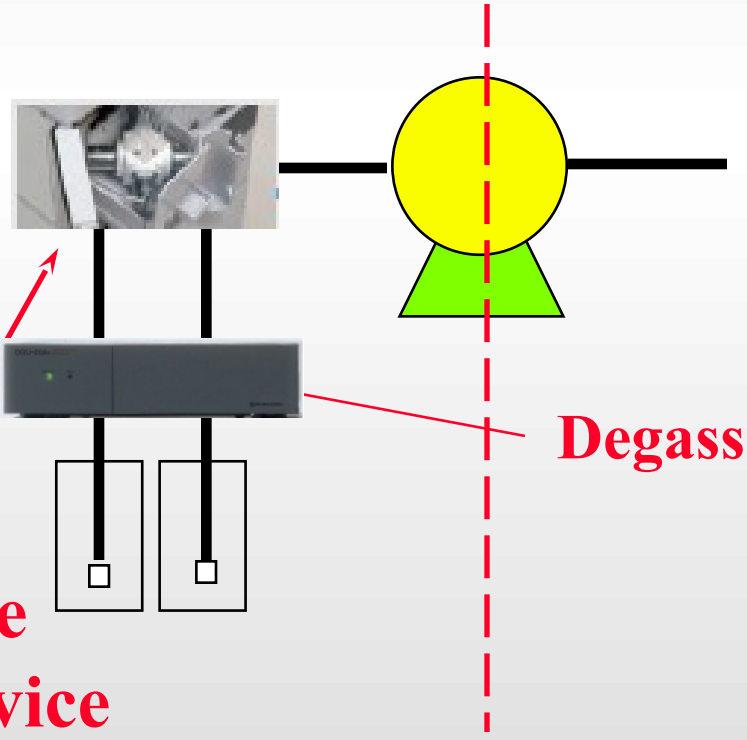
High/Low pressure gradient system

High pressure gradient system



mixer

Low pressure gradient system



Degasser

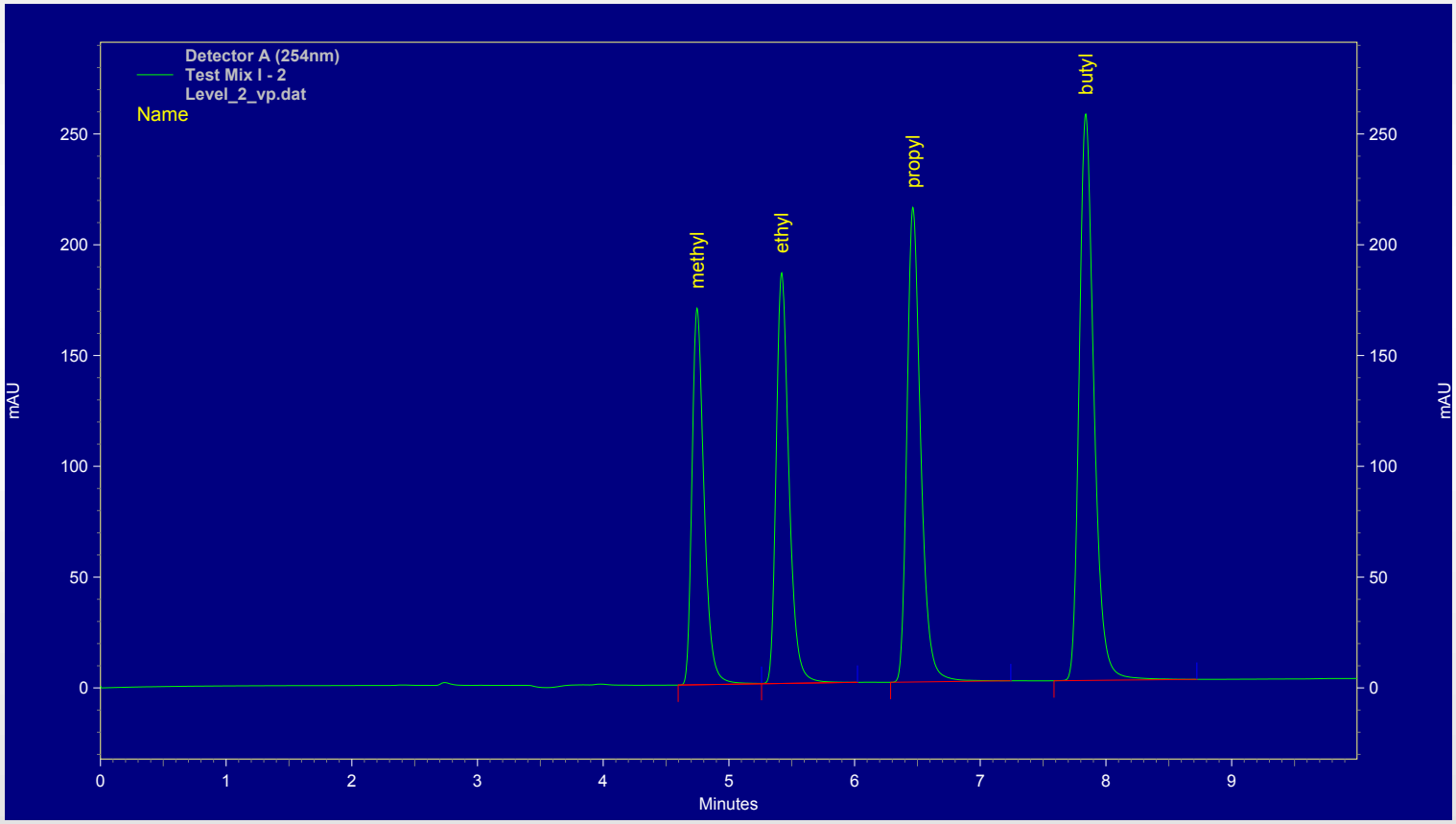
low pressure gradient device

High/Low pressure gradient system

- High pressure gradient system
  - excellent gradient accuracy
  - complicated system (more than two pumps)
- Low pressure gradient system
  - simple system
  - degasser is required

QUALITATIVE/QUANTITATIVE ANALYSIS

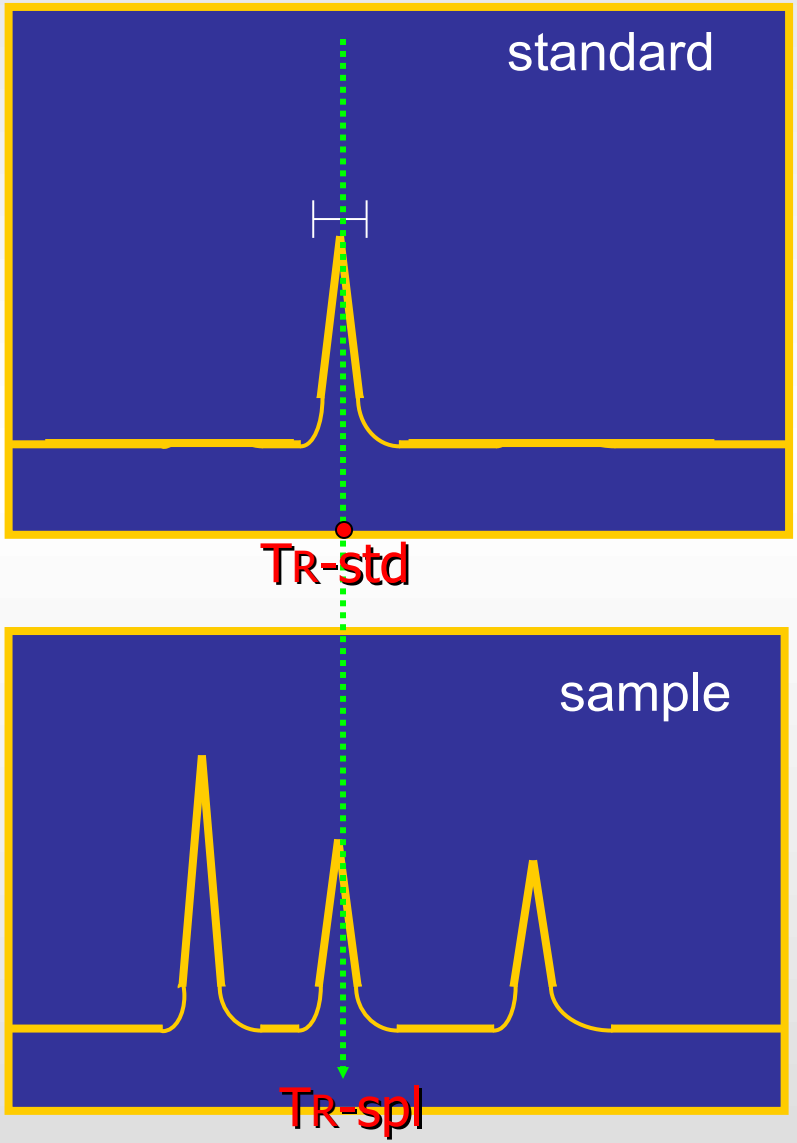
QUALITATIVE ANALYSIS



Detector Response

Retention Time

QUALITATIVE ANALYSIS



Basic Question:

Does the sample have a peak with a T_R-spl within $\pm$X% of the T_R-std?

