1. Introduction

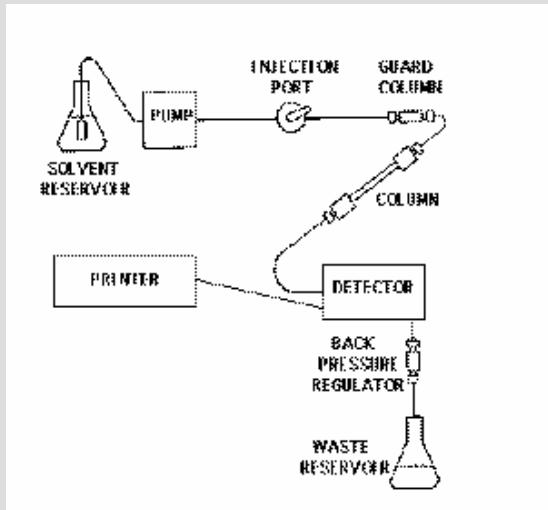
High Performance Liquid Chromatography (HPLC) is one mode of chromatography, the most widely used analytical technique. Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases.

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and has the ability to easily separate a wide variety of

chemical mixtures.

History of HPLC

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During the 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and did not achive sufficiently high resolution to distinguish between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flowthrough time, thus reducing purification times of compounds being isolated by column chromatogaphy. However, flow rates were inconsistent, and the question of whether it was better to have constant flow rate or constant pressure was debated. (Analytical Chem. vol 62, no. 19, Oct 1, 1990).



High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far above those obtained using previous techniques. Computers and automation added to the convenience of HPLC. Additional column types giving better reproducibility were introduced and such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

The past decade has seen a vast undertaking in the development of micro-columns, and other specialized columns. The dimensions of the typical HPLC column are: XXX mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 µm to 200 µm. Fast HPLC utilizes a column that is shorter than the typical column. A Fast HPLC column is about 3 mm long and is packed with smaller particles.

Currently, one has the option of selecting from a lot of columns for the separation of compounds, as well as a variety of detectors to interface with the HPLC in order to obtain optimal analysis of the compound.

Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry,in actual fact these fields currently comprise only about 50% of HPLC users(Analytical Chem. vol 62, no.19, Oct 1, 1990). Currently HPLC is used in a variety of fields and industries including the cosmetics, energy, food, and environmental industries.

What is HPLC ?

H : High
P : Performance (Pressure)
L : Liquid
C : Chromatography

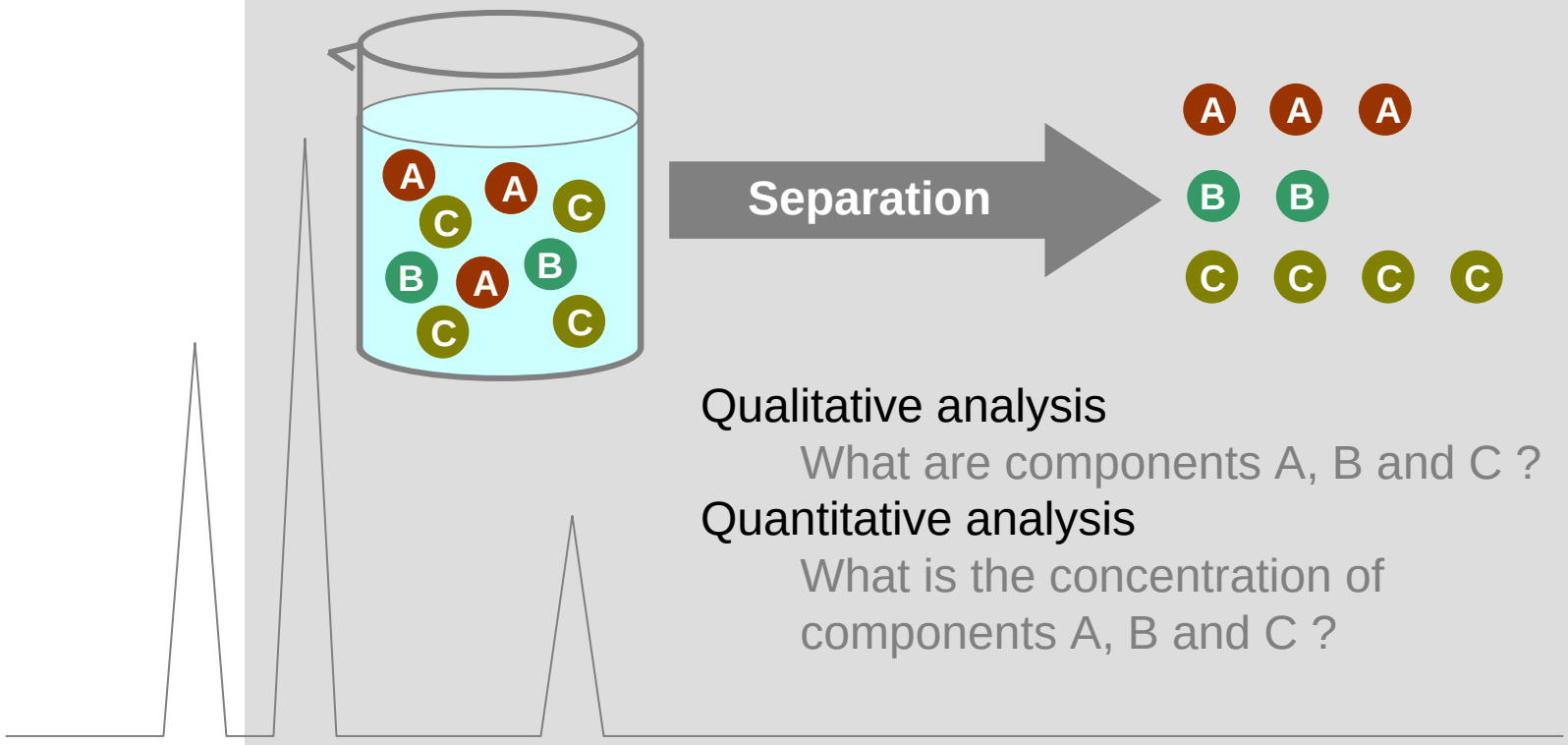
GC : Gas chromatography
TLC : Thin layer chromatography
IC : Ion chromatography

What is HPLC used for ?

1. Separation of mixed components
2. Qualitative analysis / Quantitative analysis
3. Preparation of interest components

Separation analysis and/or preparation of interest components

Separation and Analysis



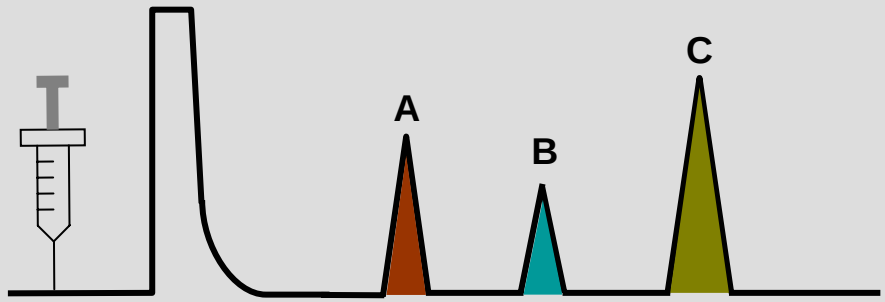
Qualitative analysis

What are components A, B and C ?

Quantitative analysis

What is the concentration of components A, B and C ?

Results obtained by HPLC

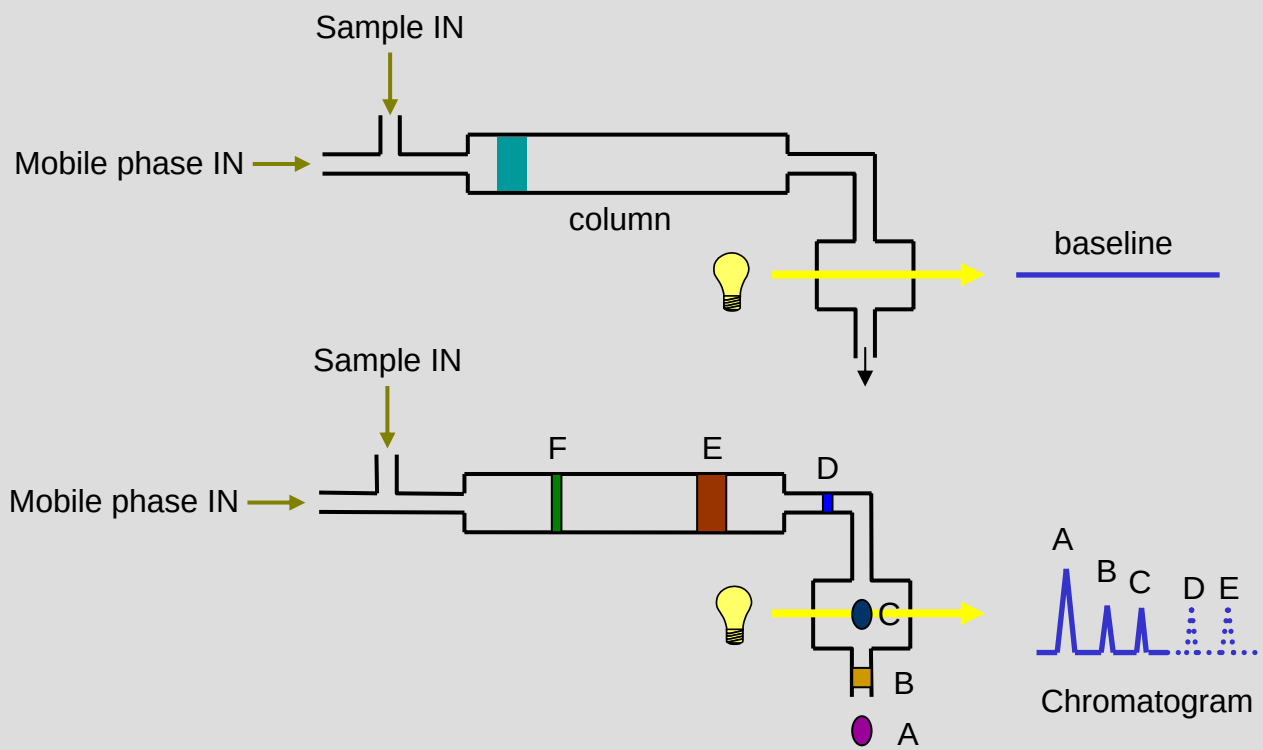


Chromatogram containing three peaks

Qualitative analysis (identification) and
Quantitative analysis (determination)
Can be performed using the information contained in the chromatogram

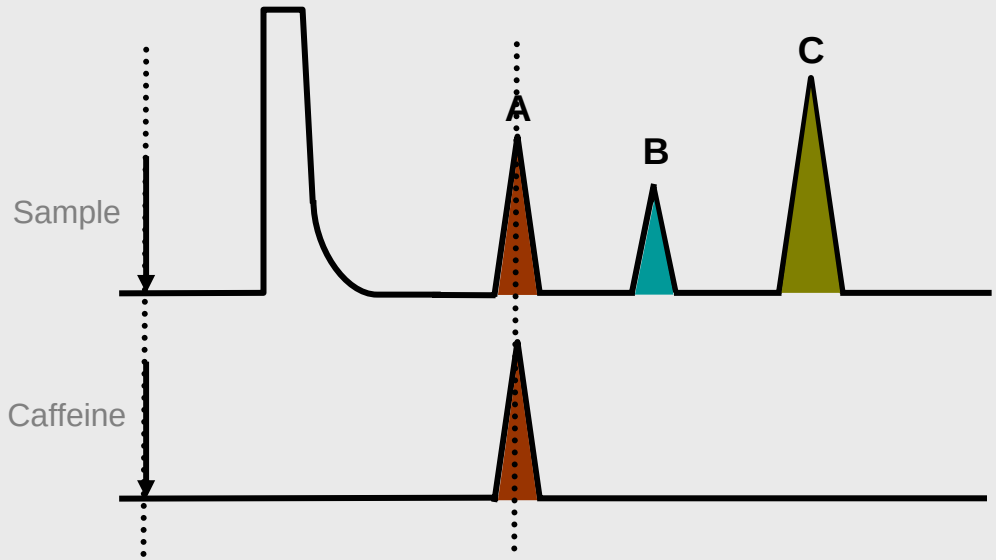
Chromatography : Method
Chromatogram : Results
Chromatograph : Instrument

Chromatogram



Identification

What is component A?

