Basic Principle of HPLC

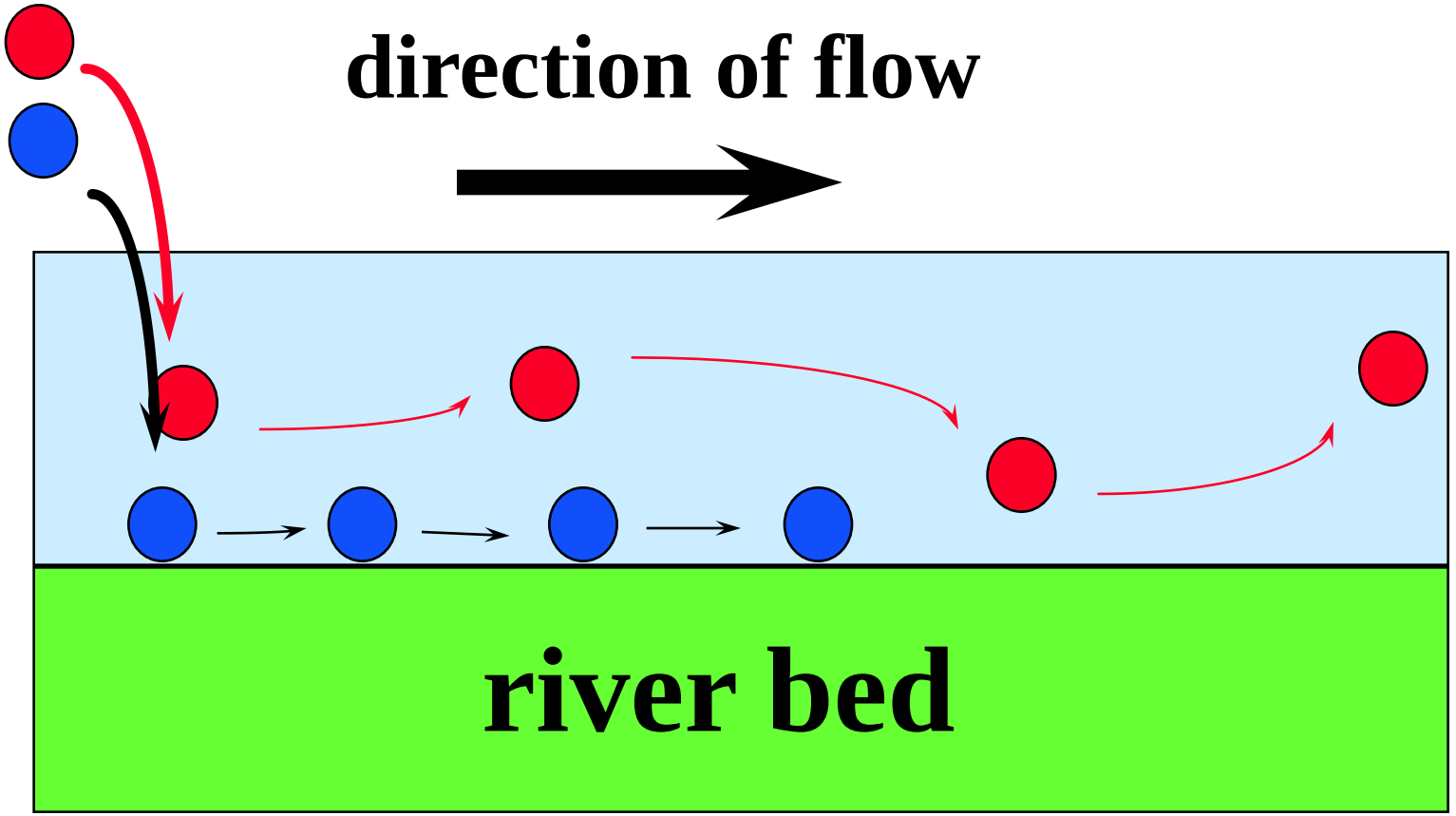
Basic Principle of HPLC

- Chromatography and HPLC
- Various HPLC modes and applications
  - Normal phase
  - Reversed phase
  - Ion exchange (IC)
  - SEC (GPC/GFC)
- HPLC system modes
  - Isocratic elution
  - Gradient elution

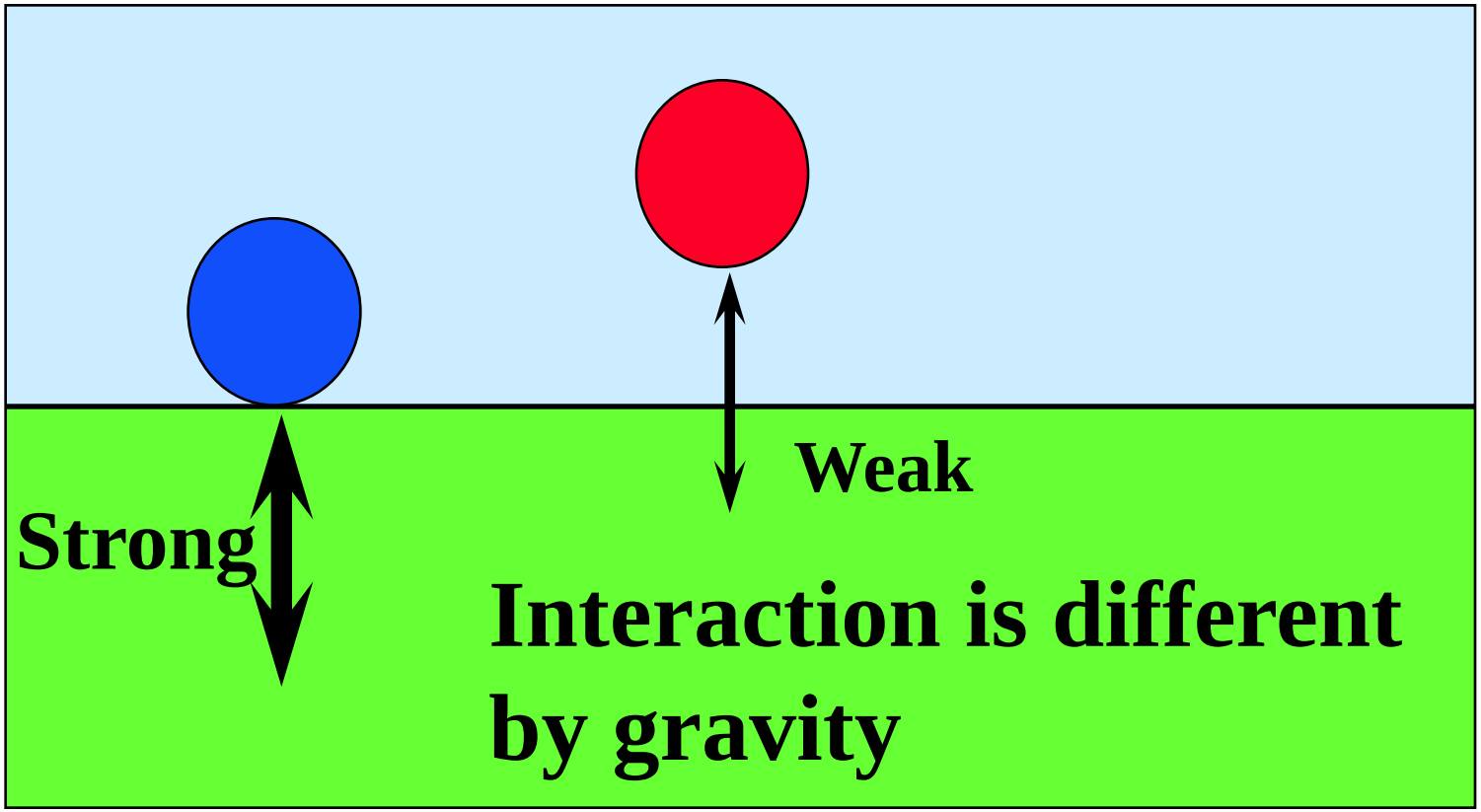
What is chromatography?

- Chromatography is one of the separation technique.
- The main purpose of chromatography is to *separate* and *quantify* the target sample in the matrix.

Why mixed sample can be separated?



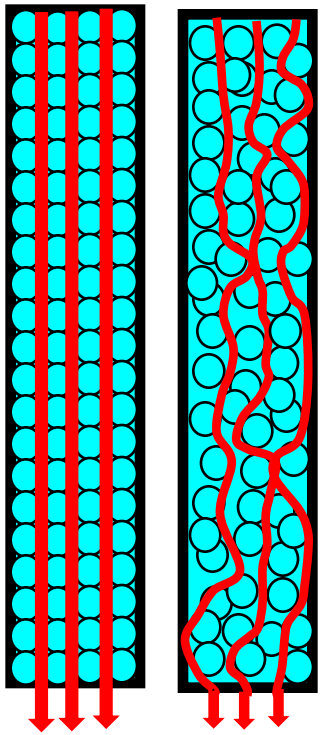
What is the difference?

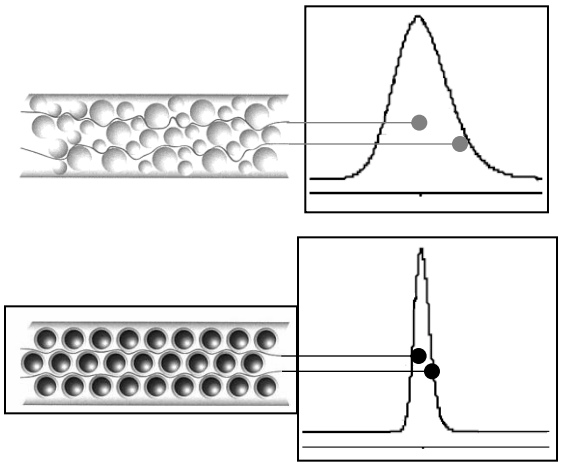


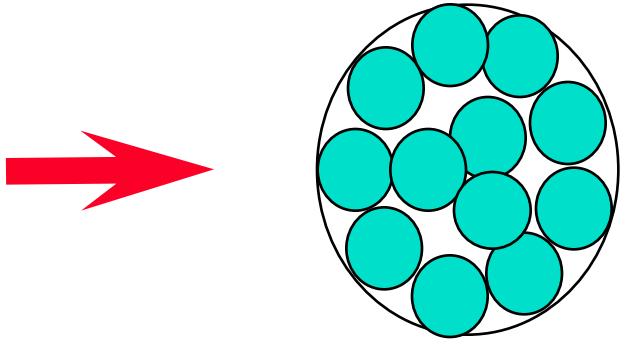
How can the separation be carried out?

- Separation can be carried out by the column.

column



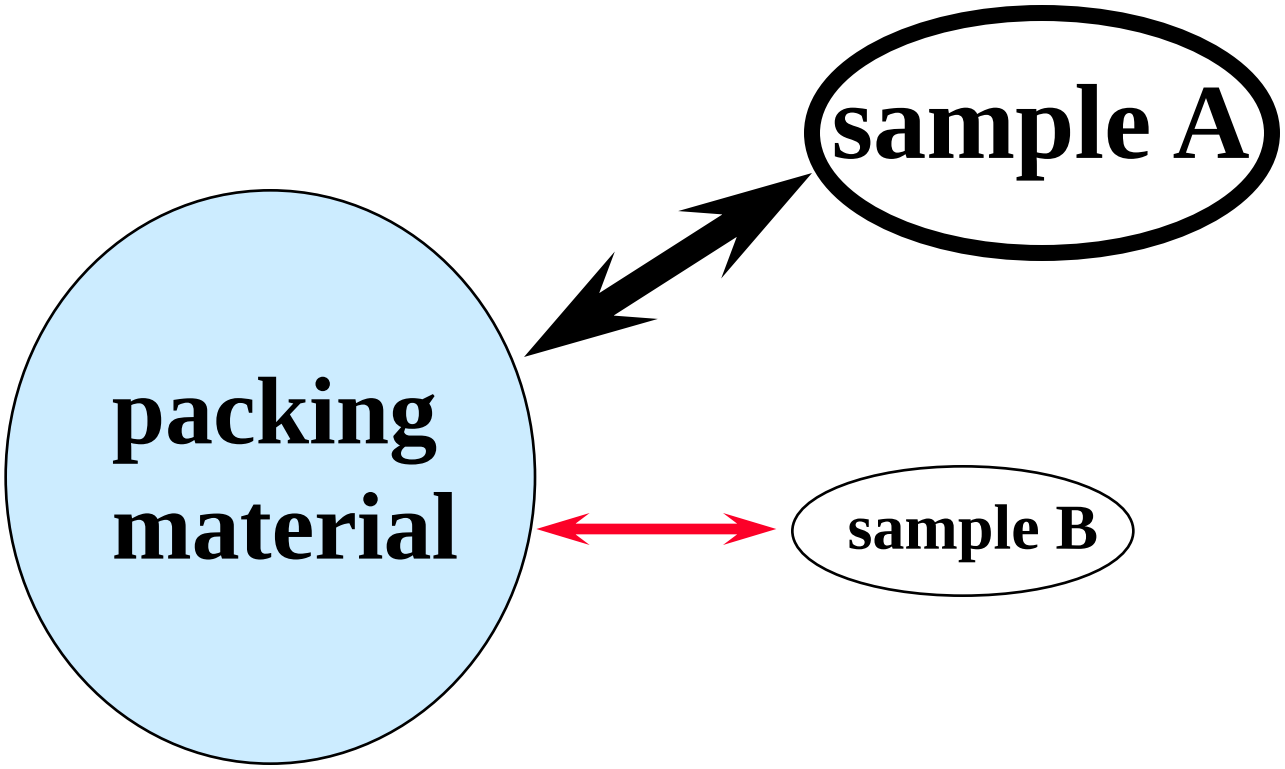




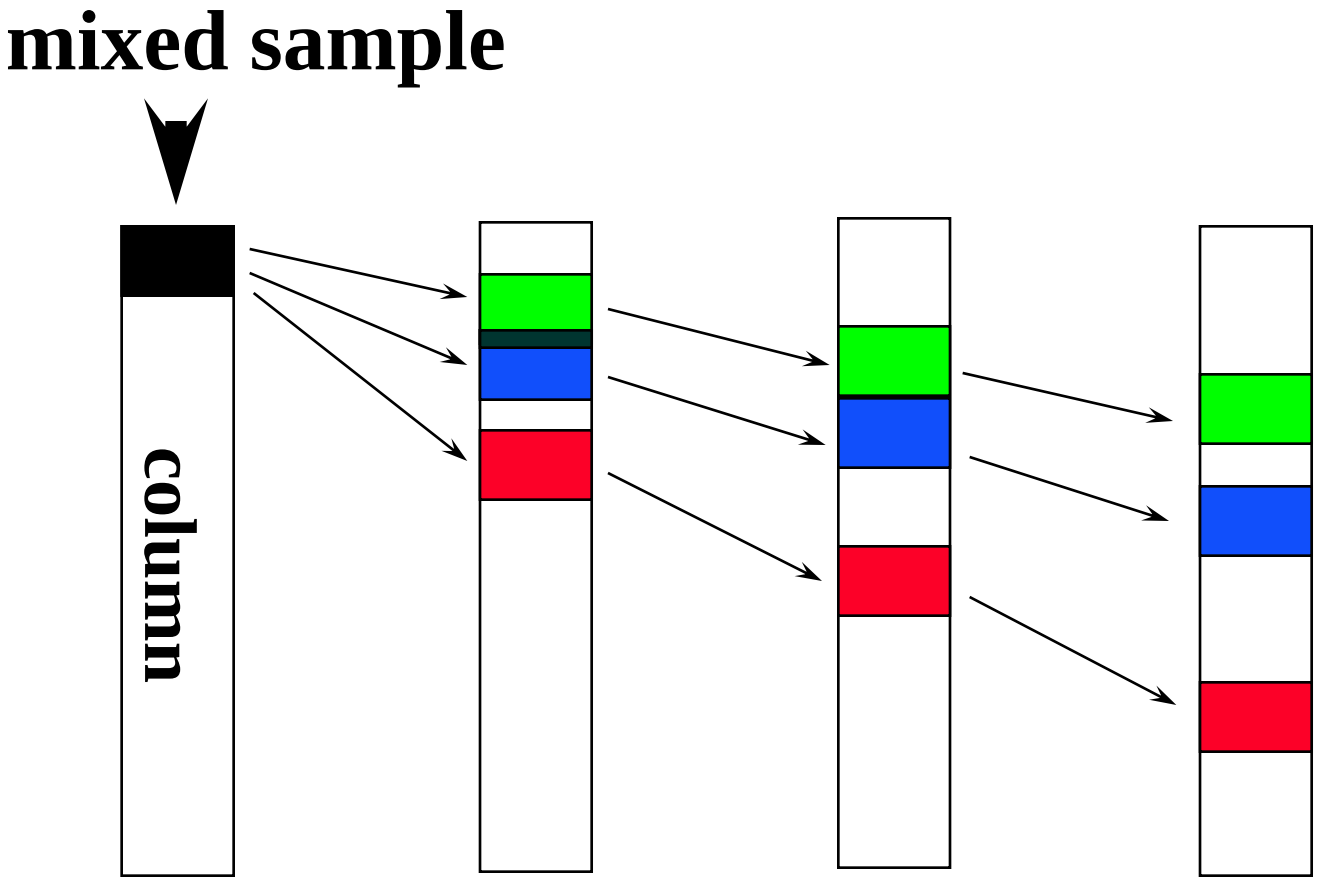
packing material, 3-5um

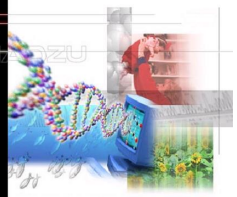
Interaction between packing material and sample

- Due to difference interaction between packing material and sample, separation can be done.



Separation can be done by the column





What kind of chromatography?

- High Performance Liquid chromatography (HPLC)
- Gas Chromatography (GC)
- Thin-Layer Chromatography (TLC)

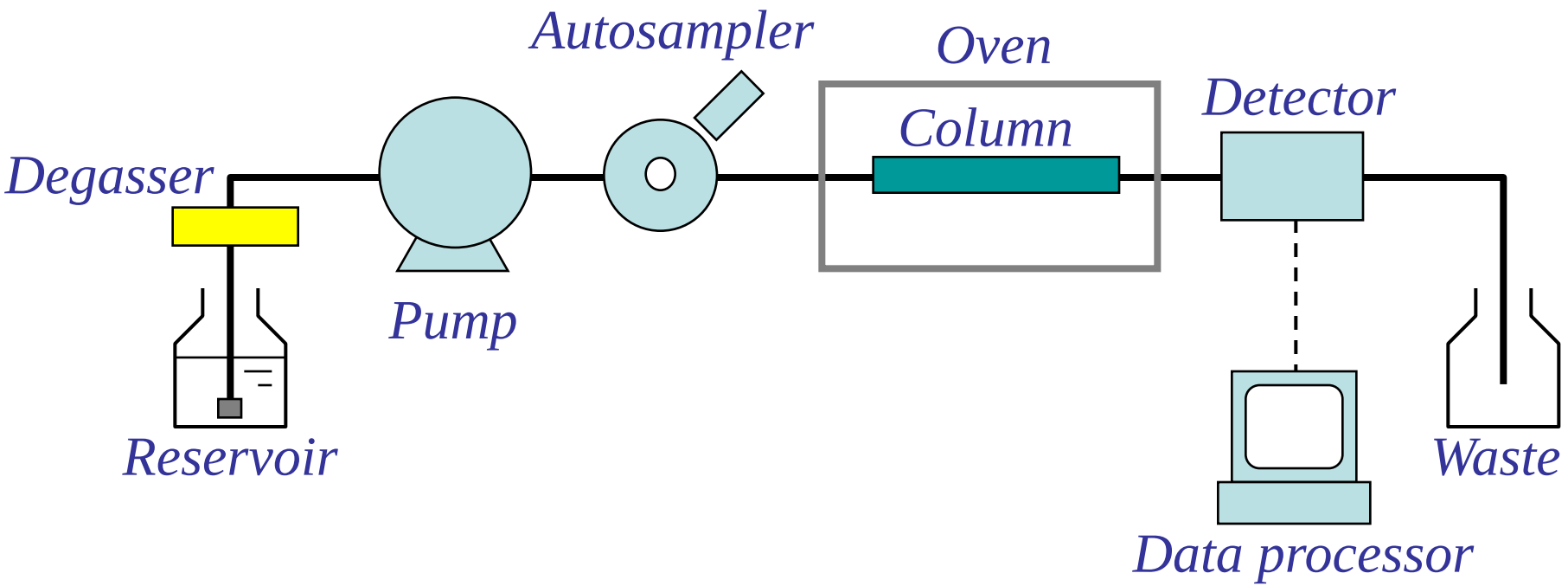
Advantage of Chromatography

- Simultaneous Analysis
- High Resolution
- High Sensitivity (ppm-ppb)
- Small Injection Volume (1-100uL)

Advantage of HPLC

- Moderate analysis condition
  - no need to vaporize the sample like GC
- Easy to fractionate the sample and purify
- Good repeatability (C.V. < 1%)

Flow-line



Separation modes of HPLC

- Normal Phase mode (NP)
- Reversed Phase mode (RP)
- Ion Exchange mode (IC)
- SEC mode (GPC / GFC)

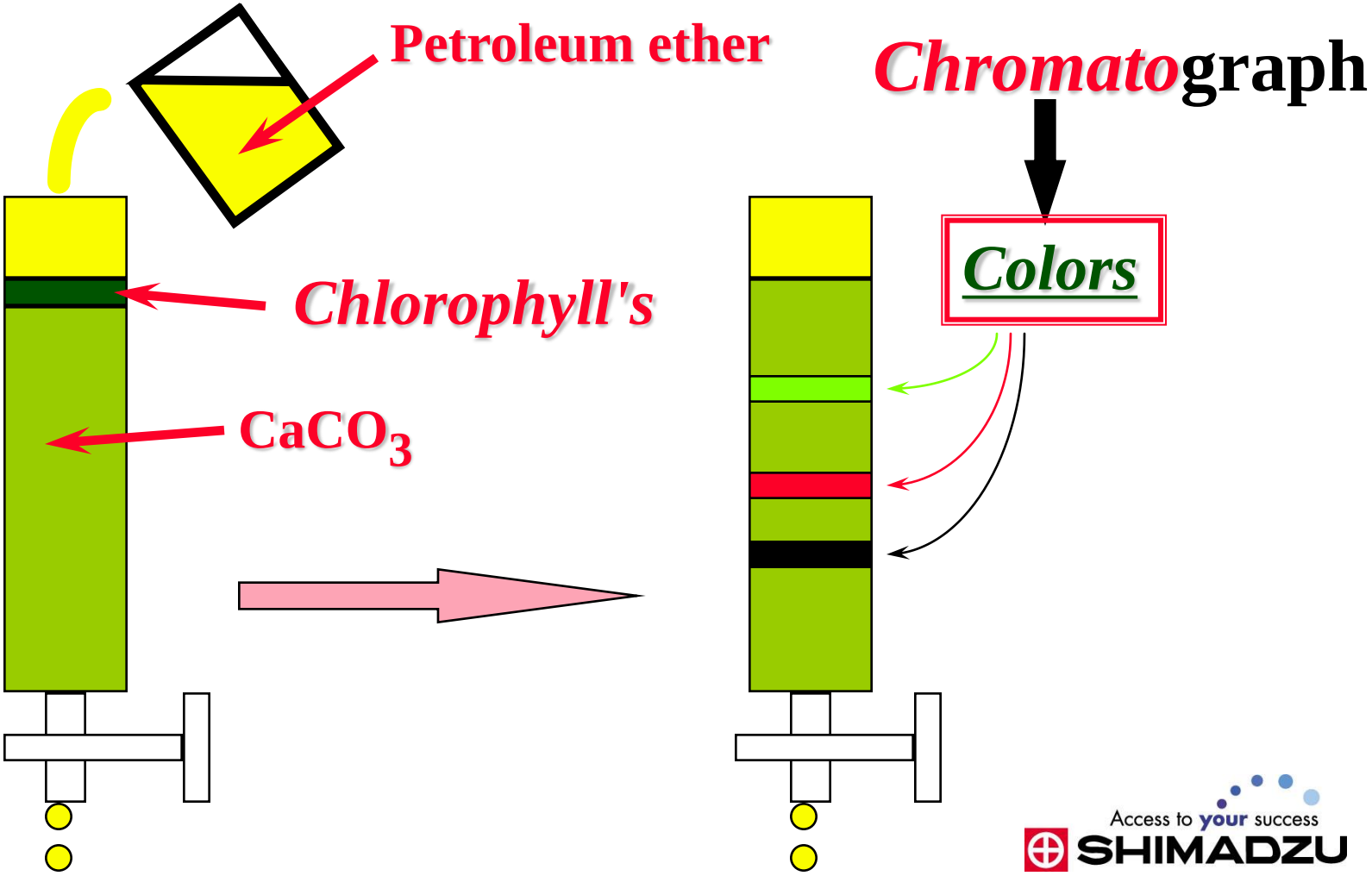
Normal Phase Mode

M. Tswett :

first developer of chromatography

Ber. Deut. Botan. Ges., 24, 384 (1906)
Adsorptionsanalyse und *chromatographische* Methode. Anwendung auf die Chemie des Chlorophylls.

Normal Phase Mode



Normal Phase Mode

- First developer used
  - –$CaCO_3$ as Separation Column
  - –Petroleum ether as Developed Solvent
- We defined this combination as

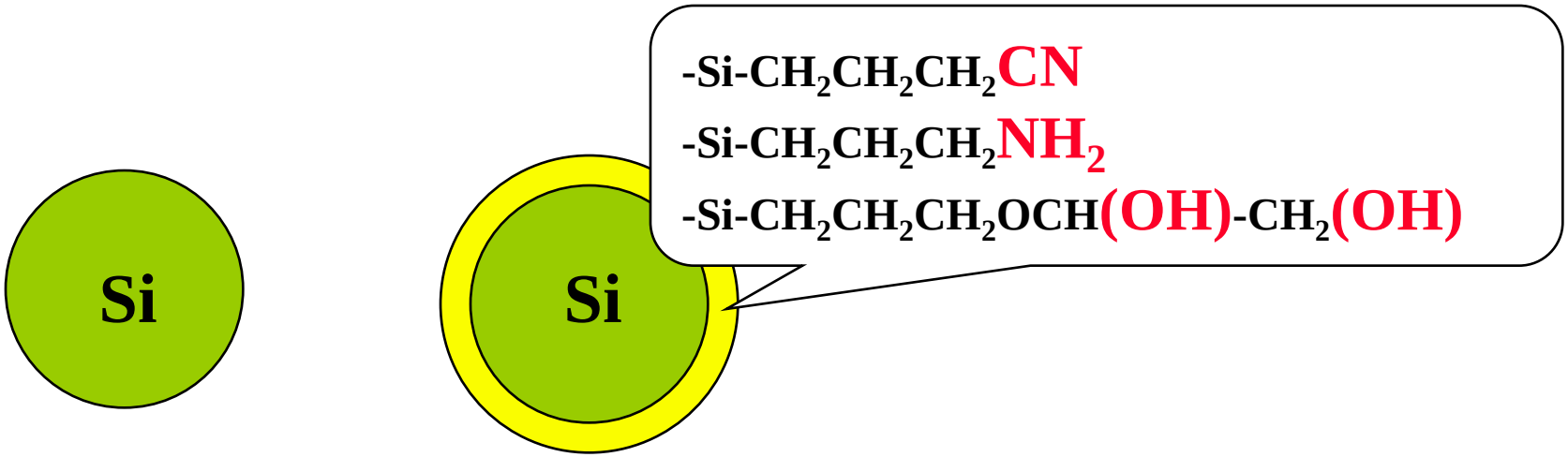
Normal Phase mode

Column (Stationary phase) : polar property

Solvent (Mobile phase) : non polar property

Normal Phase HPLC Columns

- Silica gel type : general use
- Cyano type : general use
- Amino type : for sugar analysis
- Diol type : for protein analysis

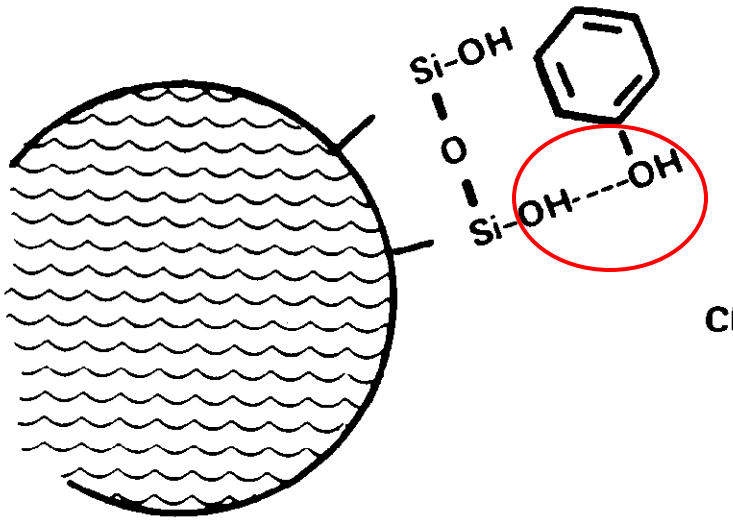


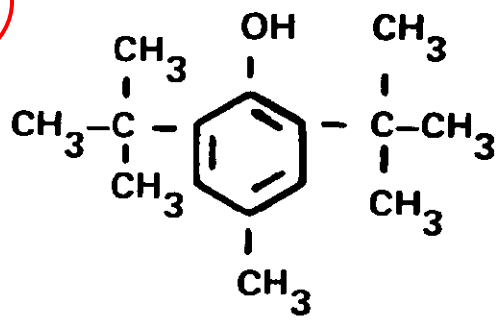
What is the interaction?