QUALITATIVE ANALYSIS

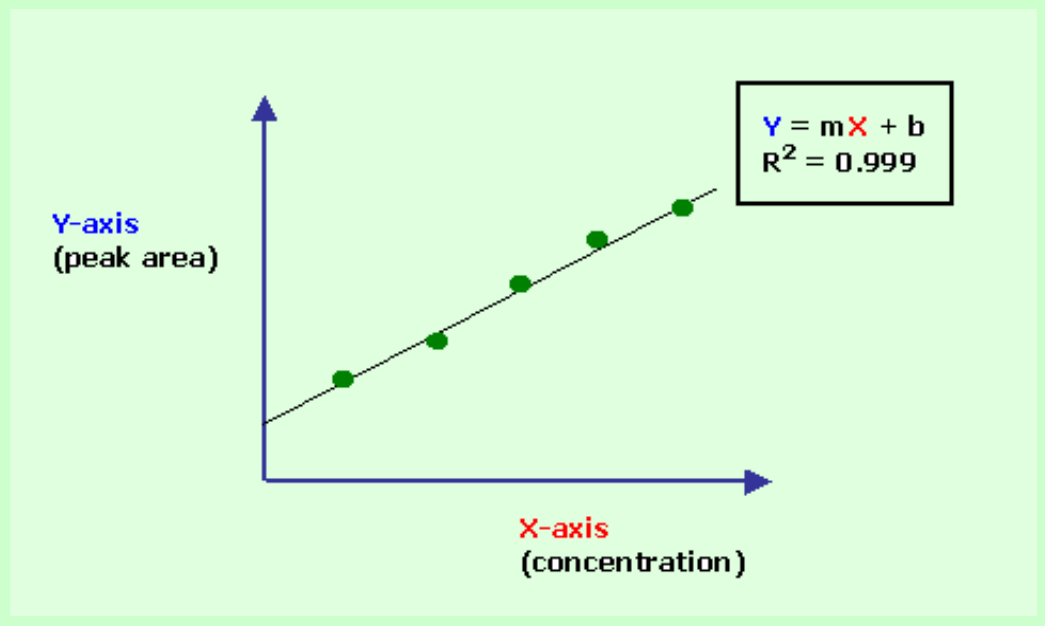
- Identification of individual components in the sample
- STANDARDS of known composition are needed
- T_R (retention time) is the qualitative data
- Directly compare the T_R of the standard and the unknown

QUANTITATIVE ANALYSIS

- **Determination of the amount / concentration of individual components separated in the sample**
- **Peak area or peak height is the quantitative data (α concentration)**
- **STANDARDS of known composition & concentrations are needed**

QUANTITATIVE TERMS

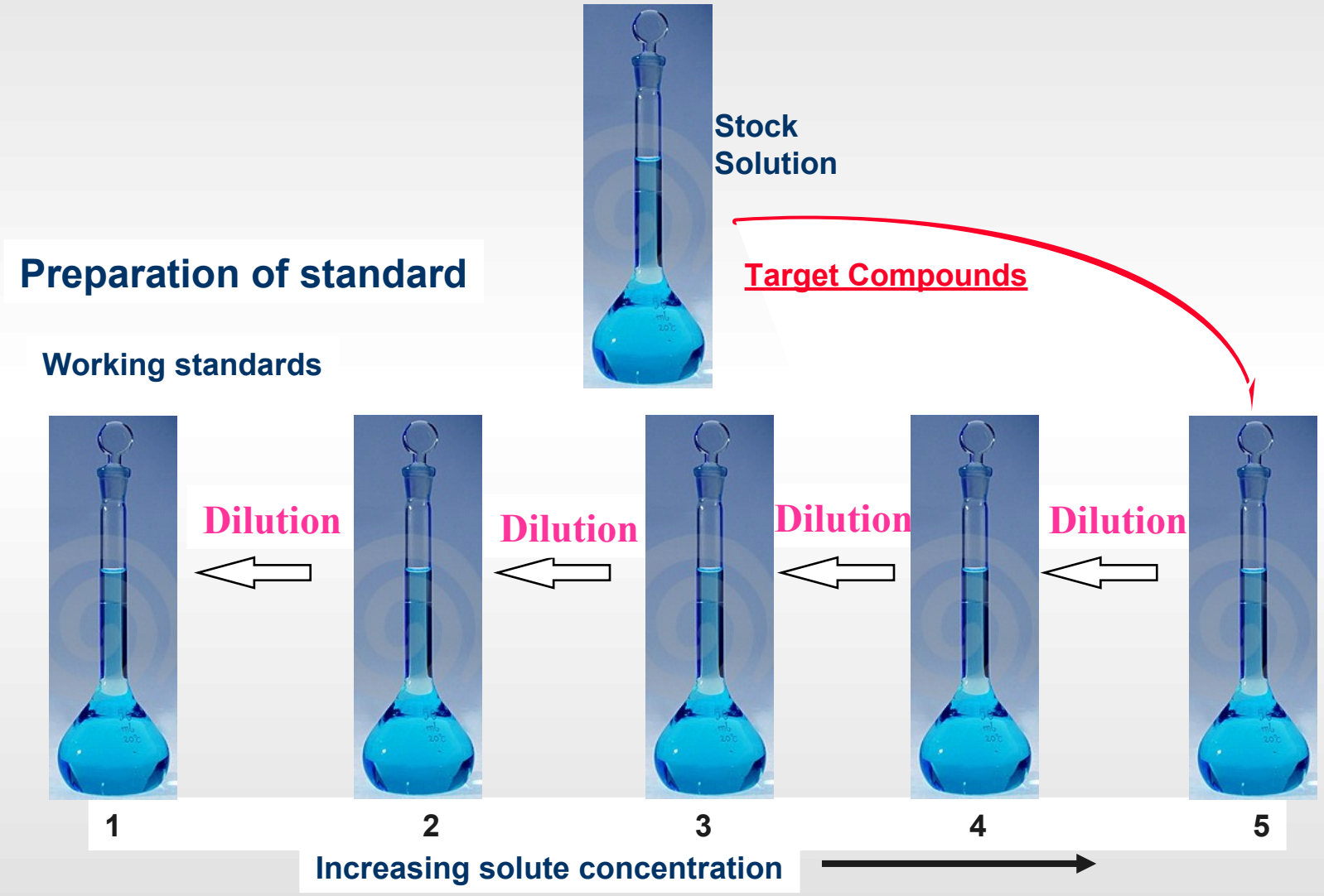
- **Calibration/Standardization**: generation of a curve that shows the relationship between concentration and peak area/height (per component)
- **External/Internal standardization method**: two of the most popular calibration methods



QUANTITATIVE TERMS

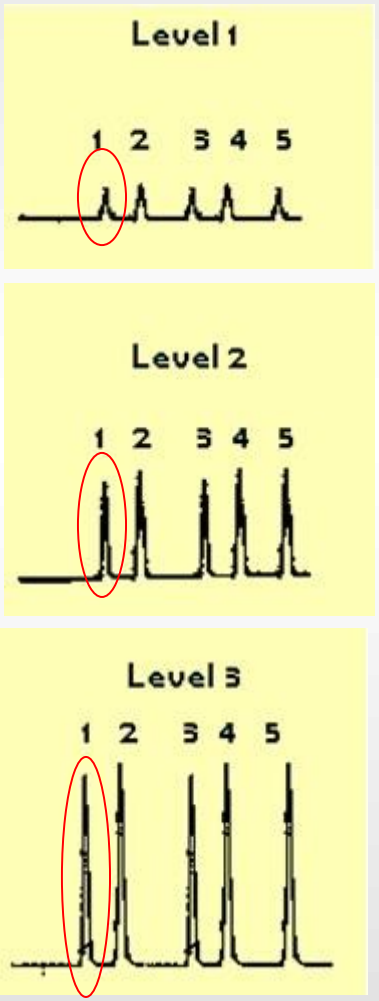
- **External Standard Method**
- **Internal Standard Method**

EXTERNAL STANDARD METHOD

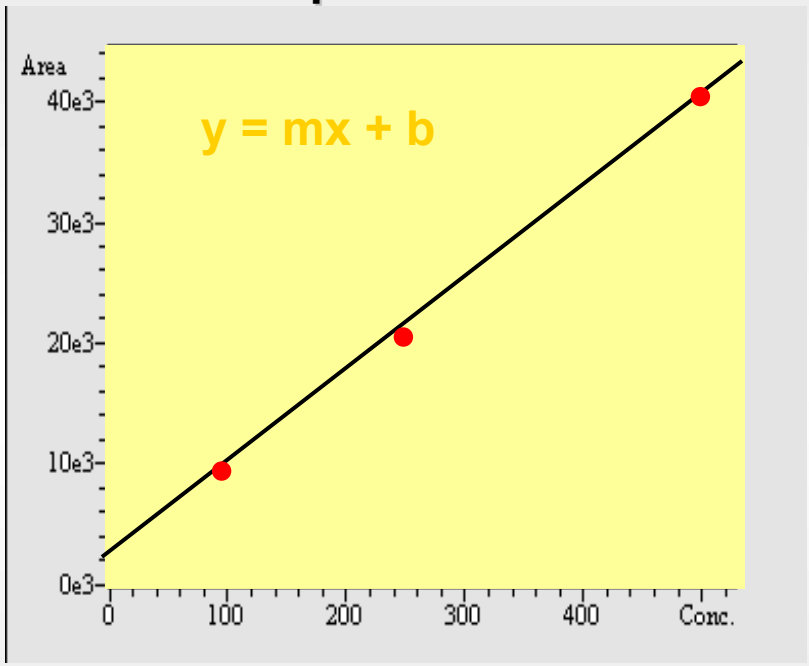


EXTERNAL STANDARD METHOD

Increasing concentration



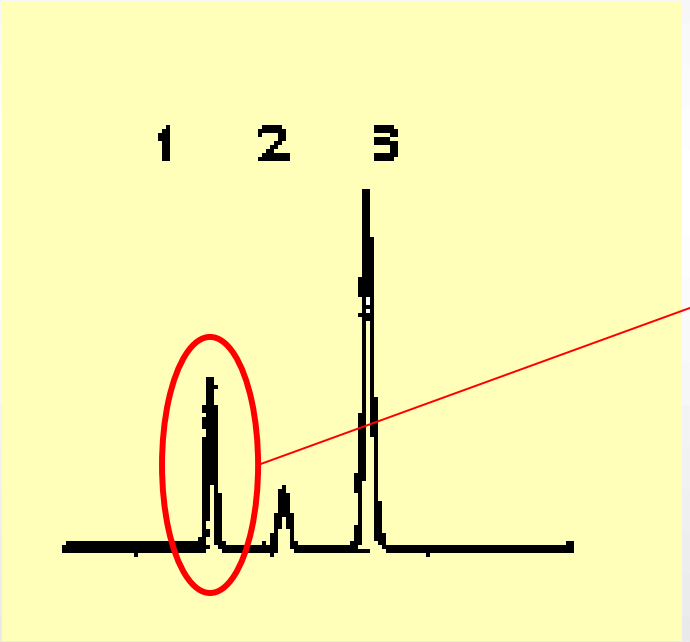
3-point calibration curve for peak # 1



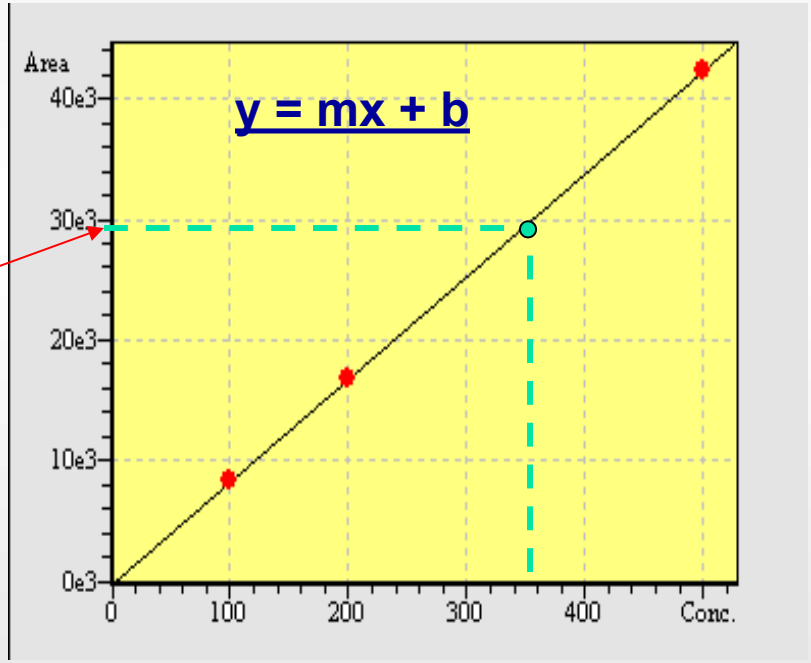
Y = AREA or HEIGHT
X = CONC.