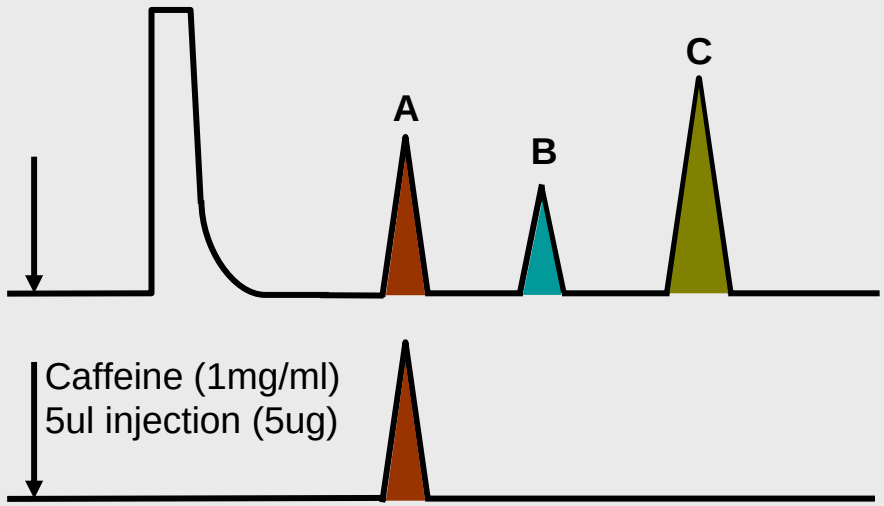


Component A elutes the same time as a caffeine peak.

Component A is identified as caffeine.

Determination

What is the concentration of component A?



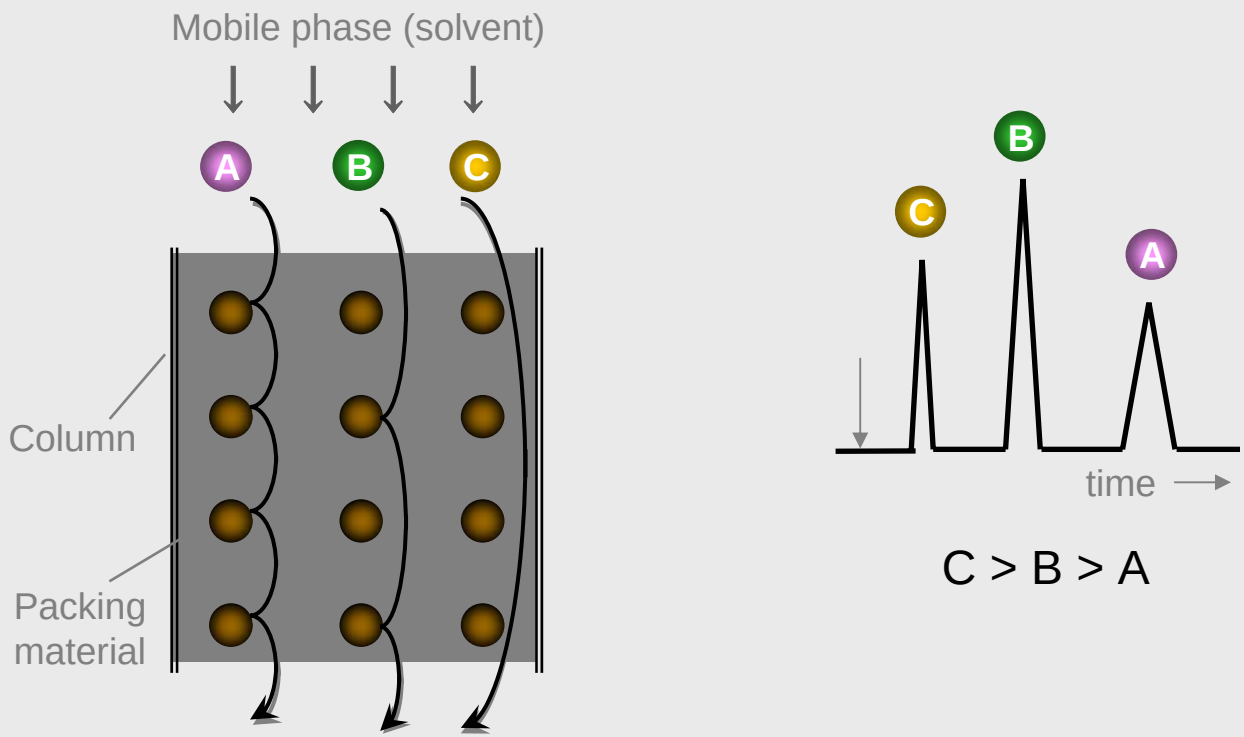
Peak area (or height) is proportional to the concentration (or amount) of the component.

The concentration of component A(caffeine) is determined by comparing the peak area with that of the standard caffeine peak.

Separation Mechanism

Separation is determined by column (packing material) and mobile phase (solvent).

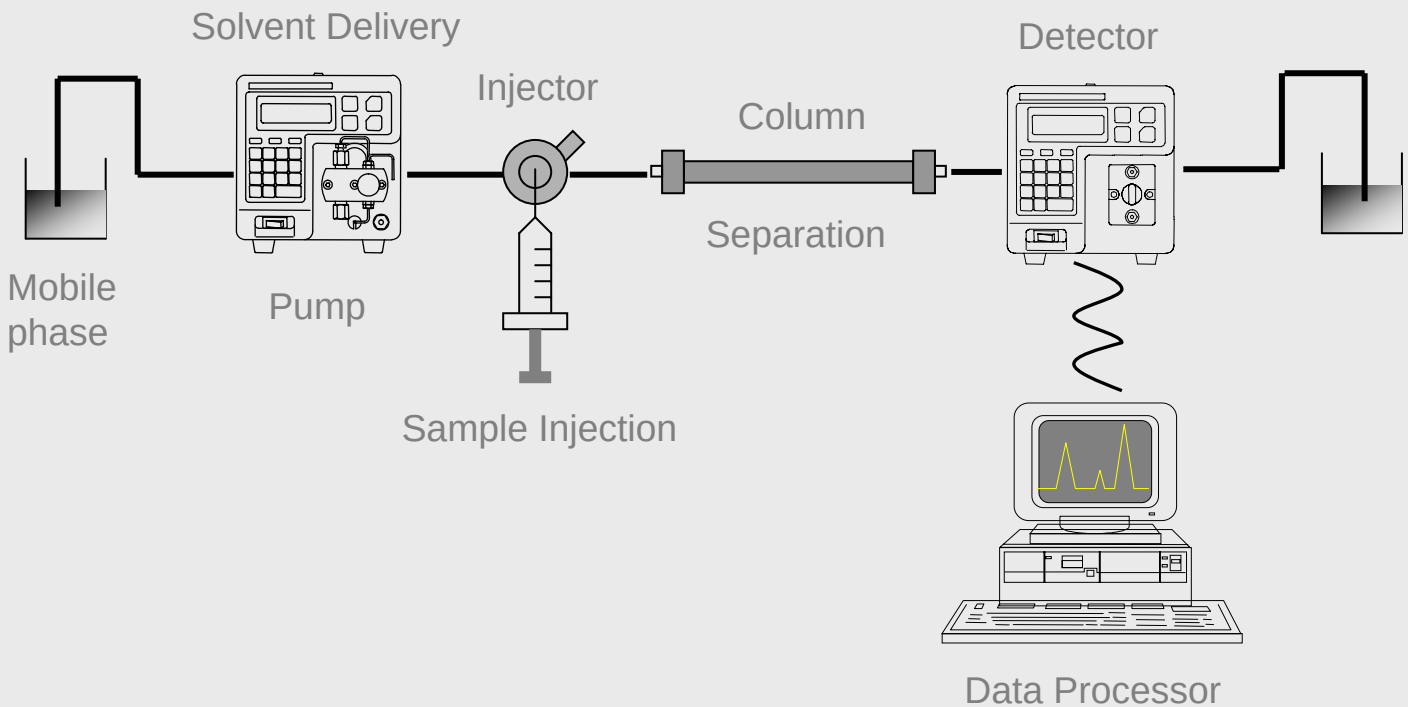
Mobile phase elutes components.
Packing materials retain components in the column.