Hydrogen bonding

HEXANE

Non-polar





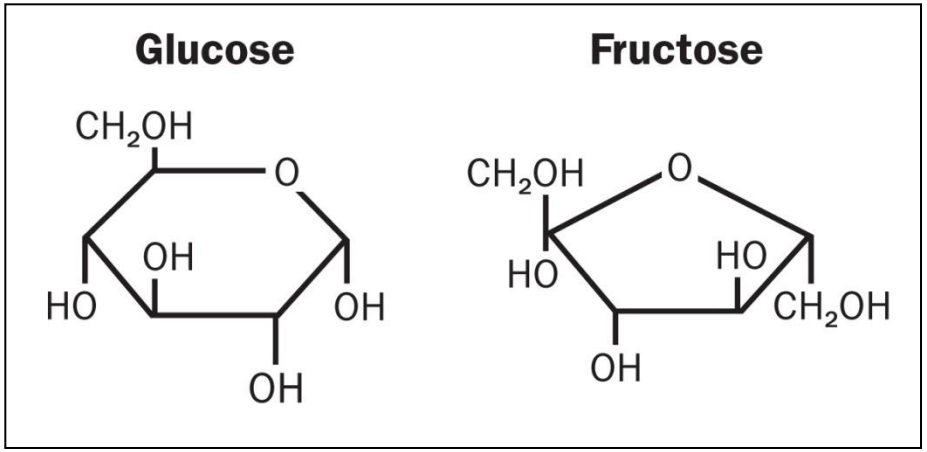
Silica gel (polar)

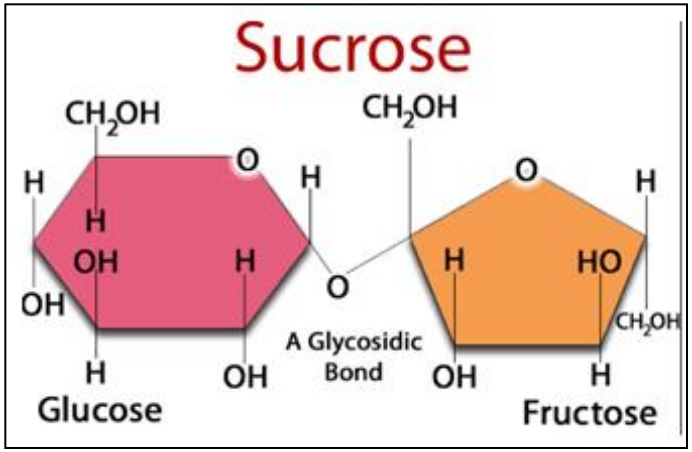
Hydrogen Bonding

- **Compounds contain**
  - **–-COOH : Carboxyl group**
  - **–-NH_2 : Amino group**
  - **–-OH : Hydroxyl group**

Hydrogen bonding – strong interaction

Note: Compounds with bulk group, hydrogen bonding may not form due to stereo hindrance



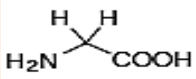


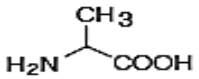
- **Compounds do not contain these groups**

No hydrogen bonding – weak interaction

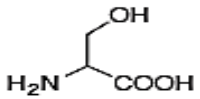
Example of samples compound

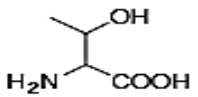
SMALL

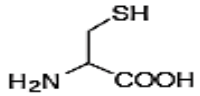

Glycine (Gly, G)
MW: 75.07


Alanine (Ala, A)
MW: 89.09

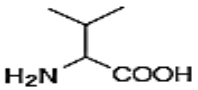
NUCLEOPHILIC

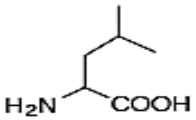

Serine (Ser, S)
MW: 105.09, $pK_a \sim 16$

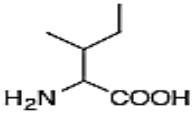

Threonine (Thr, T)
MW: 119.1, $pK_a \sim 16$

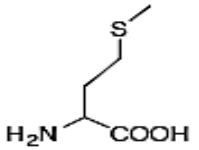

Cysteine (Cys, C)
MW: 121.2, $pK_a = 8.18$

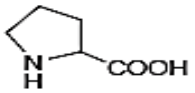
HYDROPHOBIC


Valine (Val, V)
MW: 117.1

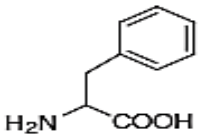

Leucine (Leu, L)
MW: 131.2

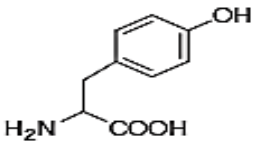

Isoleucine (Ile, I)
MW: 131.2

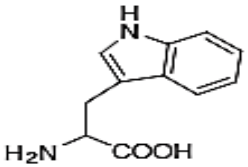

Methionine (Met, M)
MW: 149.2


Proline (Pro, P)
MW: 115.1

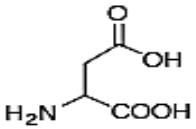
AROMATIC

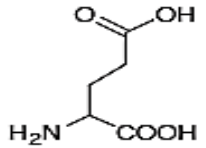

Phenylalanine (Phe, F)
MW: 165.2


Tyrosine (Tyr, Y)
MW: 181.2, $pK_a = 10.46$

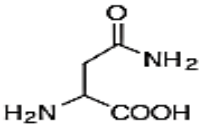

Tryptophan (Trp, W)
MW: 204.2

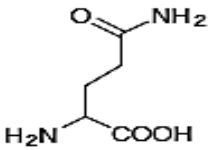
ACIDIC


Aspartic Acid (Asp, D)
MW: 133.1, $pK_a = 3.9$

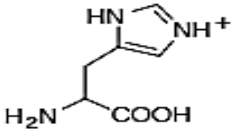

Glutamic Acid (Glu, E)
MW: 147.1, $pK_a = 4.07$

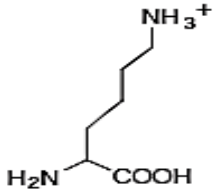
AMIDE

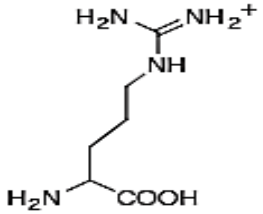

Asparagine (Asn, N)
MW: 132.1


Glutamine (Gln, Q)
MW: 146.1

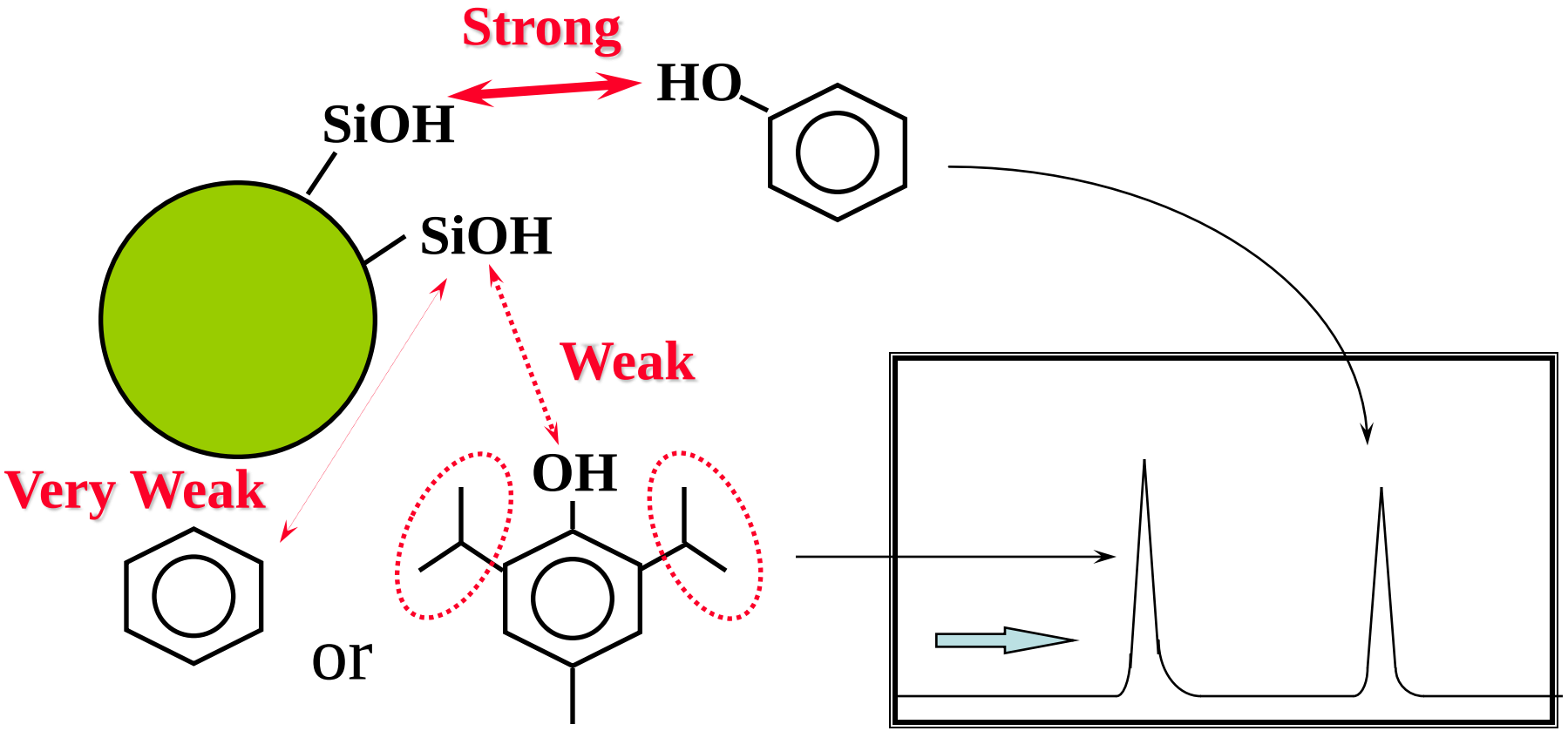
BASIC


Histidine (His, H)
MW: 155.2, $pK_a = 6.04$


Lysine (Lys, K)
MW: 146.2, $pK_a = 10.79$


Arginine (Arg, R)
MW: 174.2, $pK_a = 12.48$

Retention Time and Hydrogen bonding



Mobile phase solvents for normal phase HPLC

- Primary solvents (non-polar)
  - **Hydrocarbons (Pentane, Hexane, Heptane, Octane)**
  - **Aromatic Hydrocarbons (Benzene, Toluene, Xylene)**
  - **Methylene chloride**
  - **Chloroform**
  - **Carbon tetrachloride**

- Secondary solvents
  - **Methyl-t-butyl ether (MTBE), Diethyl ether, Tetrahydrofuran (THF), Dioxane, Pyridine, Ethyl acetate, Acetonitrile, Acetone, 2-propanol, ethanol, methanol**

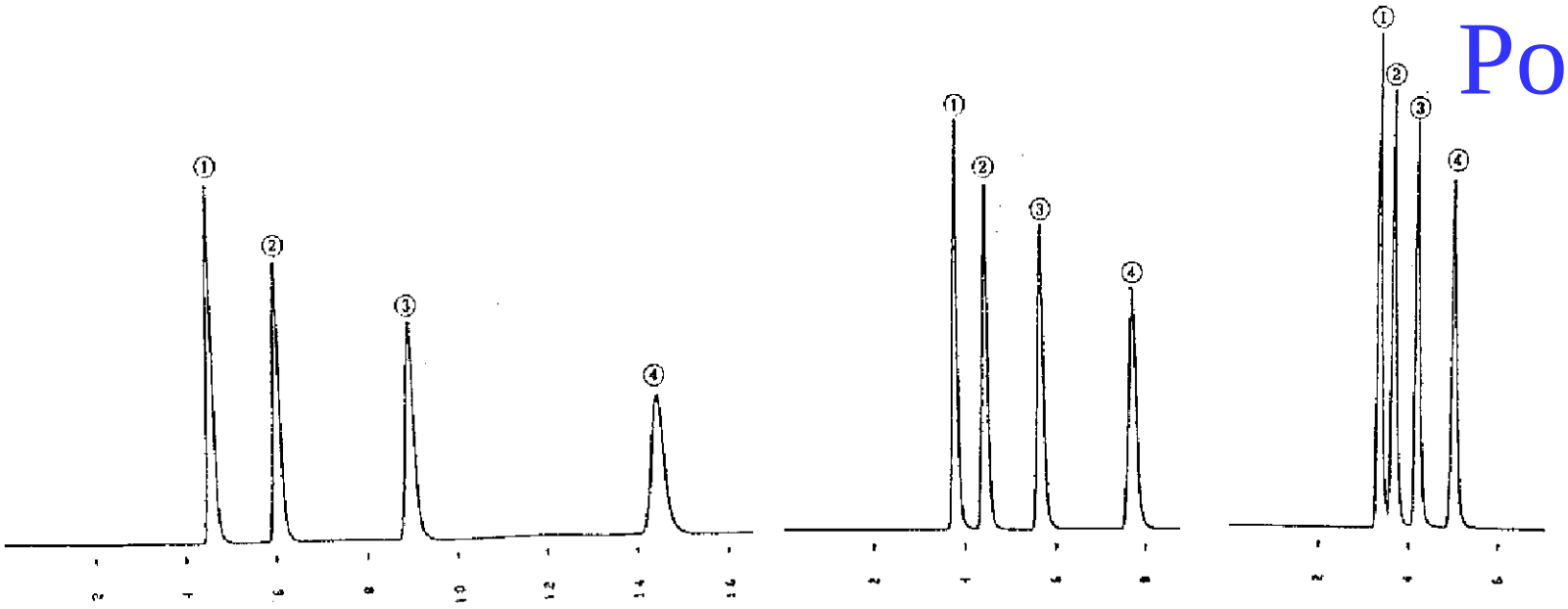
- **A primary solvent is used as mobile phase. Addition of secondary solvents is to to adjust retention time.**
- **Solvents which possess UV transparency are often preferred for easy detection.**

Increase of solvent polarity

Column = Polar

0 % **2 %** **5% / MeOH**

Polar



1 : Dioctyl phthalate
2 : Dibutyl phthalate
3 : Diethyl phthalate
4 : Dimethyl phthalate

Solvent : Hexane

Non polar

Reversed Phase mode

Column **(Stationary phase)** : Non-polar property

Solvent **(Mobile phase)** : Polar property

- Normal Phase mode -

Column : polar property

Solvent : non polar property

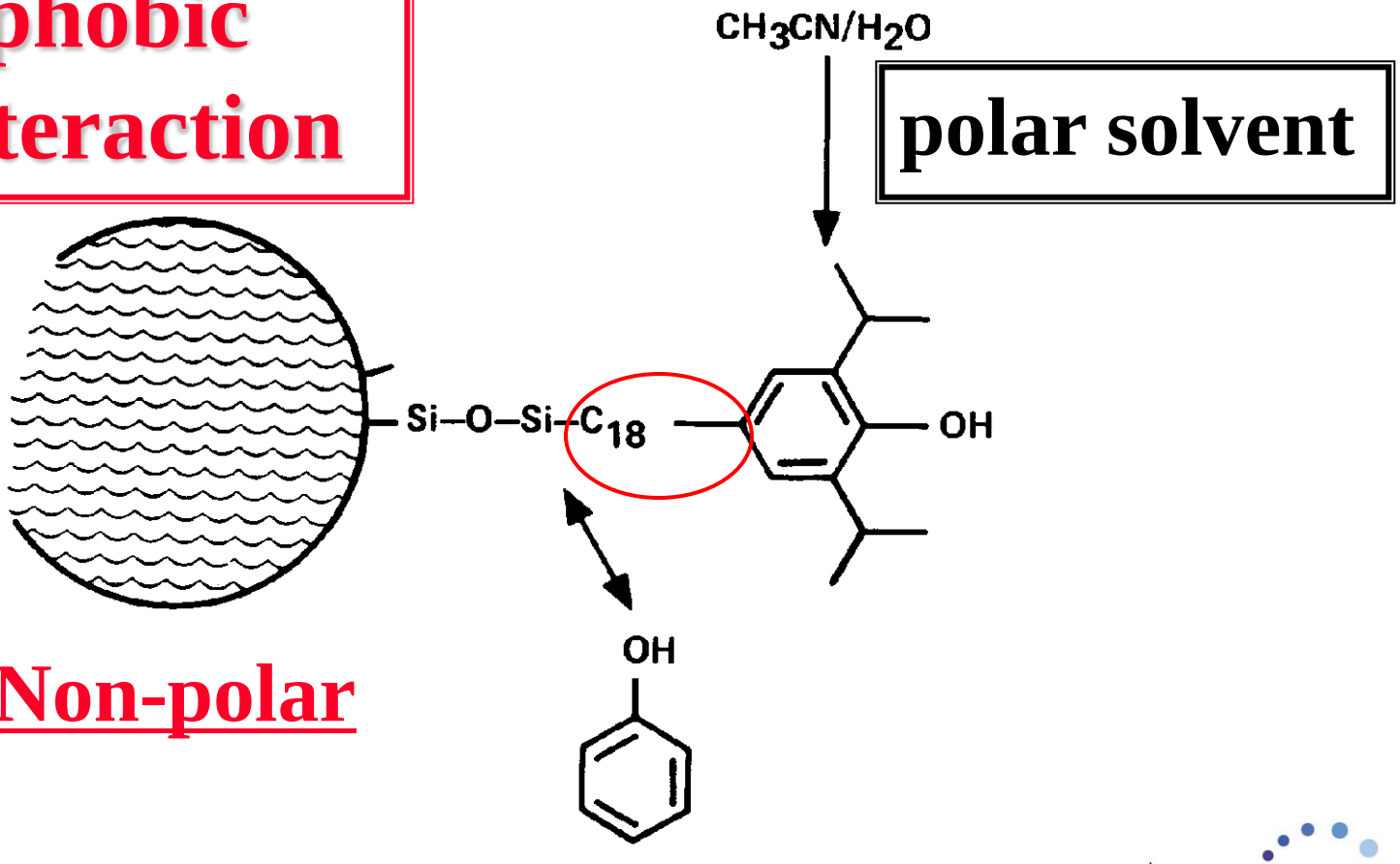
Reversed Phase HPLC Columns

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

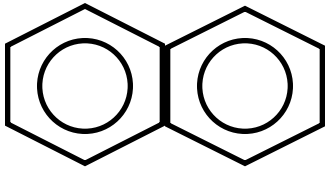
Non-polar property

What is the interaction?

Hydrophobic interaction



Hydrophobicity

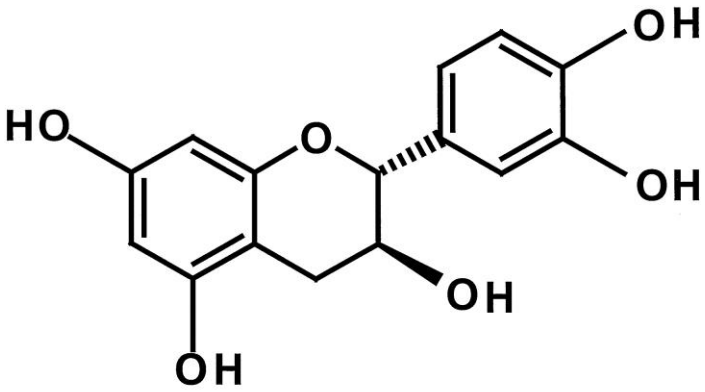
- If the sample has more
  - $–CH_3CH_2CH_2$--- : Carbon chain
  - –  : Aromatic group

Hydrophobicity is stronger.

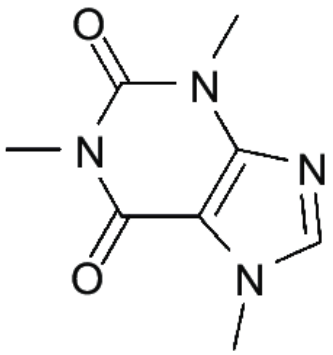
- If the sample has more
  - –-COOH : Carboxyl group
  - –-NH_2 : Amino group
  - –-OH : Hydroxyl group

Hydrophobicity is weaker.

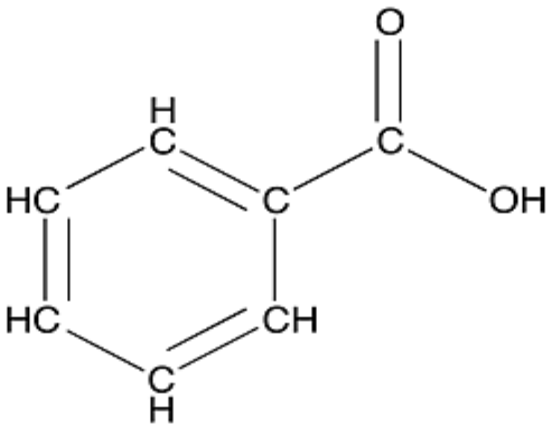
Example of samples compound



(+)-catechin



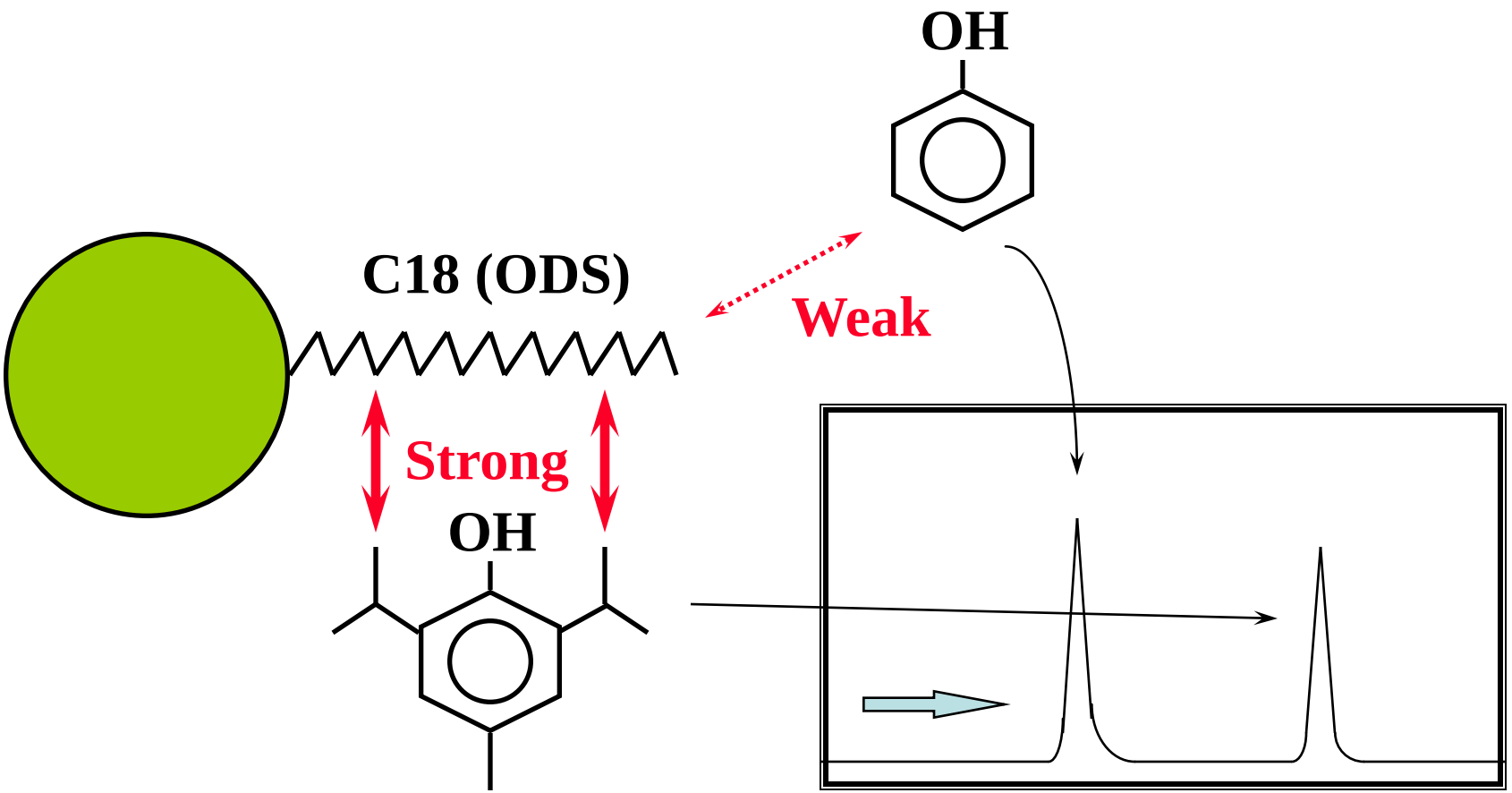
Caffeine



benzoic acid

sorbic acid

Retention Time and Hydrophobicity



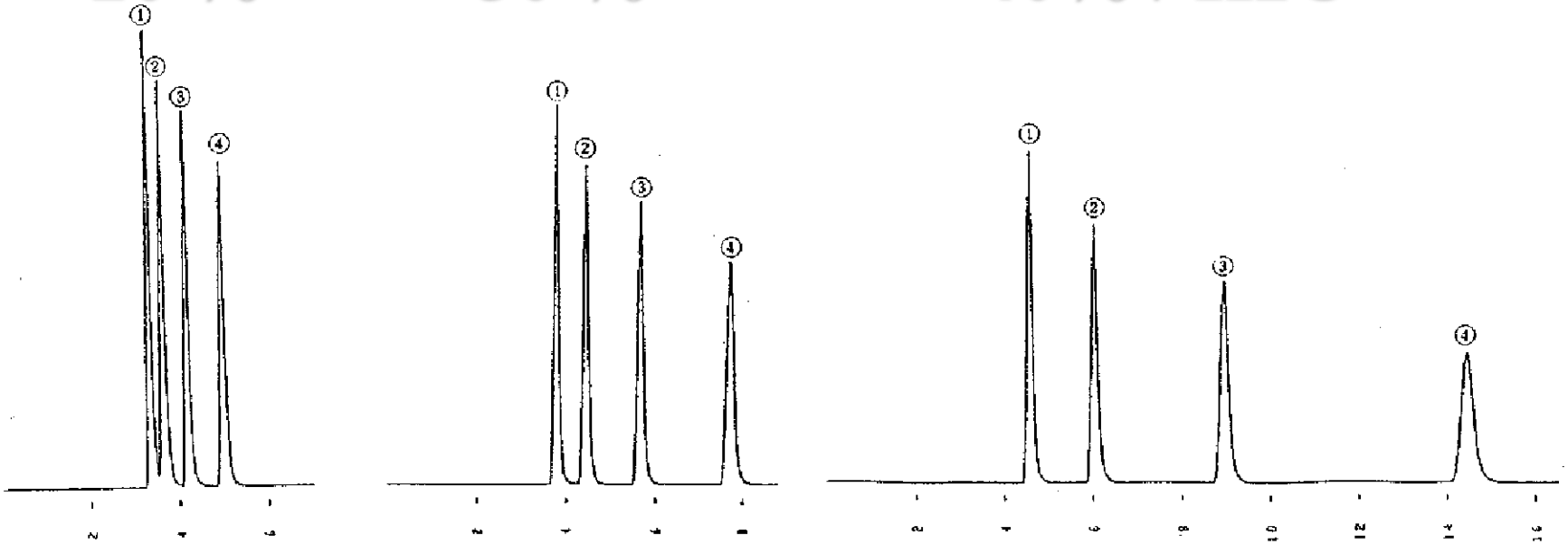
Mobile phase solvents for reversed phase HPLC

- Water (buffer) + Organic solvents
  - **When buffer is used, the concentration and pH are important factors**
  - **Methanol (MeOH), acetonitrile (ACN) or THF are common organic solvents for r.p HPLC.**

- **Optimization of water (buffer) and organic solvents' ratio is very important.**

Increase of solvent polarity

20 % **30 %** **40% / H2O**

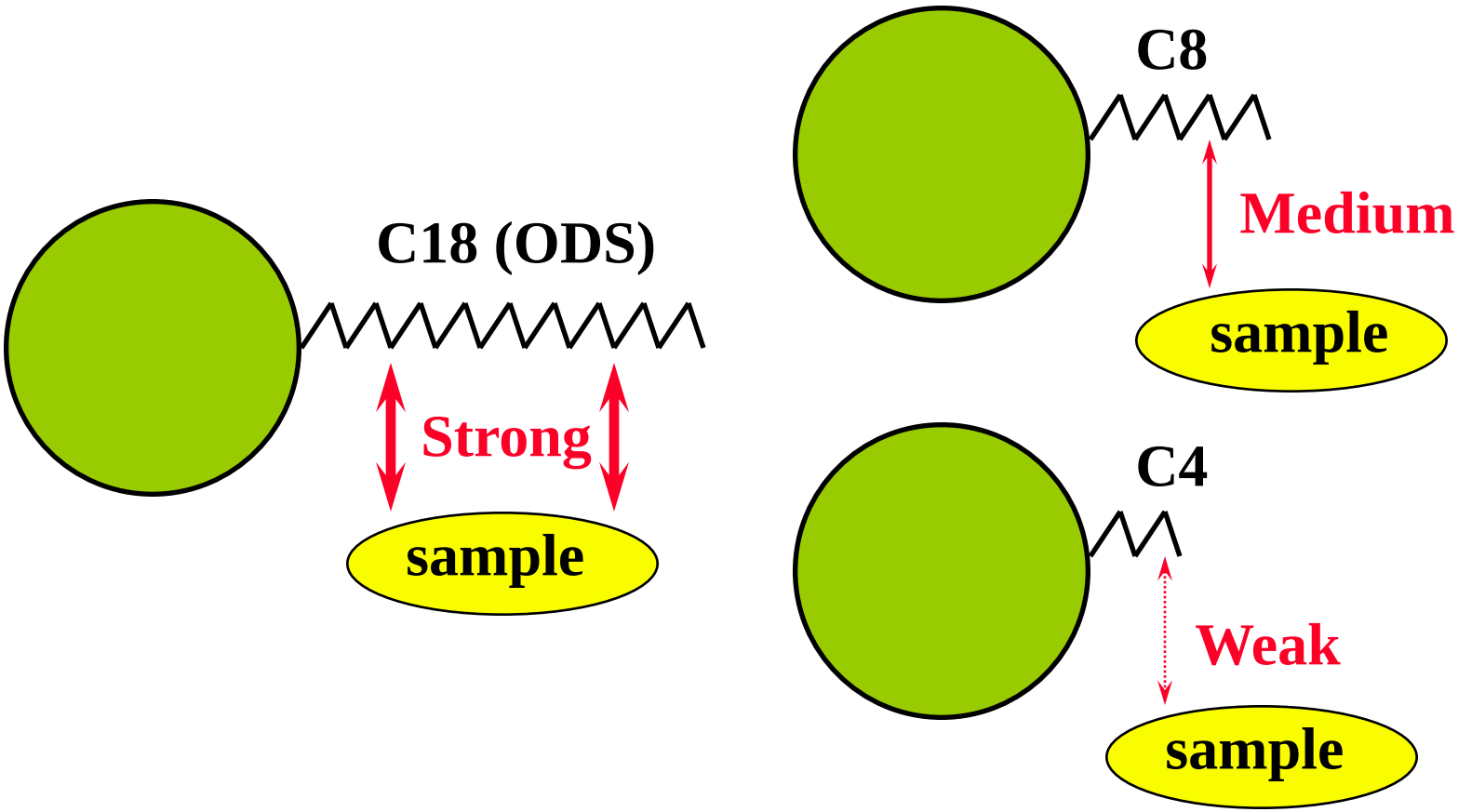


1 : p-Hydoxymethylbenzoate
2 : p-Hydoxyethylbenzoate
3 : p-Hydoxypropylbenzoate
4 : p-Hydoxybutylbenzoate

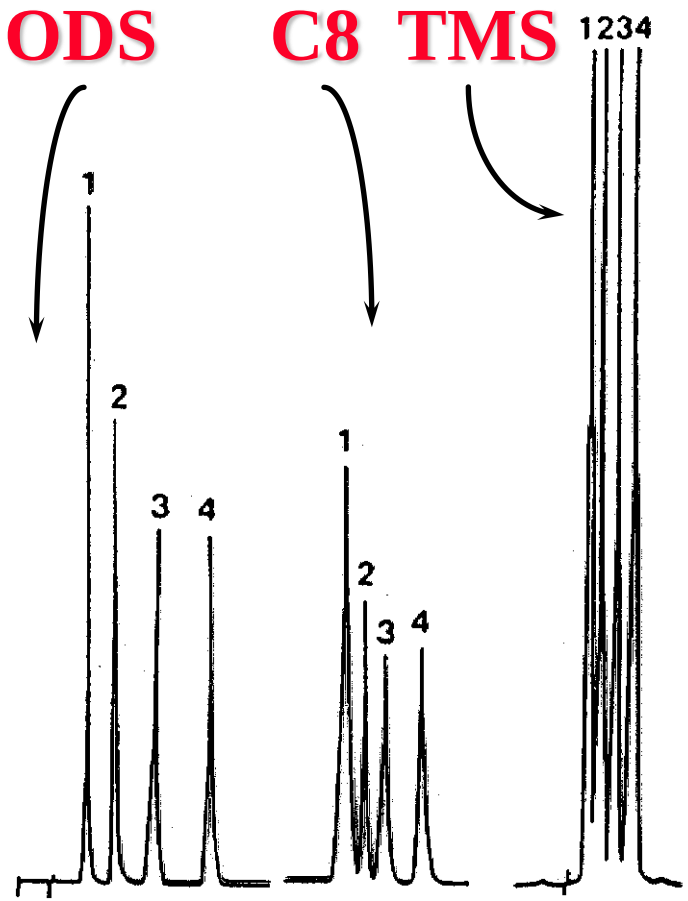
Solvent : MeOH

Access to your success
SHIMADZU

Effect of stationary phase



Effect of stationary phase

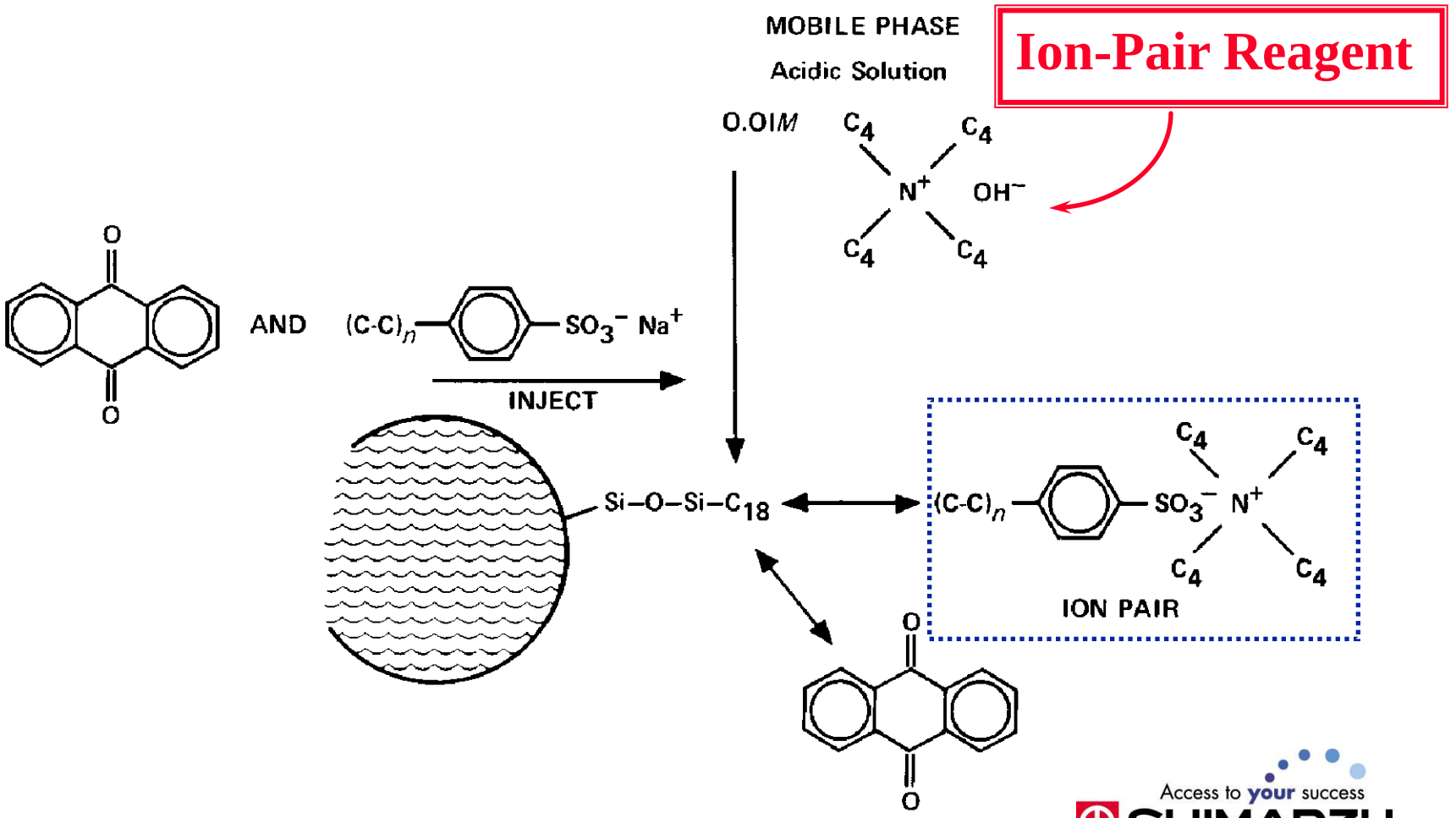


- **Analytical Conditions**
  - **Column : Shim-pack CLC-ODS**
  - **Mobile phase : MeOH : H2O = 7 :3**
  - **Flow rate : 1.0 mL/min**
  - **Temperature : 40 C**
  - **Injection volume : 10 uL**
  - **Detection : UV-254 nm**
- **Peaks**
  1. **Methyl benzoate**
  2. **Ethyl benzoate**
  3. **n-Propyl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