EXTERNAL STANDARD METHOD

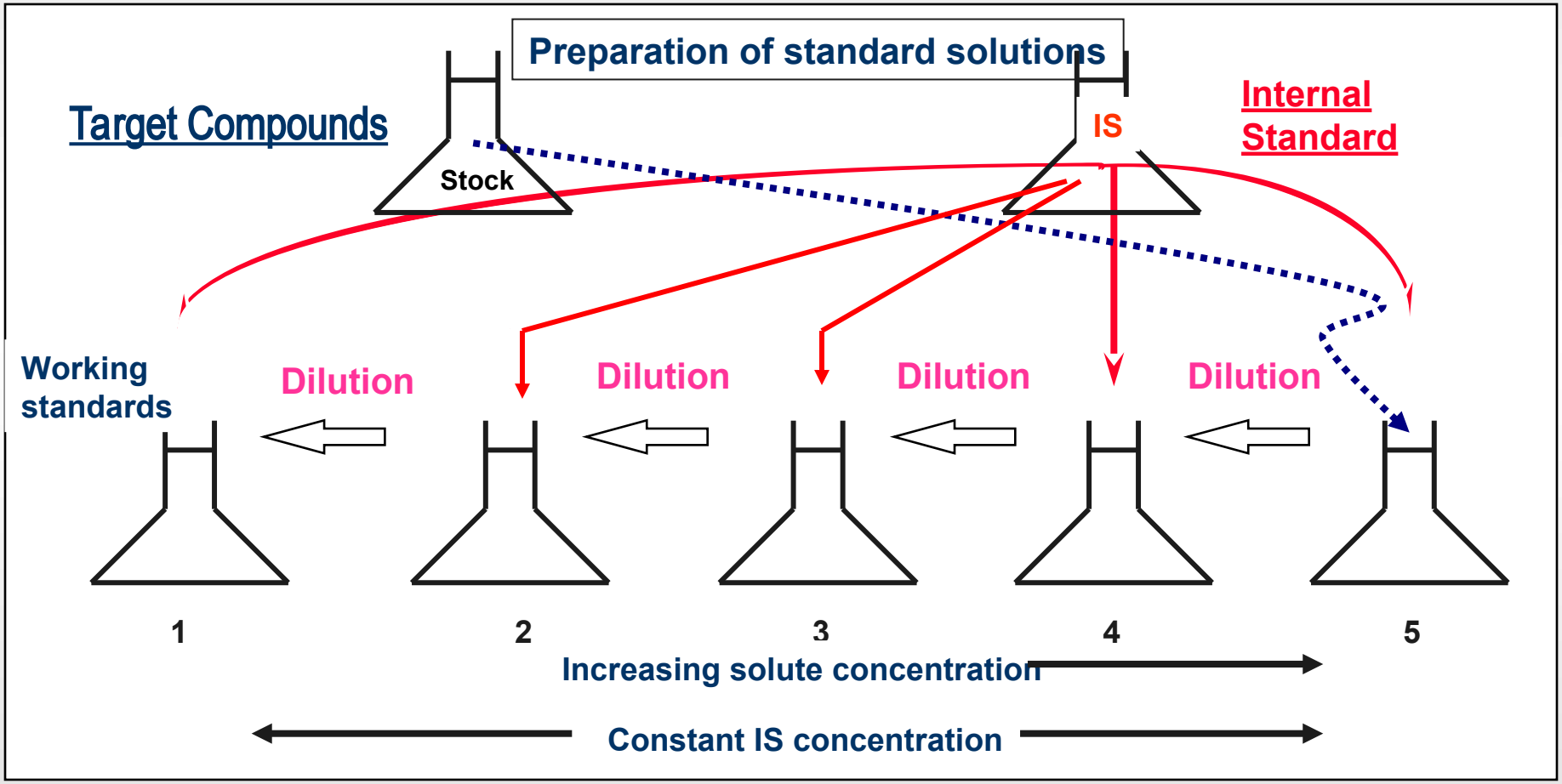
Chromatogram for sample



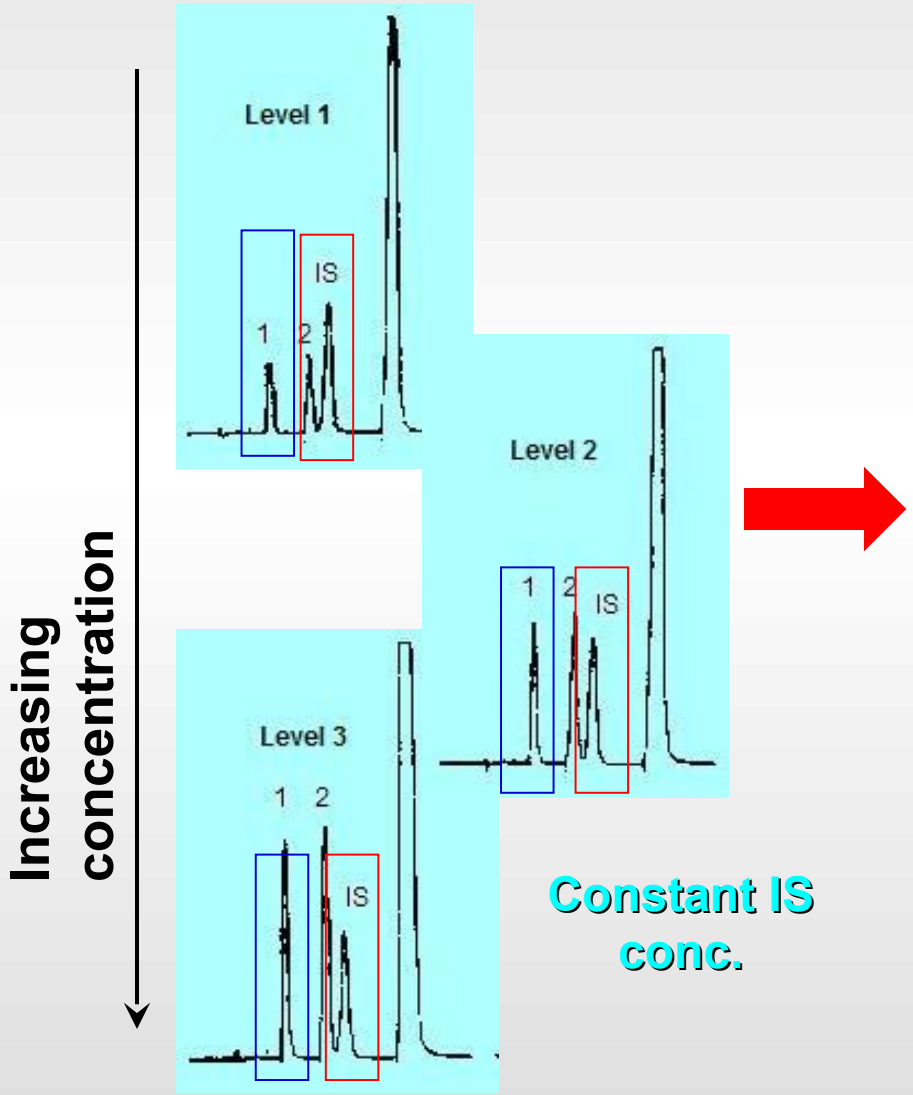
Calibration curve
(for peak 1)



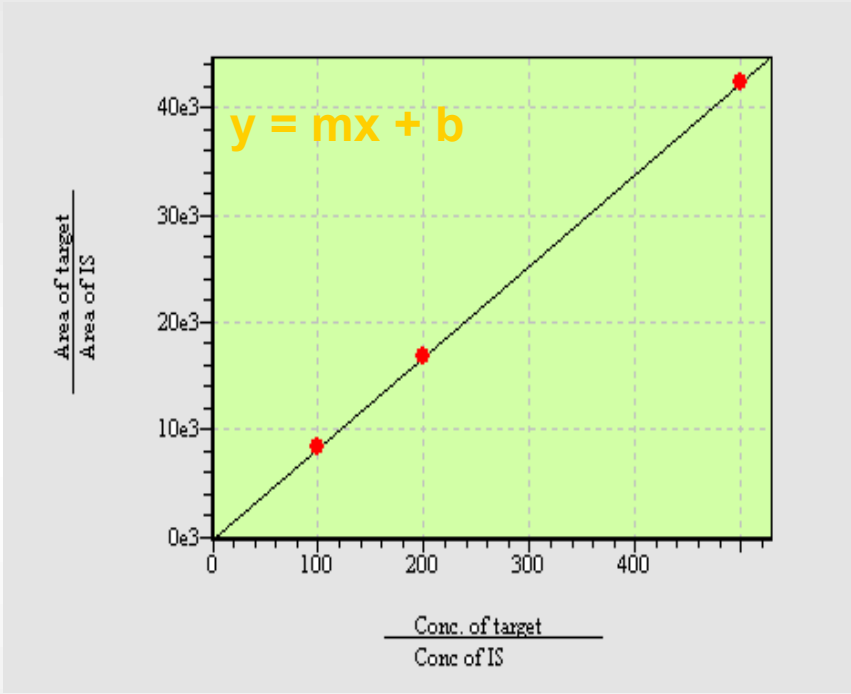
INTERNAL STANDARD METHOD



INTERNAL STANDARD METHOD



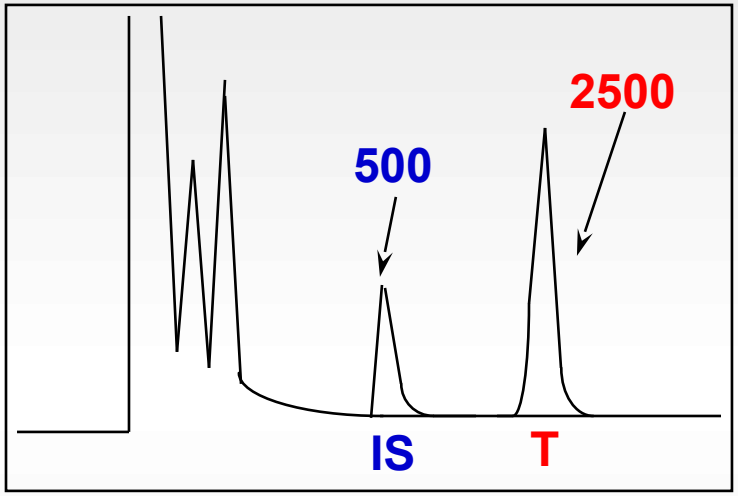
Constant IS conc.