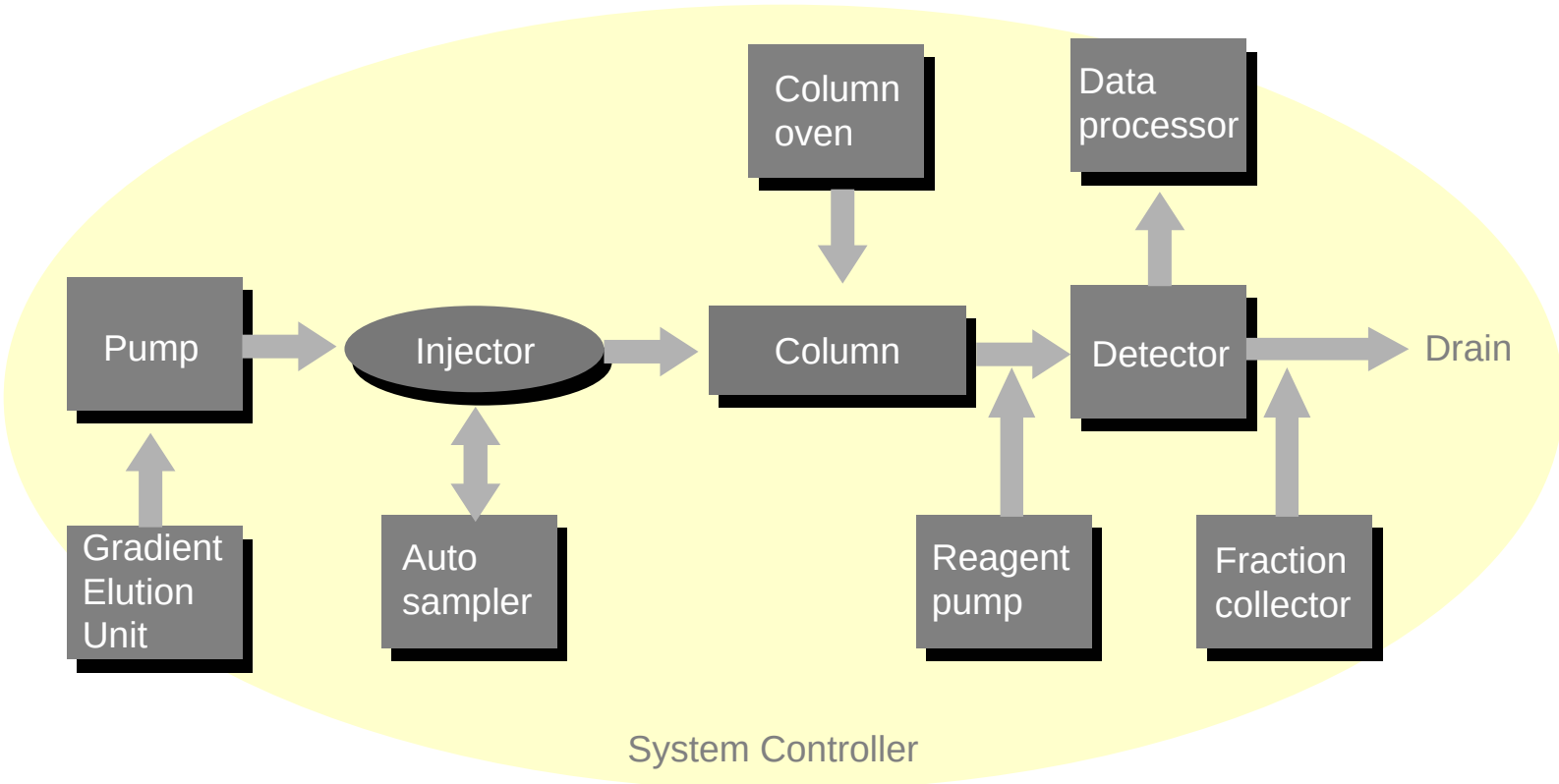
Five modes in HPLC

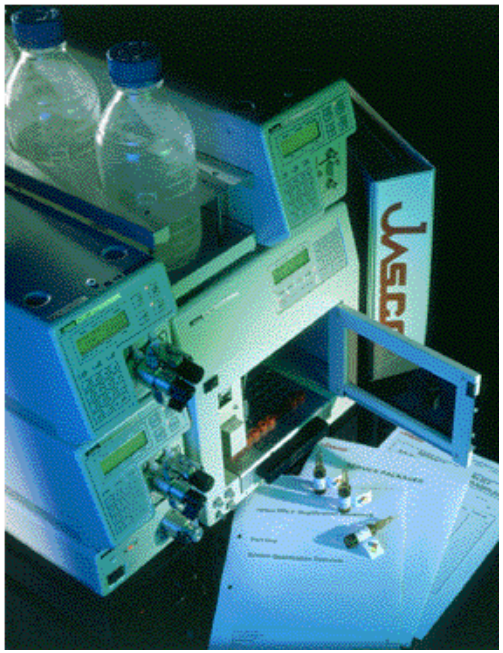
LC mode	Packing materials	Mobile phase	Interaction
Normal phase chromatography	Silica gel	n-Hexane/IPE	Adsorption
Reversed phase chromatography	Silica-C18(ODS)	MeOH/Water	Hydrophobic
Size exclusion chromatography	Porous polymer	THF	Gel permeation
Ion exchange chromatography	Ion exchange gel	Buffer sol.	Ion exchange
Affinity chromatography	Packings with ligand	Buffer sol.	Affinity

HPLC Basic Instrumentation



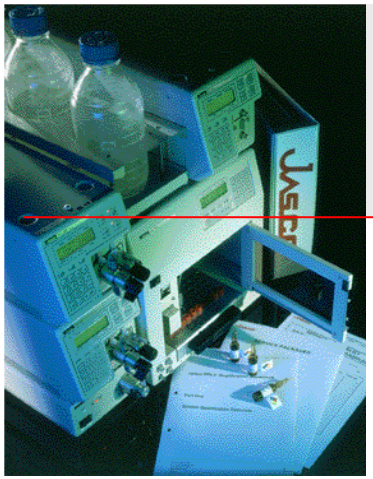
HPLC Instrumentation





The JASCO advanced technology team has again met the challenge and designed a new line of HPLC instruments, The LC-1500series more than satisfies in response to the growing demand for greatly expanded HPLC analyses in the fields of not only biochemistry, pharmaceutical and medical science, but also in the areas of among other organic and inorganic compounds, foods, agricultural sciences, polymeric and natural substances and pollution. The LC-1500 series comprises pumps, detectors, autosamplers, its own column oven and other units each having built-in intelligence and incorporating many features with much higher levels of operability and reliability in addition to multiple functions, higher performance and higher accuracy than before, making them the most advanced instruments available.

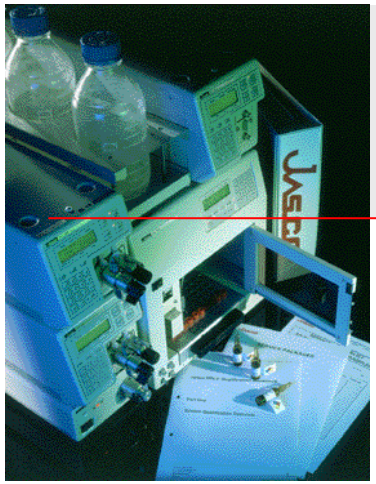
2. Parameters used in HPLC



Parameters used in HPLC

Retention parameters
Column efficiency parameters
Peak symmetry parameters
Condition for Separation

Retention : When a component in a sample interacts with the stationary phase in the column and a delay in elution occurs.
Column efficiency : Goodness of a column



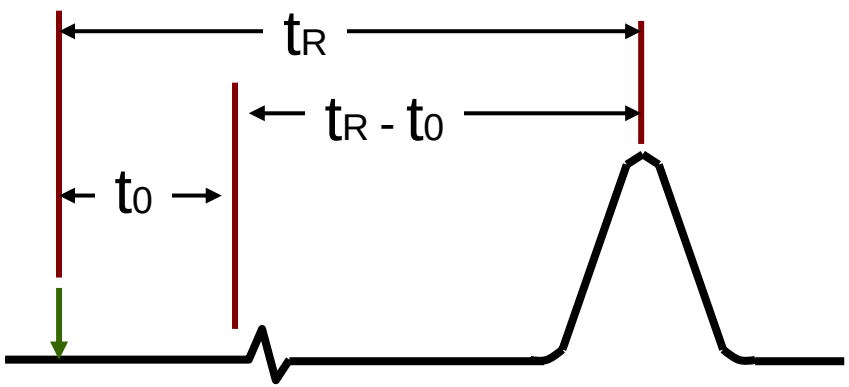
Retention parameters

t_R : retention time (the time between the injection point and the maximum detector response for correspondent compound)

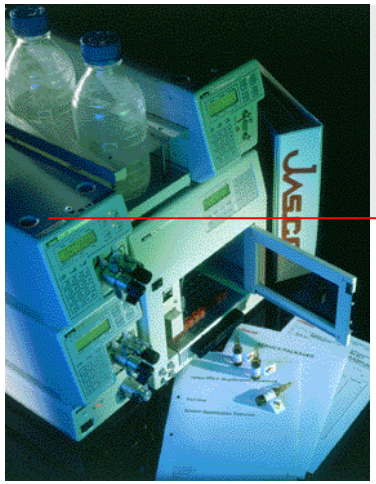
v_R : retention volume (t_R x eluent flow rate)

k' : capacity factor

t_0 : the time required for the component not retained by the column to pass through the column



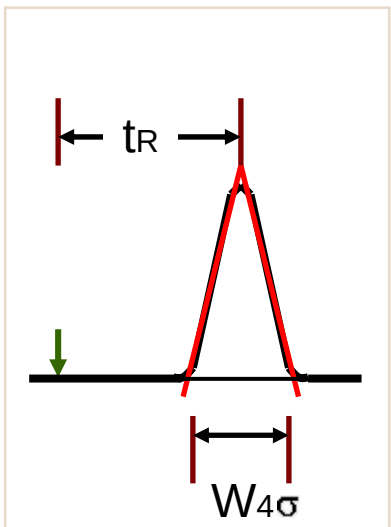
$$k' = \frac{t_R - t_0}{t_0}$$



Column efficiency

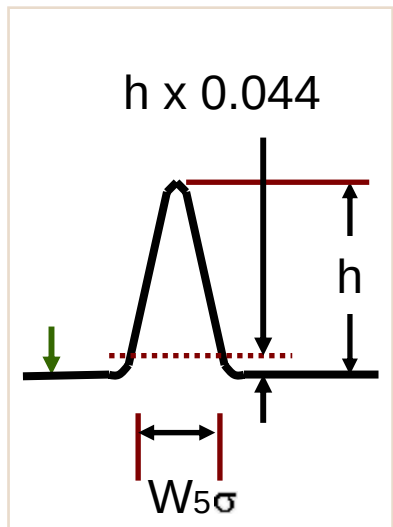
The number of theoretical plates N is given by:

4σ method



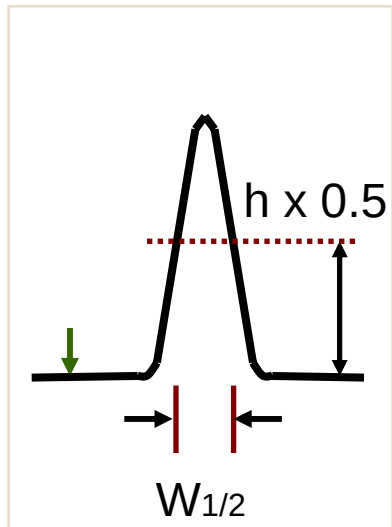
$N = 16 (t_R / W_{4\sigma})^2$

5σ method



$N = 25 (t_R / W_{5\sigma})^2$

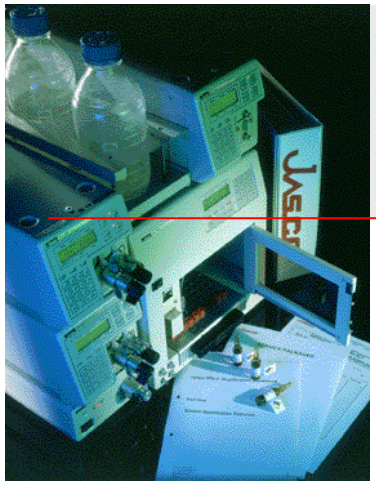
FWHM method



$N = 5.545 (t_R / W_{0.5})^2$

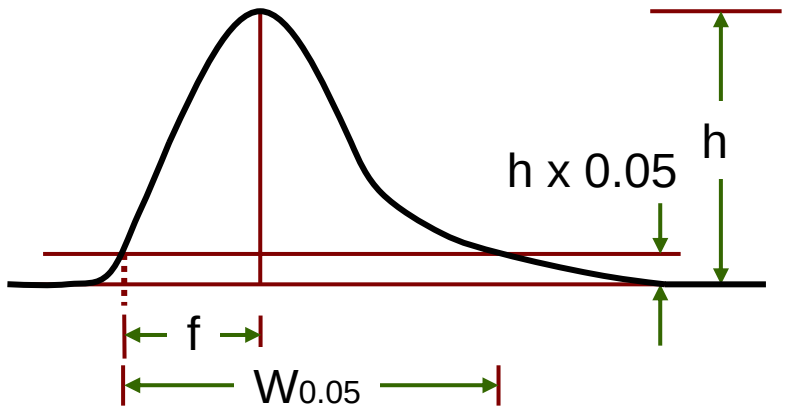
The height of the theoretical plate H is given by:

$H = L / N$ L : Column length



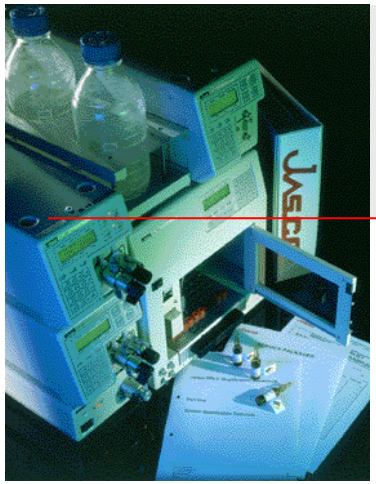
Peak symmetry

S : Symmetry factor (T : Tailing factor)