Normal vs Reversed Phase

- **Normal Phase**
  - **–good separation for stereo isomers (Vitamin E etc..)**
  - **–changeable retention time**
- **Reversed Phase**
  - **–good repeatable retention time**
  - **–rugged stationary phase**

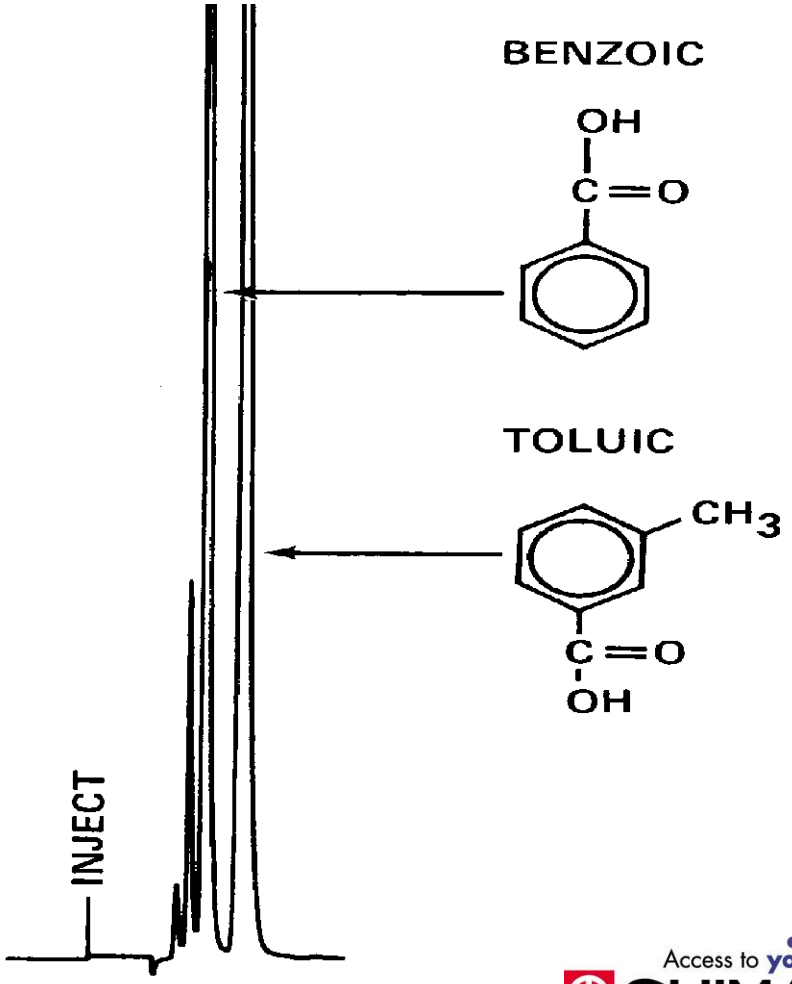
Reversed Phase Ion-Pair Chromatography



Ion-Pair Reagents

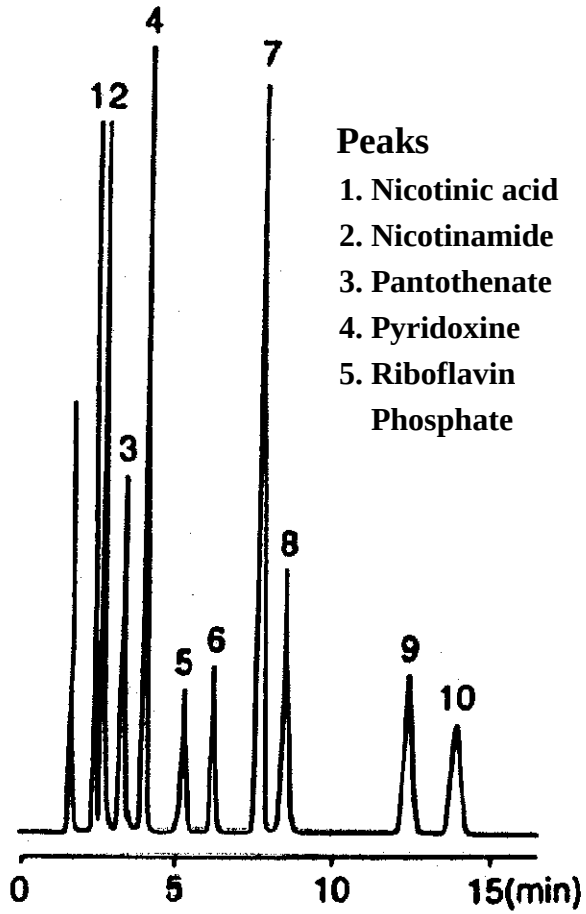
- **Anion Compounds**
  - **–Tetra-n-butylammonium hydroxide (TBA)**
- **Cation Compounds**
  - **–Butanesulfonic acid sodium salt (C4)**
  - **–Pentanesulfonic acid sodium salt (C5)**
  - **–Hexanesulfonic acid sodium salt (C6)**
  - **–Heptanesulfonic acid sodium salt (C7)**
  - **–Octanesulfonic acid sodium salt (C8)**
  - **–Decanesulfonic acid sodium salt (C10)**

Ion Pairing
Separation of Carboxylic Acids



Column: Bonded C_{18}
Mobile Phase: H_2O/MeOH
1:1 with Tetrabutyl-
Ammonium Hydroxide

Ion Pairing
Separation of Amino Compounds



Peaks

1. Nicotinic acid
2. Nicotinamide
3. Pantothenate
4. Pyridoxine
5. Riboflavin Phosphate
6. Thiamine
7. Caffeine
8. Folic acid
9. Biotin
10. Riboflavin

- **Analytical Conditions**
  - **Column : Shim-pack CLC-ODS**
  - **Mobile phase : [A] : [B] = 9 : 1**
  - **[A]**
    100 mM phosphate buffer (pH=2.1)
    0.8 mM sodium octane sulfonate
  - **[B] acetonitrile**
  - **Flow rate : 1.5 mL/min**
  - **Temperature : 40 C**
  - **Injection volume : 10 uL**

Ion Paring
Important Considerations

- Type of Ion-Pair reagents
- Concentration of Ion-Pair reagents
- pH of solvent

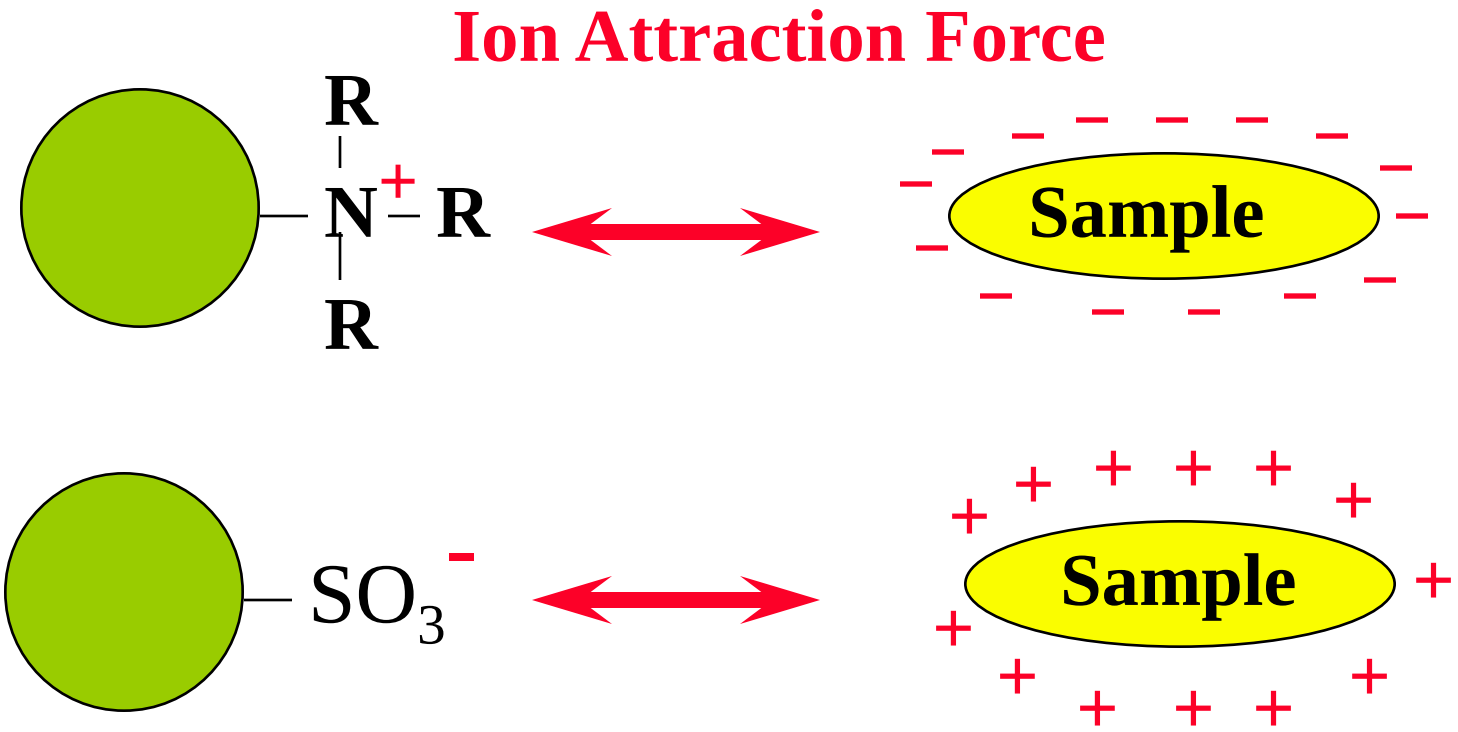
$$R\text{-}COOH \rightleftharpoons RCOO^- + H^+$$

(pKa=4.5)

$$R\text{-}NH_2 + H^+ \rightleftharpoons R\text{-}NH_3^+$$

(pKa=6.0)

Ion Exchange Mode



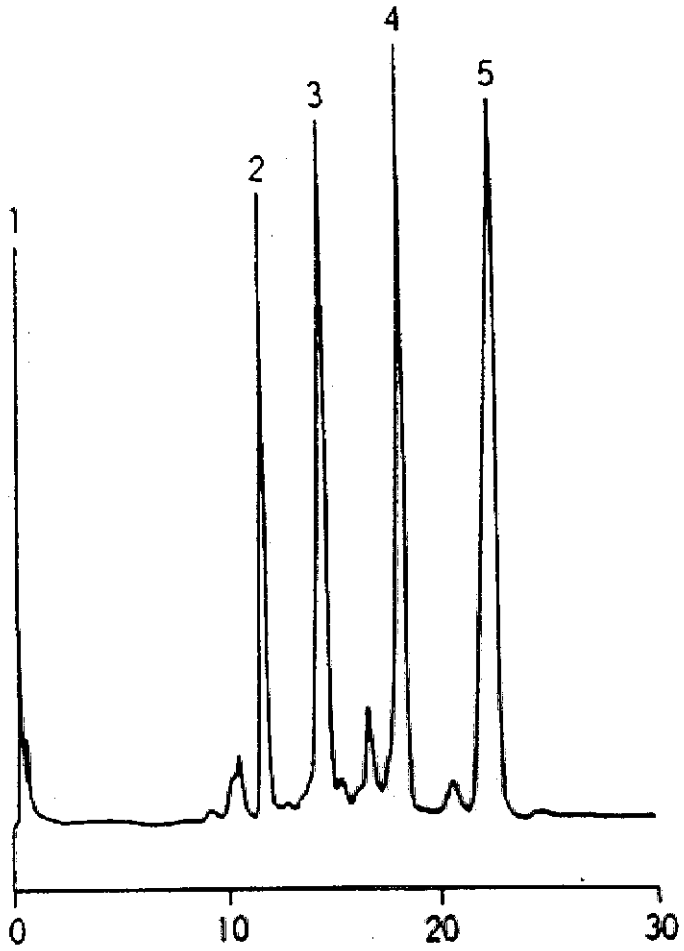
Ion Exchange Mode

- Biological Fields
  (protein, peptide, amino acid analysis)
- Ion chromatography

  –Cation Exchangers
  - Strong Cation Exchange (SCX) ($R\text{-}SO_3^-$)
  - Weak Cation Exchange (WCX) ($R\text{-}COO^-$)

  –Anion Exchangers
  - Strong Anion Exchange (SAX) (R_4N^+)
  - Weak Anion Exchange (WAX) (DEAE)

Protein Analysis using WCX column



- **Analytical Conditions**
  - **Column : Shim-pack WCX-1**
  - **Mobile phase :**
    [A] 20 mM phosphate buffer (pH=6.0)
    [B] A+0.25M sodium sulfate
    [A] - [B] 30 min linear gradient
  - **Flow rate : 1.0 mL/min**
  - **Temperature : ambient**
  - **Detector : UV-280 nm**
  - **Injection volume : 10 uL**
- **Peaks**
  1. **albumin**
  2. **myoglobin**
  3. **α-chymotrypsinogen A**
  4. **liponuclease A**
  5. **lisozyme**

Ion Exchange mode Important Considerations

- pH of Buffer solution
- Concentration of Buffer solution
- Elution Method
  - –Isocratic elution
  - –pH gradient elution
  - –increasing Ionic strength gradient

What is SEC ?

- Size Exclusion Chromatography (SEC)

➢ GPC (Gel Permeation chromatography)

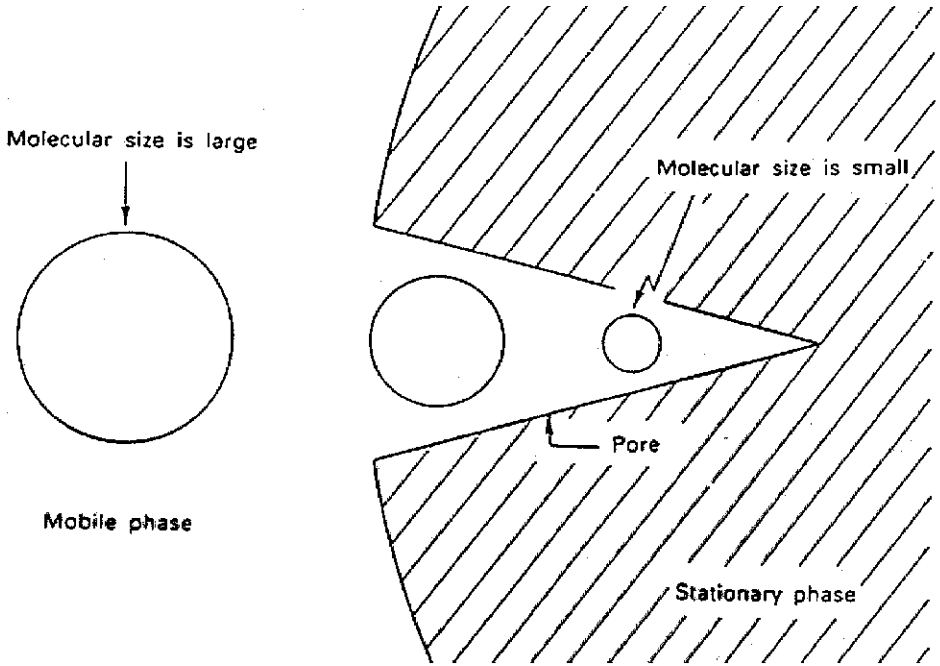
- mainly polymer science field

➢ GFC (Gel Filtration Chromatography)

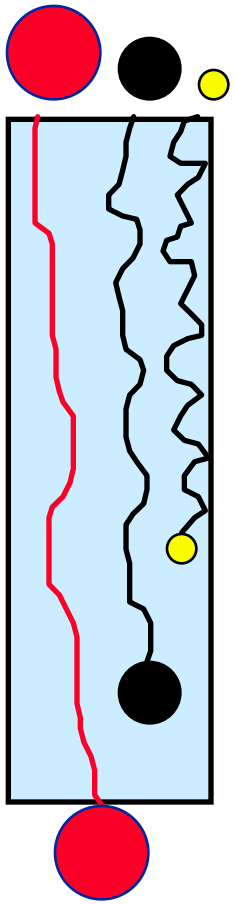
- mainly biological science field

Principle of SEC

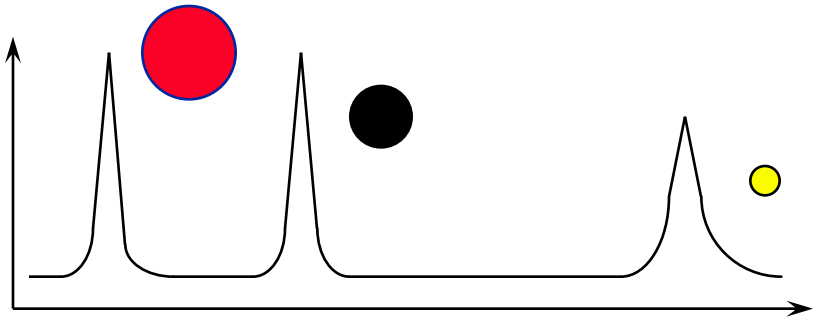
- No Interaction Force
- Difference of Traveling Time



Elution order



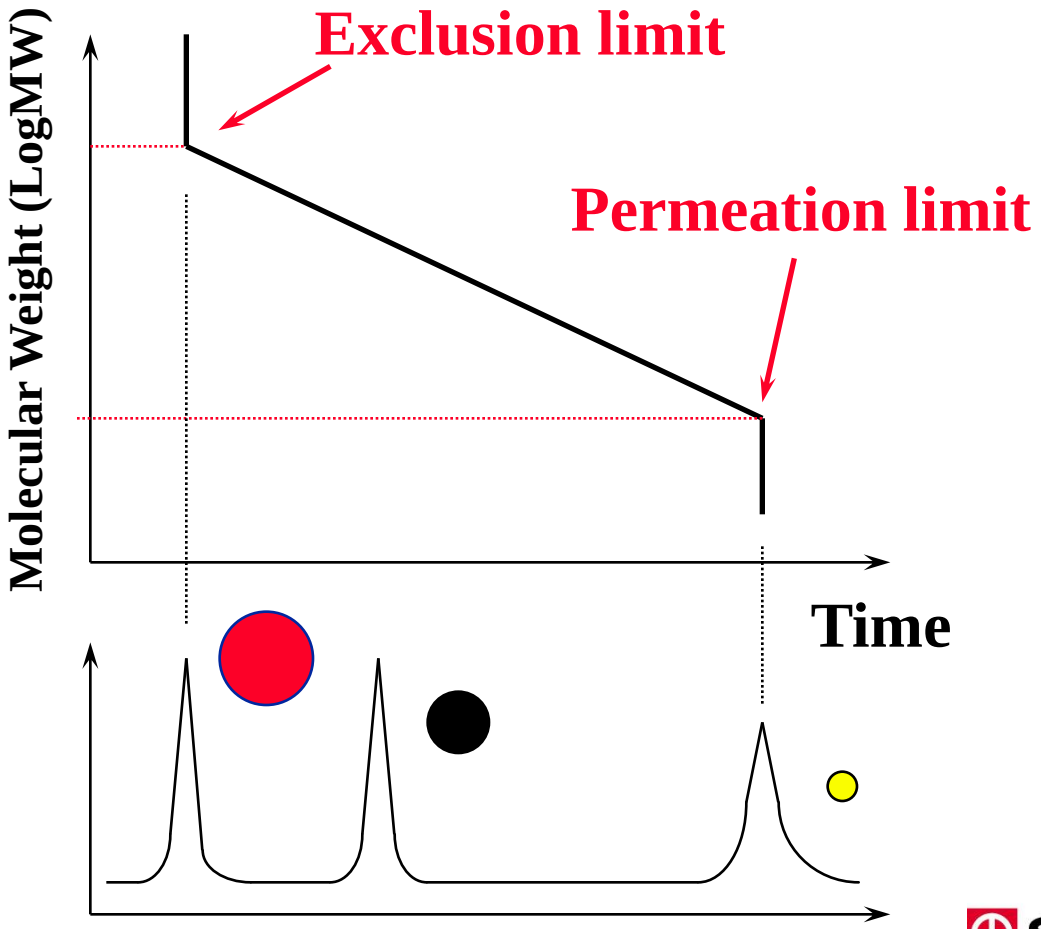
SEC
Column



Purpose of GPC / GFC

- GPC
  - –Molecular Weight measurement of polymer
- GFC
  - –Separation of protein

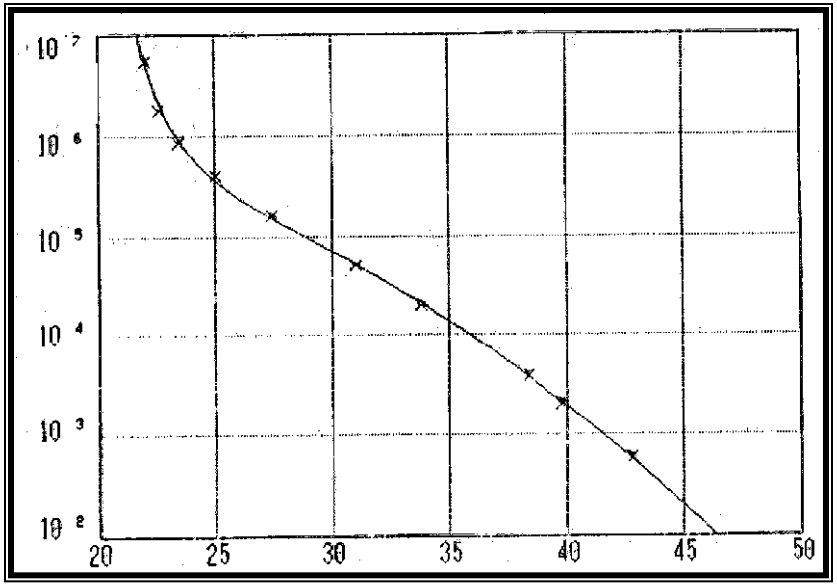
Relationship between MW and RT



Calibration Curve

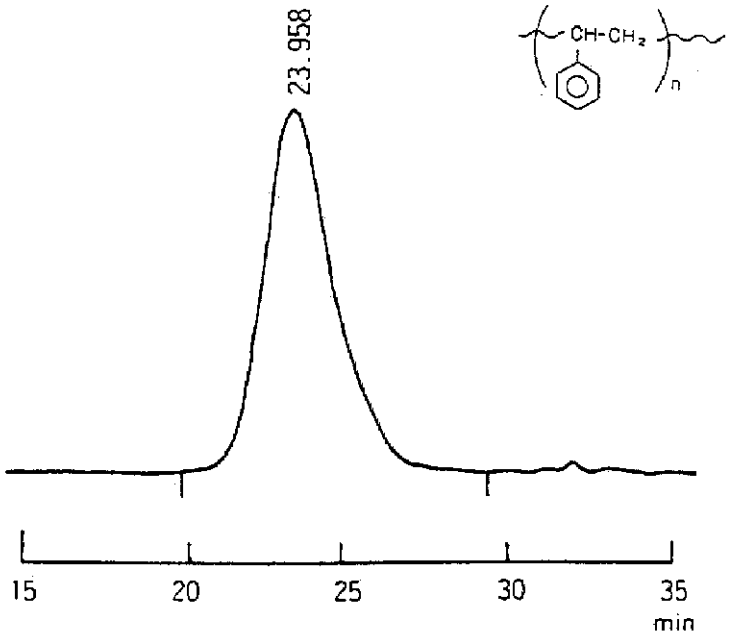
- Inject the standard polymer sample one by one to know the relationship between molecular weight and retention time.

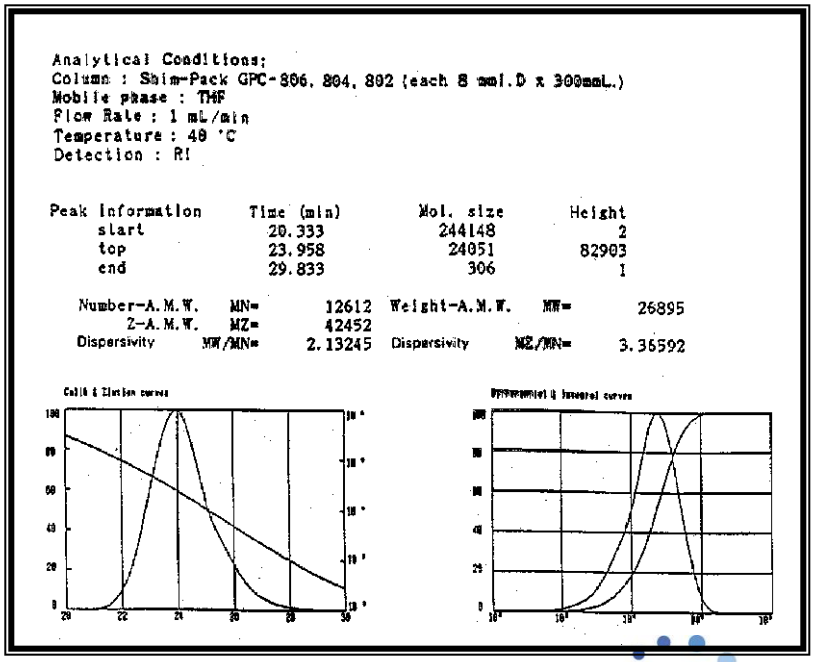
No.	time(min)	mol. wet.
1	22.0	5500000
2	22.6	1800000
3	23.4	860000
4	25.0	400000
5	27.4	160000
6	31.0	50000
7	33.8	20000
8	38.4	4000
9	39.8	2000
10	42.8	600
11	46.8	80



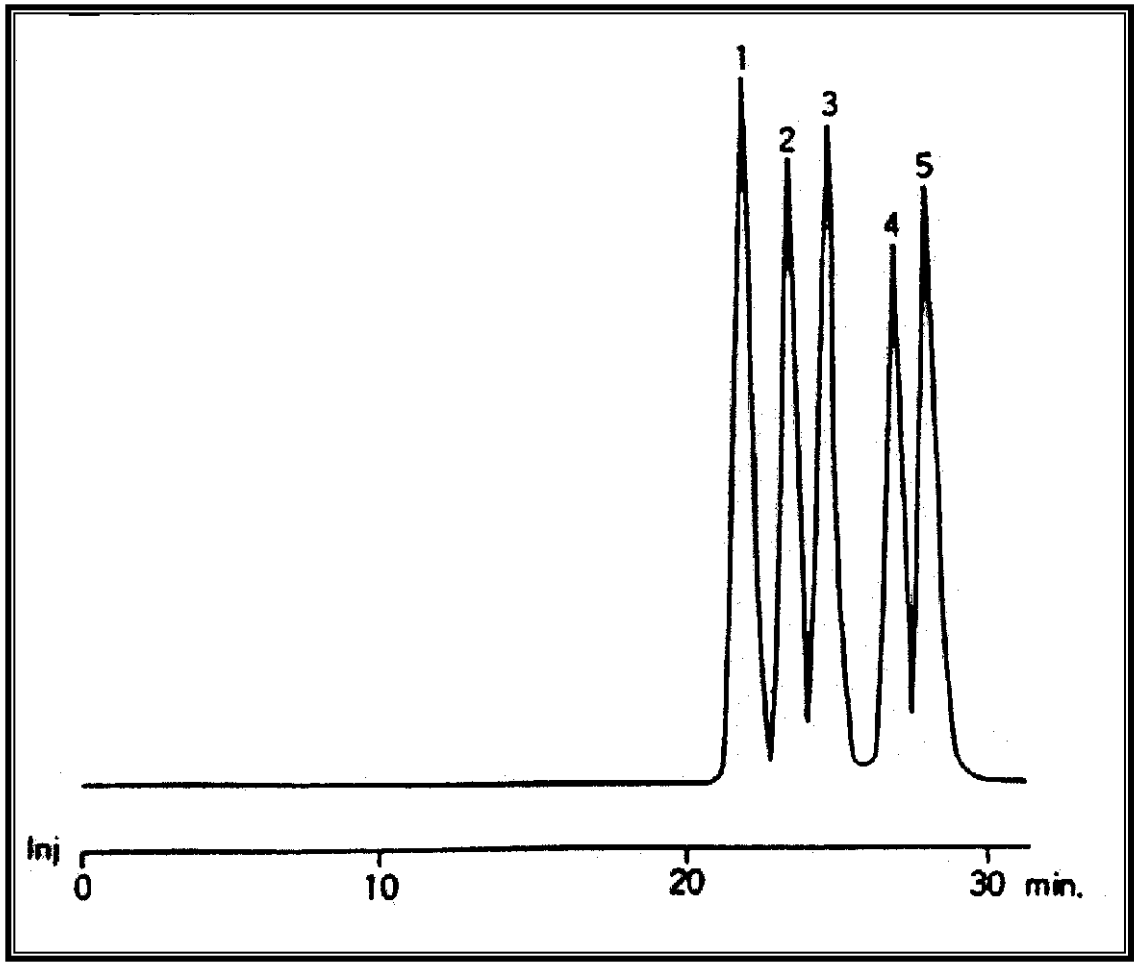
Actual Sample Injection

- To calculate the MW,
  GPC software is required.