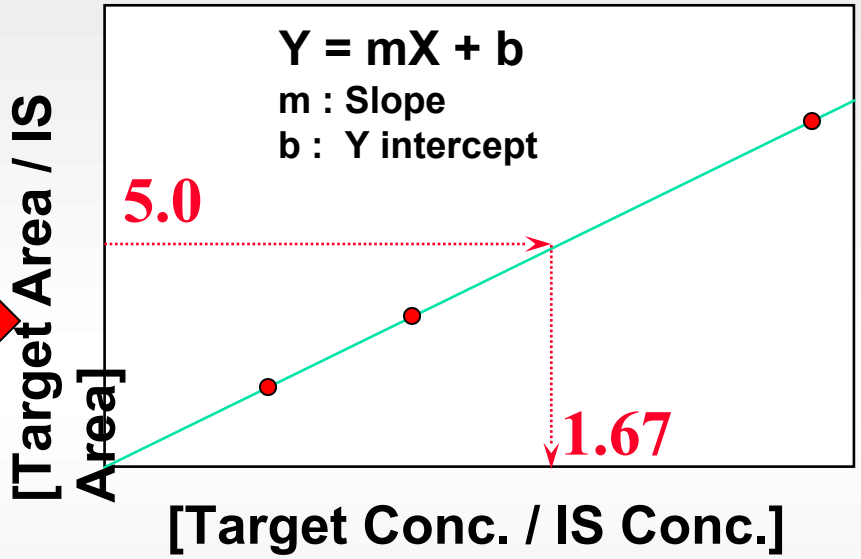


Y = AREA/ HEIGHT RATIO (target/IS)
X = CONC. RATIO (target/IS)

INTERNAL STANDARD METHOD

Calculation of Results





$$Y = mX + b$$

$$\frac{\text{Area T}}{\text{Area IS}} = m \cdot \frac{\overset{?}{\text{Conc T}}}{\text{Conc IS}} + b$$

X = Target Conc. / IS Conc.

1.67 = Target Conc./ 100 ppm

Target Conc. = 167 ppm

INTERNAL STANDARD METHOD

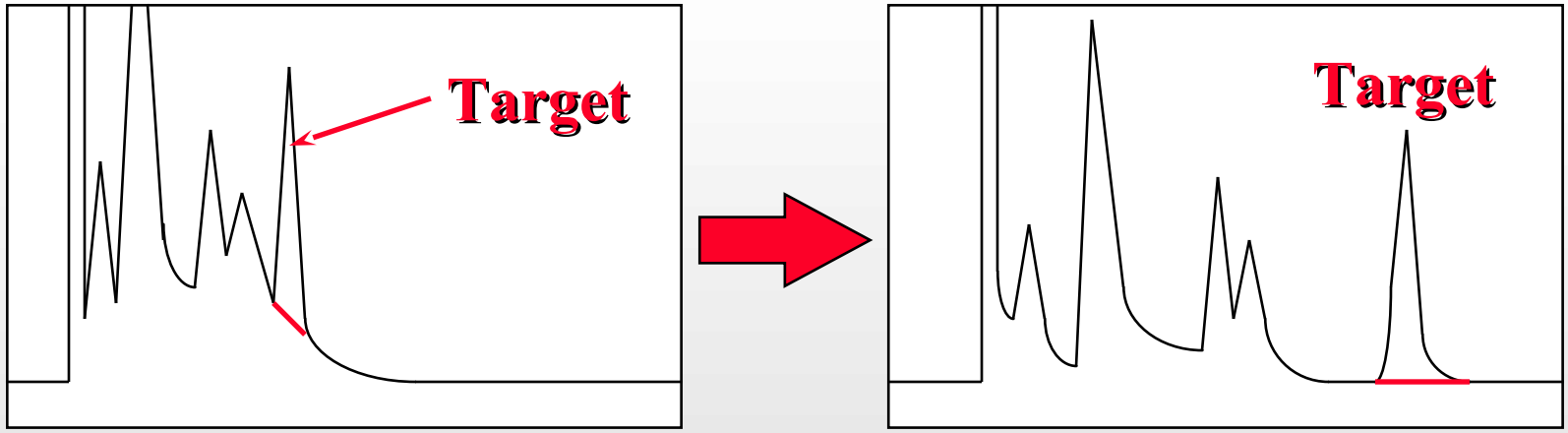
- **Properties of internal standard**

1. Must be similar in chemical nature to the target analytes
2. Must elute near the analytes of interest
3. Must not be present in the actual sample to be analyzed.
4. Must be available in pure form

- When do we use internal standard calibration?
- Is external calibration enough?

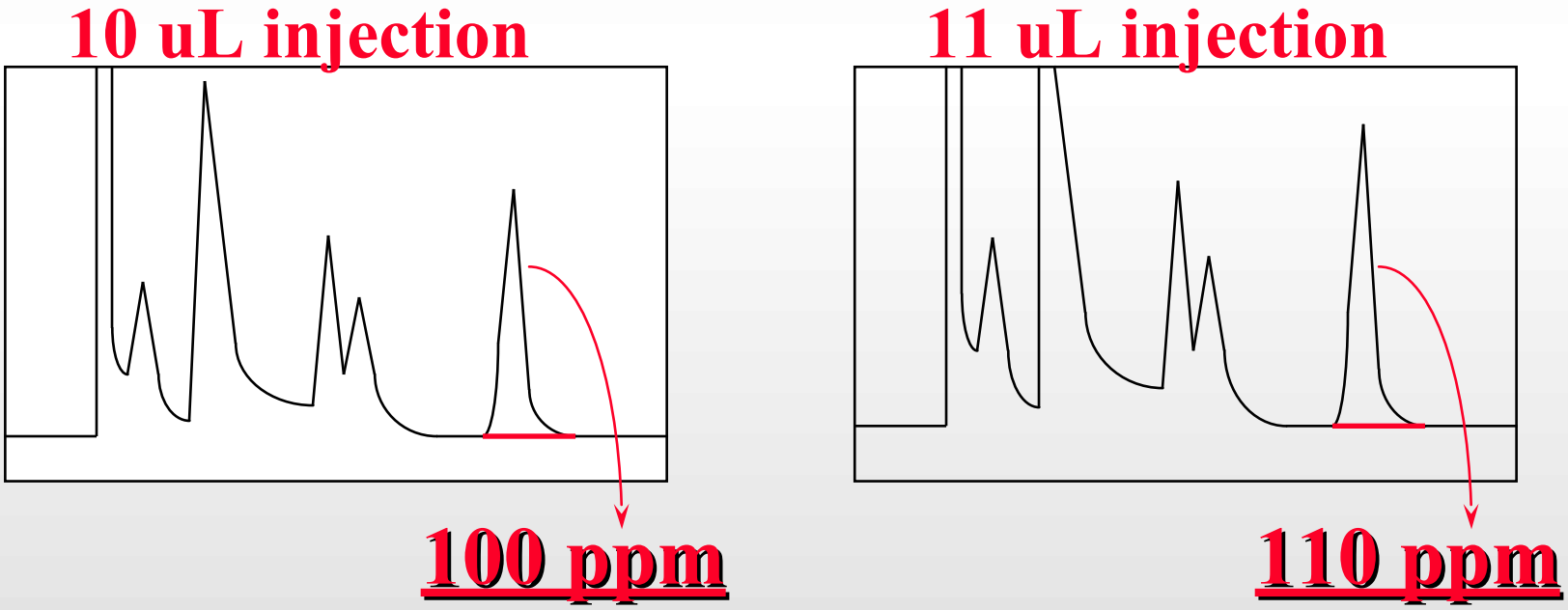
Advantage of External Standard calibration method

- Only the target compound separation can be focused.



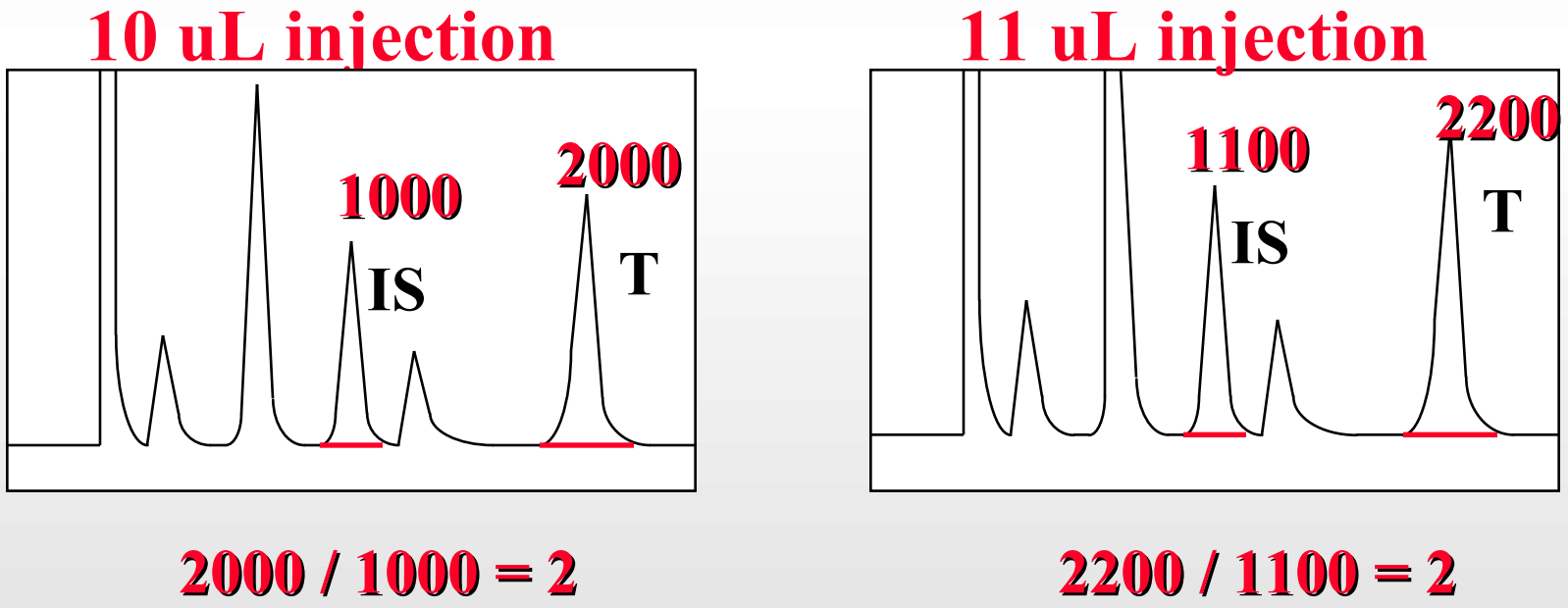
Disadvantage of External Standard calibration method

- Injection error will directly influence the quantitative result.