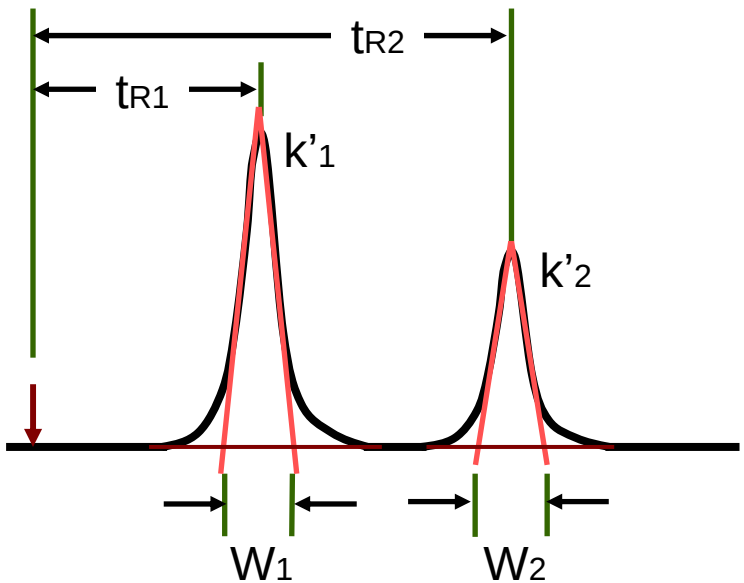


$$S = \frac{W_{0.05}}{2f}$$

S = 1 : The peak is completely symmetric.
S > 1 : Tailing
S < 1 : Leading

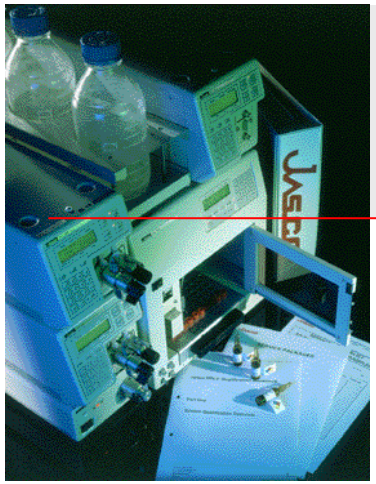


Degree of separation



Resolution : $R_s = 2 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2}$

Separation factor : $\alpha = \frac{k'_2}{k'_1}$



Condition for good separation

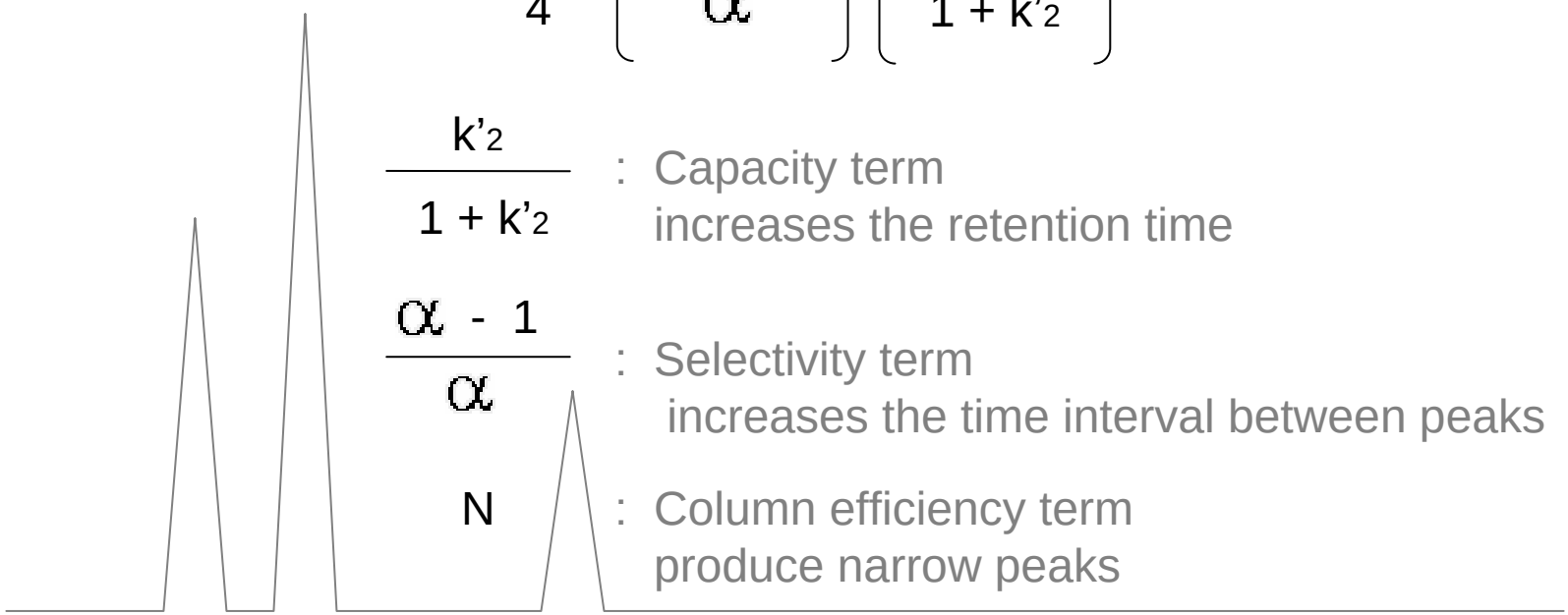
A larger R_s value means a better separation.

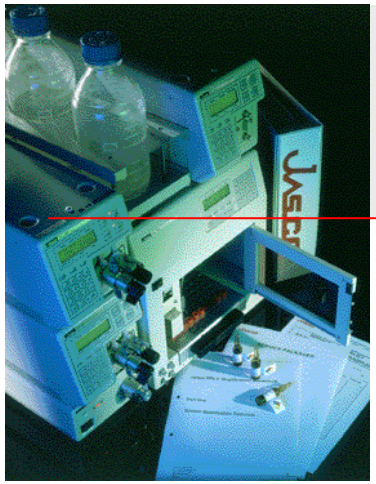
$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)\sqrt{N}$$

$\frac{k'_2}{1 + k'_2}$: Capacity term
increases the retention time

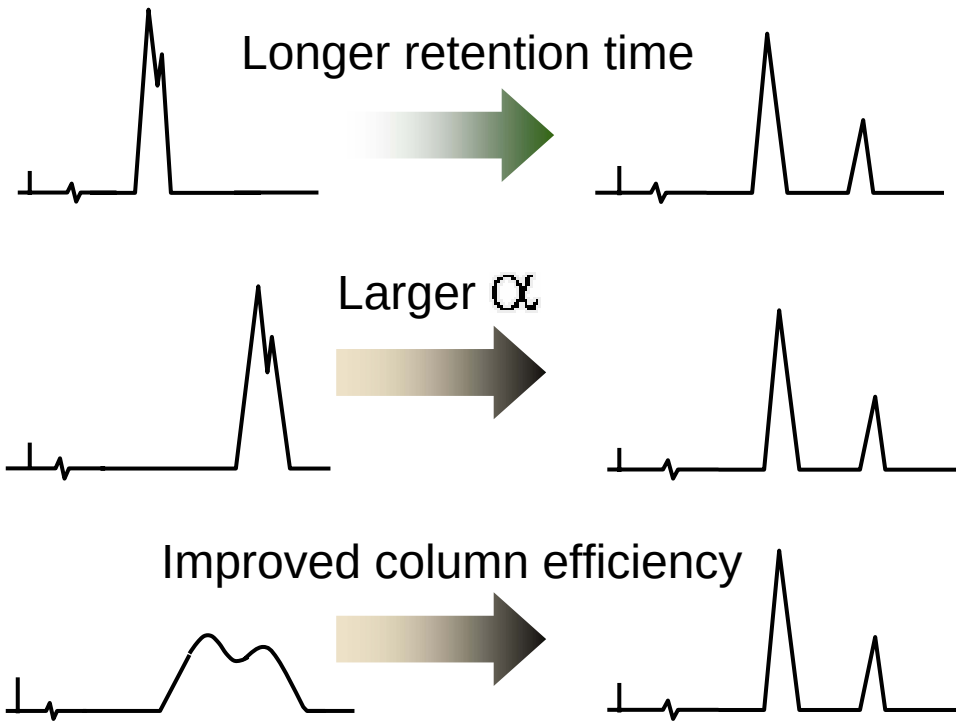
$\frac{\alpha - 1}{\alpha}$: Selectivity term
increases the time interval between peaks

N : Column efficiency term
produce narrow peaks

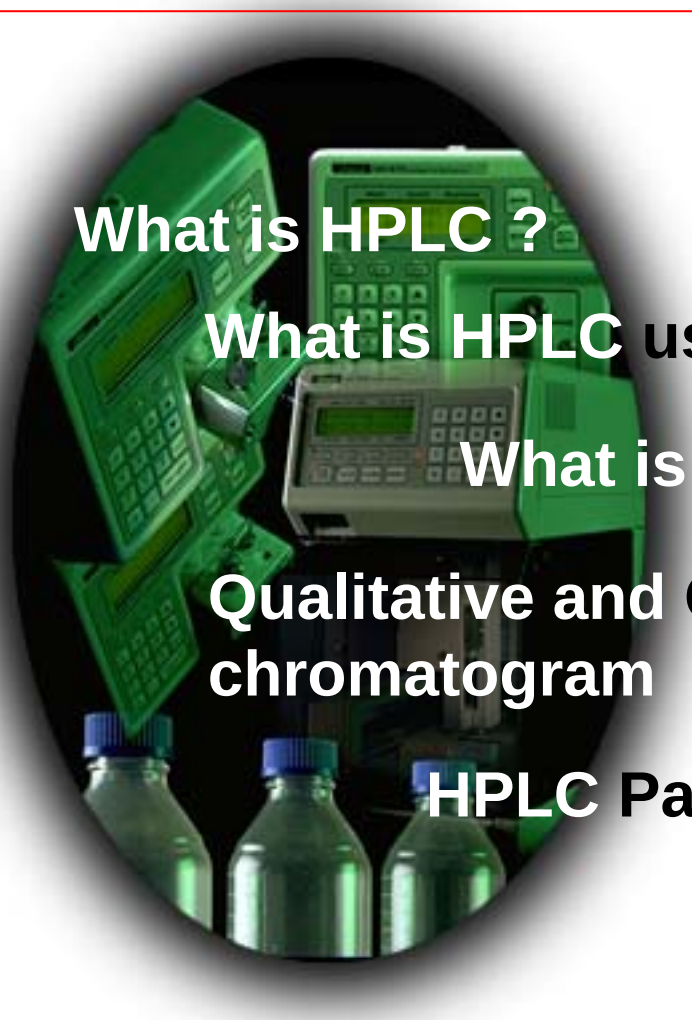




Parameters and selectivity



Review of Sections 1 and 2



What is HPLC ?

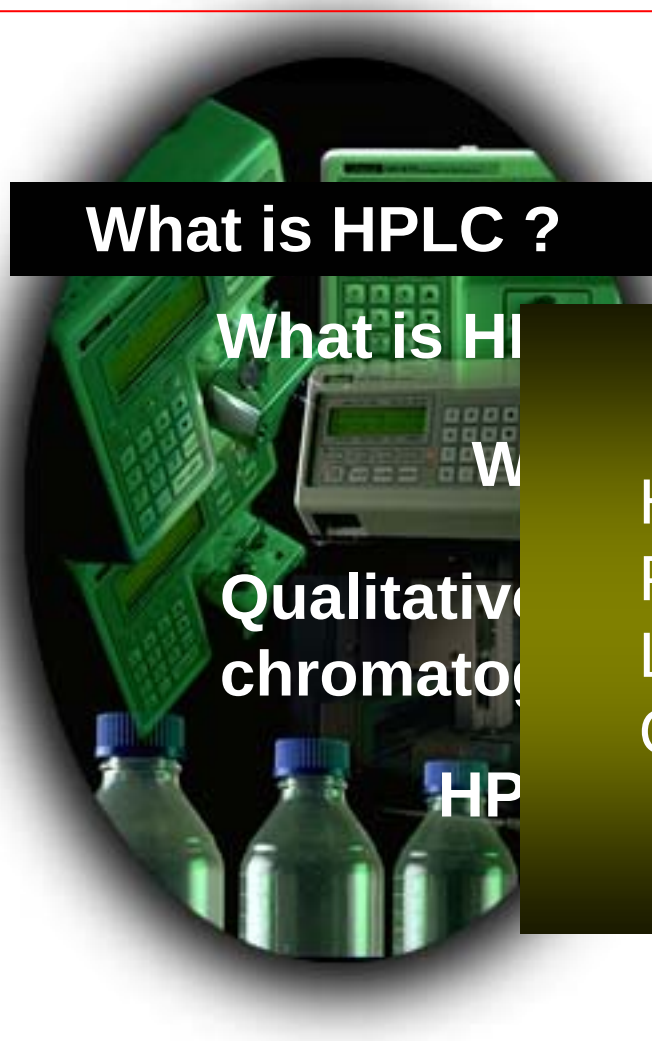
What is HPLC used for ?