Protein Separation using GFC column

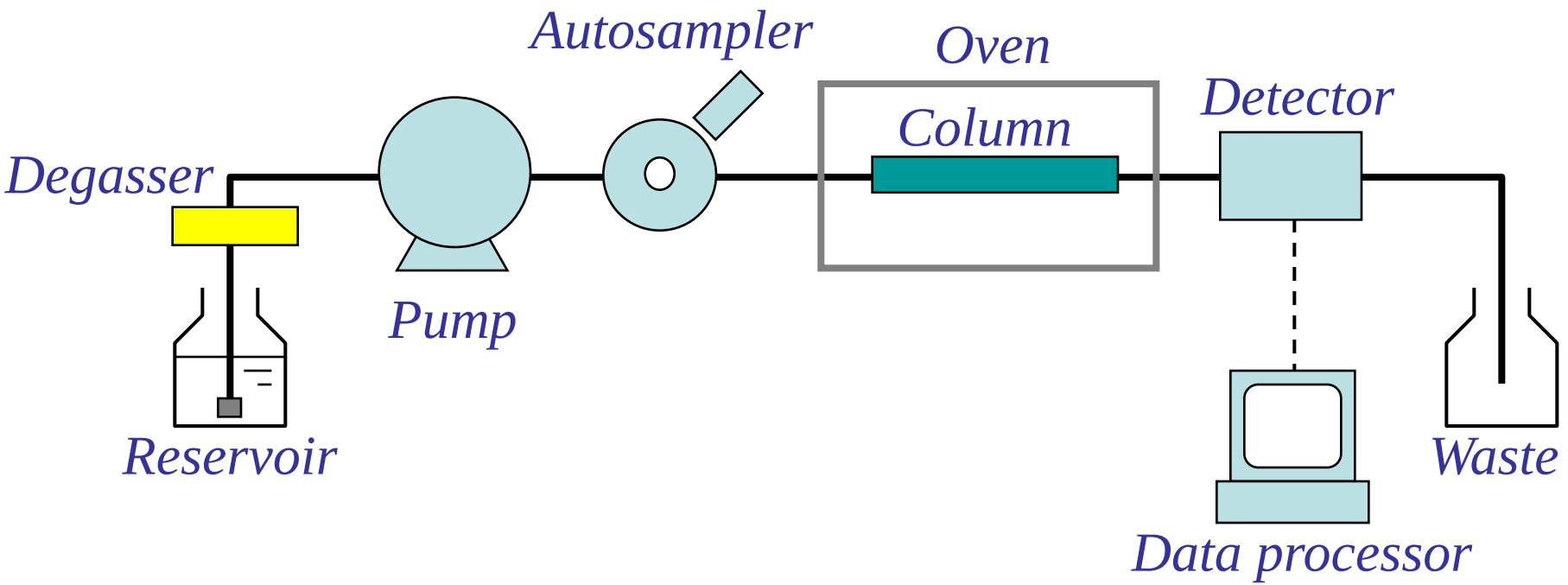


- **Analytical Conditions**
  - **Column : Asahipak GFA-50**
  - **Mobile phase :**
    0.1 M sodium phosphate
    0.1 M NaCl (pH=7.0)
  - **Flow rate : 0.5 mL/min**
  - **Temperature : ambient**
  - **Detector : UV-280 nm**
  - **Injection volume : 10 uL**
- **Peaks**
  1. **glutamate dehydrogenase**
  2. **lactate dehydrogenase**
  3. **enolase**
  4. **adenylate kinase**
  5. **cytochrome C**

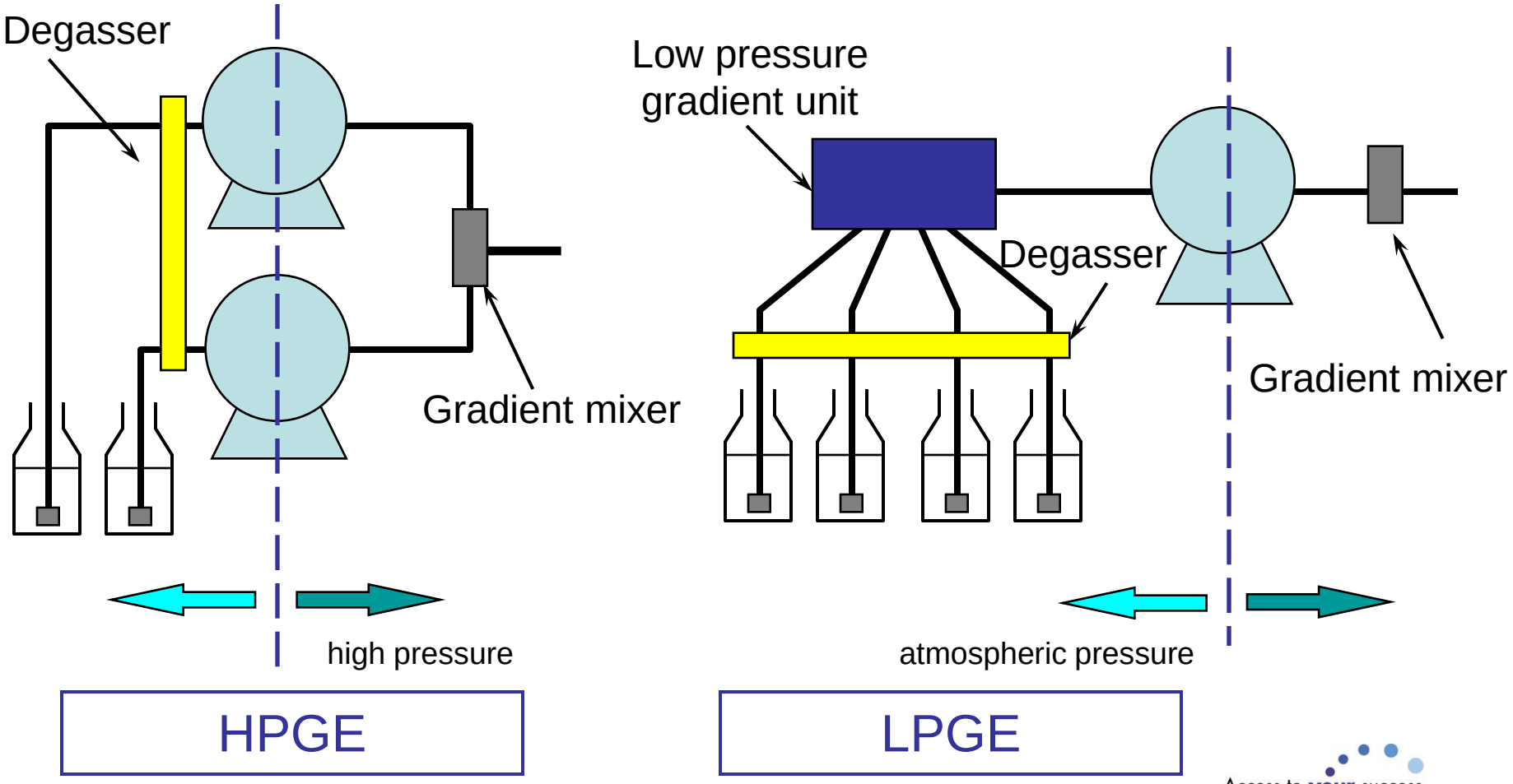
Gradient Elution in HPLC

- **Isocratic elution mode**
  - –One mobile phase with a constant composition
- **Gradient elution mode**
  - –Multi mobile phases with changing composition

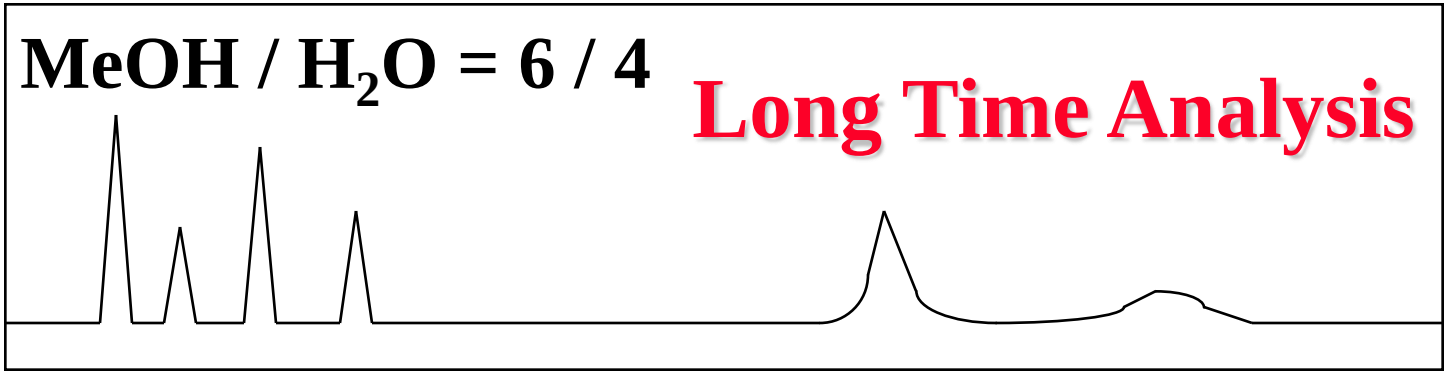
Isocratic Elution System



High / Low pressure gradient system

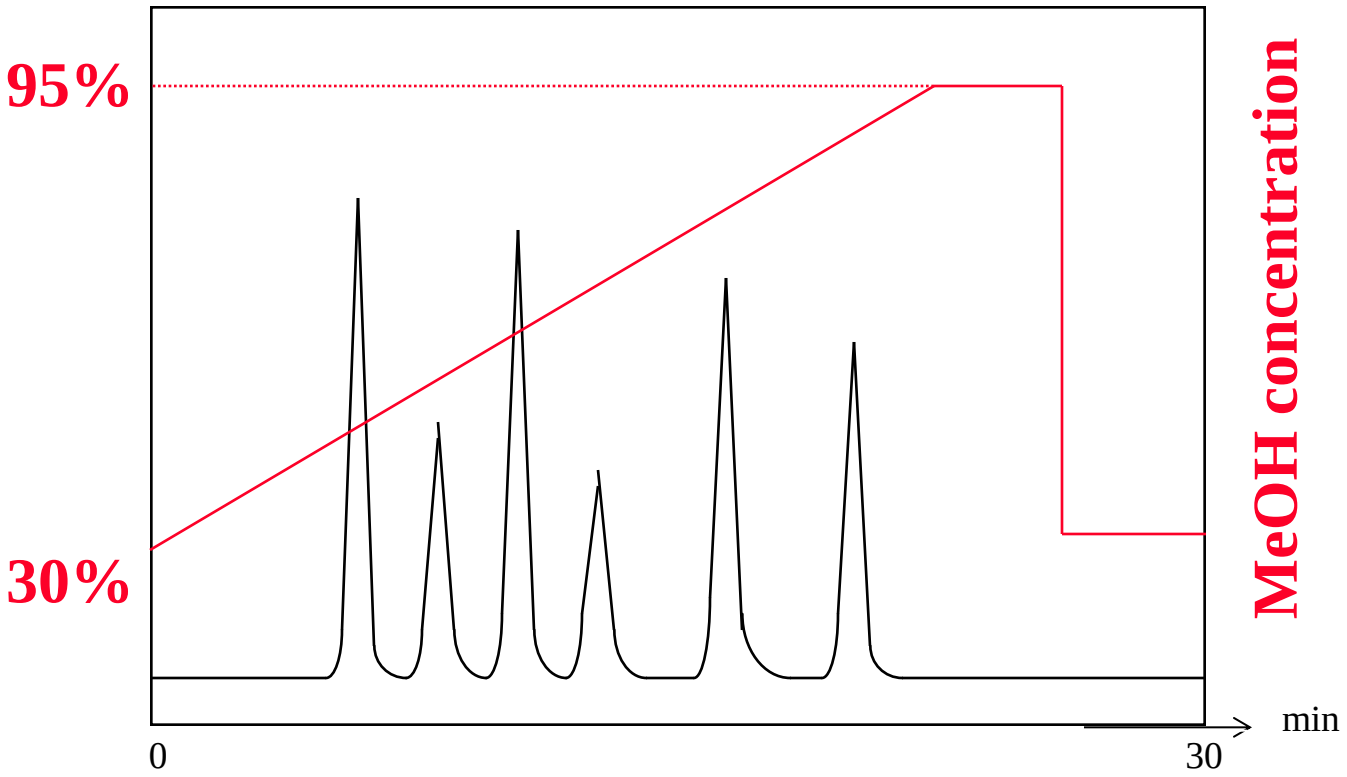


Isocratic Elution Mode



(column : ODS type)

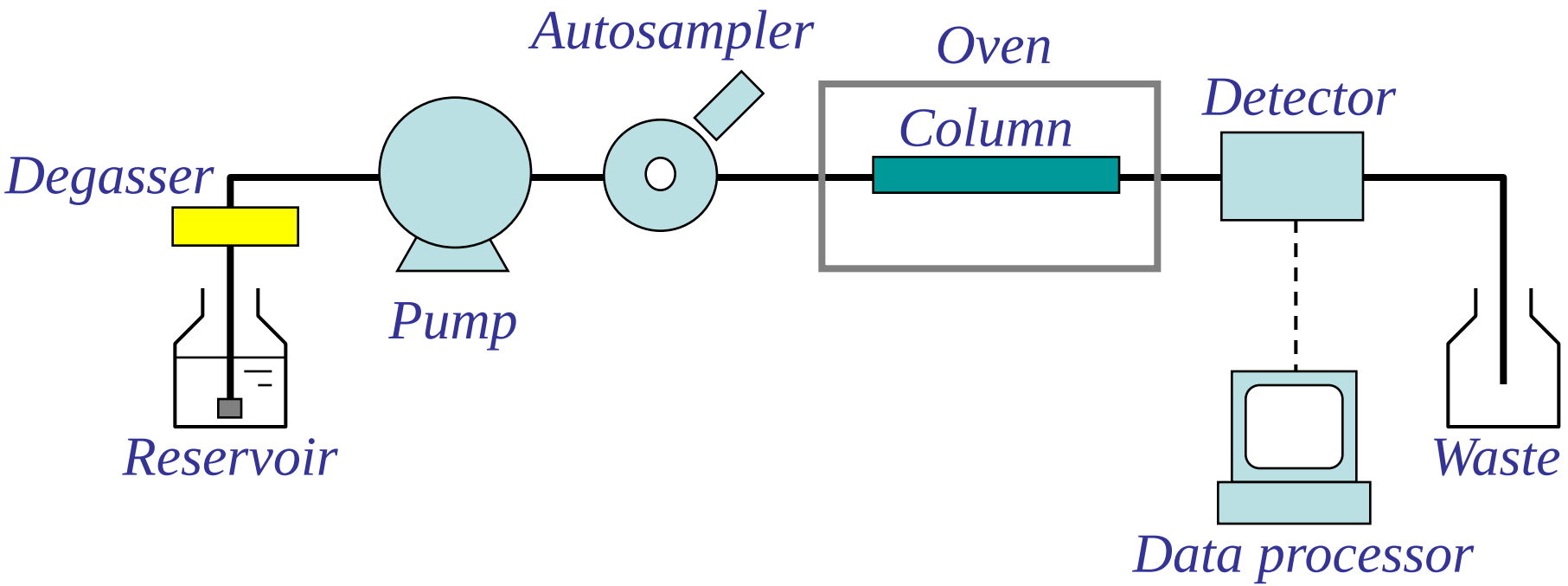
Gradient Elution Mode



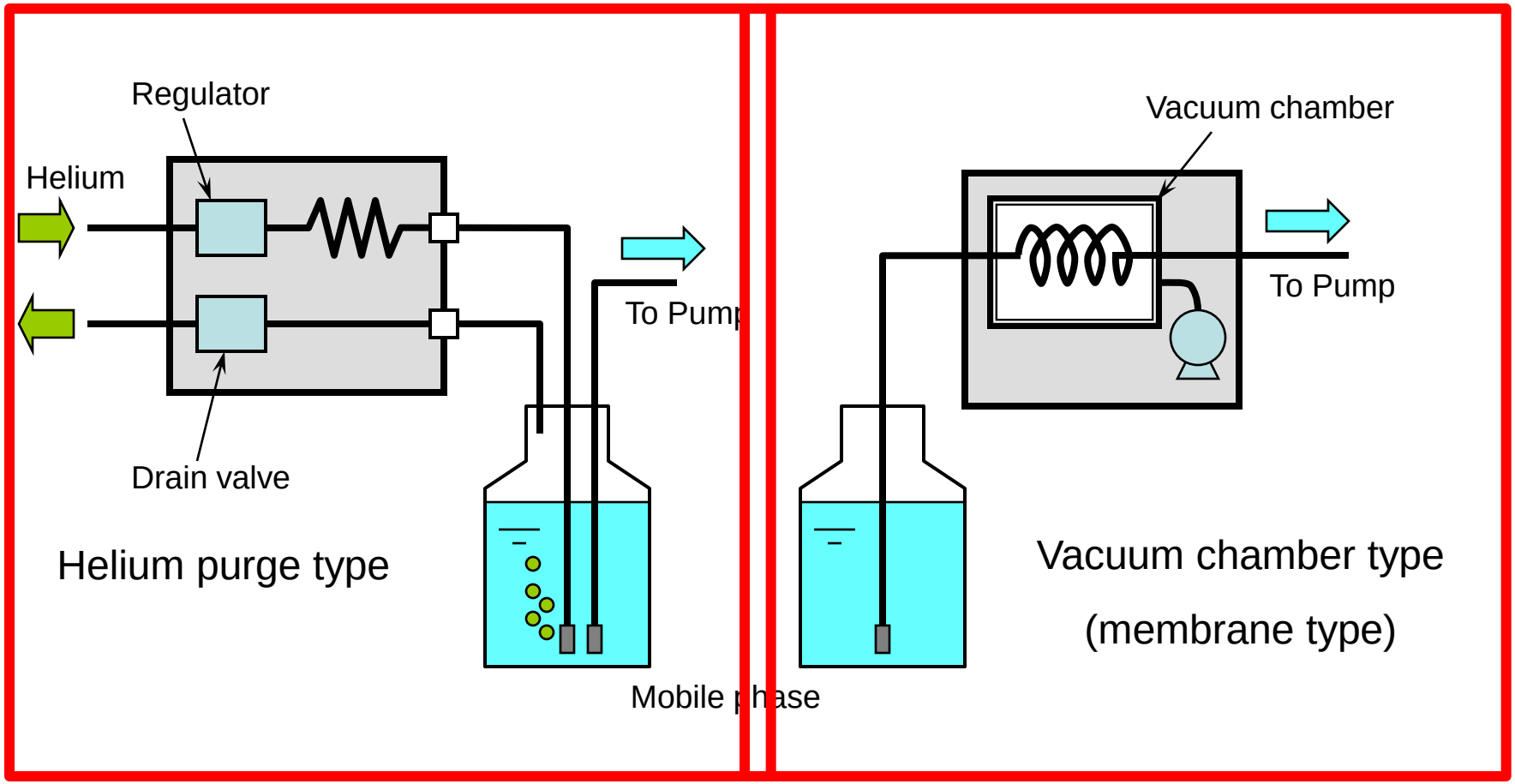
Instrumentation of HPLC

- Degasser
- Solvent delivery pump
- Sample injector
- Column oven
- Detector

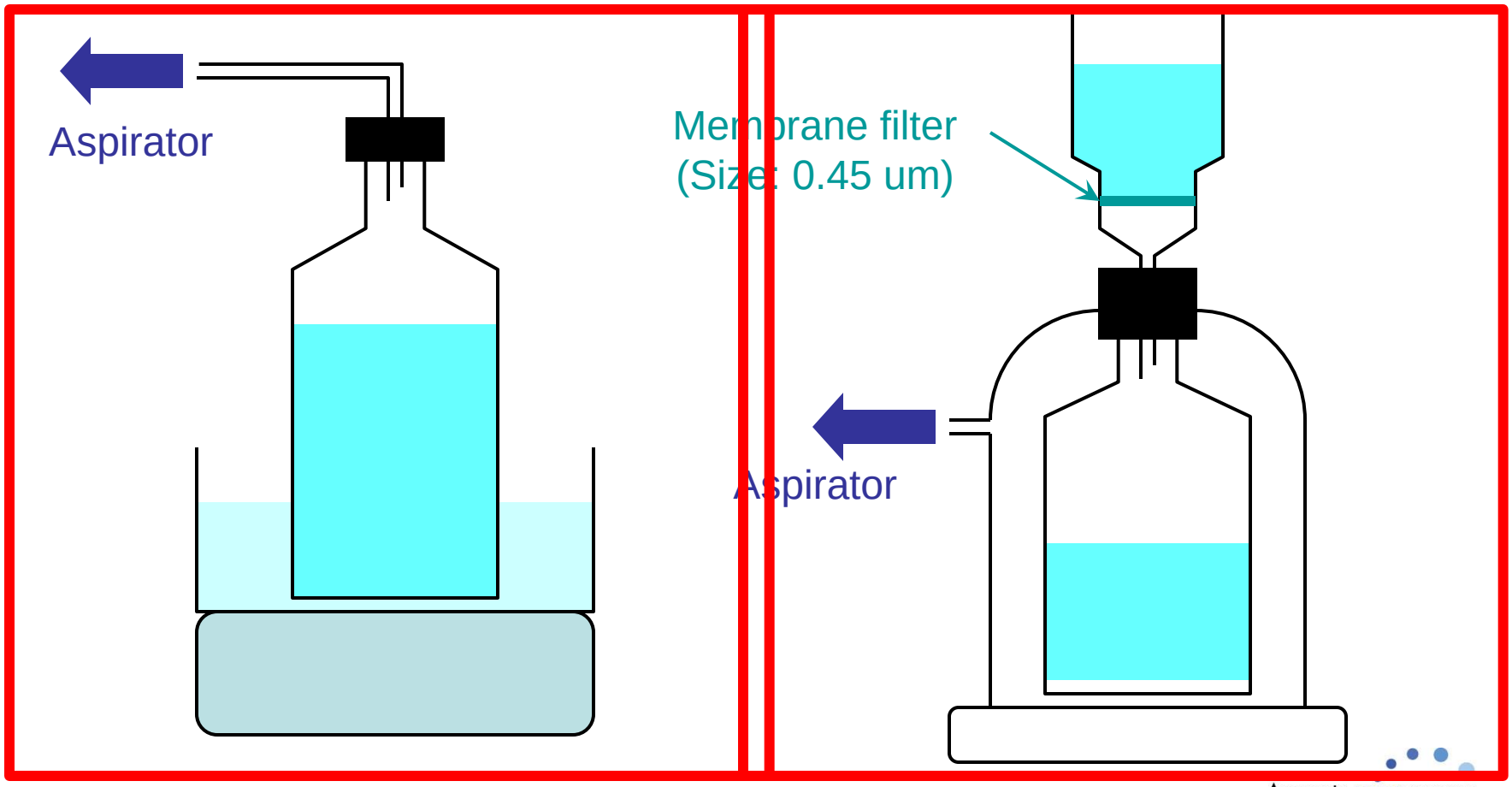
Flow-line



On-line degasser



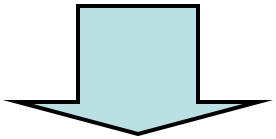
Off-line degassing & Filtration



Ultrasonic cleaning unit

Degasser

- Problems caused by dissolved air in mobile phase
  - –Unstable delivery in pump
  - –Bigger noise and large baseline-drift in detector cell

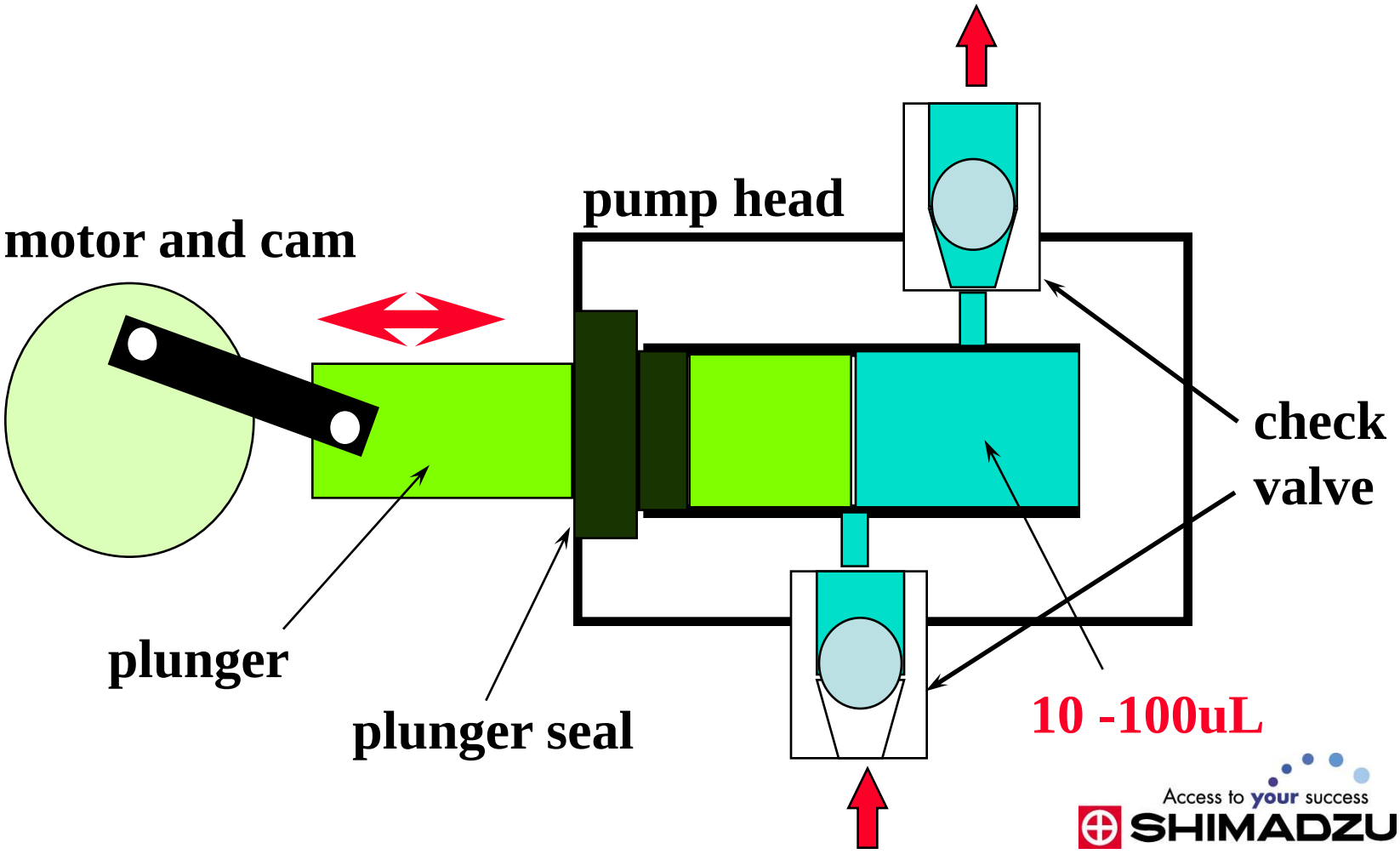


In order to avoid causing the problems, mobile phase should be degassed.

Desirable Pump Performance

- **High Pressure Resistance**
- **Precise Flow Rate**
- **Low Pressure Fluctuation**

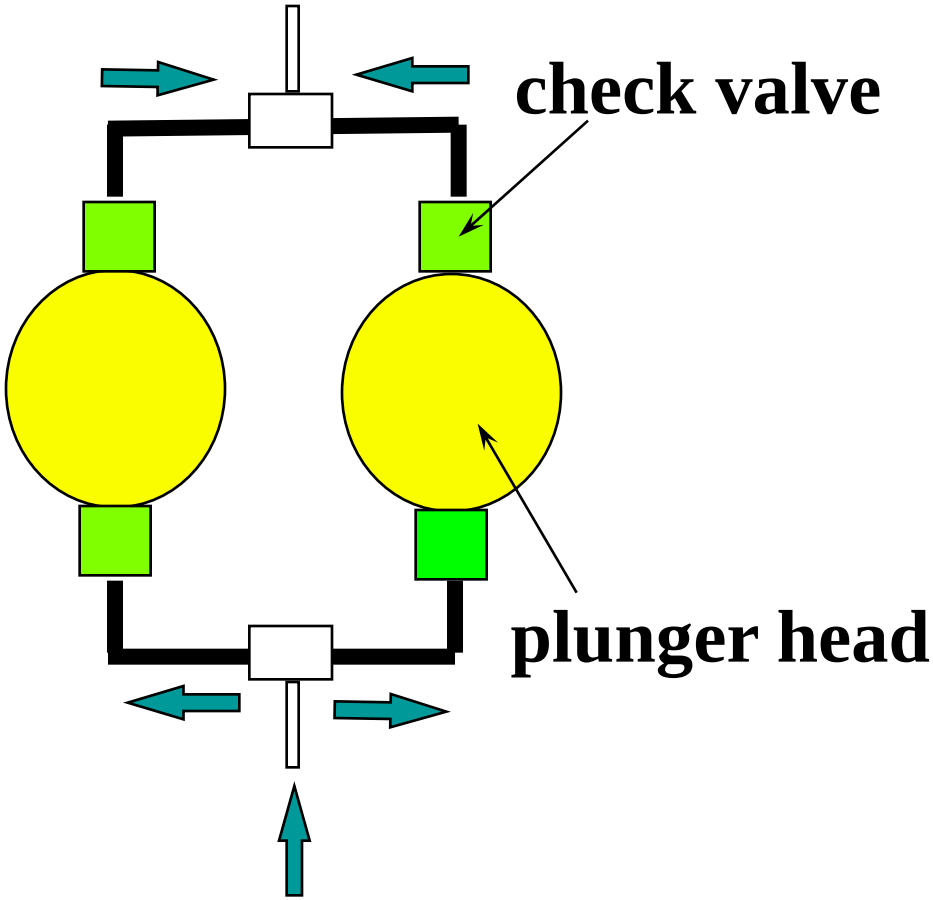
Plunger reciprocating pump



Plunger reciprocating pump

- Advantage
  - Low pressure fluctuation
  - Very easy to replace the another solvent
- Disadvantage
  - Change the plunger seal

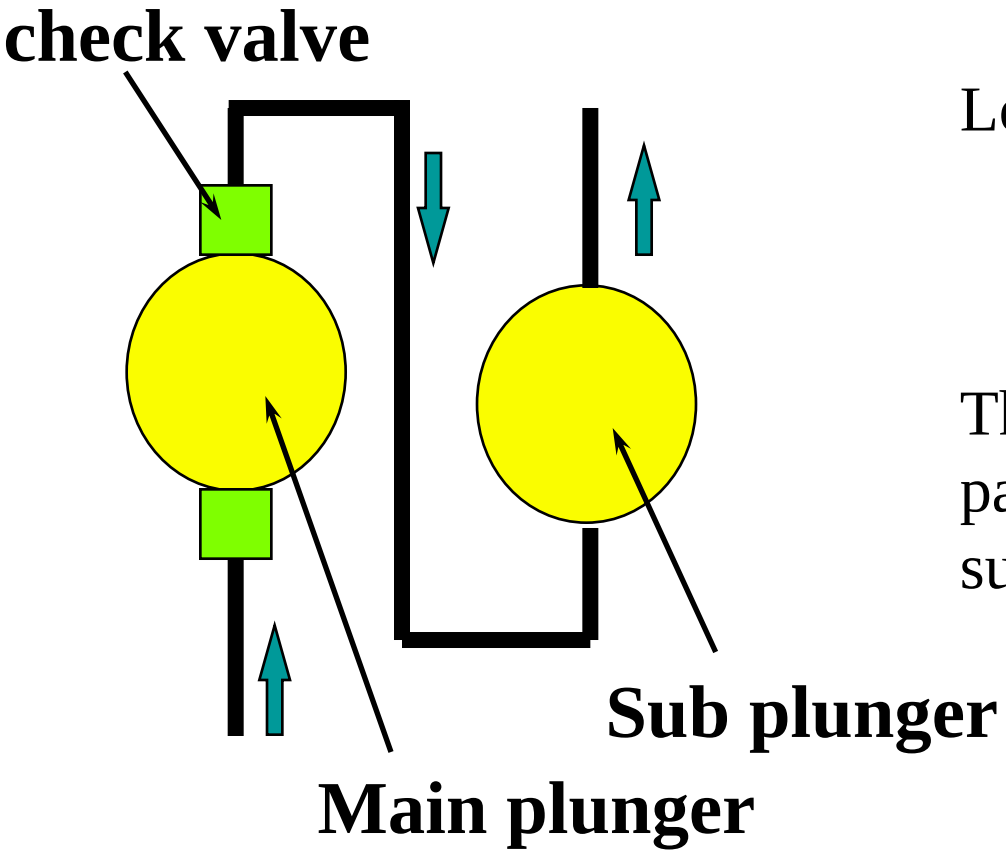
Dual plunger with parallel flow line



Very low pressure fluctuation
- Refractive index detector
- Conductivity detector
- Electrochemical detector
- MS detector

The number of maintenance parts is more.