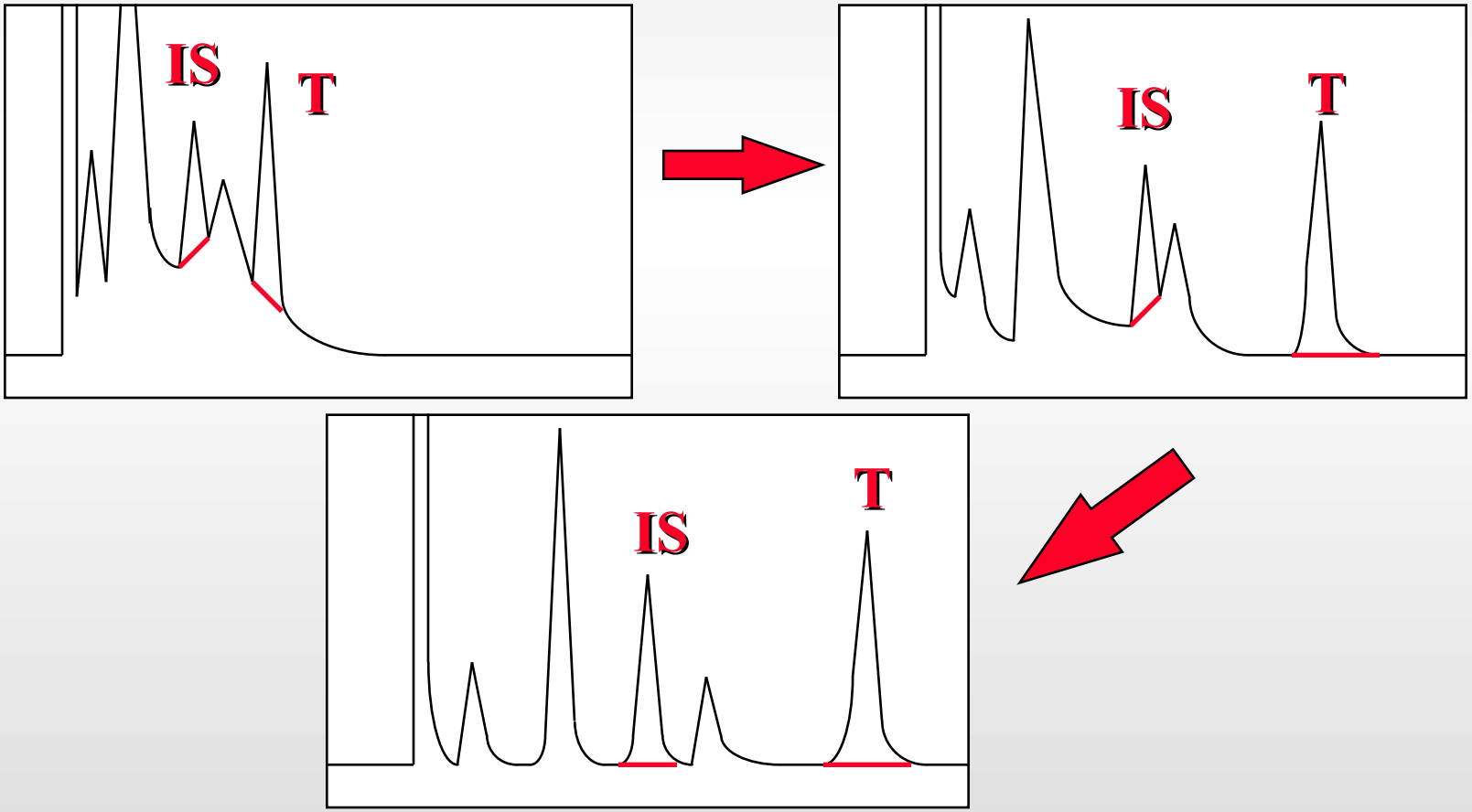
Advantage of internal standard calibration method

- Injection error can be eliminated.



Disadvantage of internal standard calibration method

- Separation is slightly difficult.



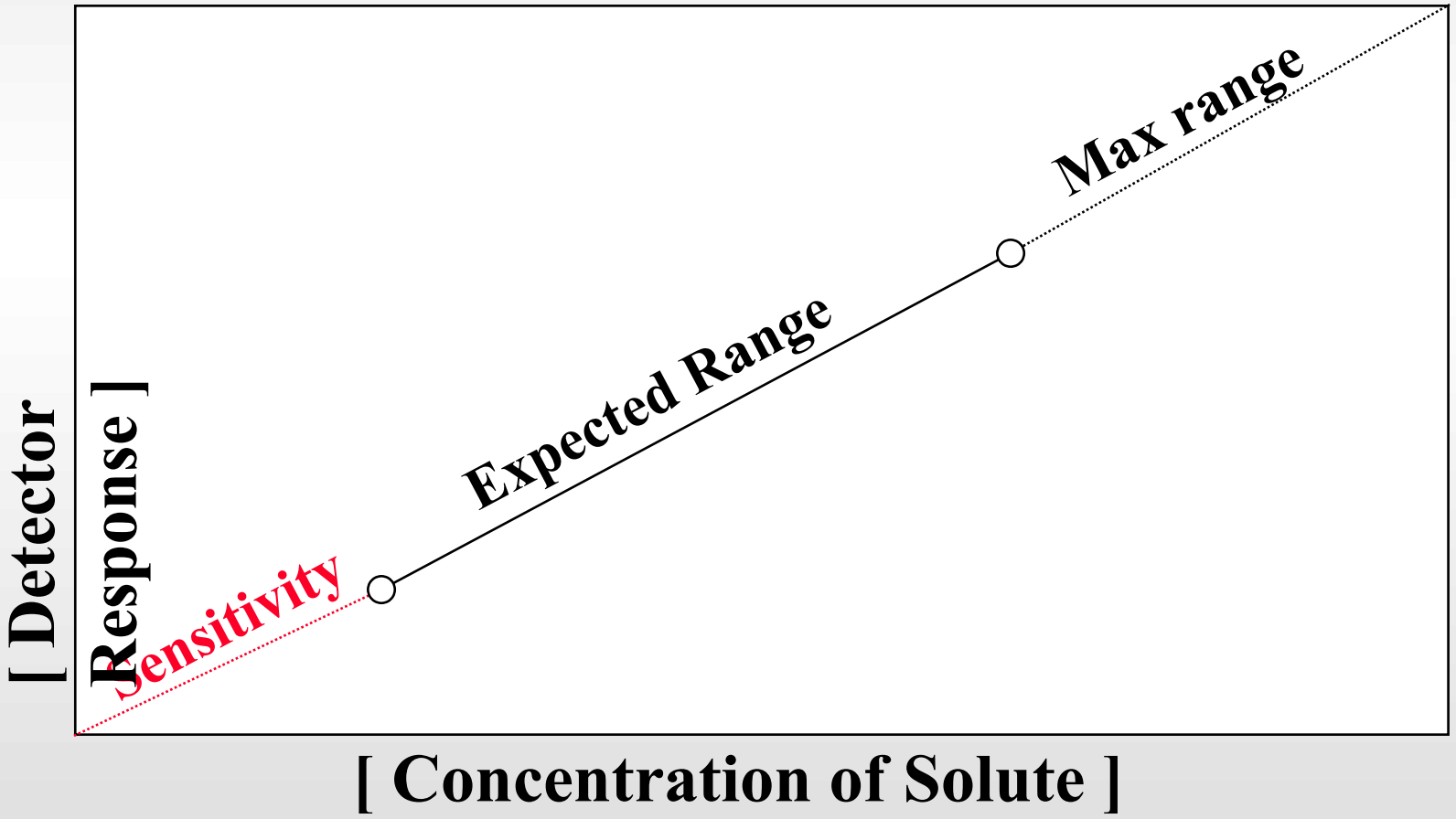
Disadvantage of IS Method

- It is difficult to look for the IS compound.
  - The chemical structure of IS compound should be similar to target compound.
  - IS sample should not exist in the actual sample.

Calibration Method

- External standard calibration
  - Separation is not difficult
  - Injection error will directly influence the quantitative result
- Internal standard calibration
  - Injection error can be eliminated
  - Recovery in the pretreatment procedure can be estimated
  - Separation is slightly difficult
  - Difficult to look for the IS compound

Accurate Quantitation - Select Appropriate Working Range



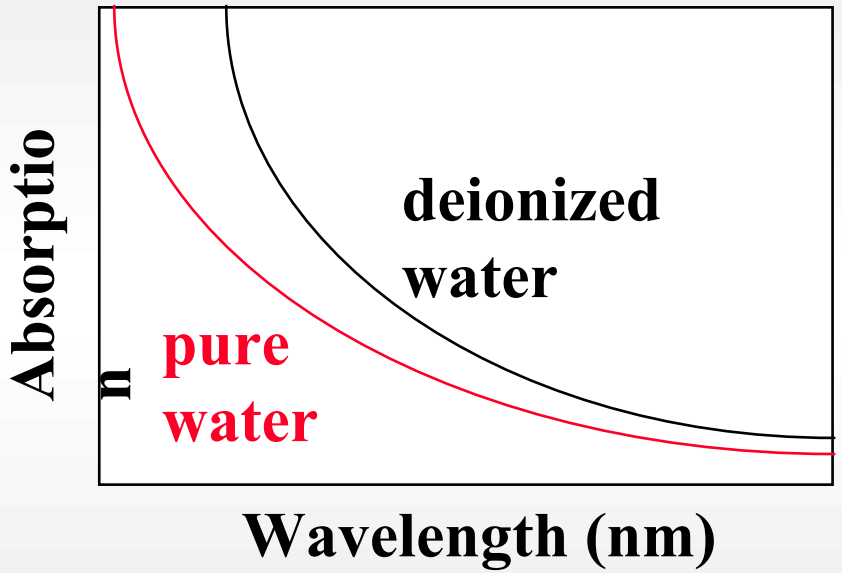
Preventive Maintenance

Common problems in HPLC

- High back pressure
- Poor baseline stability and excessive noise
- Appearance of ghost peaks
- High background absorption of solvent
- Peak tailing
- Pump leakage
- Column failure