What is Separation and Analysis ?

Qualitative and Quantitative analysis from chromatogram

HPLC Parameters

Review of Sections 1 and 2



What is HPLC ?

What is HI

W

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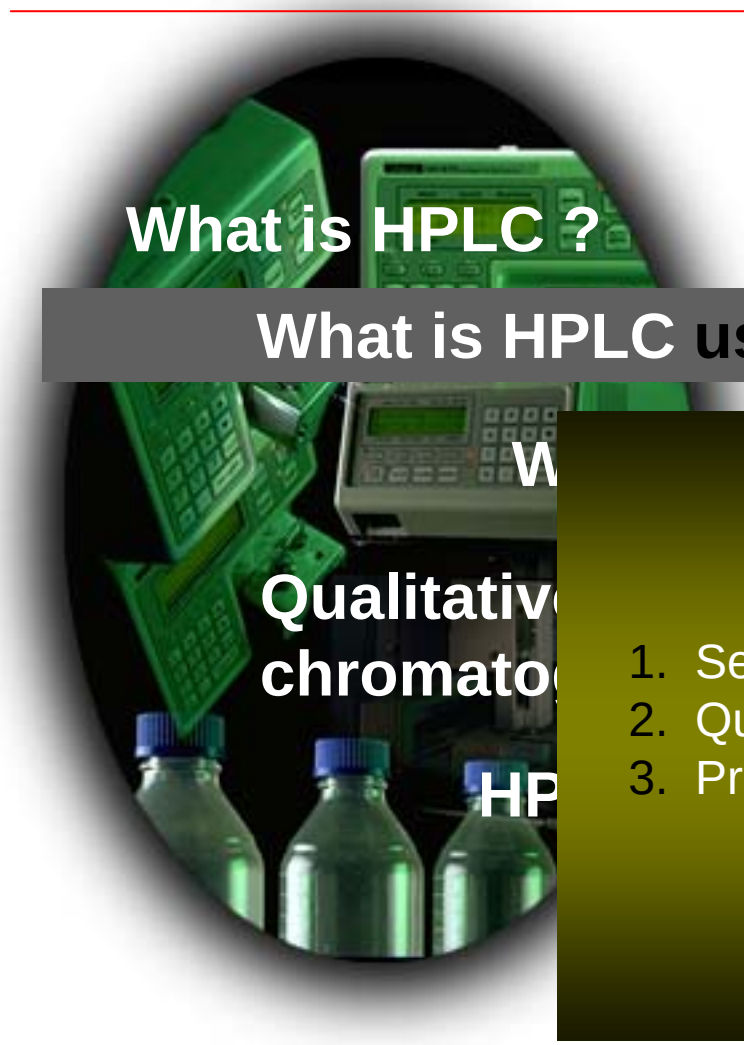
HP

H : High
P : Performance (Pressure)
L : Liquid
C : Chromatography

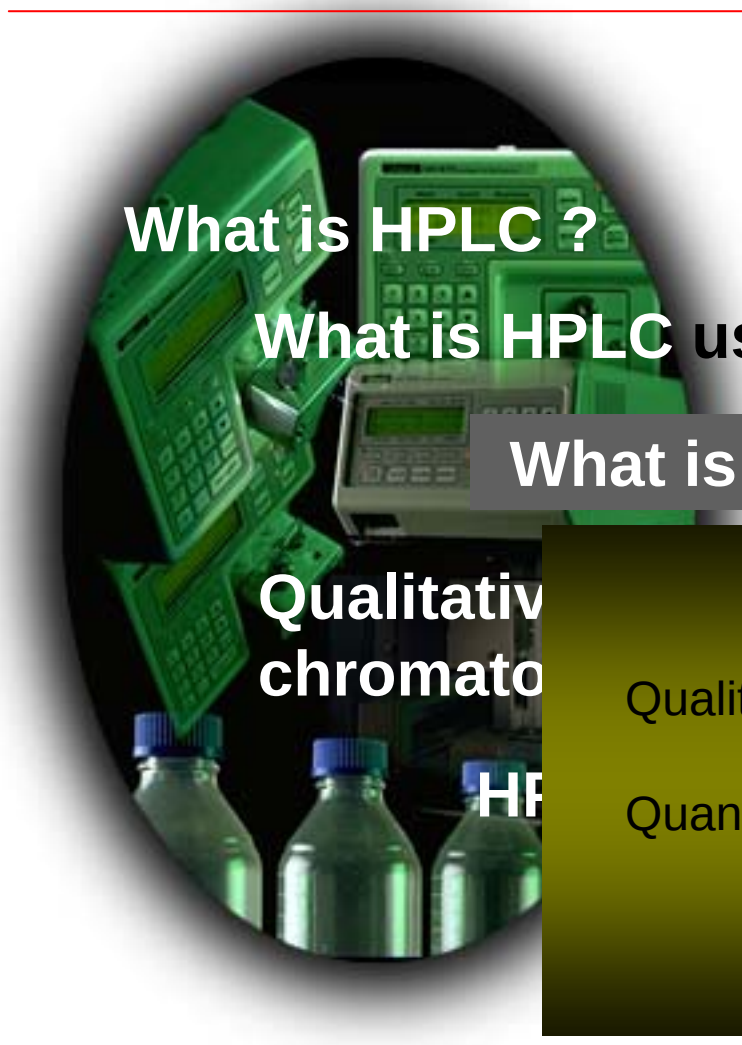
Review of Sections 1 and 2



What is HPLC used for ?

1. Separation of mixed components
2. Qualitative analysis / Quantitative analysis
3. Preparation of interest components

Review of Sections 1 and 2



What is HPLC ?

What is HPLC used for ?

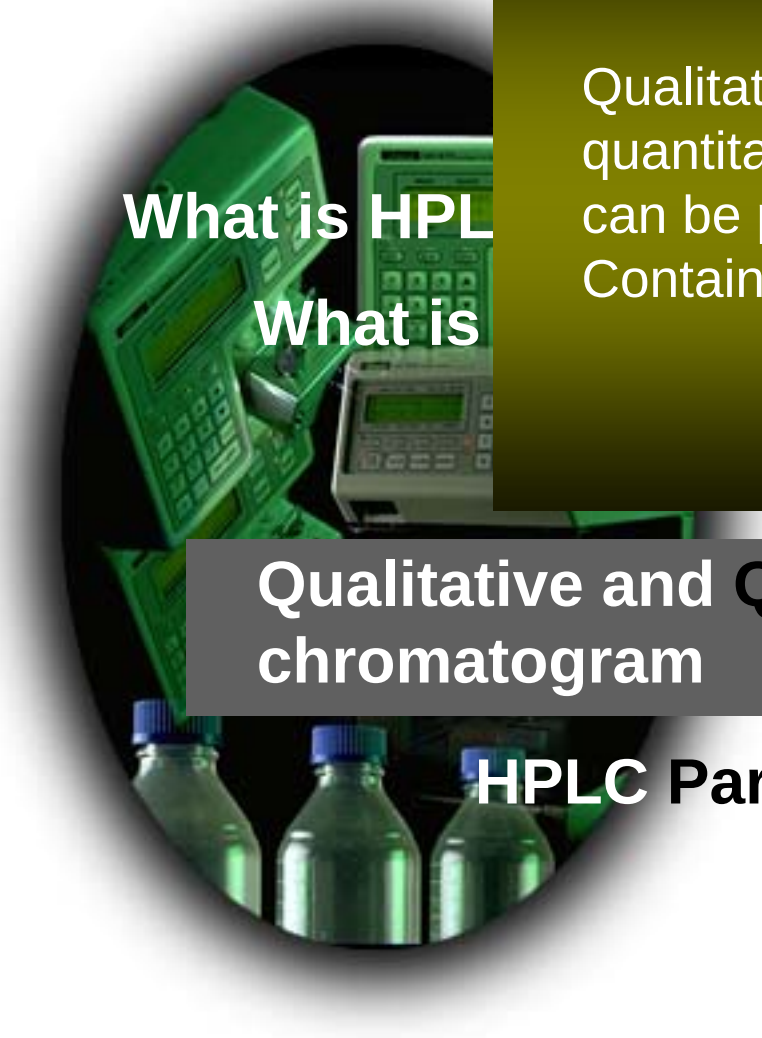
What is Separation and Analysis ?

Qualitativ
chromato

HP

Qualitative analysis
What are components A, B and C ?
Quantitative analysis
What is the concentration of components A, B and C ?

Review of Se



What is HPL

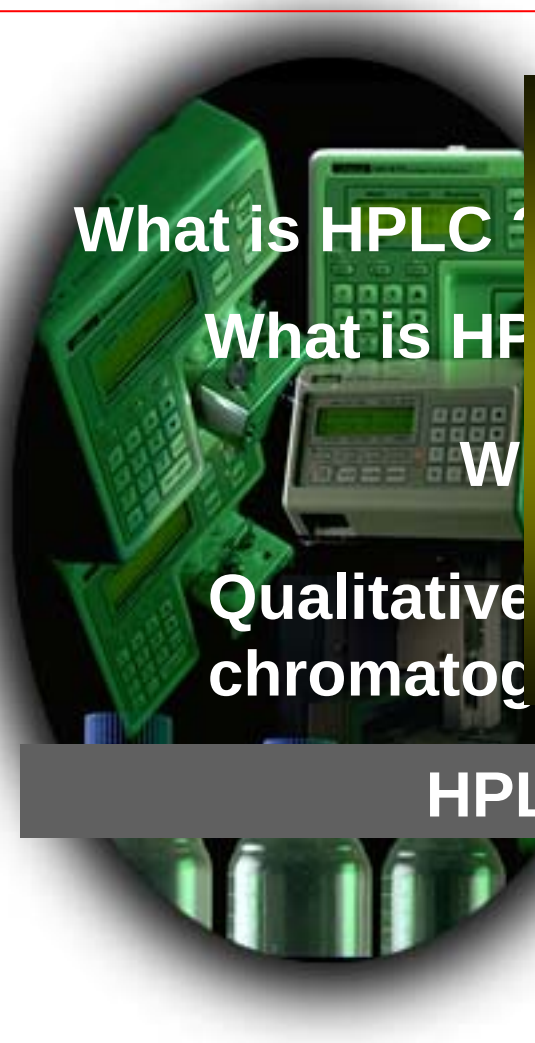
What is

Qualitative analysis (identification) and quantitative analysis (determination) can be performed using the information Contained in the chromatogram.

Qualitative and Quantitative analysis from chromatogram

HPLC Parameters

Review of Sections 1 and 2



What is HPLC

What is HP

W

Qualitative
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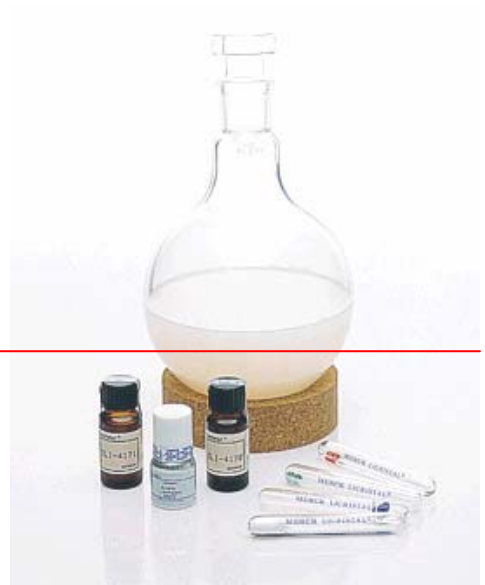
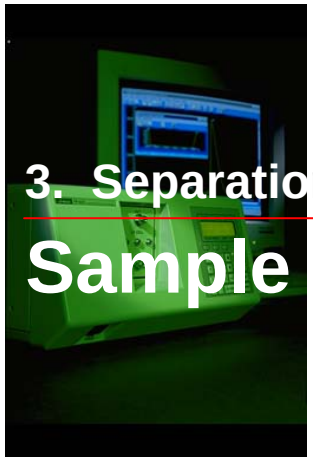
Retention parameters
Column efficiency parameters
Peak symmetry parameters
Condition for Separation

HPLC Parameters



3. Separation mode

Column and mobile phase solvent



3. Separation mode

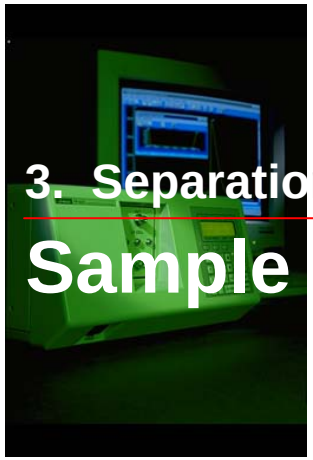
Sample and Analytical method

In which materials ?
In what concentration ?
Which sample ?
With which technique ?