Dual plunger with tandem flow line



Low pressure fluctuation
UV / PDA detector
Fluorescence detector

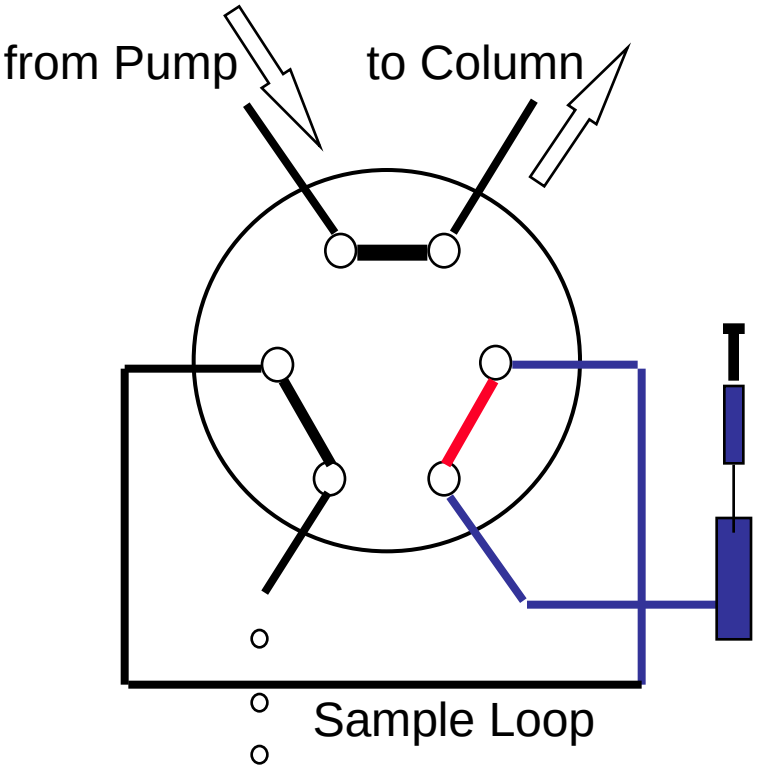
The number of maintenance parts is less. So this design is suitable for routine analysis.

HPLC Injectors

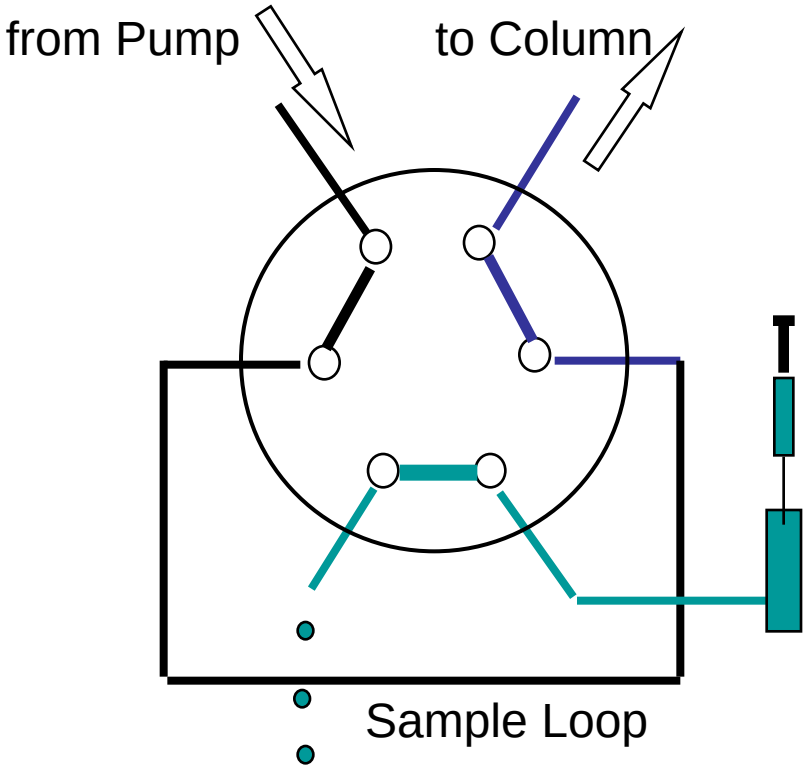
Auto injector

Manual injector

Loop injection autosampler

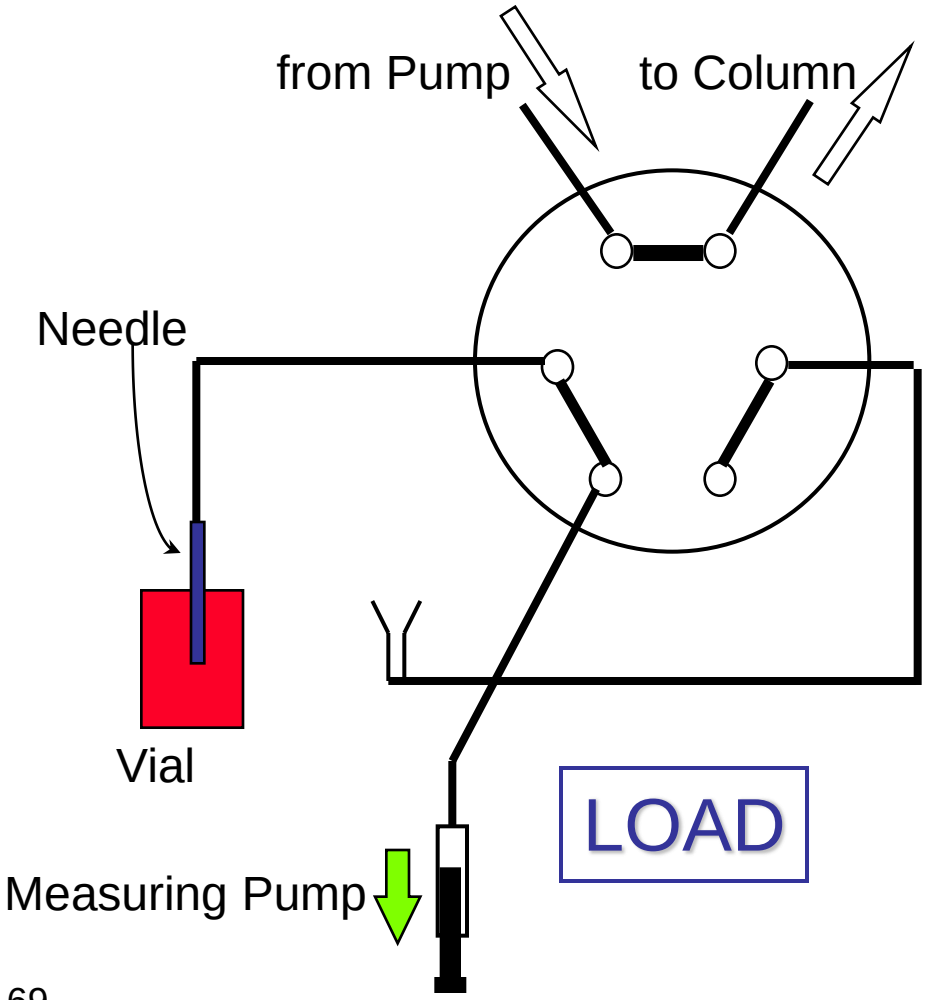


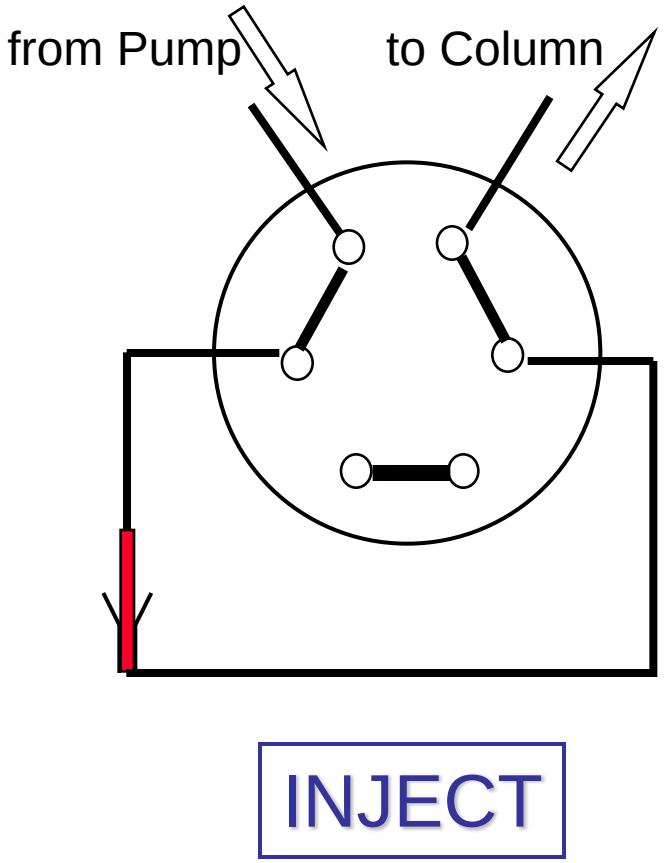
LOAD



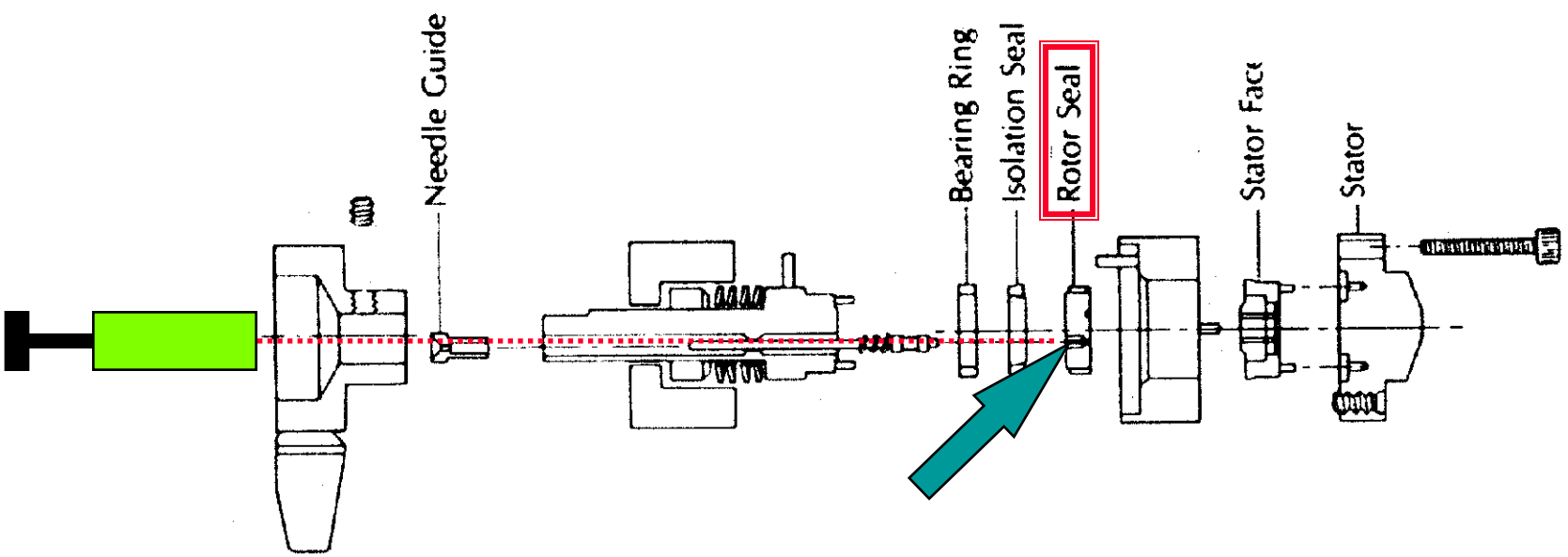
INJECT

Direct injection autosampler



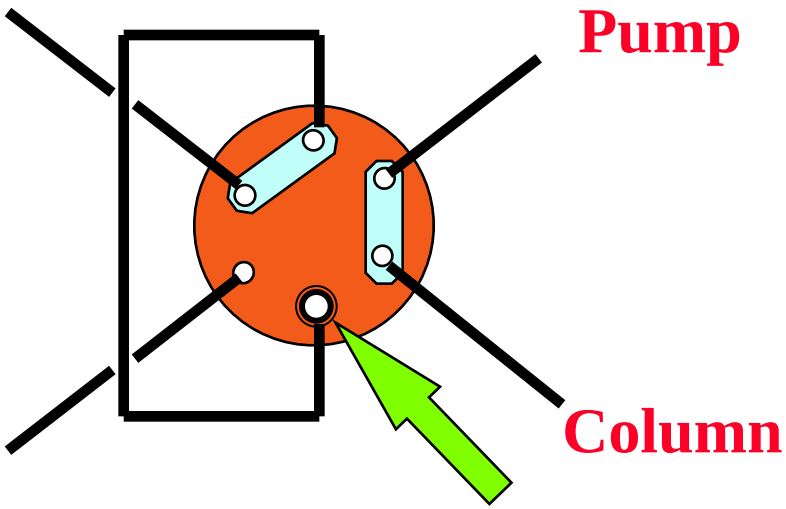


Manual Injector

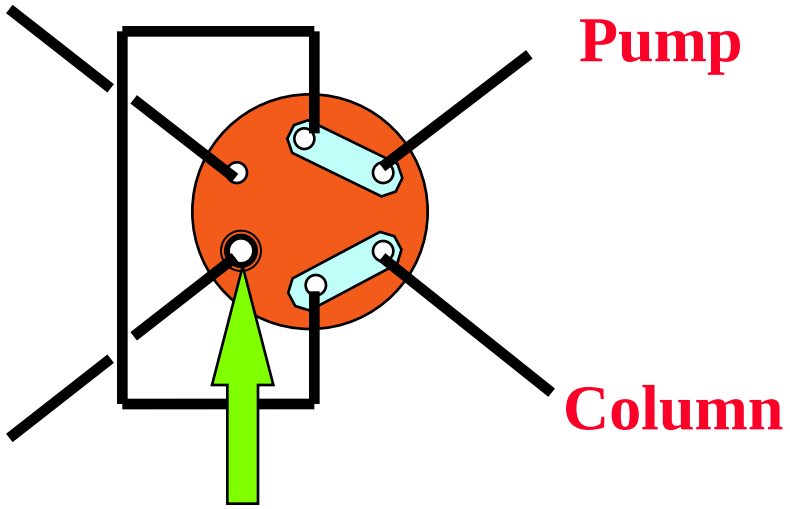


Rheodyne Manual Injector

Manual Injector

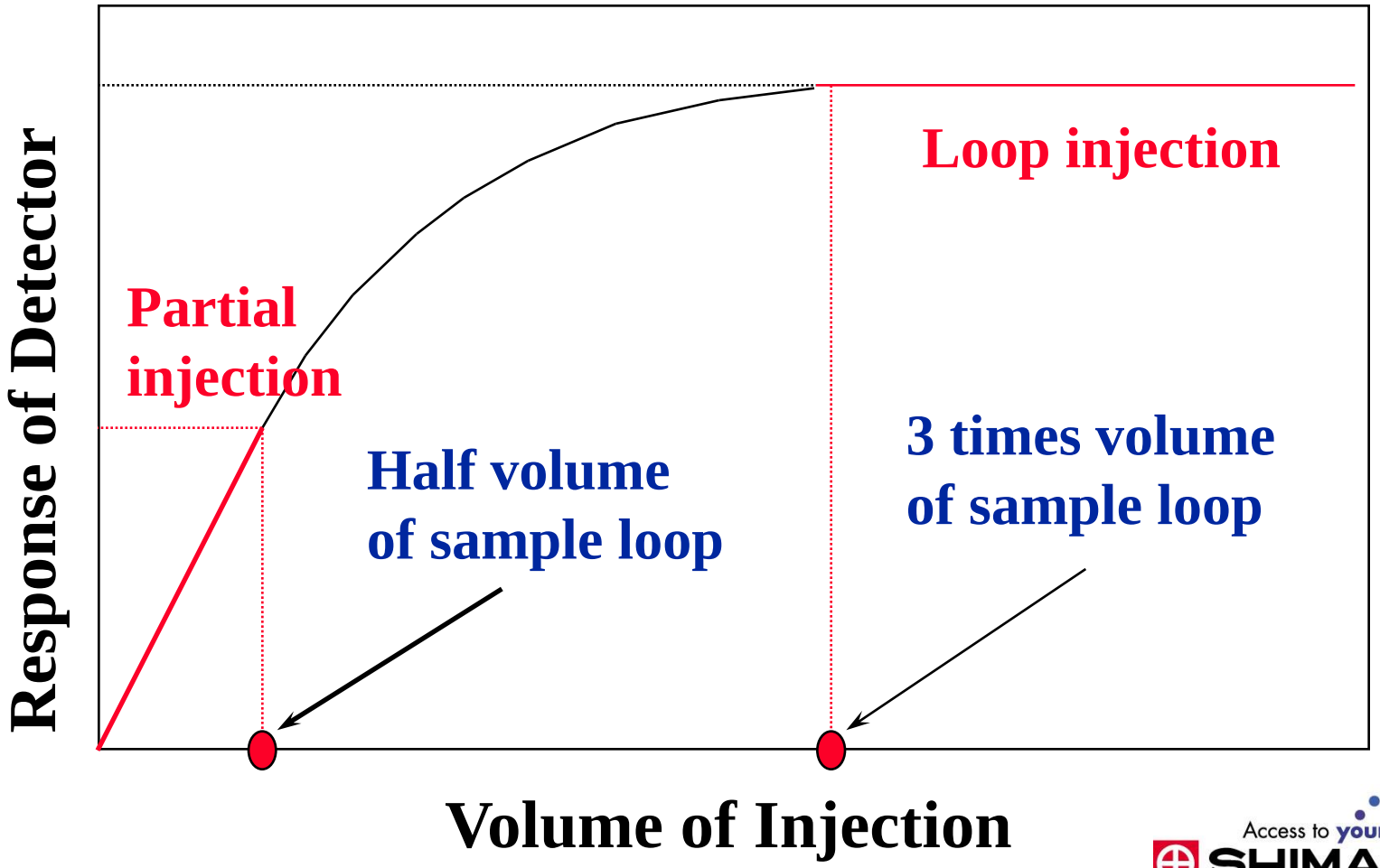


[LOAD]



[INJECT]

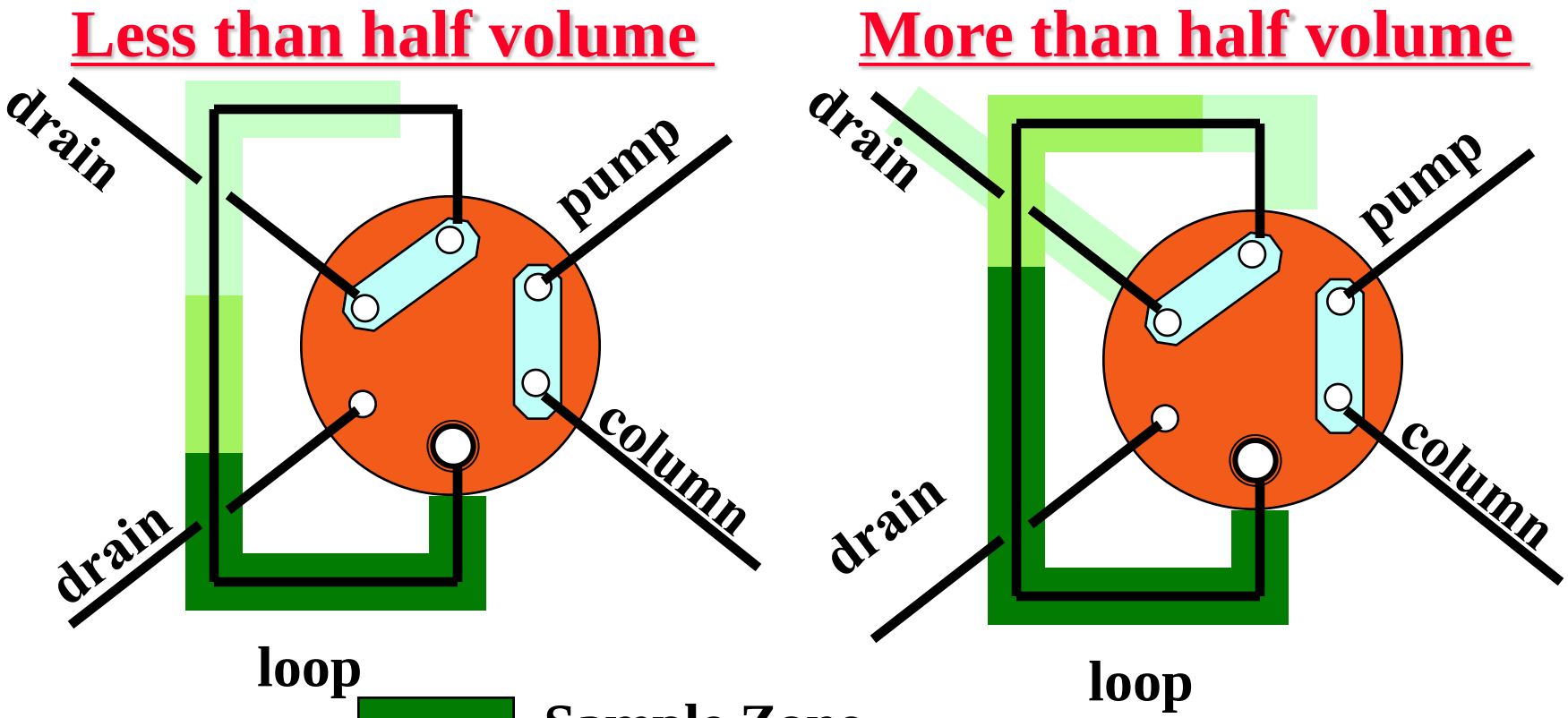
Relationship between injection volume and detector's response



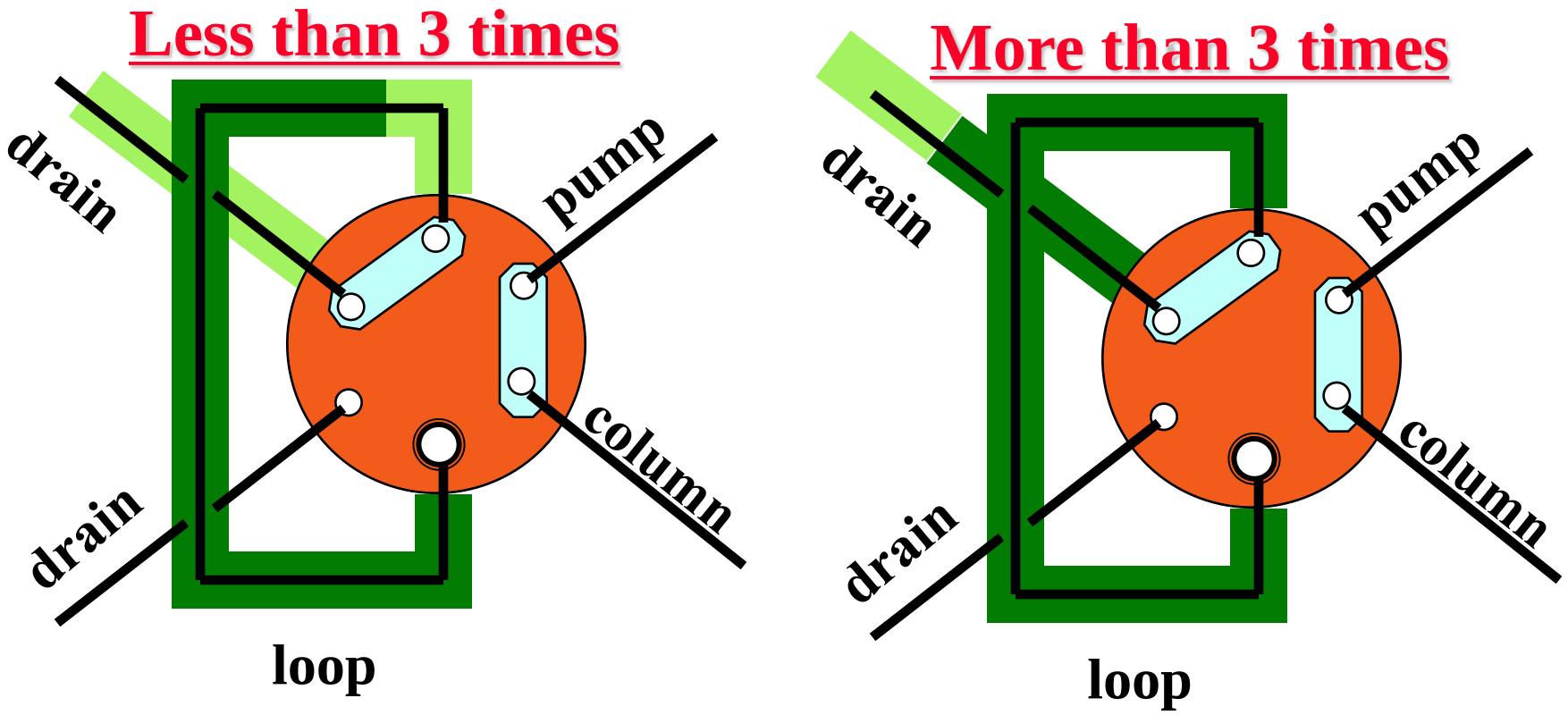
Injection Method

- Partial Injection Method
  - –better to inject less than half volume of sample loop
- Loop Injection Method
  - –better to inject more than 3 times volume of sample loop

Dispersion and Dilution Effect



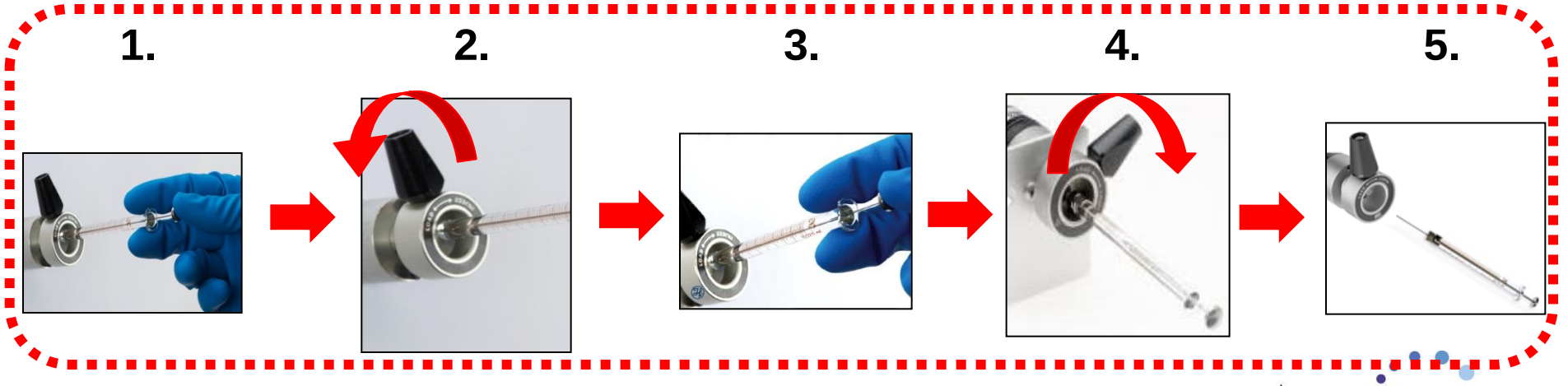
Dispersion and Dilution Effect



How to inject the sample

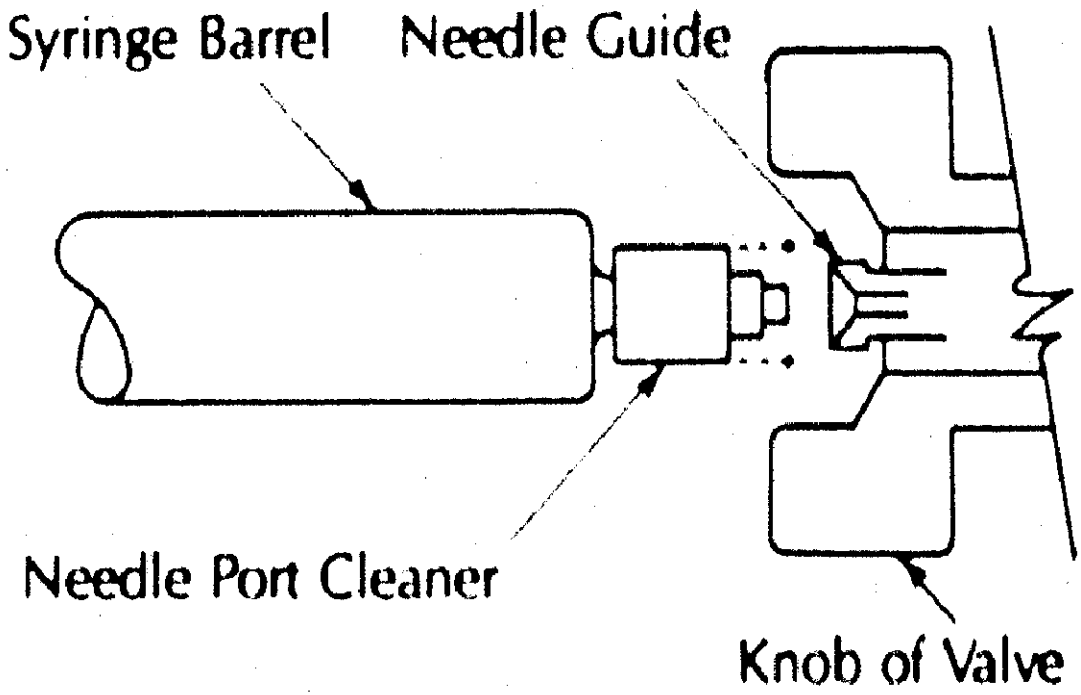
- Rheodyne Manual Injector -

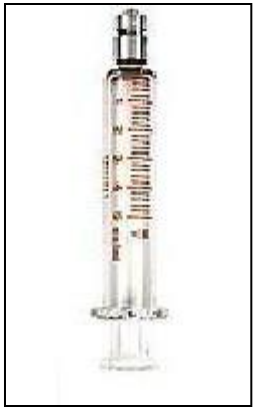
1. Insert a syringe at INJECT position.
2. Turn knob to the LOAD position.
3. Inject a sample.
4. Turn a knob to the INJECT position.
5. Remove the syringe.
6. Wash an injection port.



Washing for Injection Port

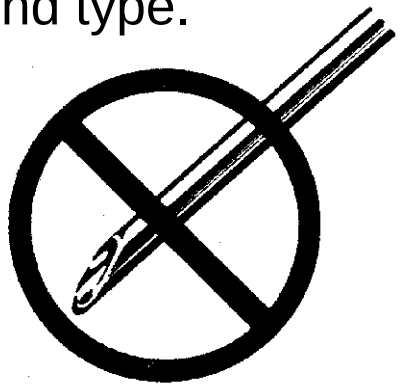
- Use of Needle Port Cleaner





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.

Column Temperature Control Devices

Column temperature control devices are functioning to keep the column temperature constant. The temperature fluctuation of column will influence retention time reproducibility.