Mobile Phase

- Water
  - Use high purity water
  - Distilled water
  - Deionized water (18.2 mega-ohm)
  - Or deionized, distilled water



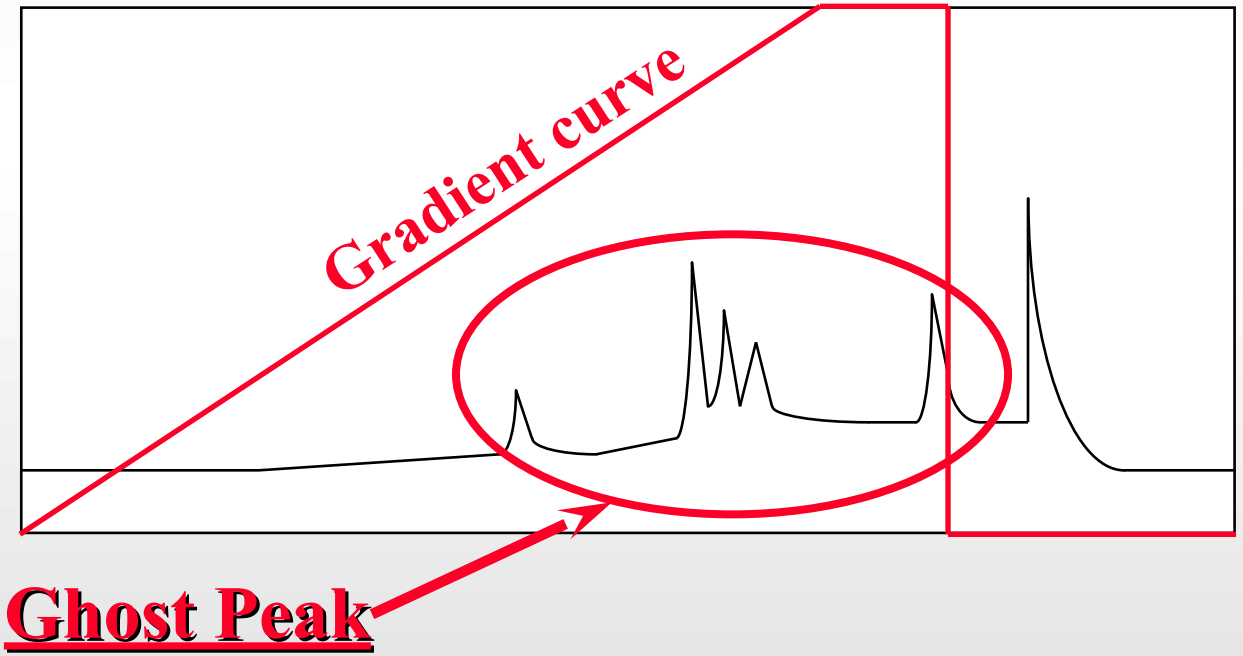
Due to existence of impurity, deionized water may show higher absorption.

Mobile Phase

- Problem with deionized water is ghost peak appear in gradient elution! (during trace analysis)

H_2O / MeOH gradient

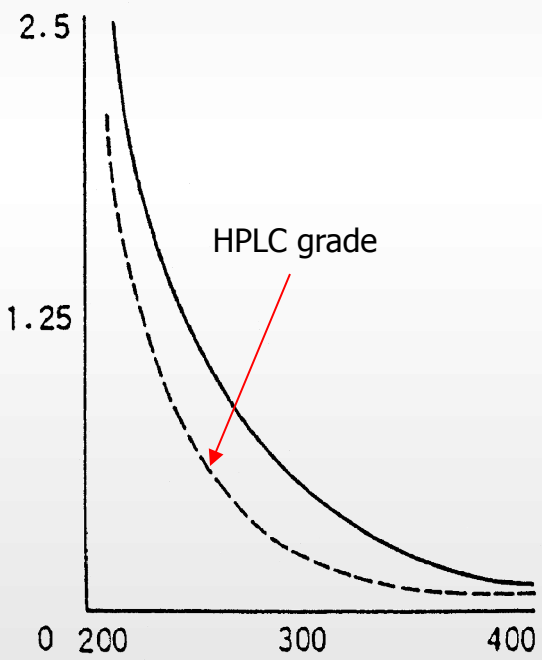
ODS Column



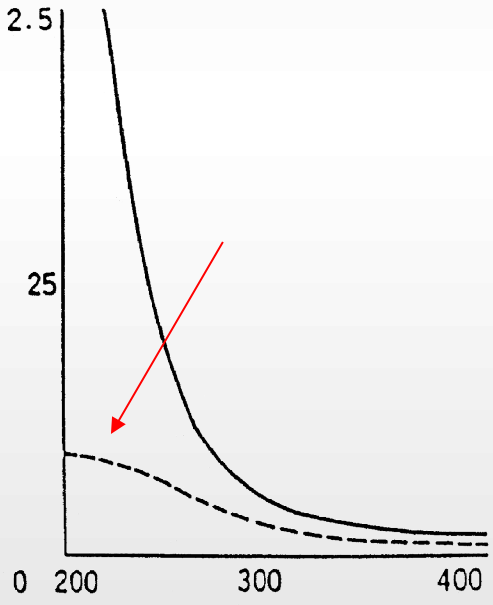
Mobile Phase

- Solvents
  - ✓Use hplc grade

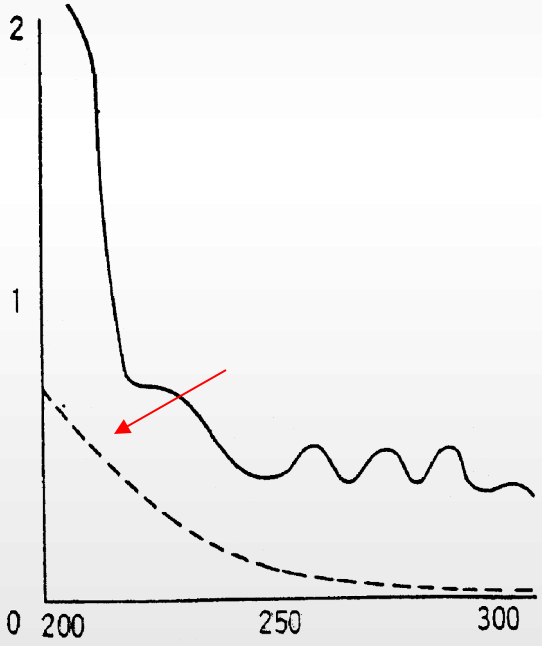
Difference Between Analytical and HPLC Grade Solvents