What is the sample ?
Concentration of the interested component
Contaminant

Characteristics of the sample
- Structure
- Molecular weight
- pKa
- Solubility

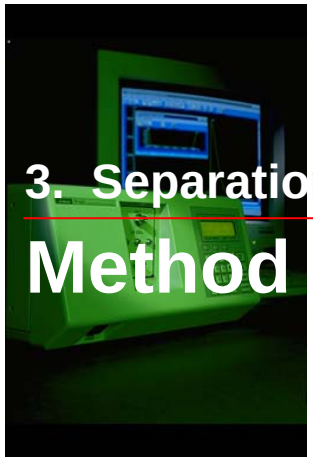
Analytical technique
- Column
- Mobile phase
- Detector
- Sample preparation



3. Separation mode

Sample information

Merck Index
Great Chemical Dictionary
Great BioChemical Dictionary
Reports based on other measurement techniques



3. Separation mode

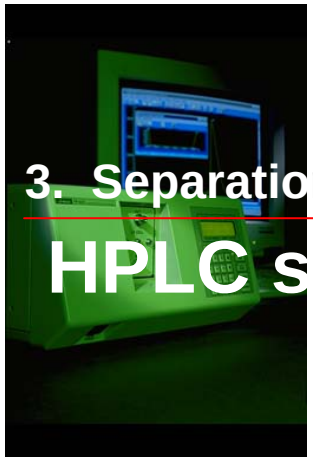
Method information

Society magazines

- Journal of Chromatography.
- Analytical Chemist

Manufacturer

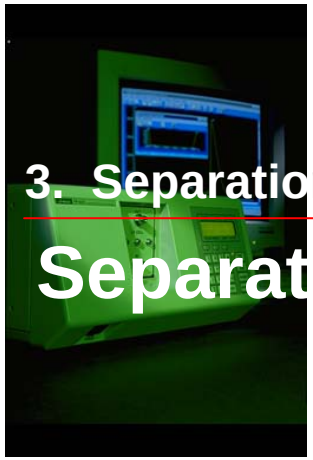
- JASCO Application data



HPLC separation mode

HPLC separation mode

Normal phase chromatography (NP)
Reversed phase chromatography (RP)
Size exclusion chromatography (SEC)
Ion exchange chromatography (IEX)
Affinity chromatography



3. Separation mode

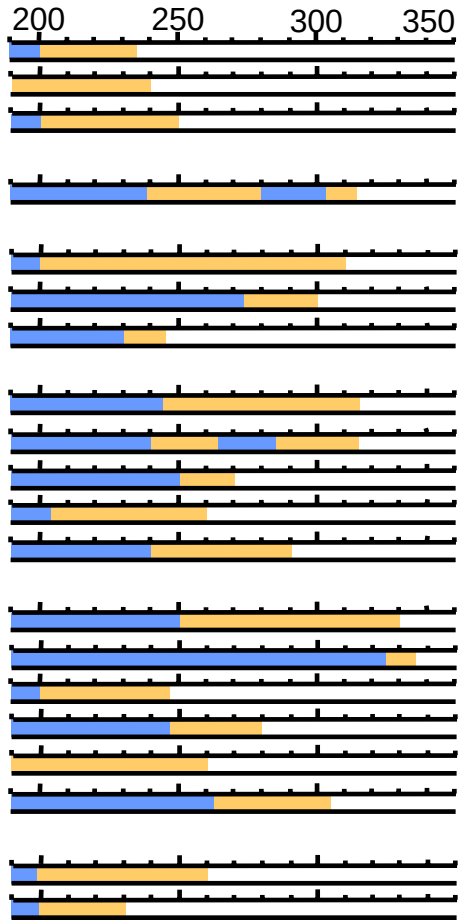
Separation modes and features

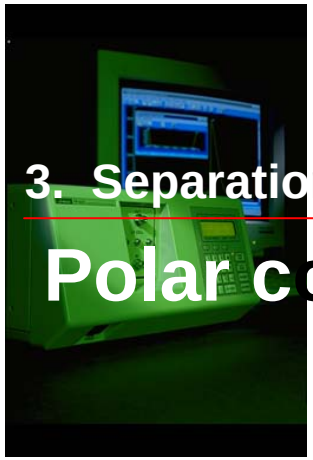
Mode	Stationary phase	Mobile phase	Interaction	Feature
Normal phase chromatography	Silica gel	Organic solvent (n-Hexane/IPE)	Adsorption	Fat-soluble
Reversed phase chromatography	Silica-ODS (Silica-C18)	MeOH/Water	Hydrophobic	Most widely used
Size exclusion Chromatography				
Non-aqueous (GPC)	Porous Polymer	Organic solvent (THF)	Gel permeation	Molecular weight distribution
Aqueous (GFC)	Aqueous porous Polymer	Buffer solution	Gel permeation	Protein Separation
Ion exchange Chromatography	Ion exchange gel	Buffer solution	Ion exchange	Separation of ionic substances
Affinity Chromatography	Packing with ligand	Buffer solution	Affinity	Purification of enzymes and proteins

GPC : Gel Permeation Chromatography
GFC : Gel Filtration Chromatography

Solvent used in HPLC and range of Application

Solvent	Polarity	E0	R.I.	b.p.	Viscosity	UV cut off
Isoctane	0.1	0.01	1.389	99	0.47	197
LCn-Hexane	0.1	0.01	1.372	69	0.30	190
Cyclohexane	-0.2	0.04	1.423	81	0.90	200
Triethylamine	1.9	0.54	1.398	89	0.36	
i-Proryl ether	2.4	0.28	1.365	68	0.38	220*
Toluene	2.4	0.29	1.494	110	0.55	285
Ethyl ether	2.8	0.38	1.350	35	0.24	218
Benzene	2.7	0.32	1.498	80	0.60	280
Methylene chloride	3.1	0.42	1.421	40	0.41	233
n-Butanol	3.9	0.7	1.397	118	2.6	210
n-Propanol	4.0	0.82	1.385	97	1.9	240
Tetrahydrofuran	4.0	0.57	1.405	66	0.46	212*
Ethyl acetate	4.4	0.58	1.370	77	0.43	256
i-Propanol	3.9	0.82	1.384	82	1.9	205
Chloroform	4.1	0.40	1.443	61	0.53	245
Methylethyl ketone	4.7	0.51	1.376	80	0.38	329
Dioxane	4.8	0.56	1.420	101	1.2	215
Acetone	5.1	0.56	1.356	56	0.30	330
Ethanol	4.3	0.88	1.359	78	1.08	210
Acetic acid	6.0		1.370	118	1.1	
Acetonitrile	5.8	0.65	1.341	82	0.34	190
Dimethylformamide	6.4		1.428	153	0.80	268
Dinethylsulfoxide	7.2	0.75	1.477	189	2.00	268
Methanol	5.1	0.95	1.326	65	0.54	205
Water	10.2		1.333	100	0.89	

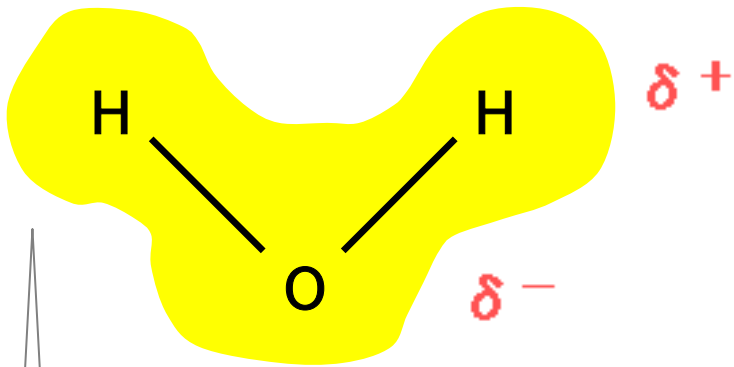




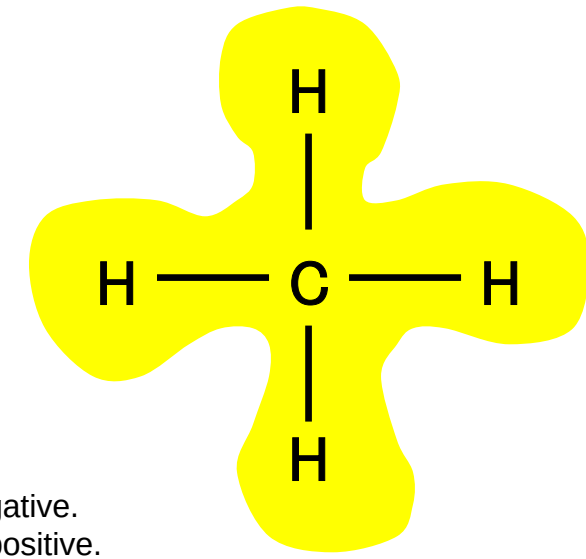
3. Separation mode

Polar compounds

Polar compound



Non-polar compound



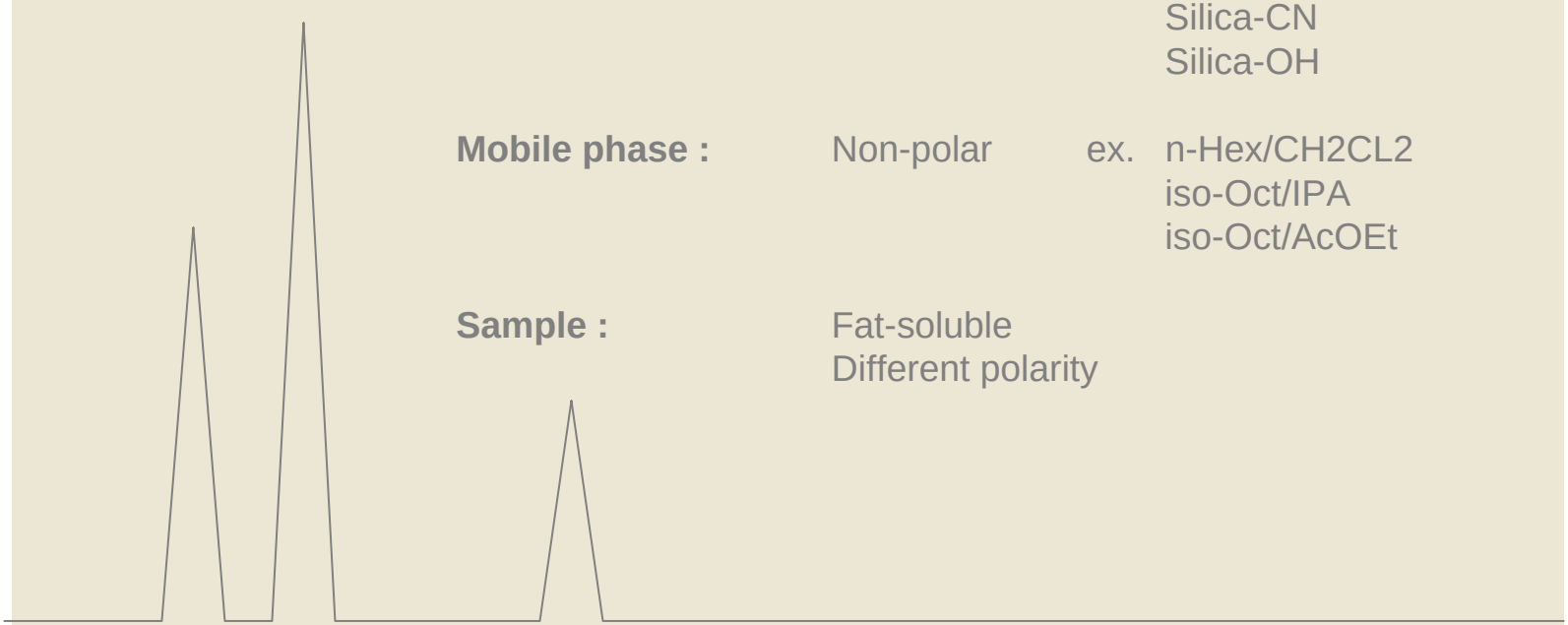
Bonding electrons are not shared evenly.
The end of the bond with electrons becomes partially negative.
The end of the bondwithout electrons becomes partially positive.