CTO-10ASvp

CTO-20A/20AC

CTO-40S

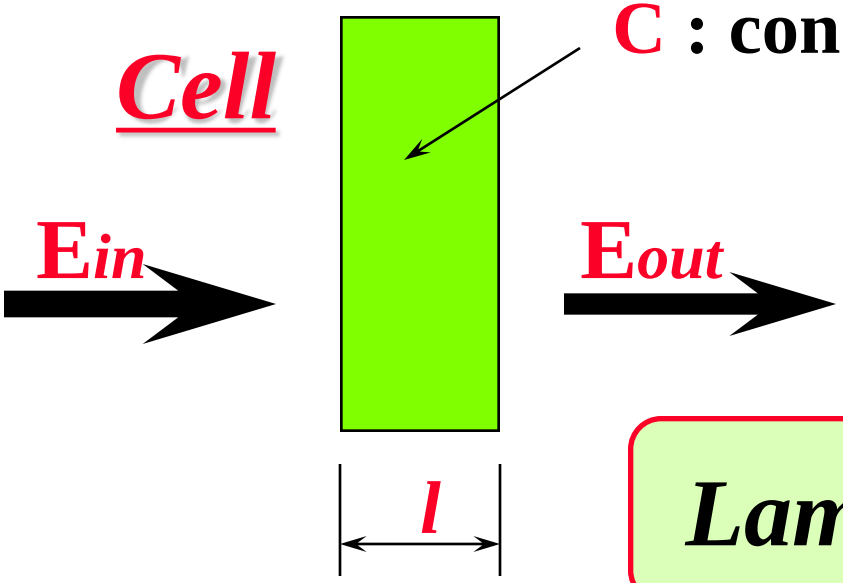
CTO-40C

- **Column Oven (heat /or cool)**
- **Heated Column Jacket**
  - **–Aluminum block**
- **Insulated Column Jacket**
  - **–Water bath**

Detectors for Detectors

- Ultraviolet / Visible detector (UV/VIS)
- Photodiode Array detector (PDA)
- Fluorescence detector (RF)
- Conductivity detector (CDD)
- Refractive Index detector (RID)
- Electrochemical detector (ECD)
- Evaporative Light Scattering detector (ELSD)
- Mass spectrometer detector (MS)

Ultraviolet / Visible detector

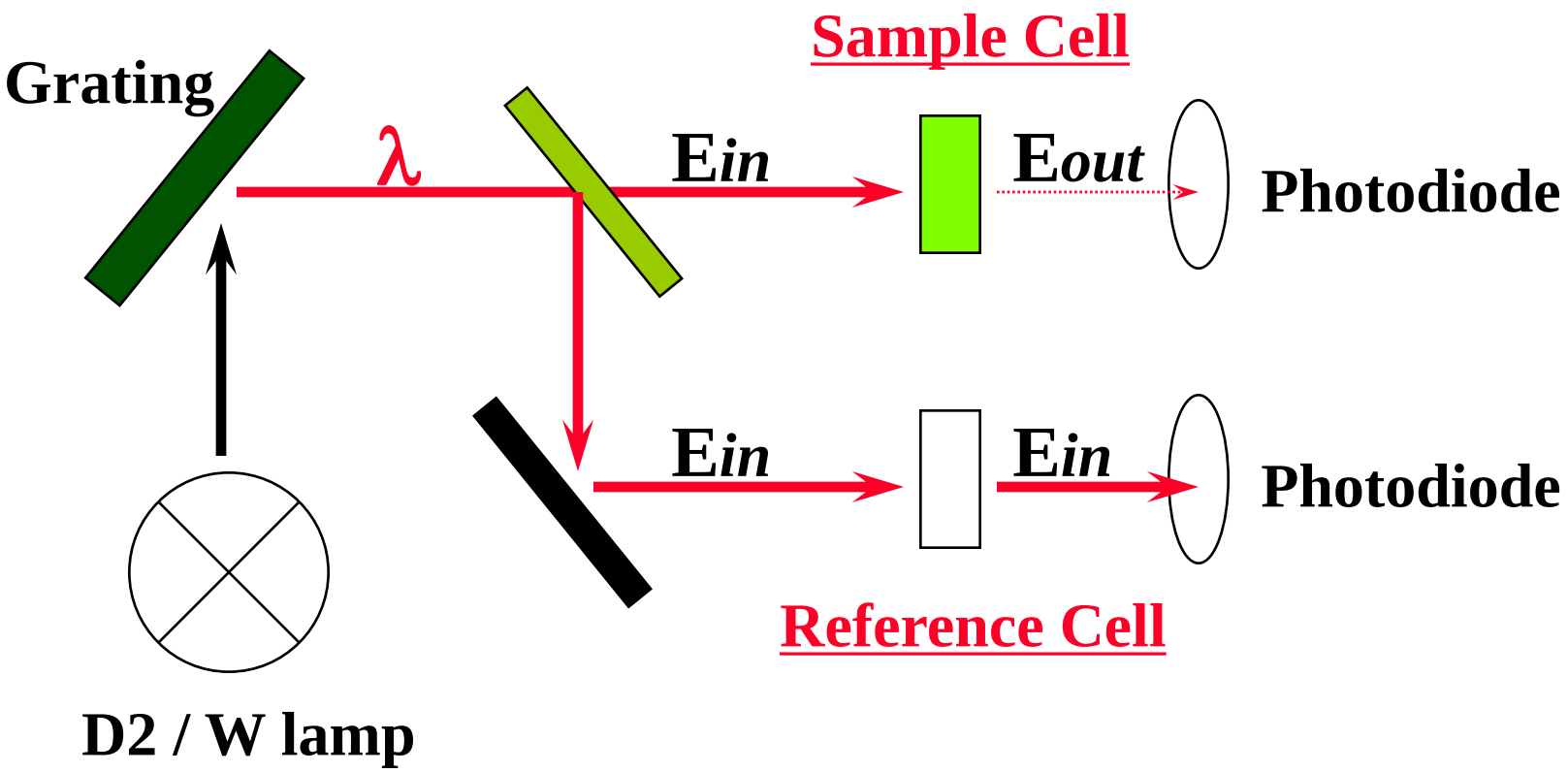


Lambert-Beer's law

$$A = \varepsilon C l = -\log (E_{out} / E_{in})$$

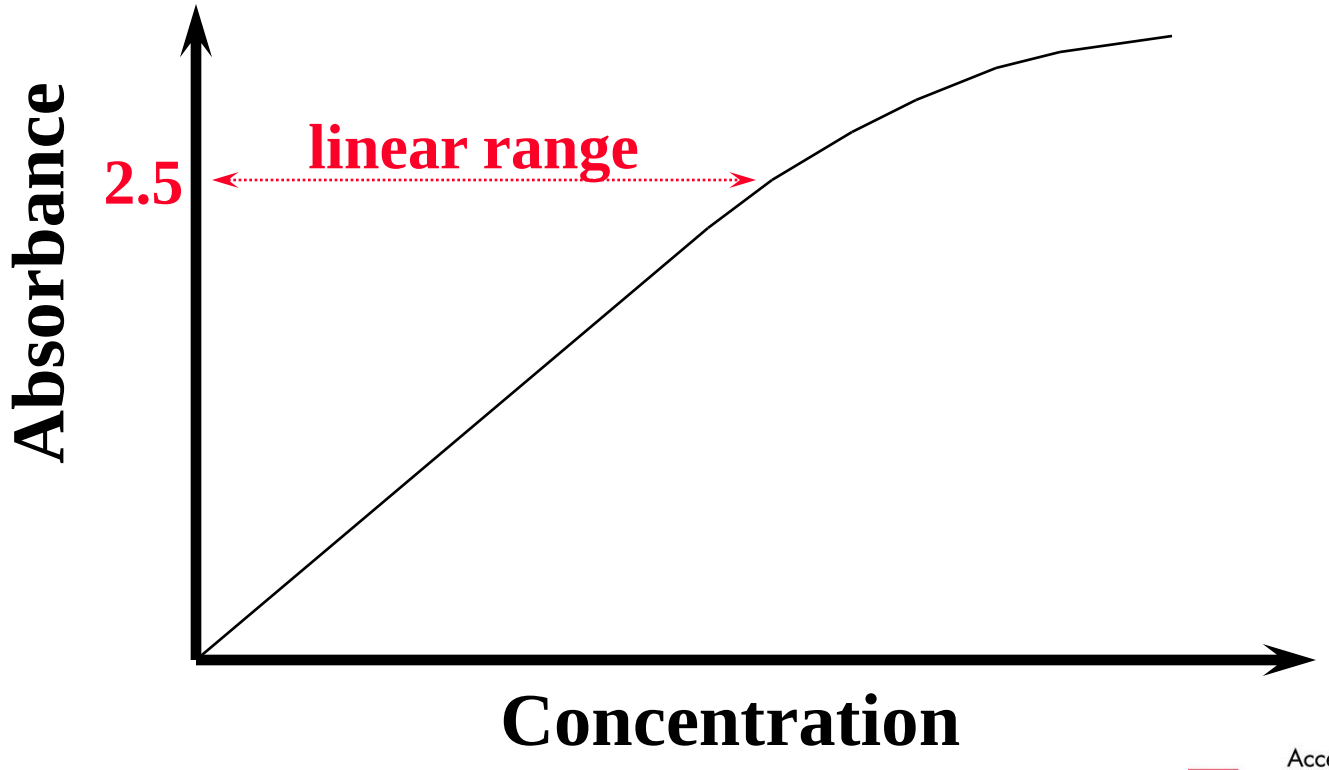
(A : absorbance)

Ultraviolet / Visible detector



Lambert-Beer's law

$A = \varepsilon C l = -\log (E_{out} / E_{in})$

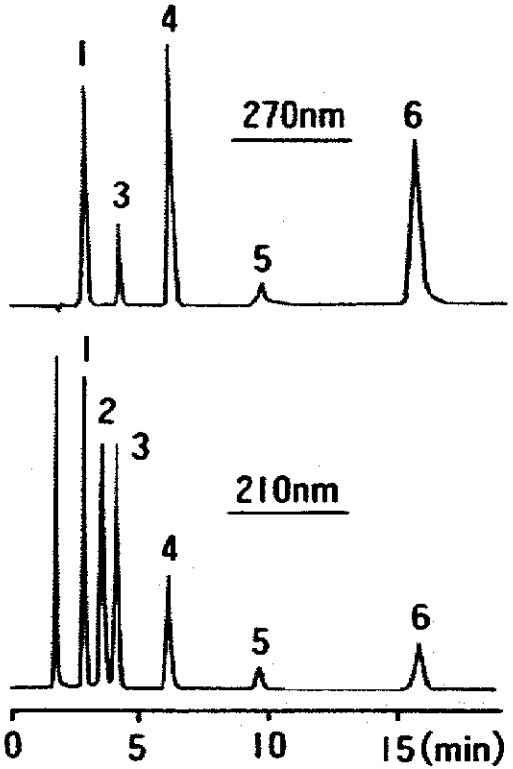


Additional Functions

- Dual Wavelength mode
- Ratio Plot mode
- Wavelength Time Program mode
- Wavelength Scan mode

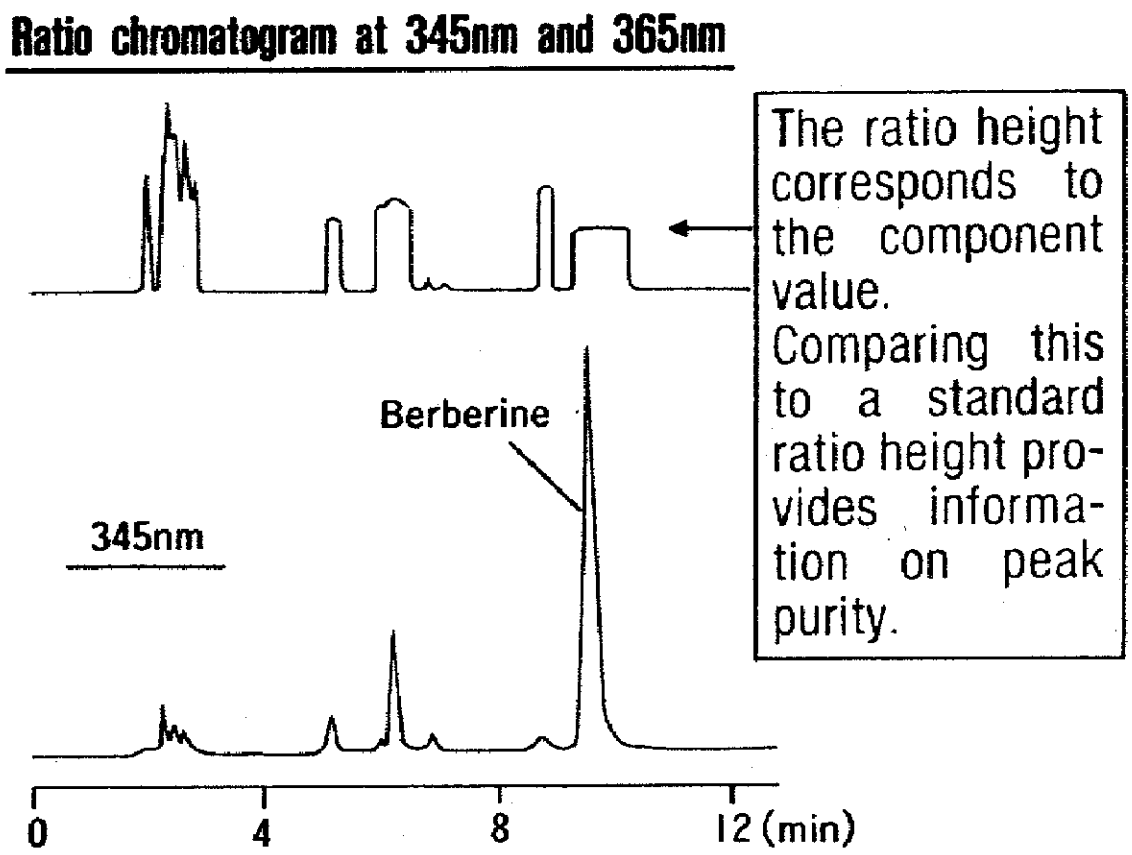
Dual Wavelength Mode

1. Nicotinamide
2. Pantothenic acid
3. Pyridoxine
4. Thiamine
5. Folic acid
6. Riboflavin



Simultaneous dual-wavelength measurement of water soluble vitamins

Ratio Plot Mode

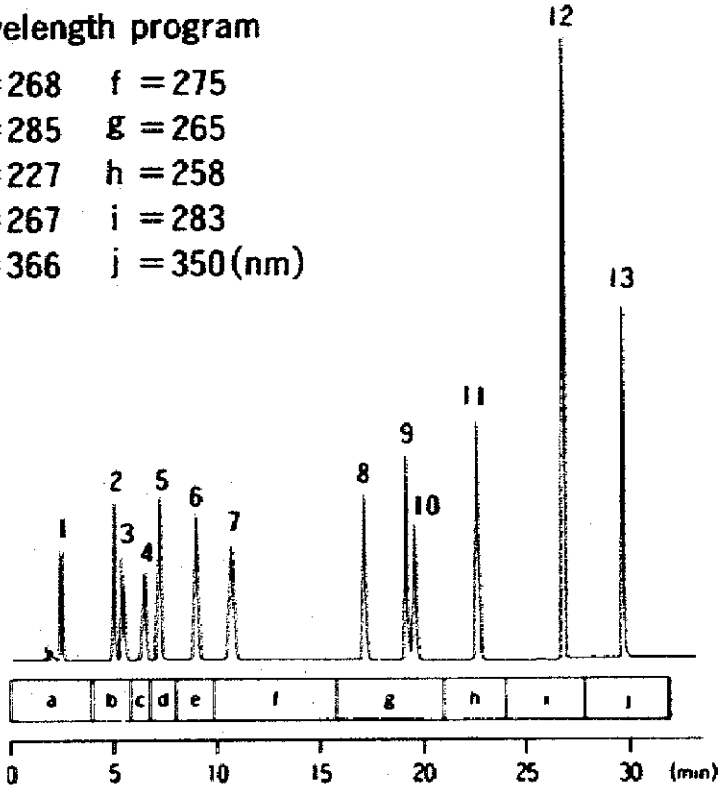


Ratio chromatogram of coptis japonica powder

Wavelength Time Program Mode

Wavelength program

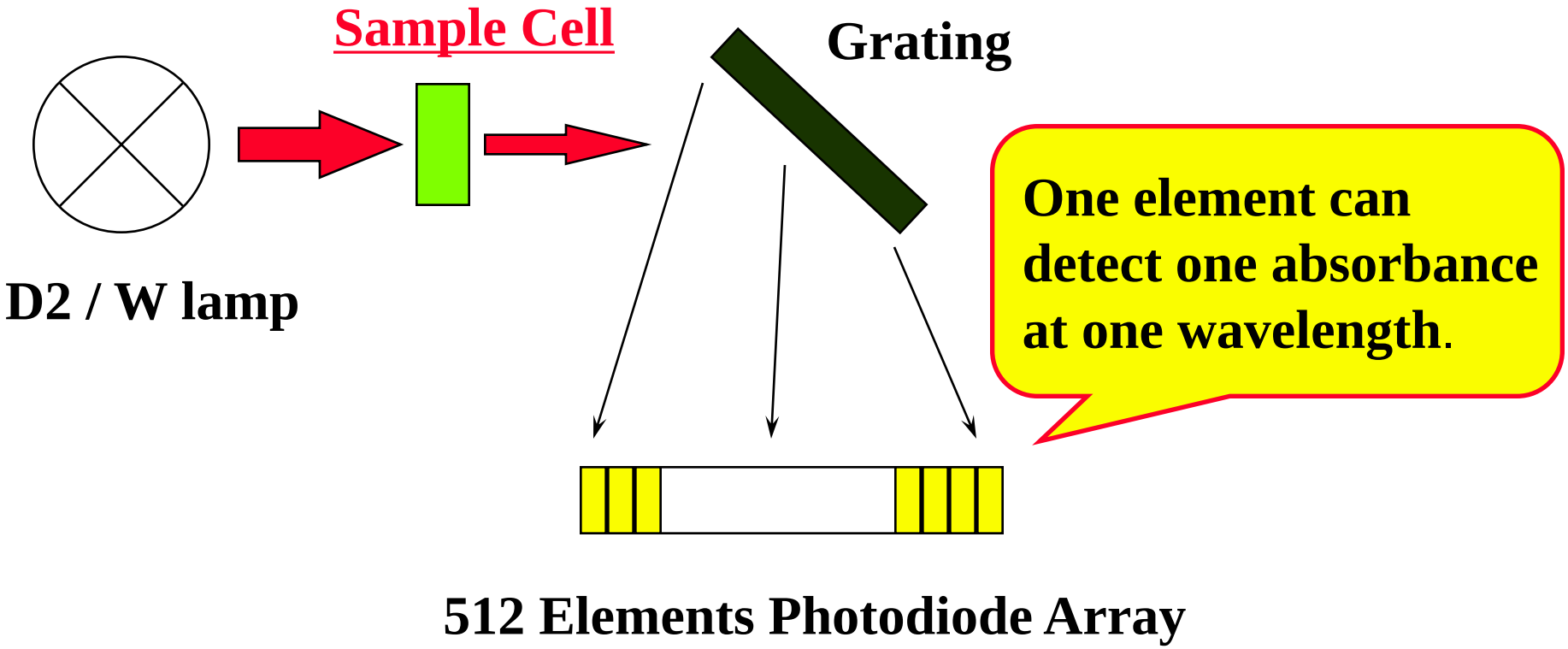
a = 268 f = 275
b = 285 g = 265
c = 227 h = 258
d = 267 i = 283
e = 366 j = 350 (nm)



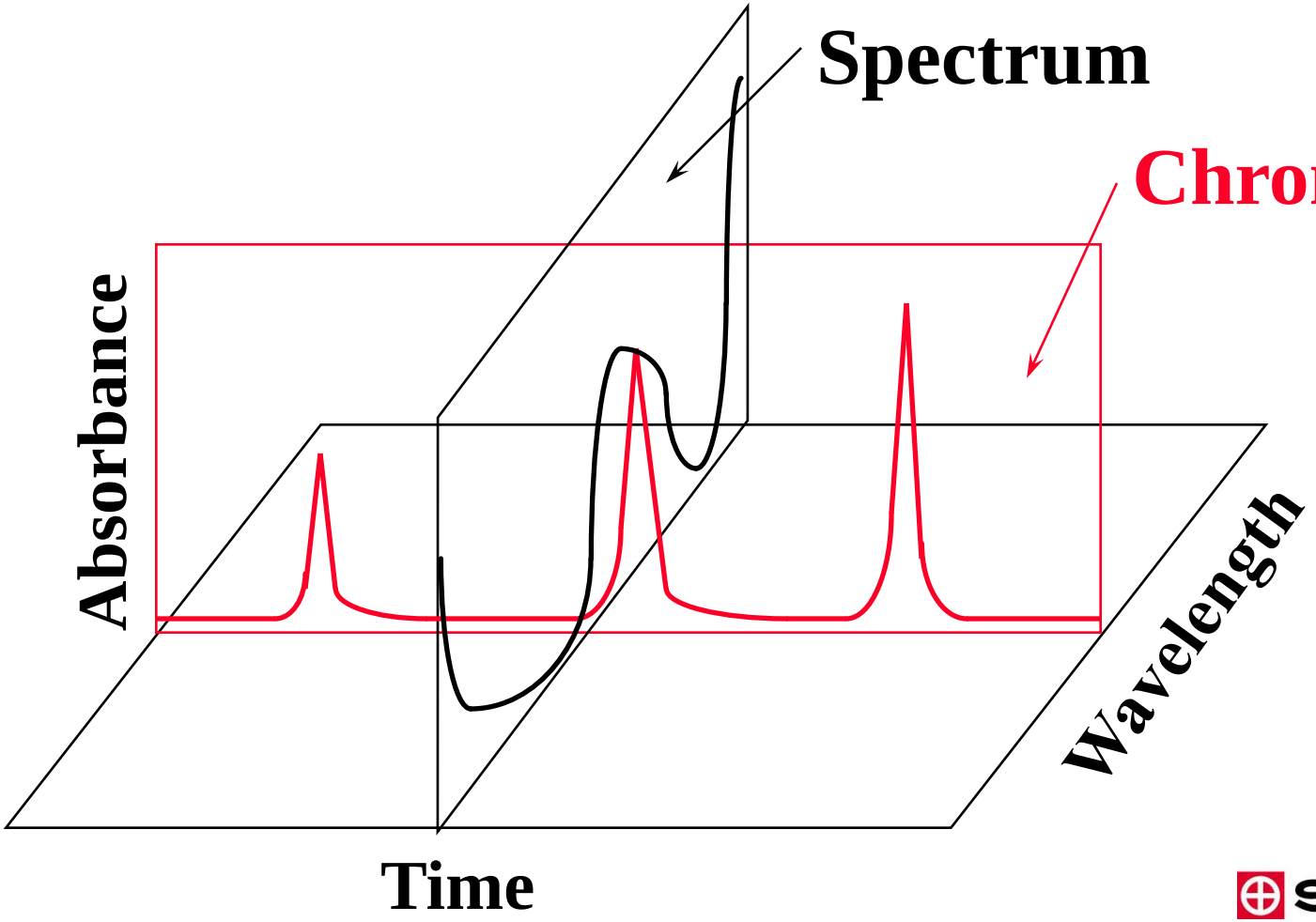
1. Olaquindox
2. Carbadox
3. Sulfamerazine
4. Thiamphenicol
5. Sulfadimidine
6. Furazolidone
7. Sulfamonomethoxine
8. Oxolinic Acid
9. Sulfadimethoxine
10. Sulfaquinoxaline
11. Nalidixic Acid
12. Piromidic Acid
13. Nicarbazin

Wavelength chromatogram analysis of antibacterial drugs

Photodiode Array detector



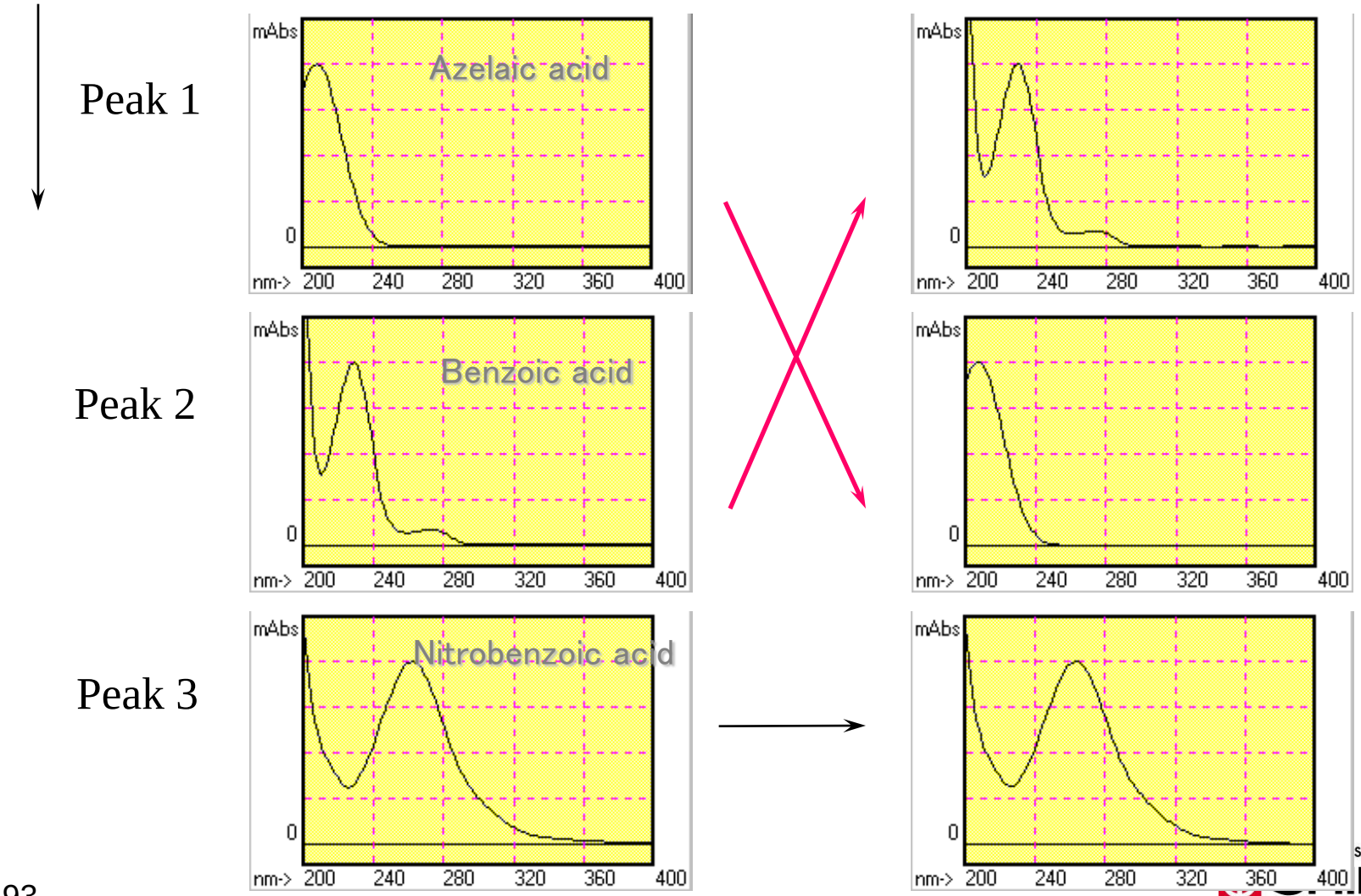
Photodiode Array detector



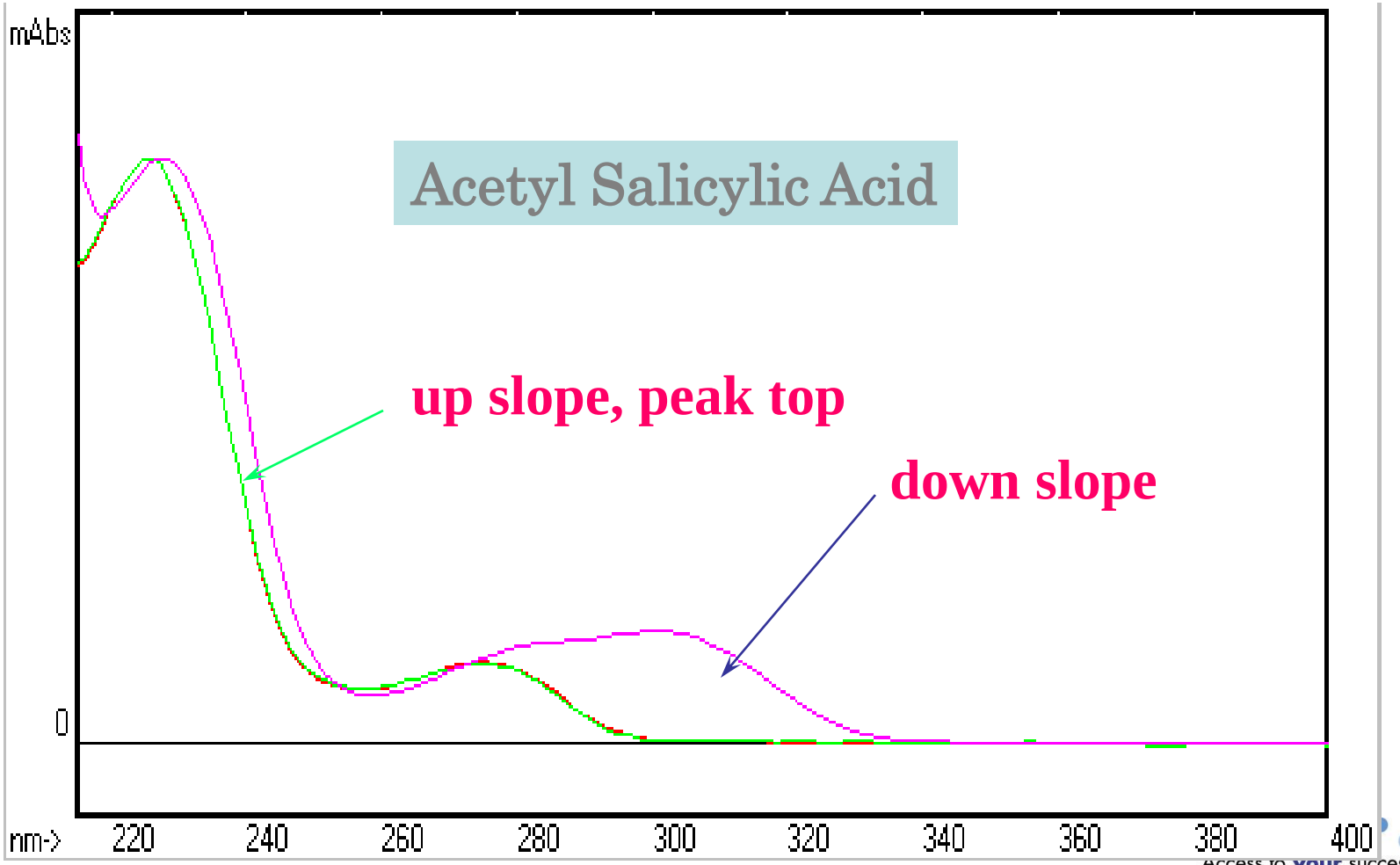
Photodiode Array detector

- **UV spectra of peaks are obtained, which are supportive for identification.**
- **By checking peak purity, one can know if any impurity is present.**