Methanol



Acetonitrile



Hexane

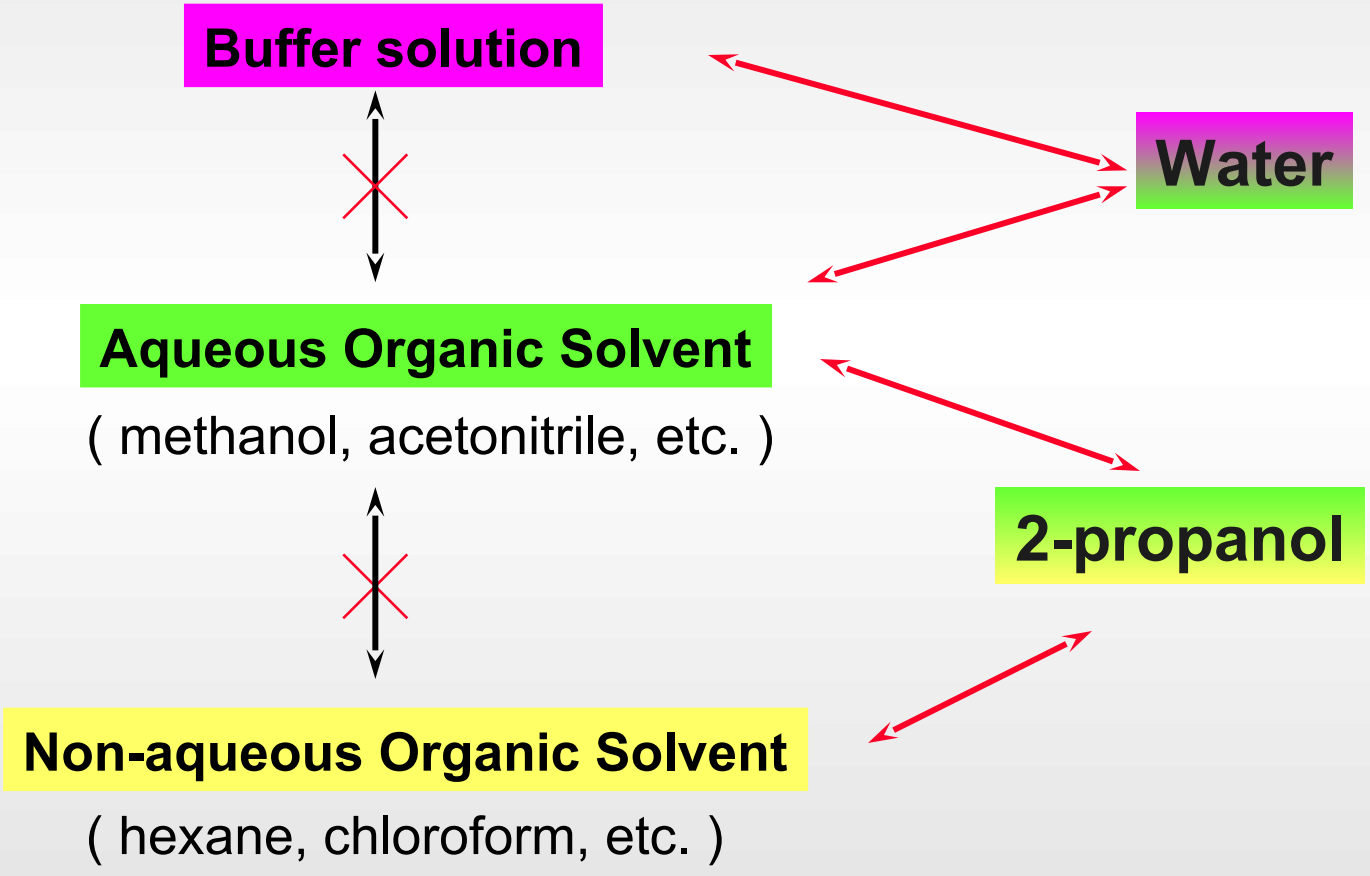
Mobile Phase

Use solvents with low background absorption

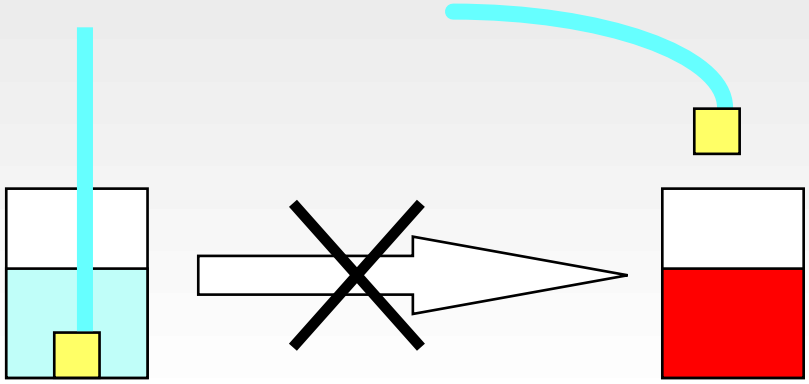
Cut-off Point for HPLC Solvents

Wavelength	190	195	200	205	210	215	220	230	235	240	245	250	254
Acetonitrile	1.000	0.150		0.070	0.040		0.020						0.010
1-Butanol						1.000	0.500	0.200					0.025
Chloroform											1.000	0.320	0.150
Cyclohexane			1.000	0.880	0.670								0.014
Ethanol				1.000	0.650		0.350						0.040
Ethyl Acetate													1.000
Ethyl Eter							1.000						0.070
Heptane			0.750				0.200						0.014
Hexane		1.000			0.250		0.080						0.014
Methanol				1.000			0.300	0.150					0.025
Methylene Chloride								1.000	0.700	0.200			0.100
Pentane	1.000		0.300		0.100								0.014
2-Propanol				1.000			0.250	0.130					0.025
THF					1.000	0.600		0.300					0.100

Changing the mobile phase

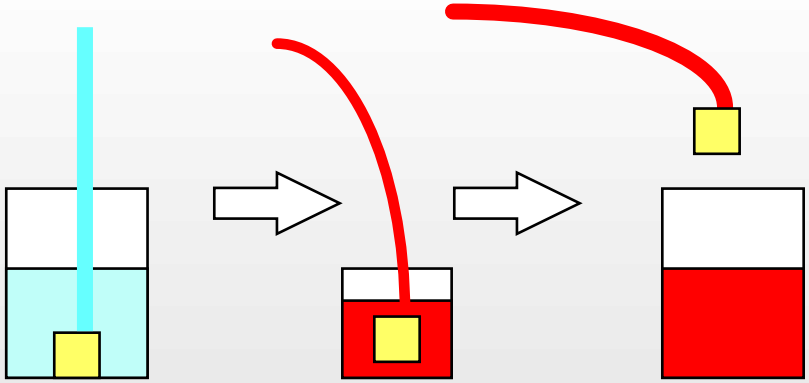


Changing the mobile phase

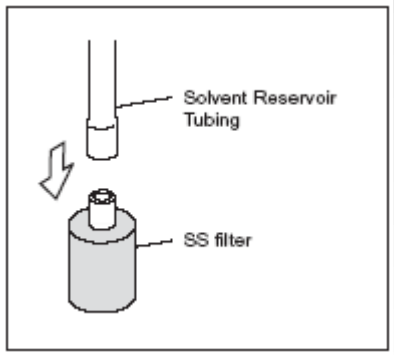


Old mobile phase

New mobile phase



Rinse suction filter
in fresh mobile phase



Pipe

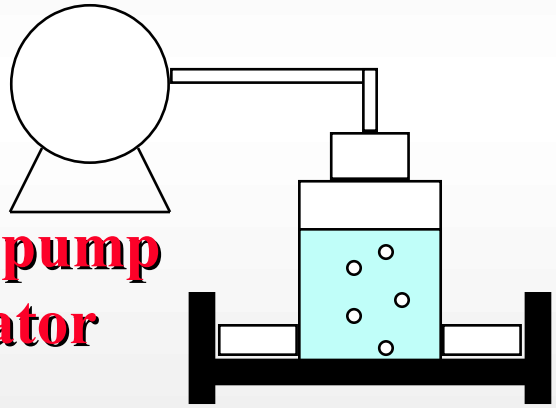
~20 cm
Do not touch directly

Suction Filter

Degassing

Eliminates dissolved gases which can contribute to baseline noise and pump problems.

Off line degassing



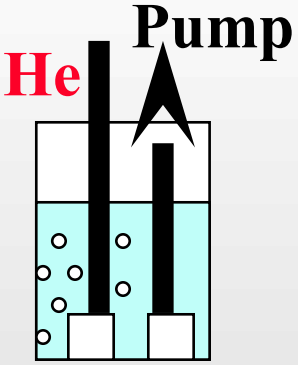
Vacuum pump or Aspirator

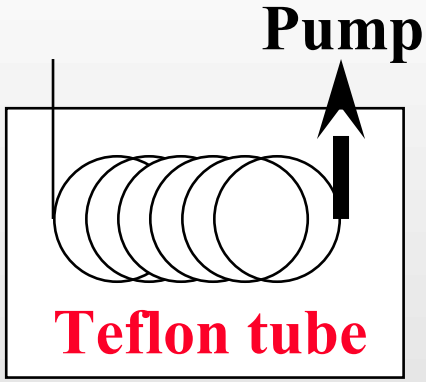
Ultrasonic bath

On line degassing

He purging

Vacuum Chamber





Vacuum Chamber

Reasons for Column Failure

- Plugged Frit or Column Packing
- Adsorbed Sample & Solvent Impurities
- Mechanical Shock, forming Voids
- Chemical Attack of Packing Material

Plugged Frit or Column Packing

Back-Pressure too High!

- Shortens column lifetime
- Separation sometimes affected
- Caused by particulates in the sample and or mobile phase

Adsorbed Sample/Solvent Impurities

- Accumulated impurities/contaminants retained in the column can cause peak tailing.

Prevent Column Clogging

Avoid Clogging!

- Mobile phase/buffer solutions “must” be filtered using by 0.45 or 0.2 um membrane filter.
- Samples must be filtered using 0.45 or 0.2 um membrane filter.
- Don’t leave buffer inside the column when not in use

Remedies for Clogging/Contamination

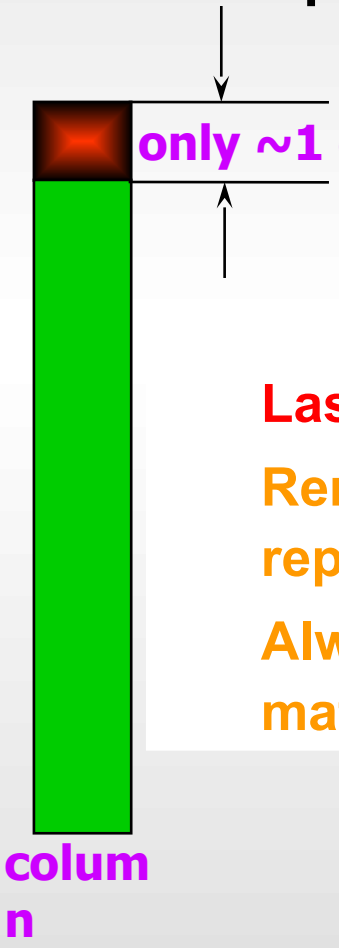
- Washing

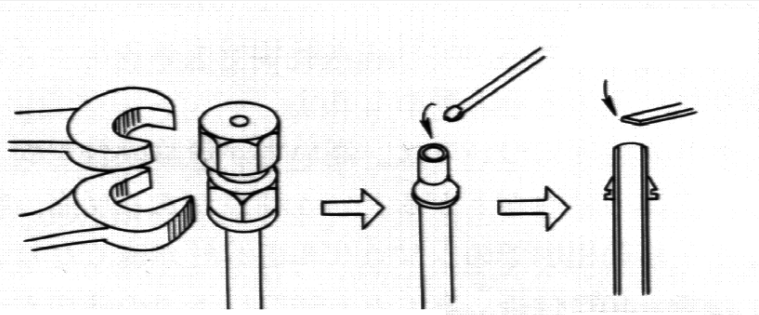
  For reversed phase column, connect the column in reverse and flow the washing solvents slowly (in sequence).

  - 1. Wash with mobile phase without buffer salts
  - 2. Wash with methanol or acetonitrile
  - 3. Wash with THF or isopropanol
  - 4. Wash with hexane
  - 5. Wash with THF or isopropanol
  - 6. Wash with methanol or acetonitrile

Remedies for Clogging/Contamination

- Repacking

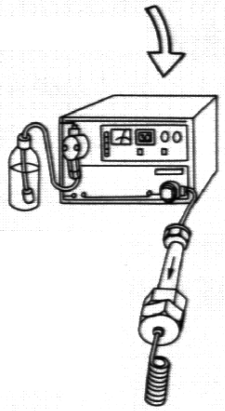




Last resort:

Remove the contaminated part and repack with clean packing material.

Always use the same size and type of material used originally in the column.