Polar compounds are soluble in polar solvents.
Non-polar compounds are soluble in non-polar solvents.

3. Separation mode

Normal Phase Chromatography

Interaction :	Adsorption		
Packing materials :	Polar	ex.	Silica gel Silica-NH2 Silica-CN Silica-OH
Mobile phase :	Non-polar	ex.	n-Hex/CH2CL2 iso-Oct/IPA iso-Oct/AcOEt
Sample :	Fat-soluble Different polarity		

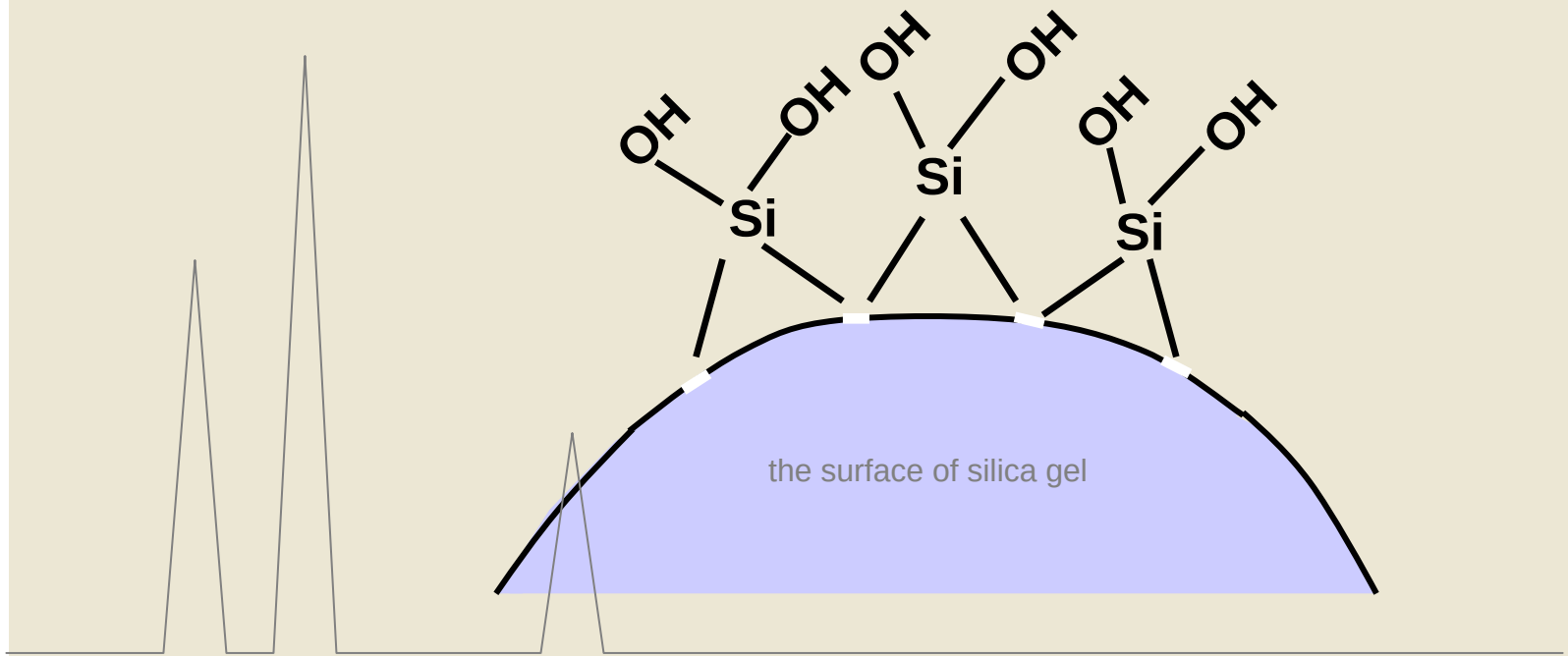


3. Separation mode

Normal Phase Chromatography

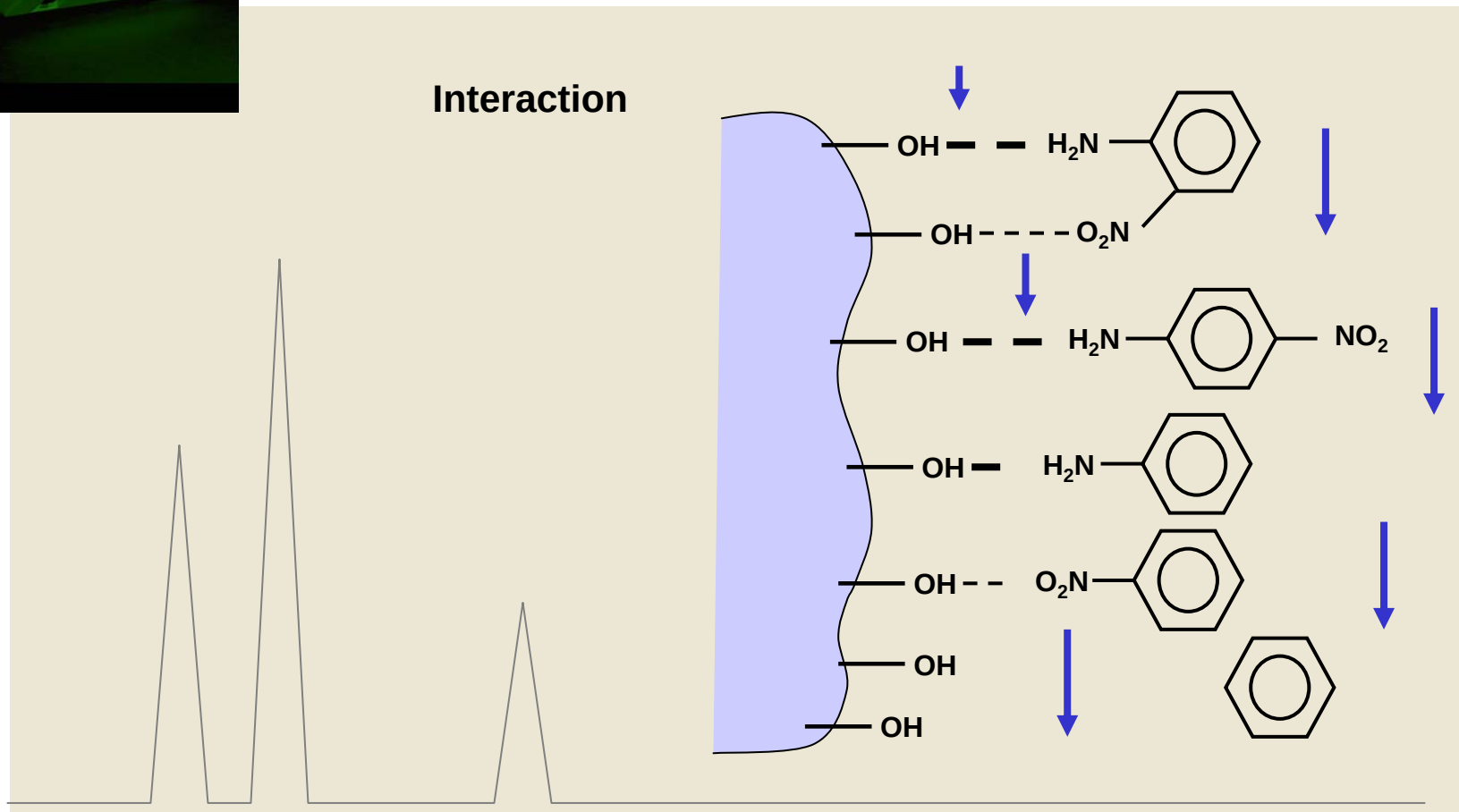
Packing material

The most popular packing material is silica gel.
It is believed that silanol radicals (-Si-OH) on the surface of silica gel act as the active site and the sample is separated.



3. Separation mode

Normal Phase Chromatography



3. Separation mode

Normal Phase Chromatography

Mobile phase solvents

n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

Low

Polarity

High

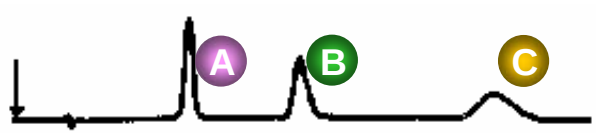
Normal Phase Chromatography

Retention behavior

Low

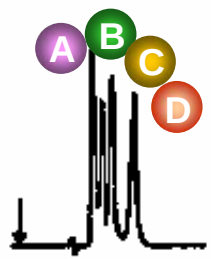
Polarity of
Mobile phase

High



n-Hex/AcOEt(60/40)

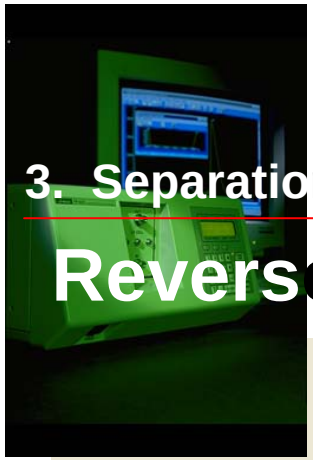
n-Hex/AcOEt(50/50)



n-Hex/AcOEt(30/70)