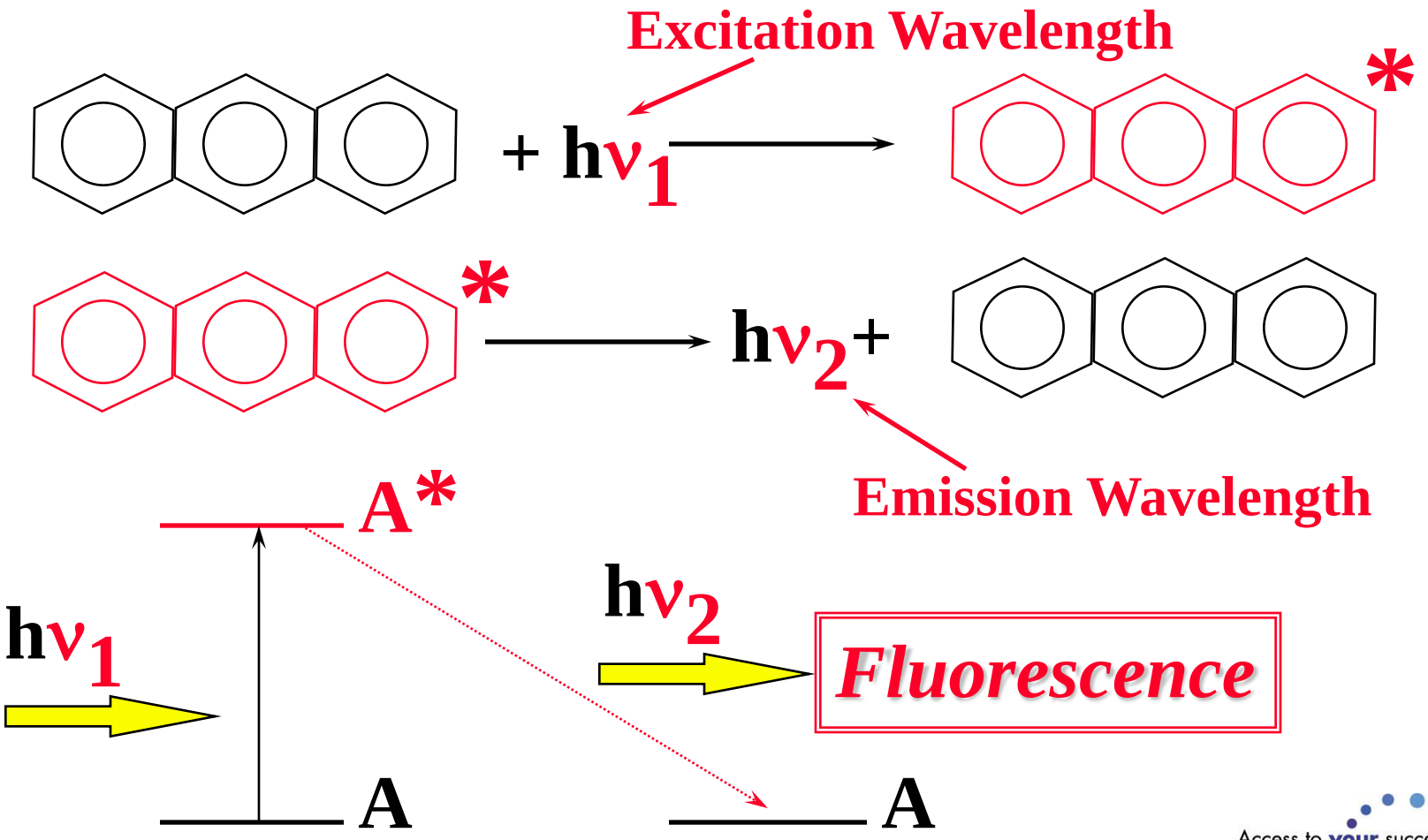
Identification by Spectrum



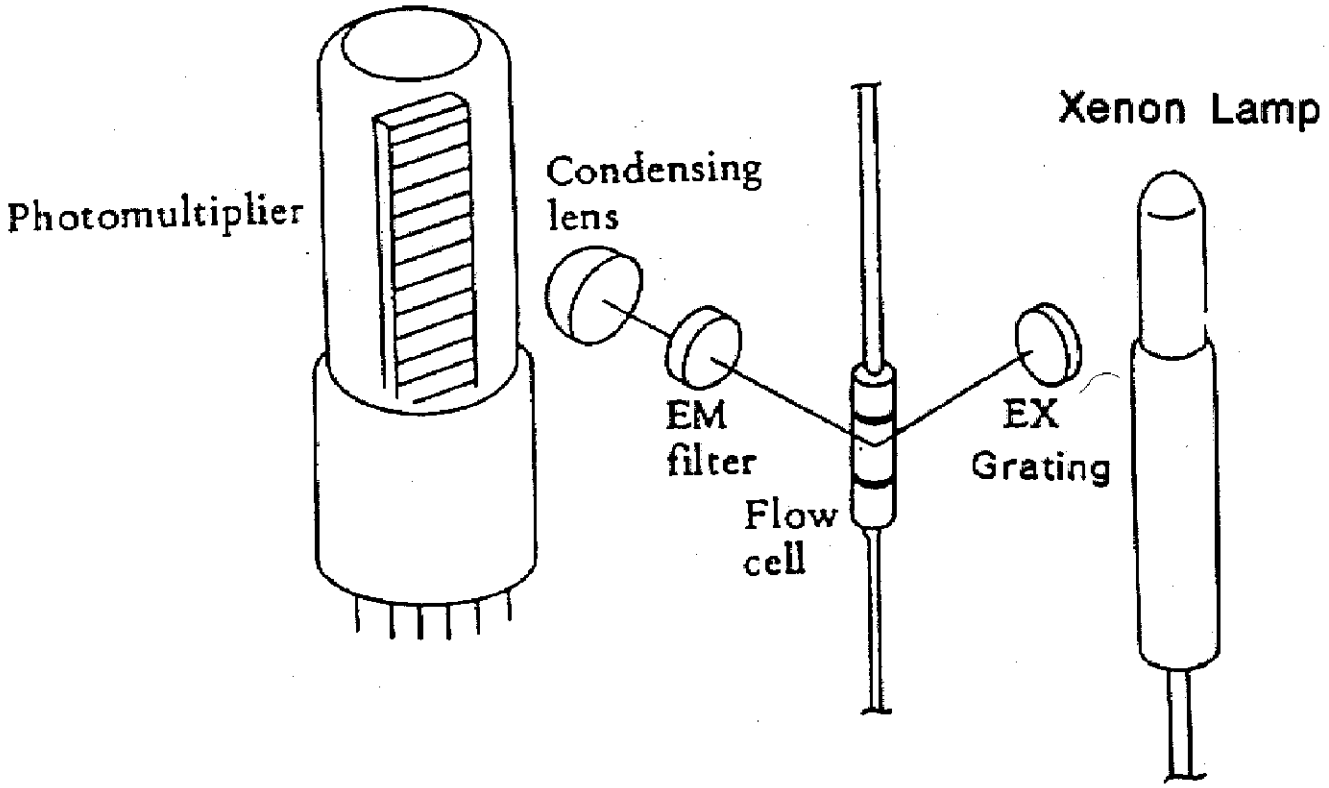
Comparison by 3 Point Spectrum



Fluorescence detector

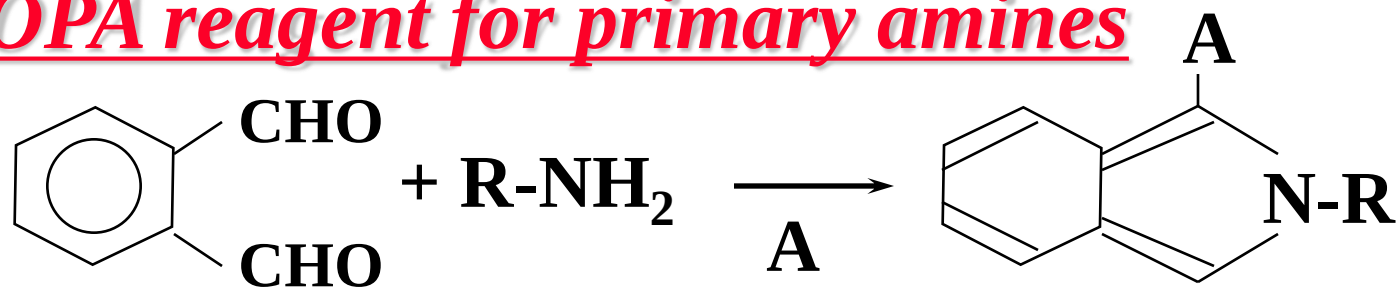


Cell design of Fluorescence detector



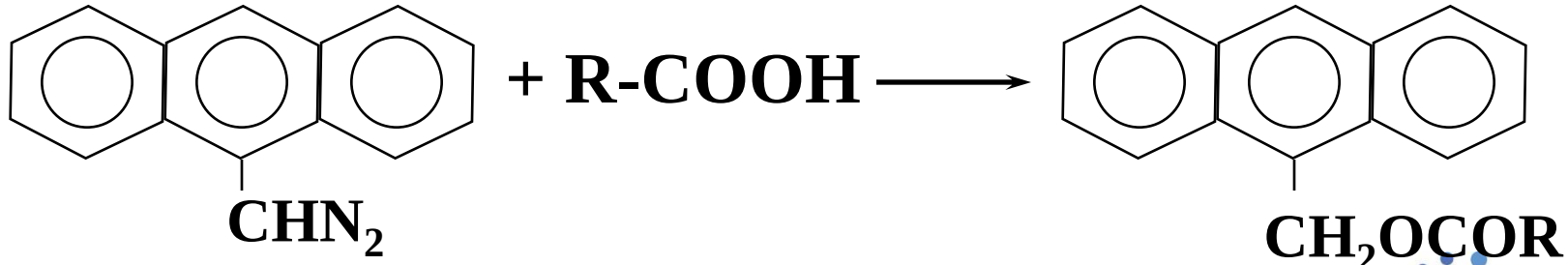
Derivatization Reagents

OPA reagent for primary amines



o-phthalaldhyde (OPA)

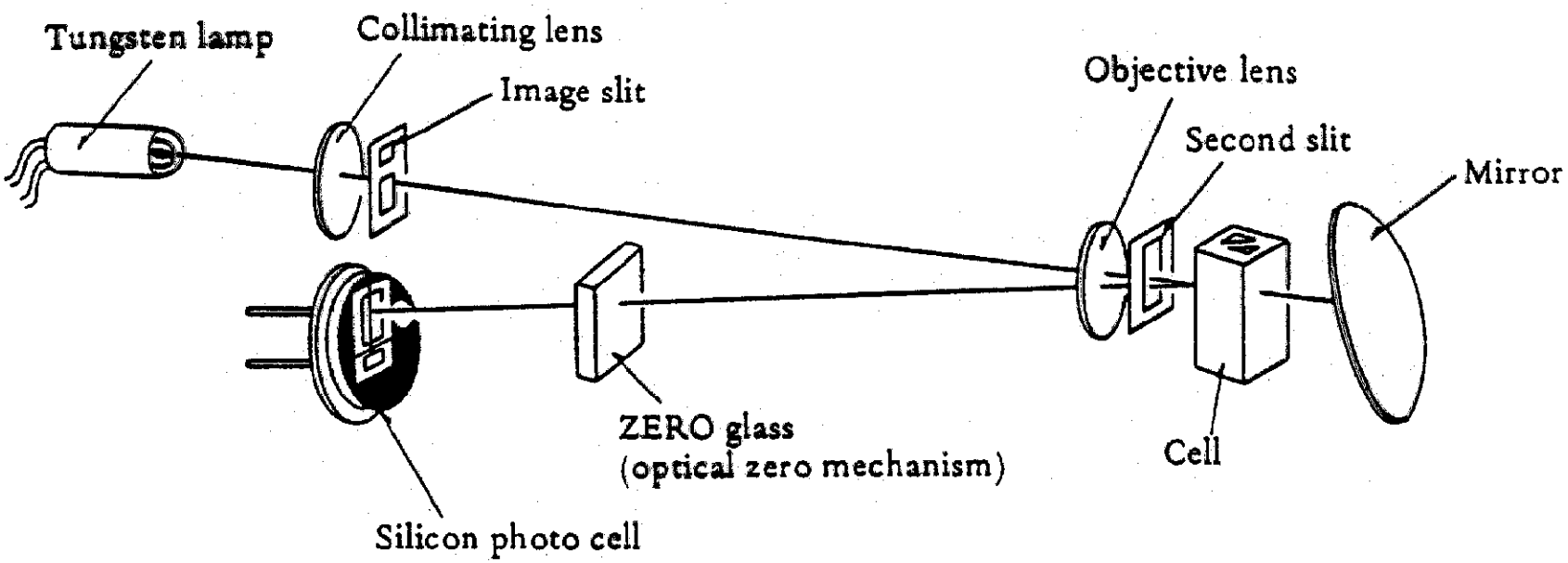
ADAM reagent for fatty acids



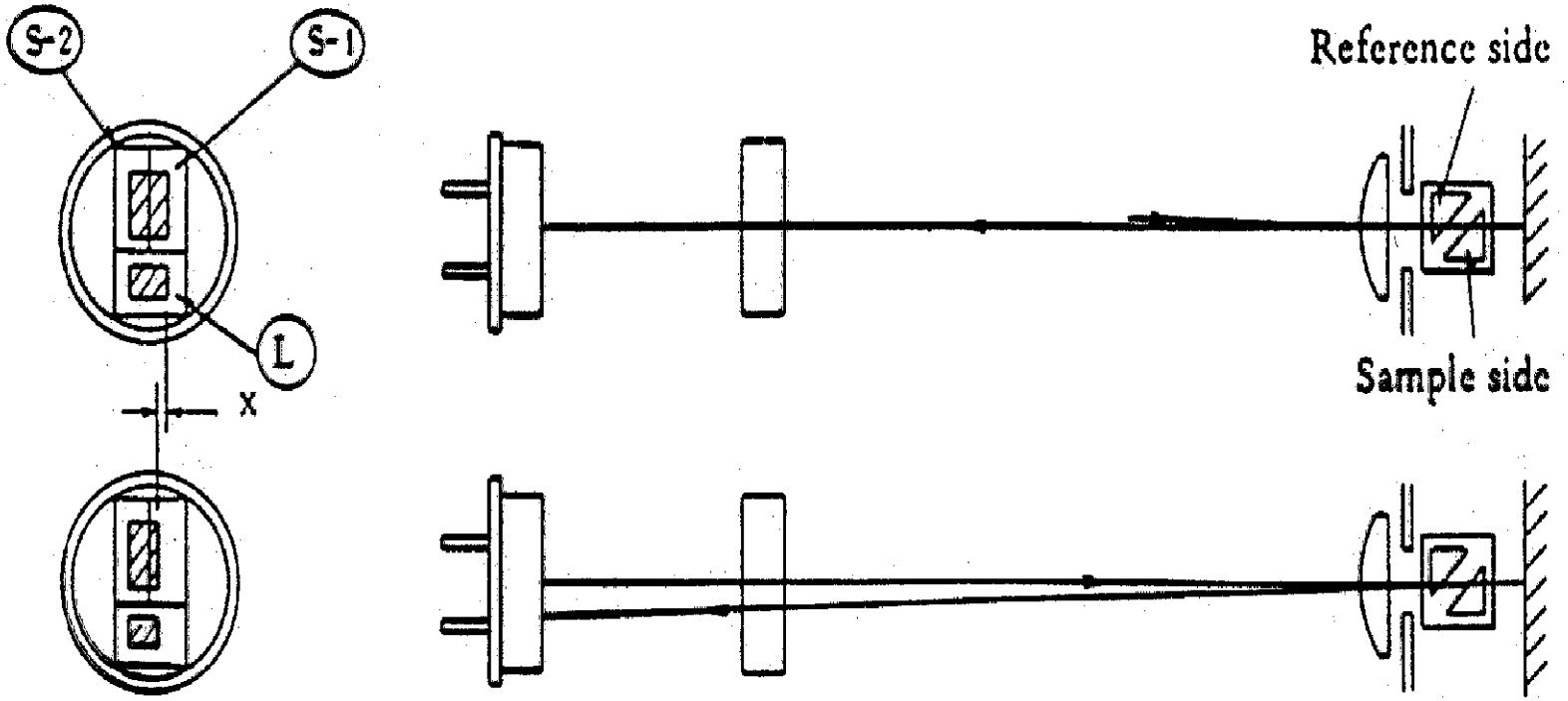
9-anthryldiazomethane (ADAM)

Refractive Index detector

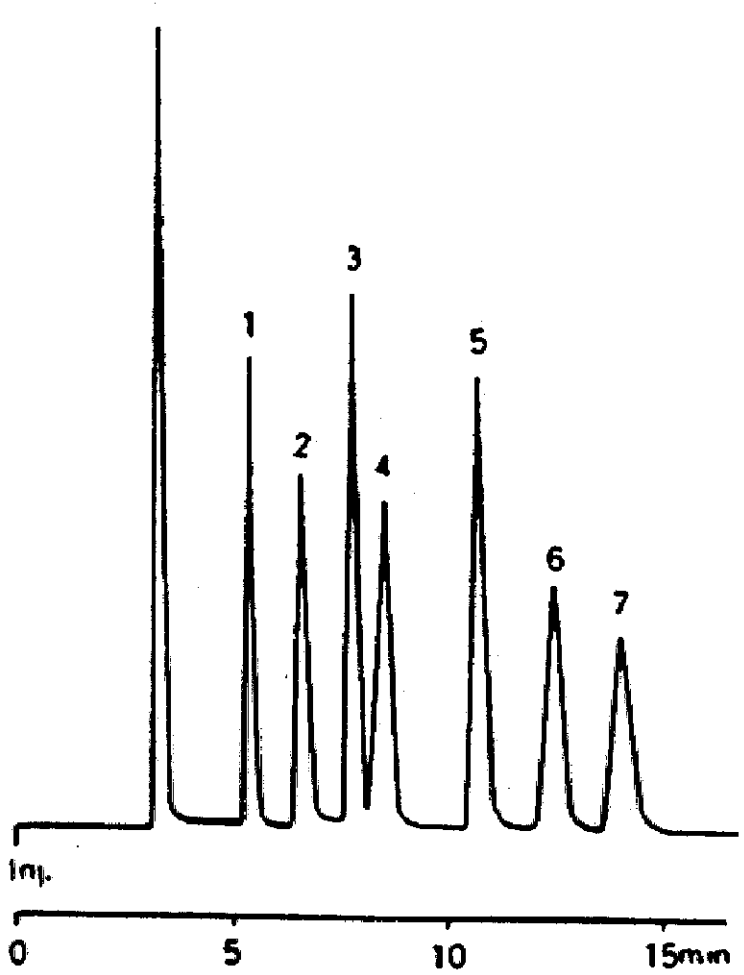
Optical System



Refractive Index detector

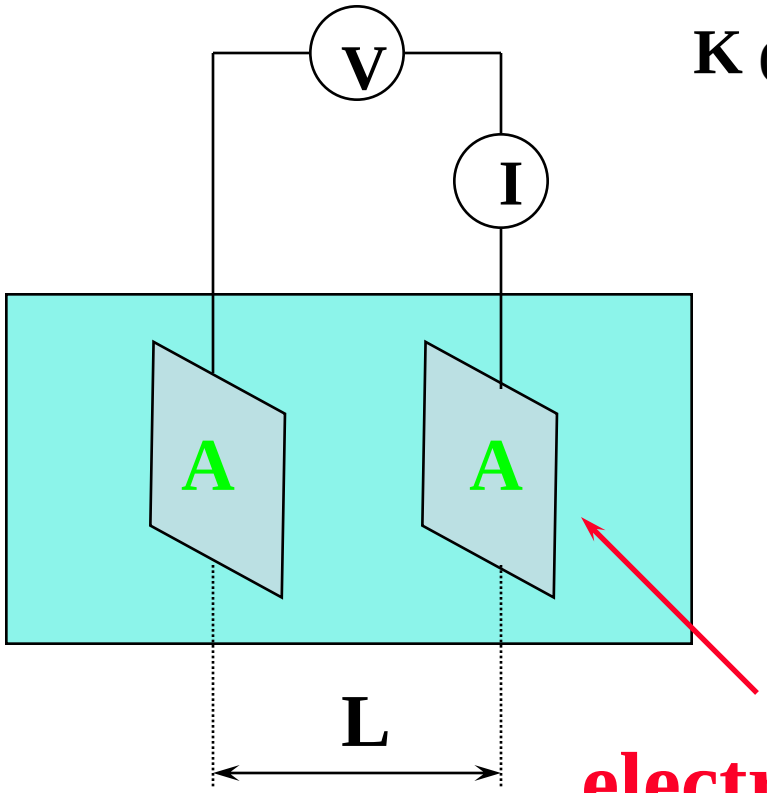


Chromatogram of sugars



- Analytical Conditions
  - –Column : Shim-pack CLC-NH2
  - –Mobile phase : Acetonitrile / water
    = 7 / 3
  - –Flow rate : 1.0 mL/min
  - –Temperature : Ambient
- Peaks
  1. Glycerol
  2. Xylose
  3. Fructose
  4. Glucose
  5. Sucrose
  6. Manose
  7. Lactose

Conductivity detector



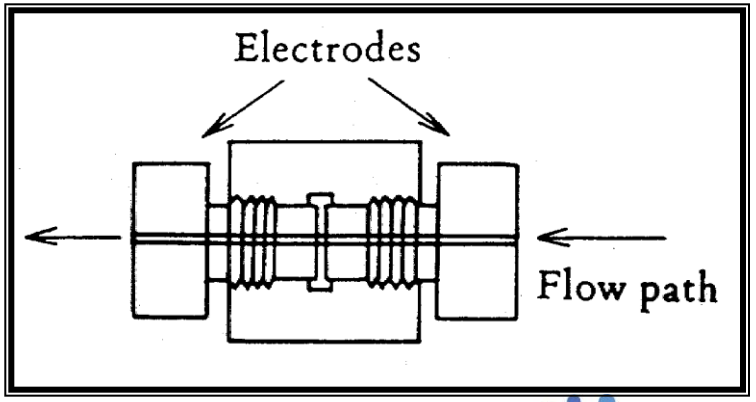
K (conductivity) = I [A] / E [V]

=A [cm^2] / L [cm] * k

(k : specific conductivity)

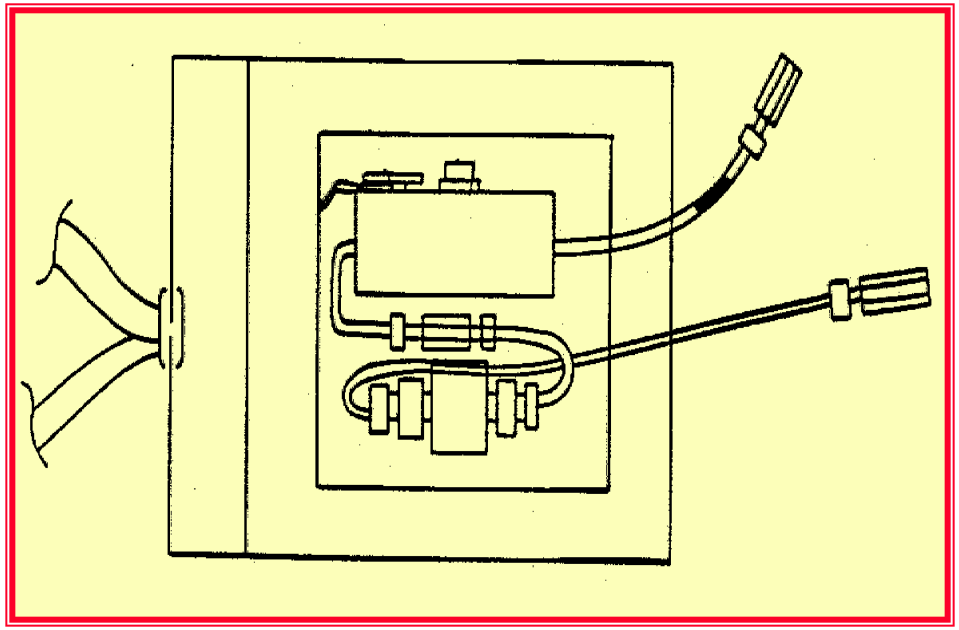
k= (I/E)*(L/A)

electrode



Conductivity detector

- Conductivity is very affected to temperature.
- Must keep the cell in the temperature control devise.

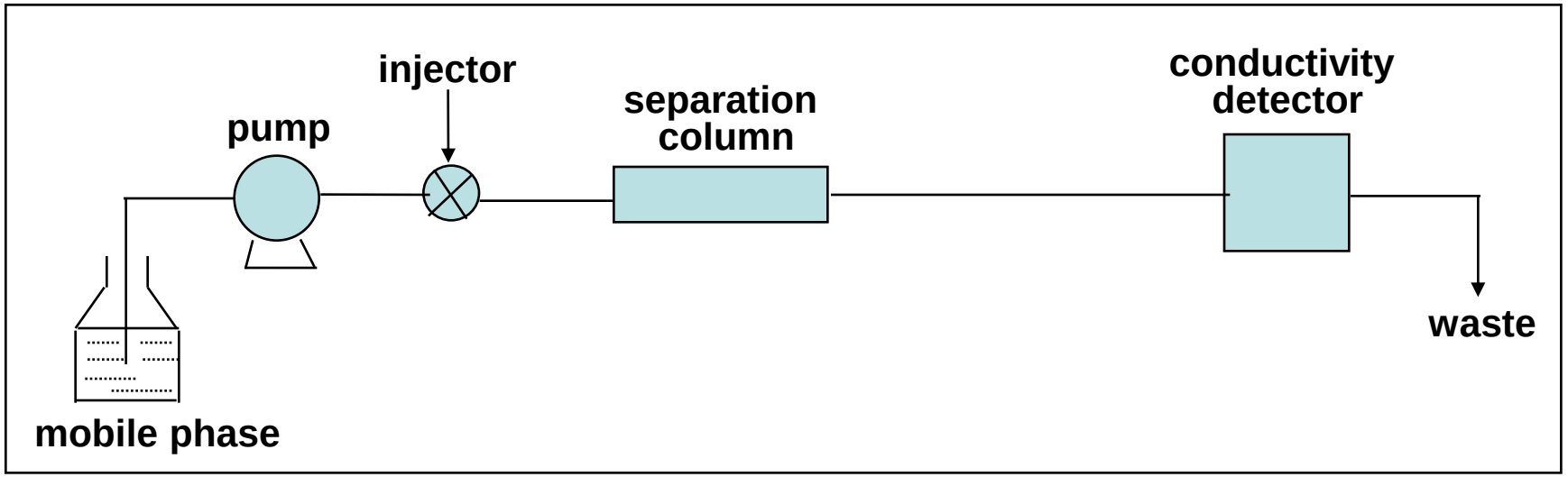


Type of Ion Chromatography

- Non-Suppresser type
  - –simple system (easy maintenance)
  - –low conductivity mobile phase
- Suppresser Type
  - –low back grand current
  - –difficult maintenance

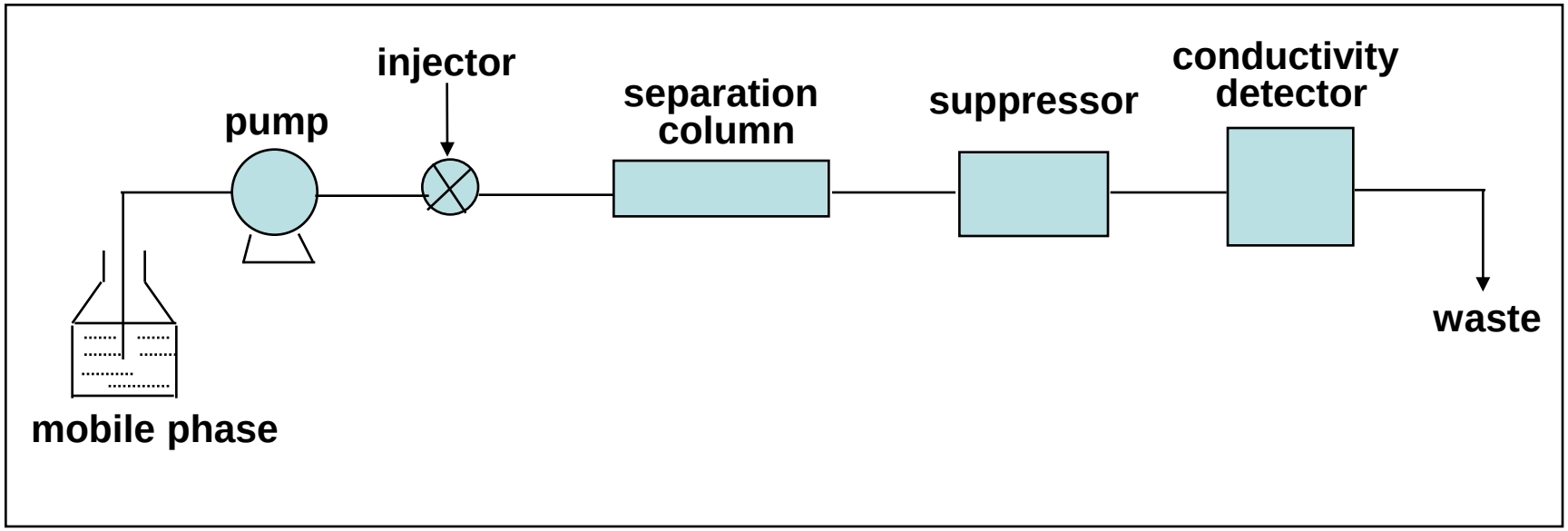
Non-Suppressor Type Ion Chromatography

Non-Suppressor Type Ion Chromatography

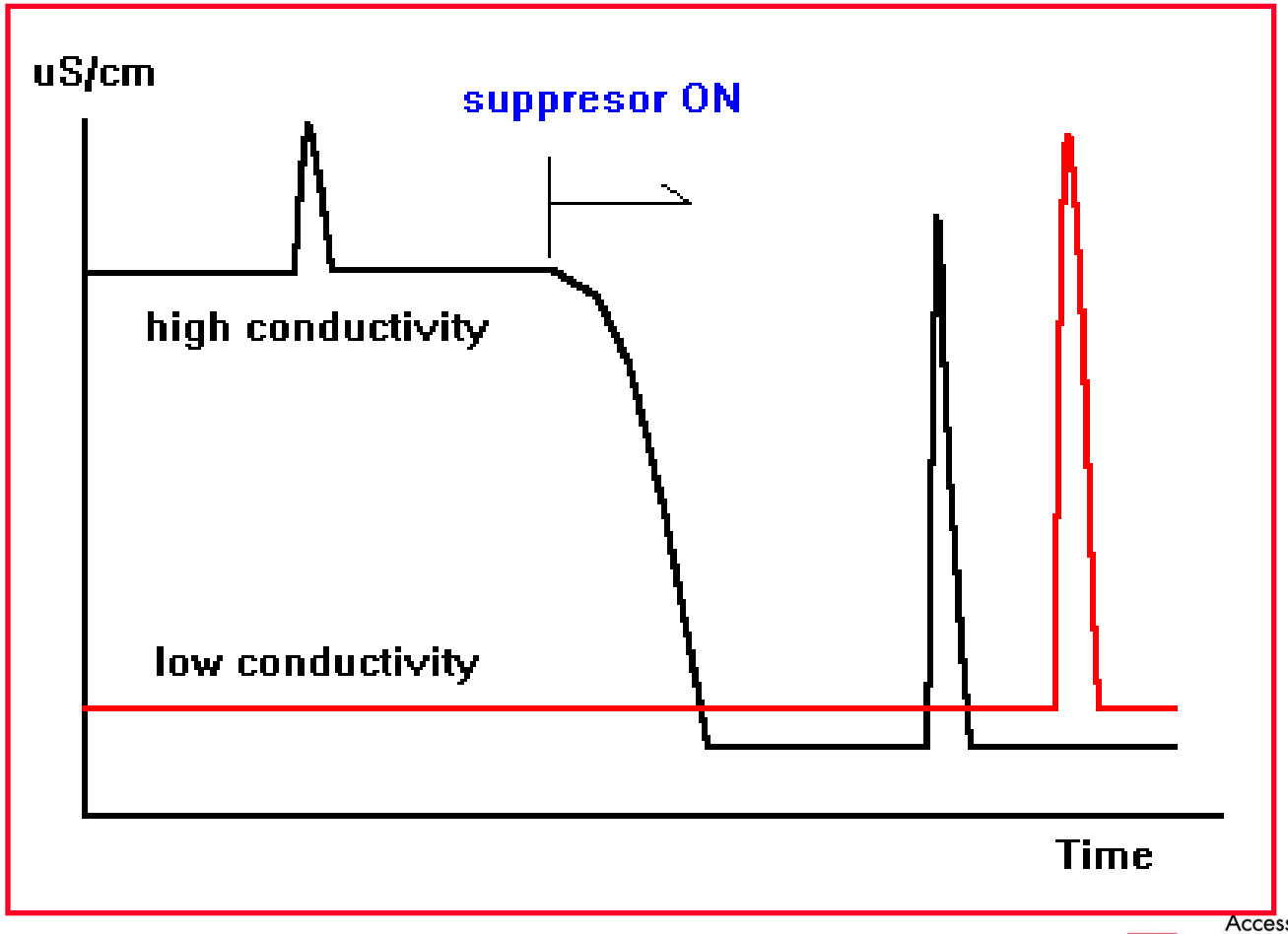


Suppressor Type Ion Chromatography

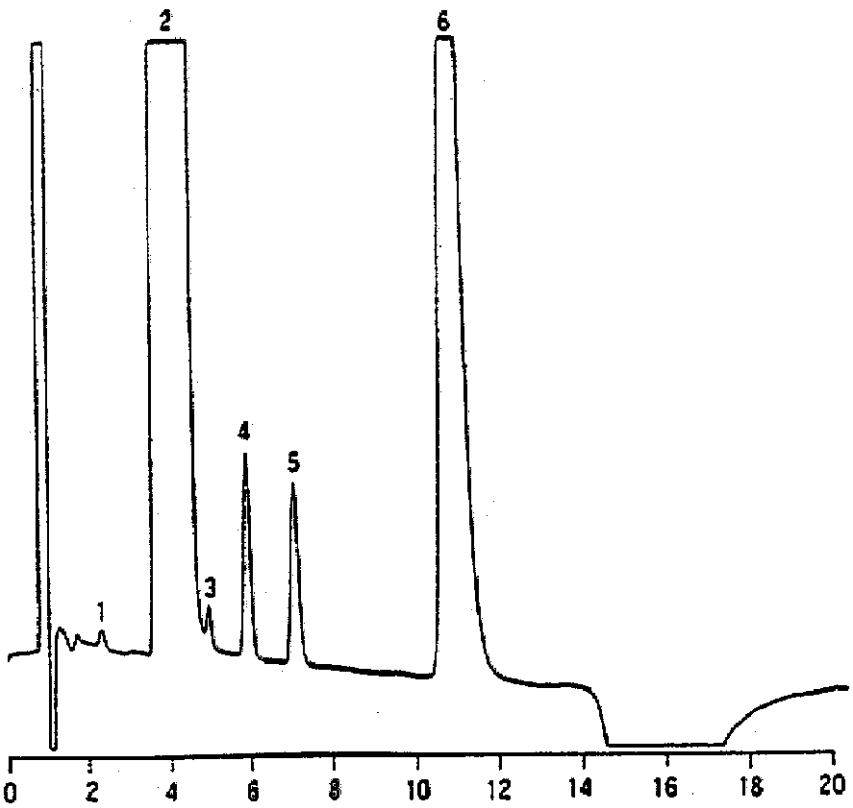
Suppressor Type Ion Chromatography



Conductivity and Peak Response



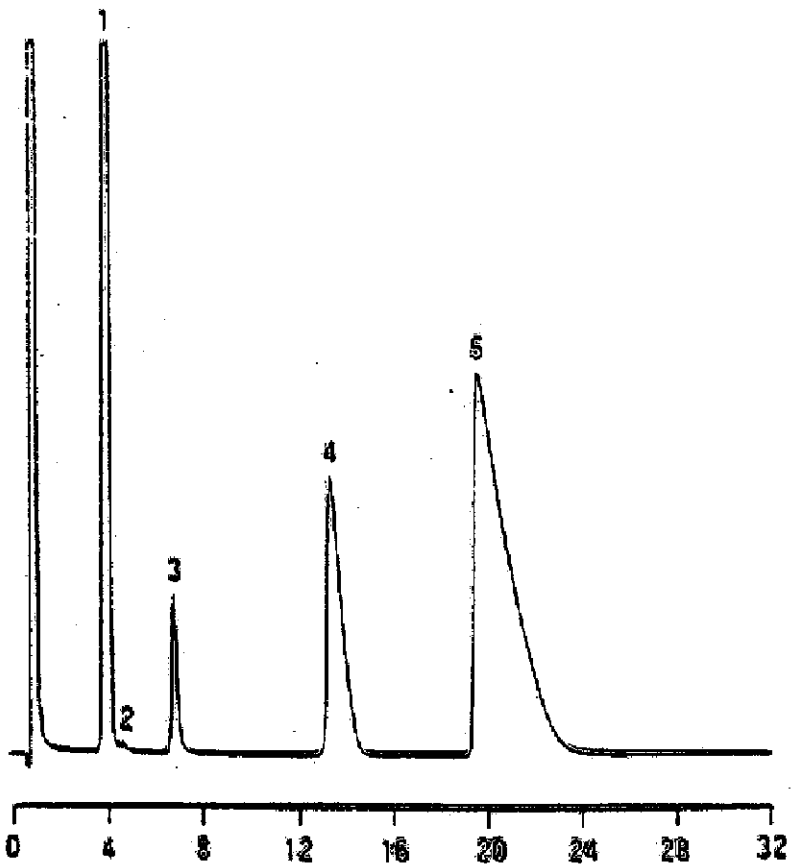
Chromatogram of Anion in the brine



- Analytical Conditions
  - –Column : Shim-pack IC-A3
  - –Mobile phase :
    - 8.0 mM p-hydroxybenzoic acid
    - 3.2 mM Bis-Tris *
  - –Flow rate : 1.5 mL/min
  - –Temperature : 40 C
  - –Injection Volume : 100 uL
- Peaks
  - –1. F (1.4 ppm)
  - –2. Cl (10200 ppm)
  - –3. NO2 (10 ppm)
  - –4. Br (43 ppm)
  - –5. NO3 (44 ppm)
  - –6. SO4 (431 ppm)