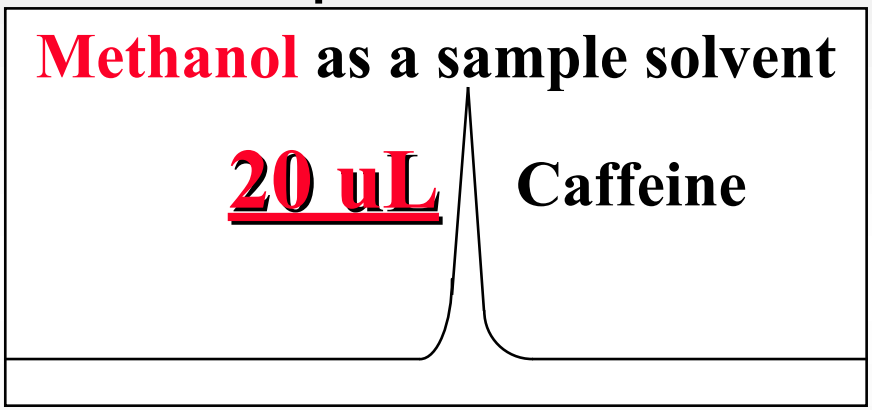


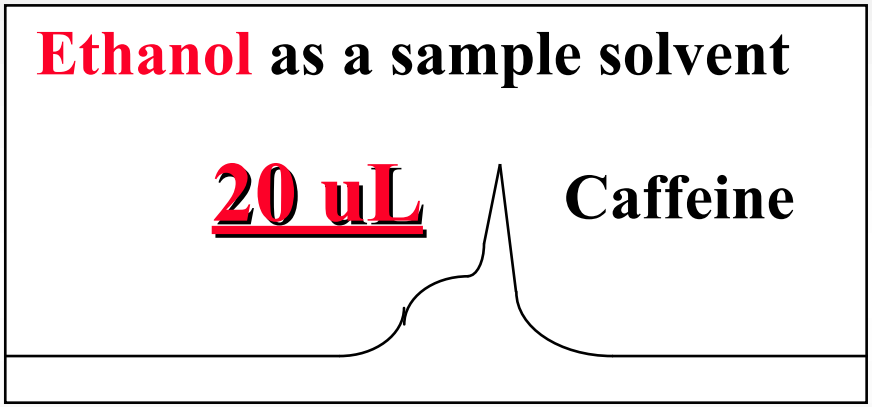
Causes of Tailing Peaks

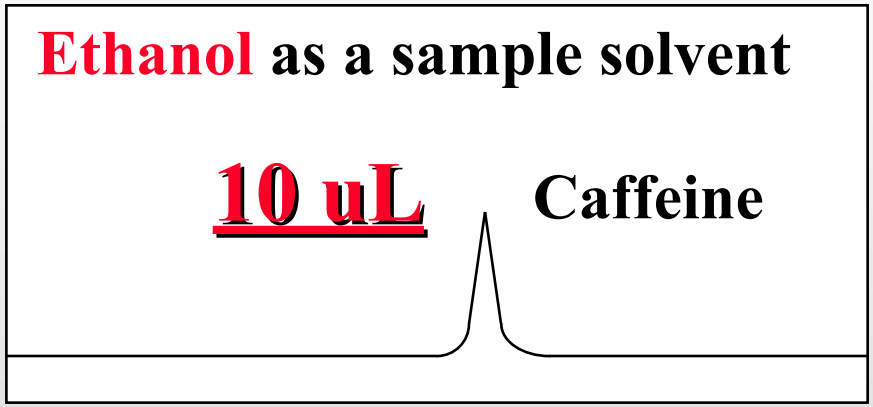
- Build-up of garbage on the column inlet
- Extra column effects (dead volume)
- Sample Overload
- Incorrect solvents for the sample
- Secondary retention effects
  - Silanol group
  - Residual heavy metal

Incorrect solvent for sample

- Avoid selecting a high soluble solvent as sample solvent.



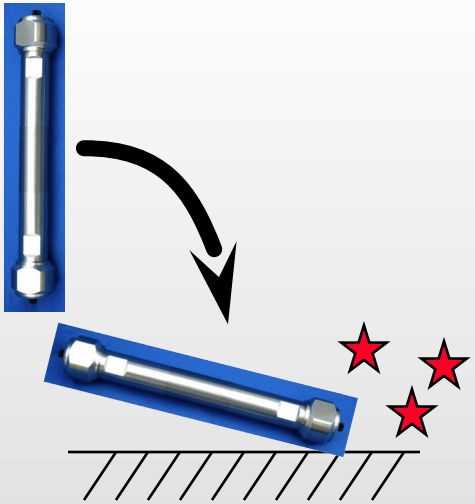




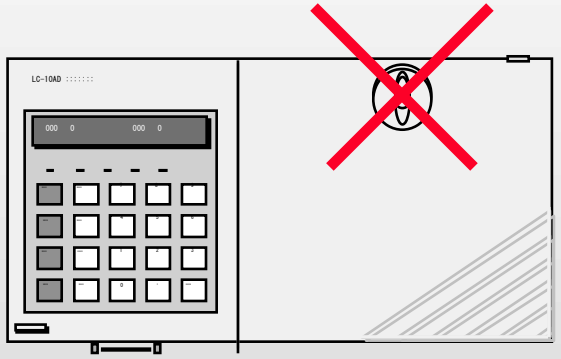
- **Better inject small amount of sample.**

Mechanical Shock, forming Voids

- Do not subject the column to shock, such as drop of column or sudden release of pressure. These mechanical shocks will form voids.

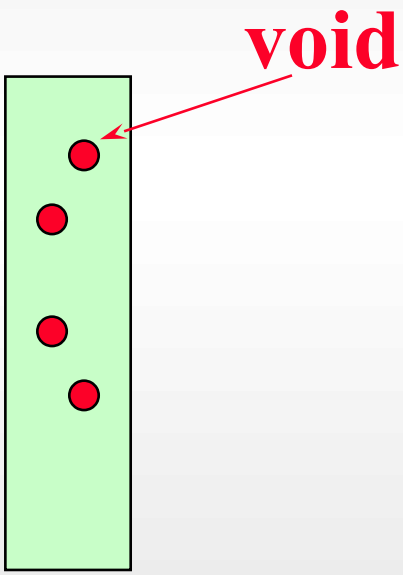


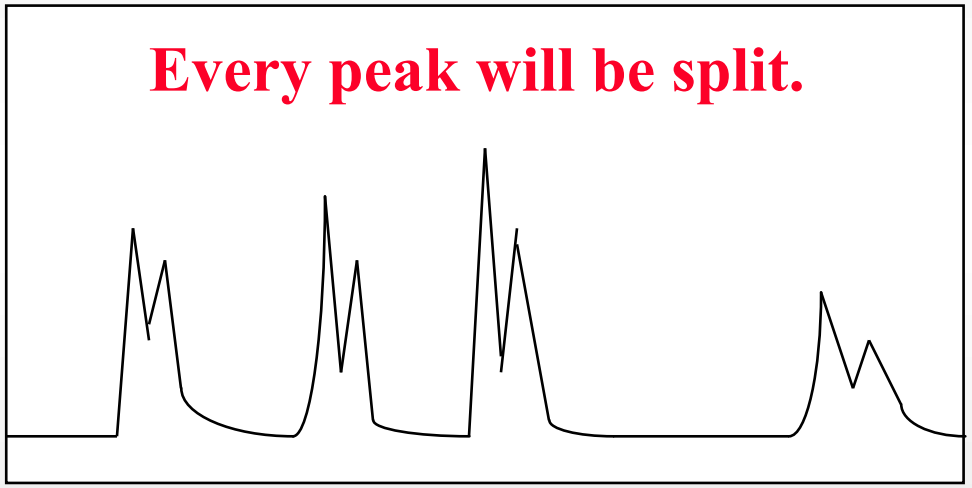
Do not open drain valve during operation



Voids

- Voids will cause split peaks.





Hard to repair!!!

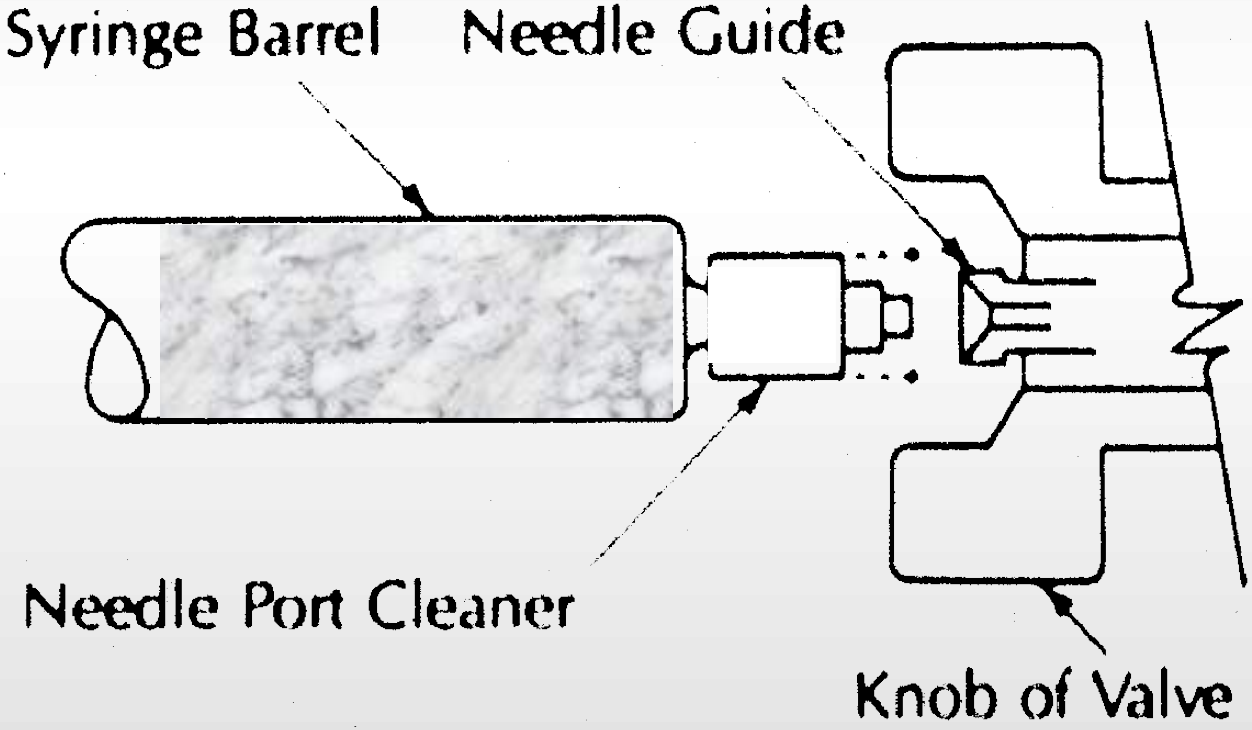
Precautions for the Packing Material

	Silica gel	Polymer
pH range	2 - 7.5	wider range
Organic solvent	all solvent	limited compatibility
Pressure	less than 250 kgf/cm2	low pressure
Temperature	better to set	possible to set
	at less than 60degC	at high temperature

Reminder: **Always read the literature or information sheet that comes with the column!**

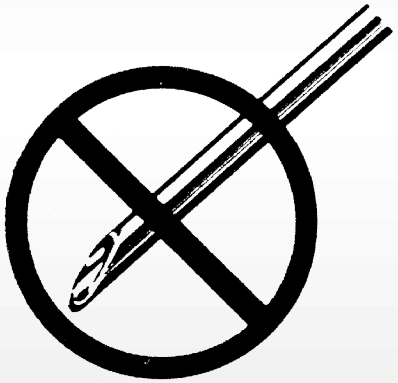
Washing for Injection Port

- Use of Needle Port Cleaner



Caution

- Do not use pointed or beveled needle tip.
  - Must use square end type.



- Do not use more than pH 10 solution.
  - Must change rotor seal.

Maintenance Tips

- Pump
  - check for leaks
  - wash plunger seal after using buffers
- Injector
  - wash injection port after every injection
  - select a suitable washing solvent
- Column
  - do not store in buffer solution
  - Always wash
- Detector
  - check life-time of lamp or electrode