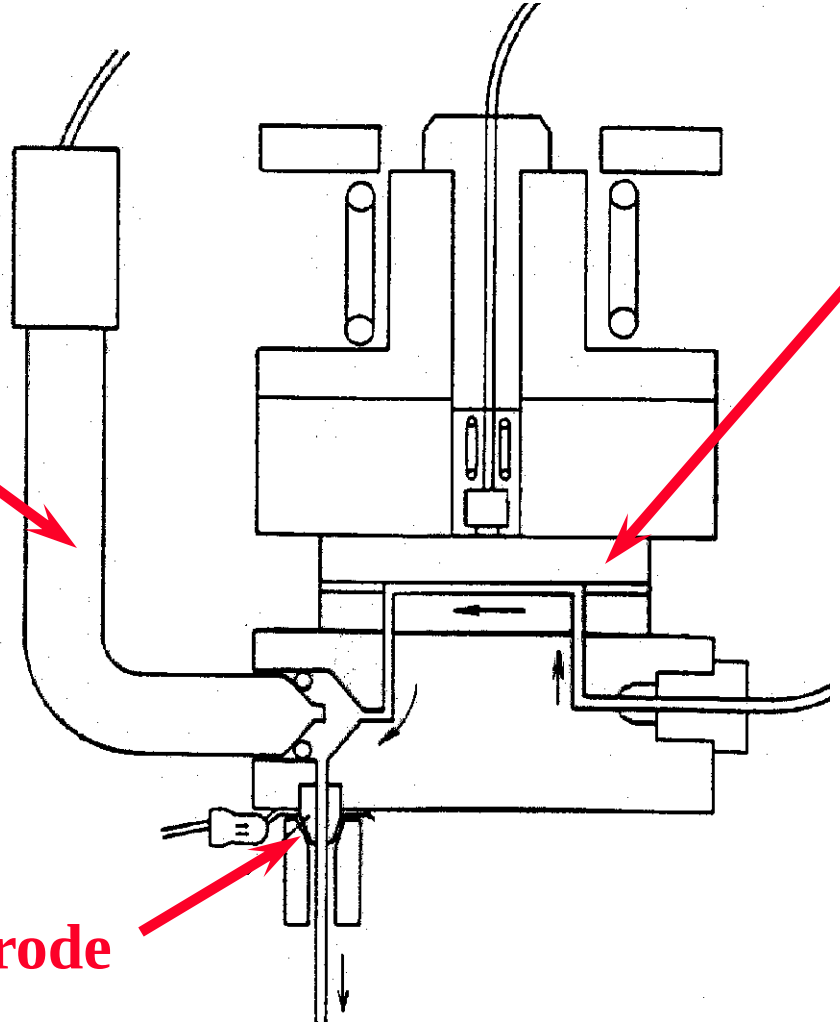
Bis-Tris : bis (2-hydroxyethyl) iminotris (hydroxymethyl) methane

Chromatogram of Cations in Tap water

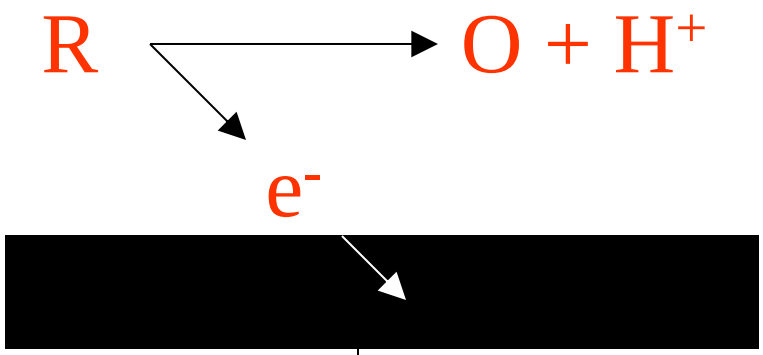


- **Analytical Conditions**
  - **Column : Shim-pack IC-C3**
  - **Mobile phase : 2.0 mM Oxalic Acid**
  - **Flow rate : 1.0 mL/min**
  - **Temperature : 40 C**
  - **Injection volume : 100 uL**
- **Peaks**
  - **1. Na (8.25 ppm)**
  - **2. NH4 (0.01 ppm)**
  - **3. K (1.66 ppm)**
  - **4. Mg (2.22 ppm)**
  - **5. Ca (11.85 ppm)**

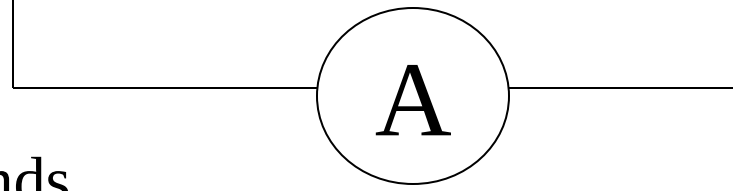
Electrochemical detector



Principal of ECD detection



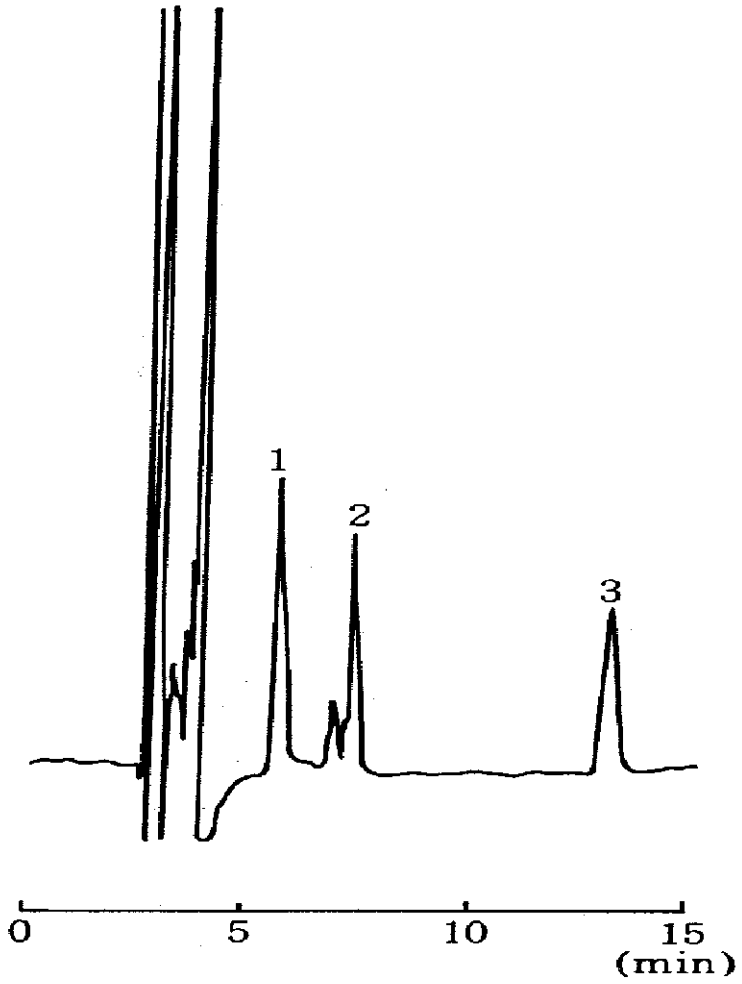
Electrode
Glassy Carbon (GC)
Pt, Ag, Au



[Applications]

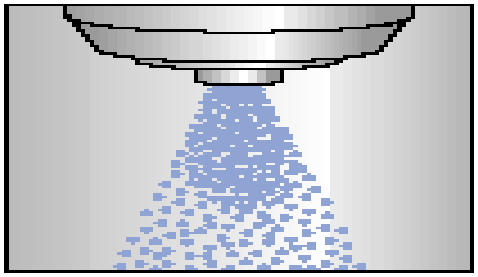
GC : phenol compounds
general use
Pt : H_2O_2
Ag : halogen ion
Au : sugar analysis

Chromatogram of Catecolamines

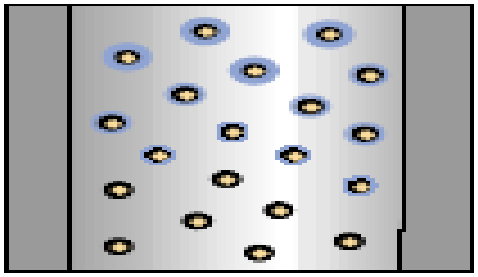


- **Analytical Conditions**
  - **Column : Shim-pack CLC-ODS**
  - **Mobile phase :**
    80 mM phosphate buffer (pH=2.7)
    100 mM NaNO3, 200 mg/l SOS
    5 mg/l EDTA, 4 % acetonitrile
  - **Flow rate : 1.0 mL/min**
  - **Applied Potential : + 0.8 V**
  - **Temperature : 40 C**
  - **Injection volume : 10 uL**
- **Peaks**
  - **1. Noradrenalin (5 ppb)**
  - **2. Adrenalin (5 ppb)**
  - **3. Dopamine (5 ppb)**

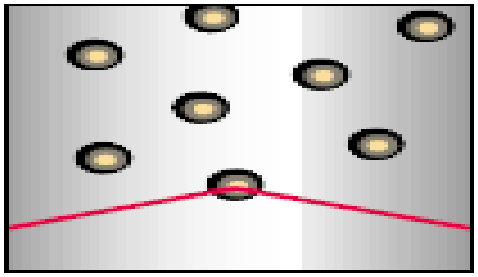
Principle of ELSD



1. **Nebulization:** Inside the nebulizer, the column effluent passes through a needle, mixes with nitrogen gas, and forms a dispersion of droplets.



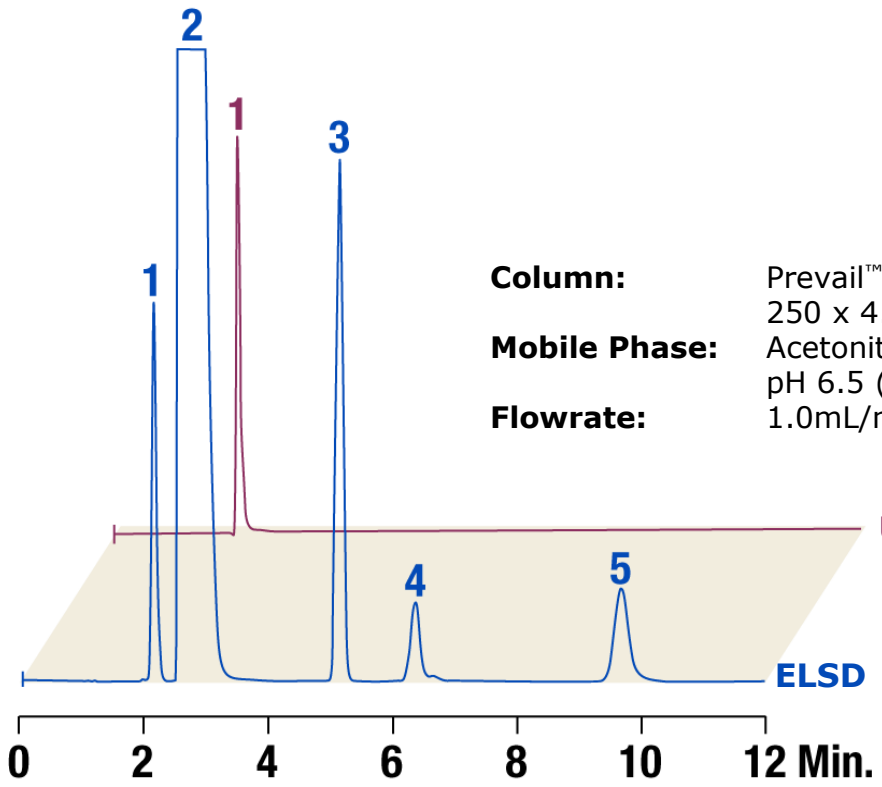
2. **Mobile Phase Evaporation:** The droplets pass through a heated "drift tube" where the mobile phase evaporates leaving a fine mist of dried sample particles in solvent vapor.



3. **Detection:** The sample particles pass through a flowcell where they are hit with a laser light beam. Light scattered by the sample particles is detected generating an electrical signal.

Example:

ELSD vs UV for an Antihistamine Formulation



1. Diphenhydramine
2. PEG, Gelatin
3. Sorbitan
4. Glycerol
5. Sorbitol

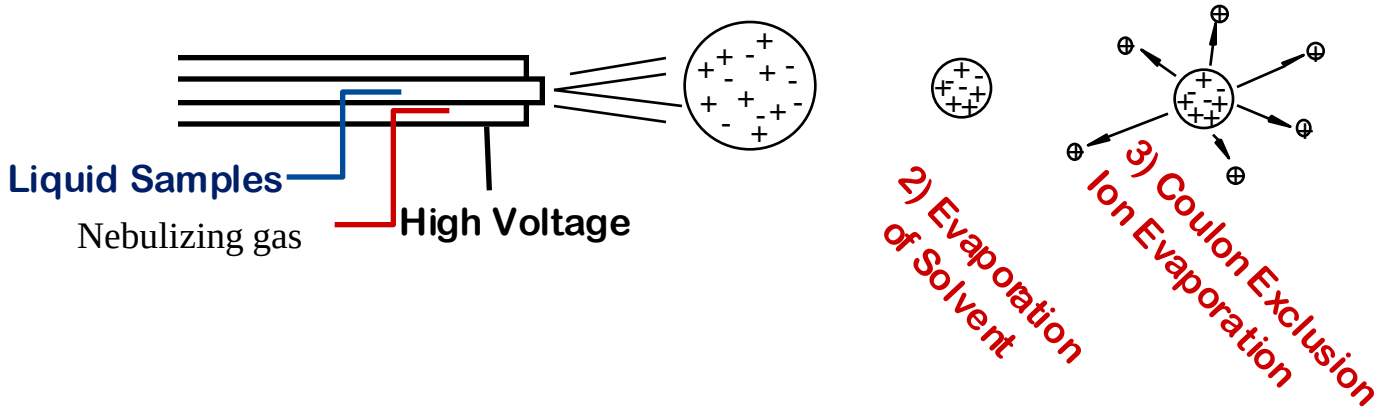
Column: Prevail™ Carbohydrate ES, 5µm, 250 x 4.6mm

Mobile Phase: Acetonitrile:0.1% Ammonium Carbonate, pH 6.5 (80:20)

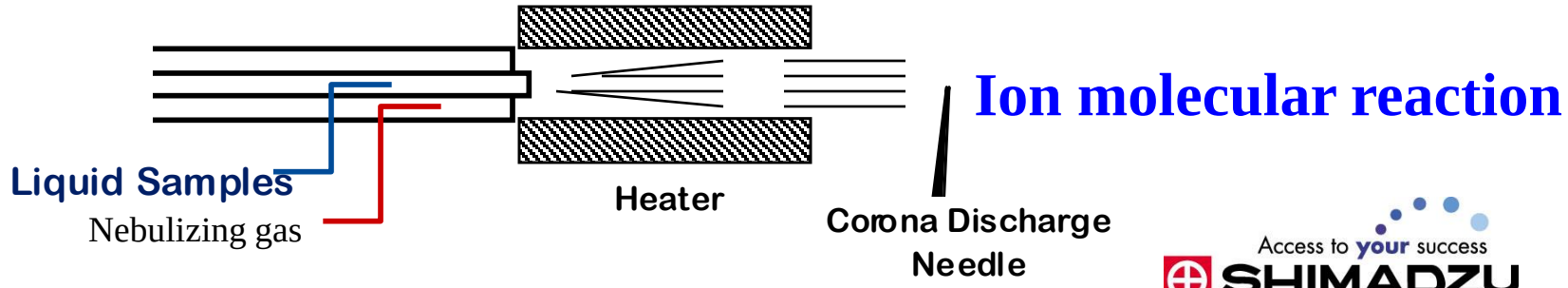
Flowrate: 1.0mL/min

LC/MS/MS
Atmospheric Pressure Ionization

Pneumatically Assisted ElectroSpray(ESI)



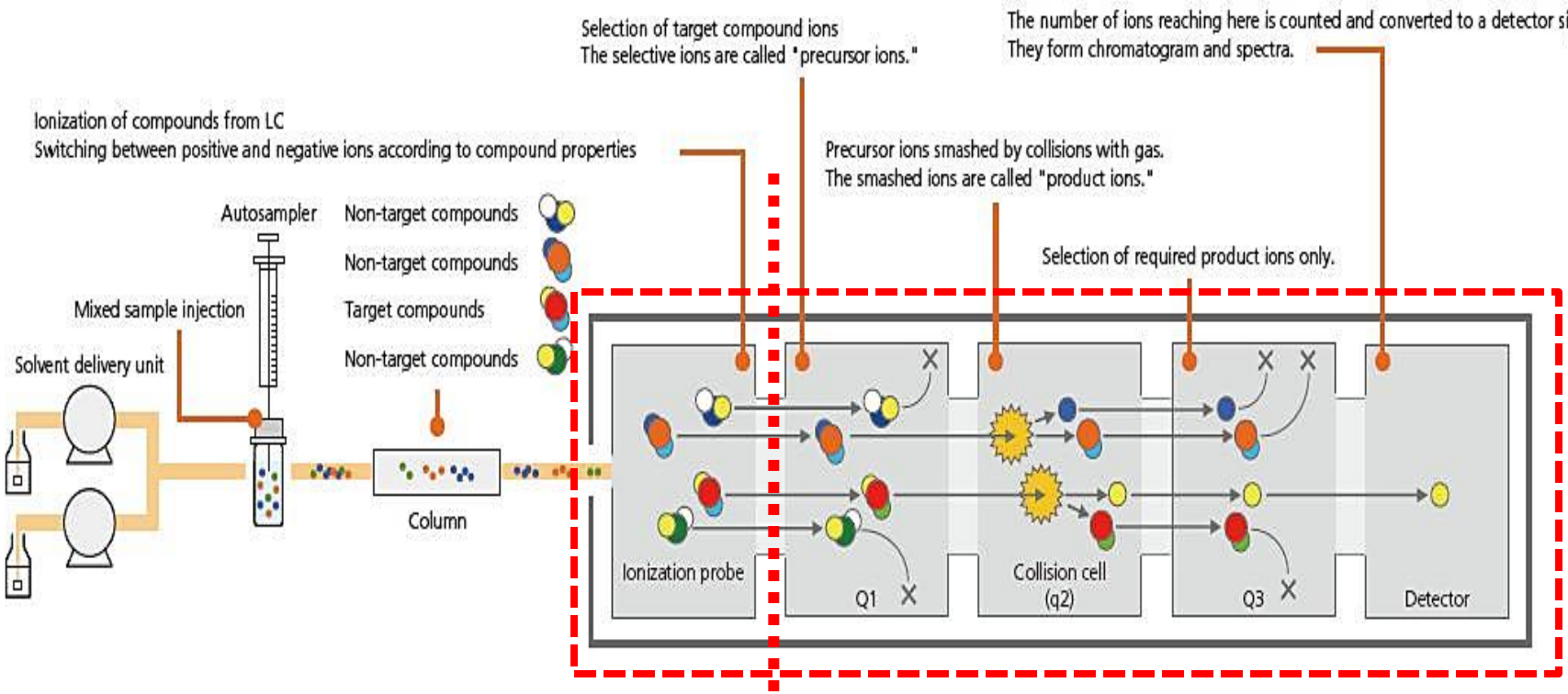
Atmospheric Pressure Chemical Ionization(APCI)



What kind of compounds can be analyzed ?

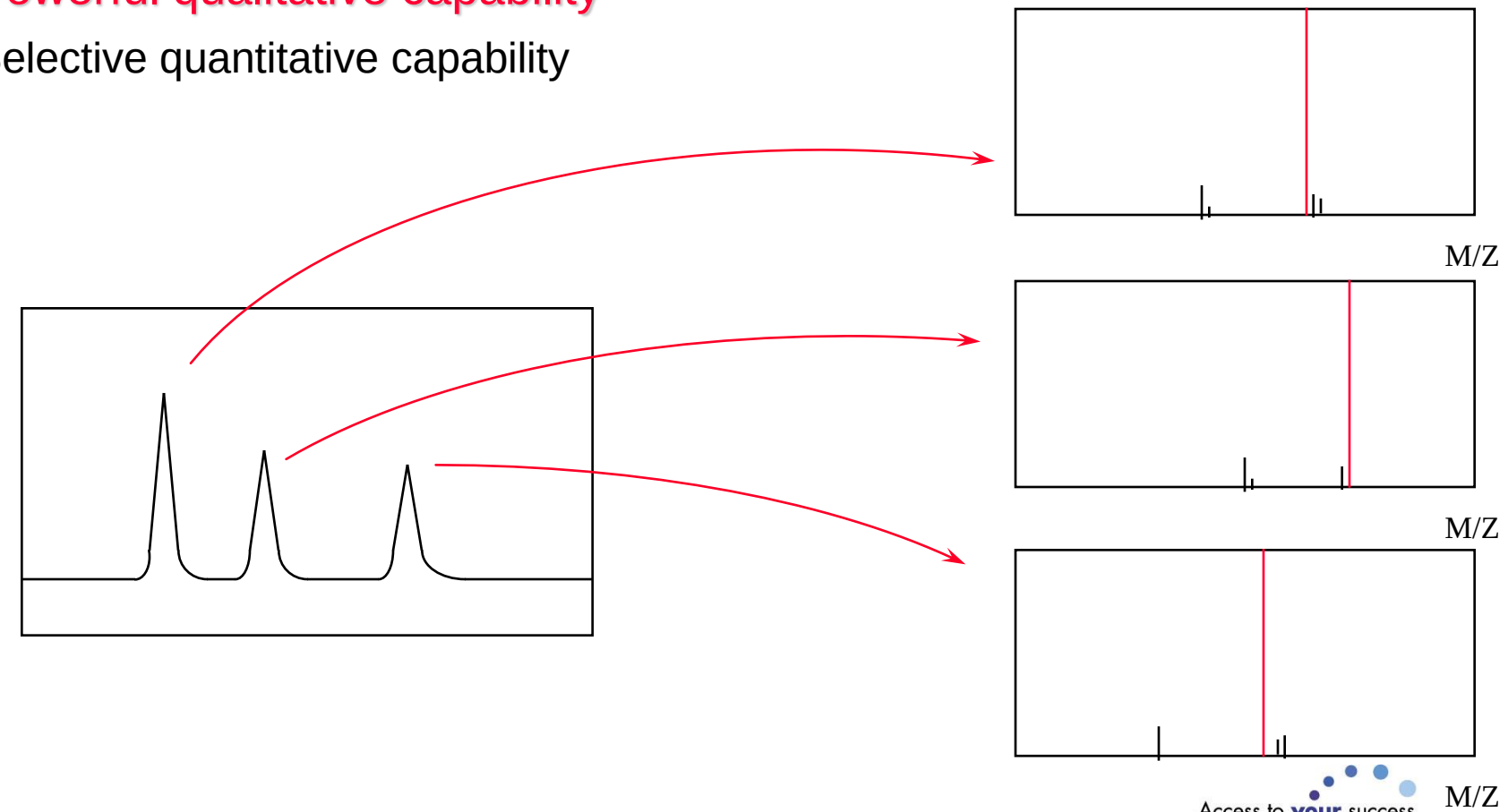
- **ESI**
  - –drugs and their metabolites
  - –peptides
  - –proteins
  - –many kinds of natural product
    (-OH, -NH2,-COOH, SO2, PO3 etc.)
- **APCI**
  - –pesticides
  - –steroids
  - –drugs

Design of LC/MS/MS



What kind of benefits LC/MS users can get ?

- Powerful qualitative capability
- Selective quantitative capability



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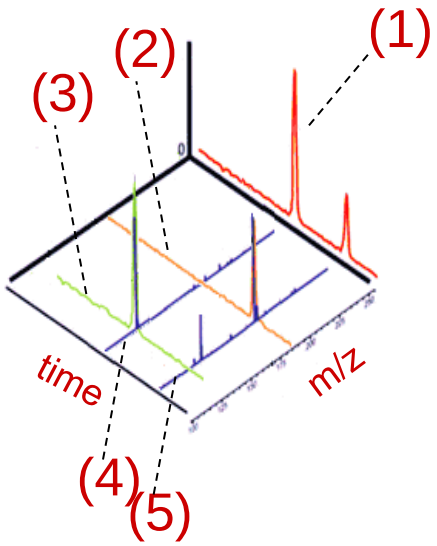
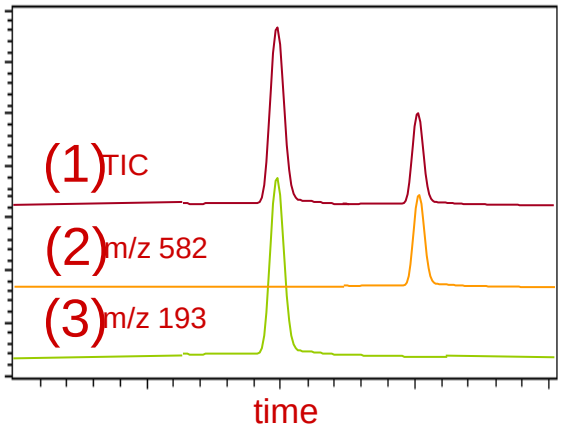
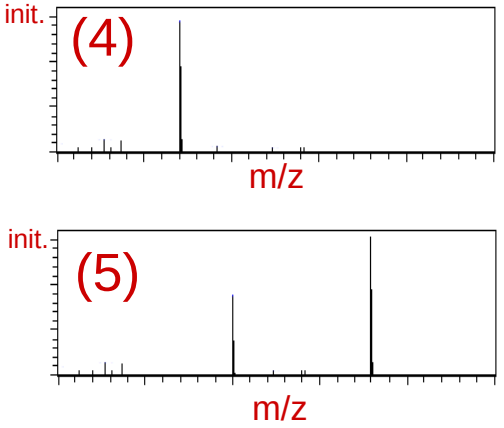




MS spectra



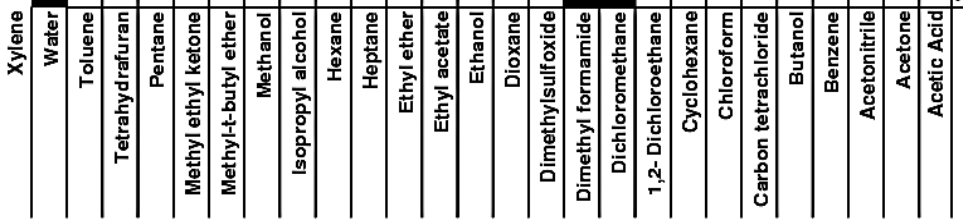
Comparison of each detector

	UV/ VIS	RF	RID	CDD	ECD	MS
LOD	1 ppb	10 ppt	100 ppb	1 ppb	10 ppt	1 ppb
GC	Yes	Yes	No	No	No	Yes

LOD : Limit of detection (S/N=3)
GC : Gradient Compatibility

PDA sensitivity is slightly less sensitive than normal UV detector.

	Polarity Index[1]	Viscosity (cP)	UV (nm) Cutoff[2]	Solubility in Water (%)
Acetic Acid	6.2	1.26	230	100
Acetone	5.1	0.32	330	100
Acetonitrile	5.8	0.37	190	100
Benzene	2.7	0.65	280	0.18
Butanol	4.0	0.73	254	0.43
Carbon tetrachloride	1.6	0.97	263	0.08
Chloroform	4.1	0.57	245	0.815
Cyclohexane	0.2	1.00	200	0.01
1,2- Dichloroethane	3.5	0.79	225	0.81
Dichloromethane	3.1	0.44	235	1.6
Dimethyl formamide	6.4	0.92	268	100
Dimethylsulfoxide	7.2	2.00	268	100
Dioxane	4.8	1.54	215	100
Ethanol	5.2	1.20	210	100
Ethyl acetate	4.4	0.45	260	8.7
Ethyl ether	2.8	0.32	220	6.89
Heptane	0.0	0.39	200	0.0003
Hexane	0.0	0.33	200	0.001
Isopropyl alcohol	3.9	2.30	210	100
Methanol	5.1	0.60	205	100
Methyl-t-butyl ether	2.5	0.27	210	4.8
Methyl ethyl ketone	4.7	0.45	329	24
Pentane	0.0	0.23	200	0.0004
Tetrahydrafuran	4.0	0.55	215	100
Toluene	2.4	0.59	285	0.051
Water	9.0	1.00	200	100
Xylene	2.5	0.61	290	0.018



[1] The polarity index is a measure of the relative polarity of a solvent and is useful for identifying suitable mobile phase solvents. The polarity index increases with polarity. For reverse phase chromatography eluent strength decreases as its polarity increases

[2] UV cutoff, the wavelength at which the solvent absorbance in a 1 cm path length cell is equal to 1 AU (absorbance unit) using water in the reference cell.

Solvent Miscibility and Viscosity Chart adapted from Paul Sadek The HPLC Solvent Guide Wiley-Interscience, 2002.

Mobile phases, stationary phase, analyte and samples must be compatible

Solvent Polarity Chart

Relative Polarity	Formula	Group	Solvents
Non-polar	R-H	Alkanes	Petroleum ethers, hexanes, ligroin
	Ar-H	Aromatics	Toluene
	R-O-R	Ethers	Diethyl ether
	R-X	Alkyl halides	Tricholoromethane, chloroform
	R-COOR	Esters	Ethyl acetate
	R-CO-R	Aldehydes and ketones	Acetone, MEK
	R-NH2	Amines	Pyridine, triethylamine
	R-OH	Alcohols	MeOH, EtOH, IPA, Butanol
	R-COHN2	Amides	Dimethyformamide
	R-COOH	Carboxylic Acid	Ethanoic Acid
Polar	H-O-H	Water	