Polarity of sample components

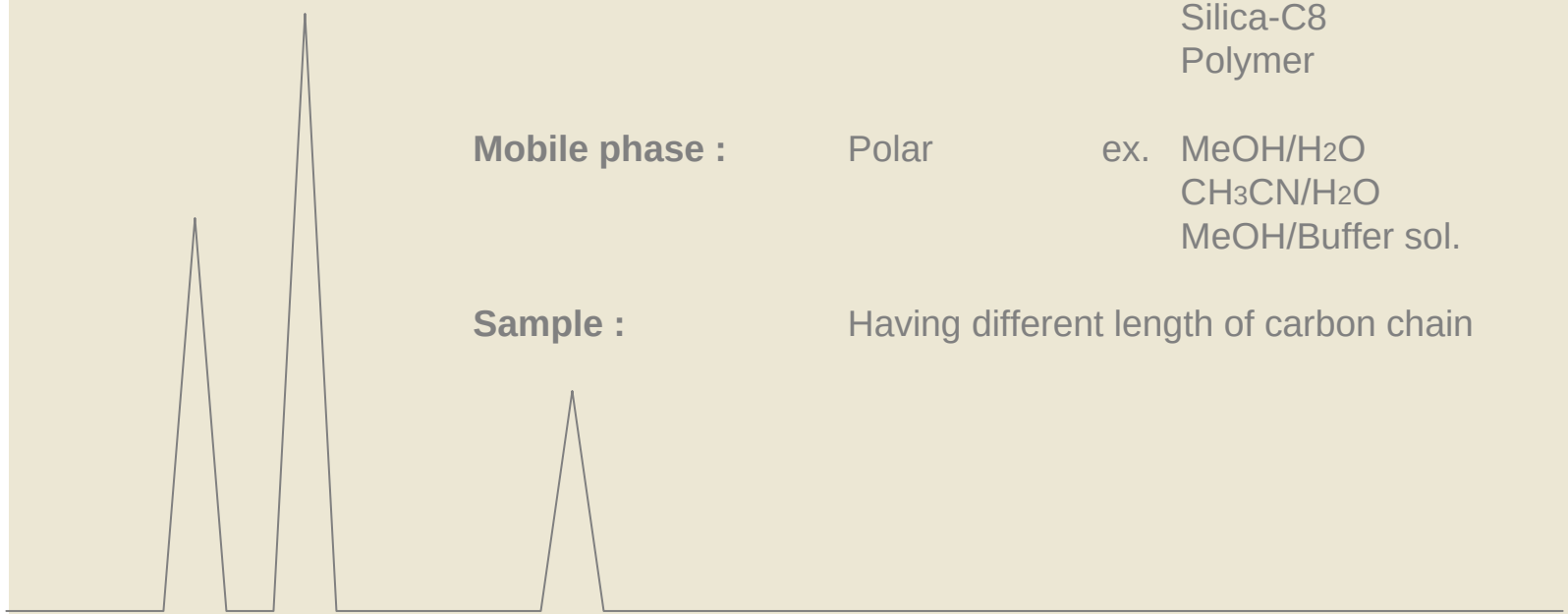
A < B < C < D

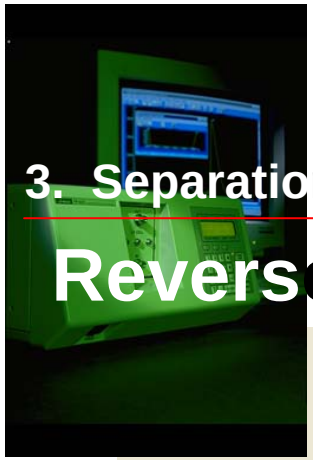


3. Separation mode

Reversed Phase Chromatography

Interaction :	Hydrophobic		
Packing materials :	Non-polar	ex.	Silica-C18 Silica-C8 Polymer
Mobile phase :	Polar	ex.	$MeOH/H_2O$ CH_3CN/H_2O MeOH/Buffer sol.
Sample :	Having different length of carbon chain		



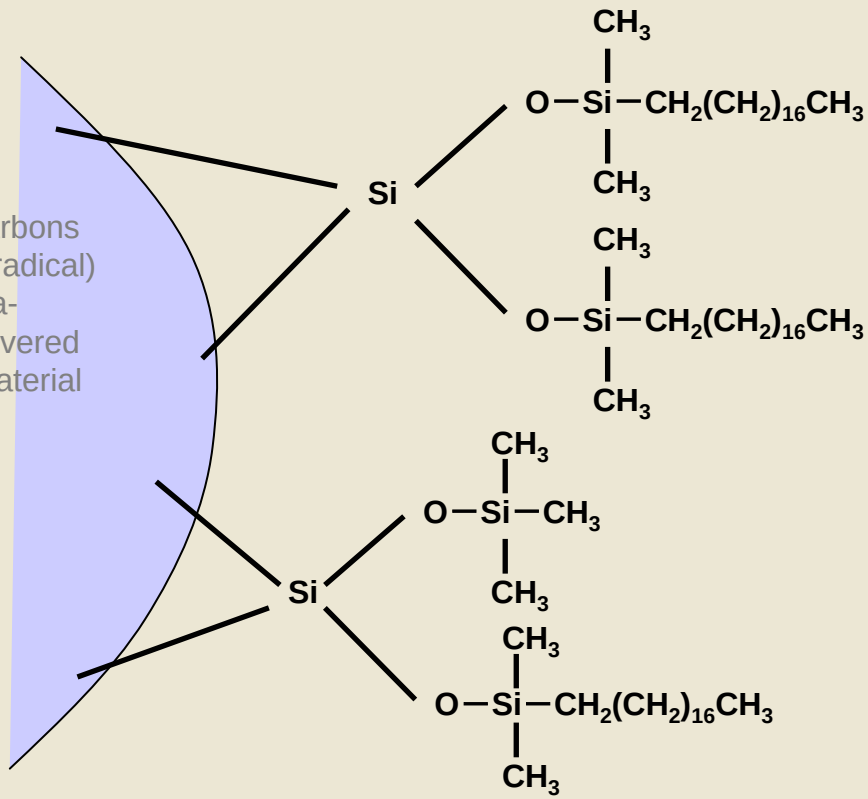


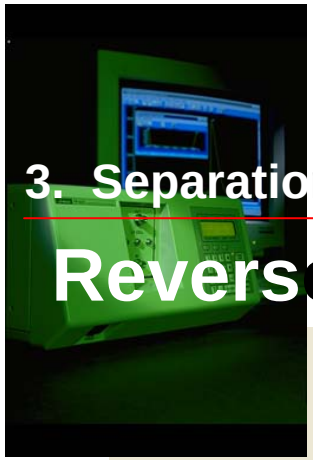
3. Separation mode

Reversed Phase Chromatography

Silica-C18 Packing materials

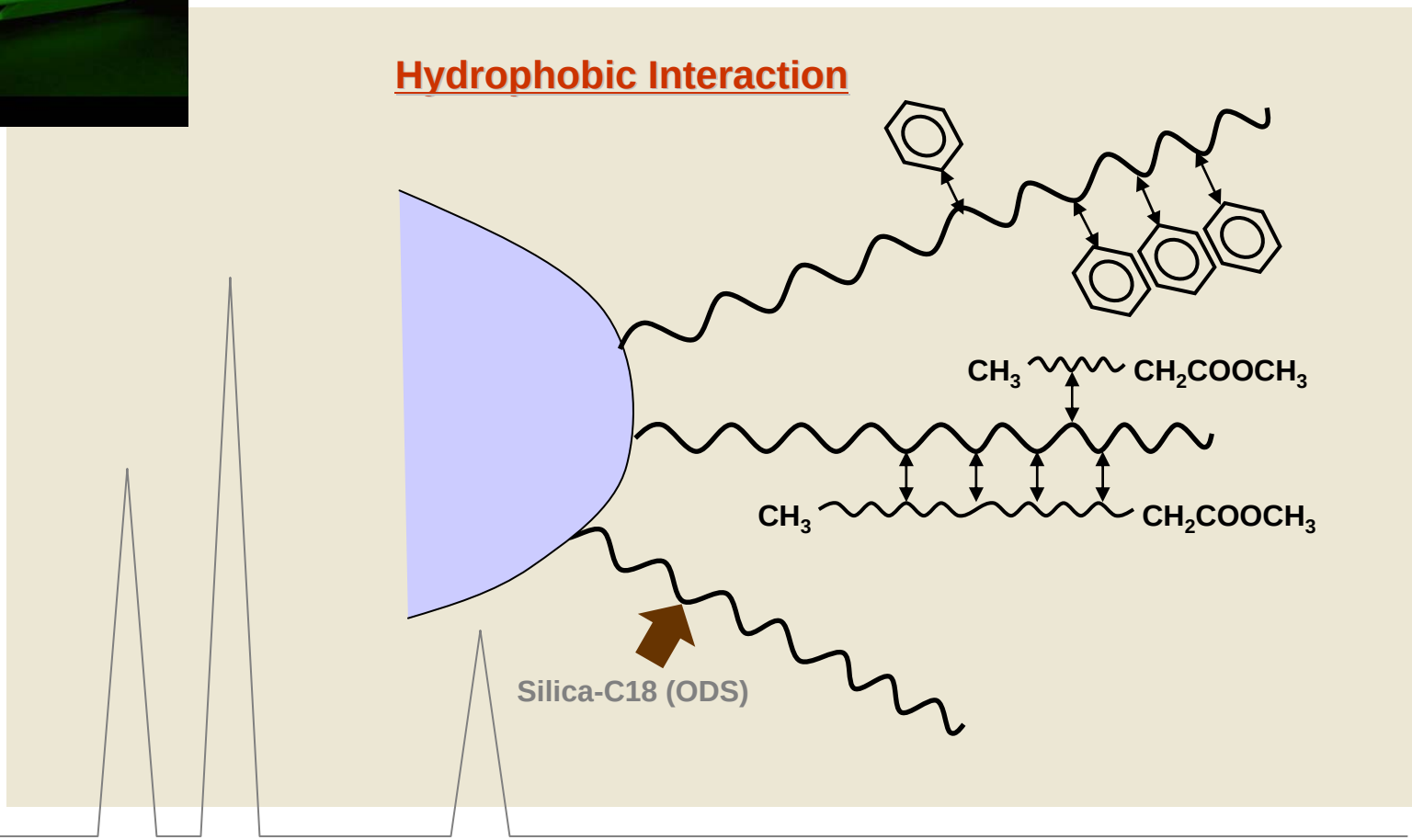
Commonly used packing materials are hydrocarbons having 18 carbon atoms (called the Octadecyl radical) which are chemically bonded to silica gel (Silica-ODS).Since the surface of the Silica-ODS is covered with hydrocarbon, the polarity of the packing material itself is very low.

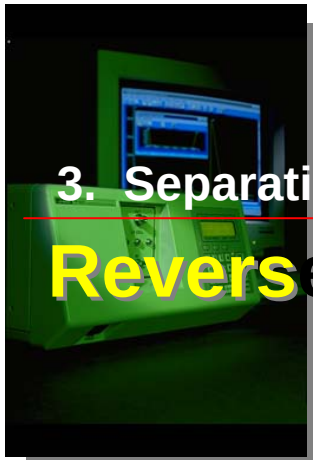




3. Separation mode

Reversed Phase Chromatography





3. Separation mode

Reversed Phase Chromatography

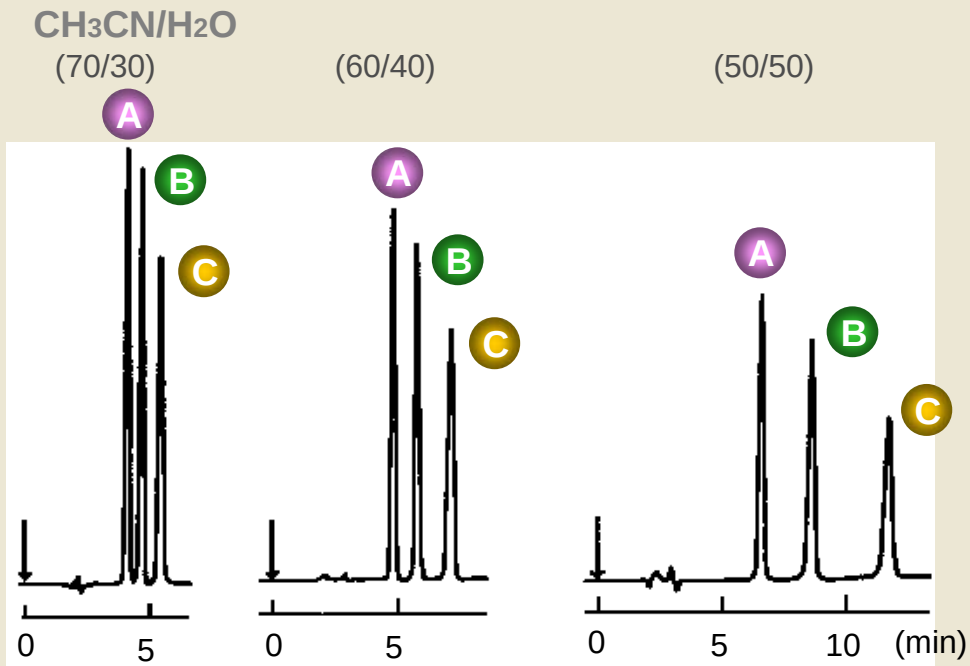
Mobile phase solvents

Main solvent :	MeOH - H2O CH3CN - H2O
Sub solvent :	EtOH IPA THF DMF
Additive :	Acid Salt Ion-pairing agent

3.Separation mode

Reversed Phase Chromatography

Retention behavior in reversed phase HPLC



Low ← Polarity of Mobile phase → High

Column : Finepak SIL C18

Carbon chain length of sample

A < B < C

A : p-Hydroxy ethyl benzoate

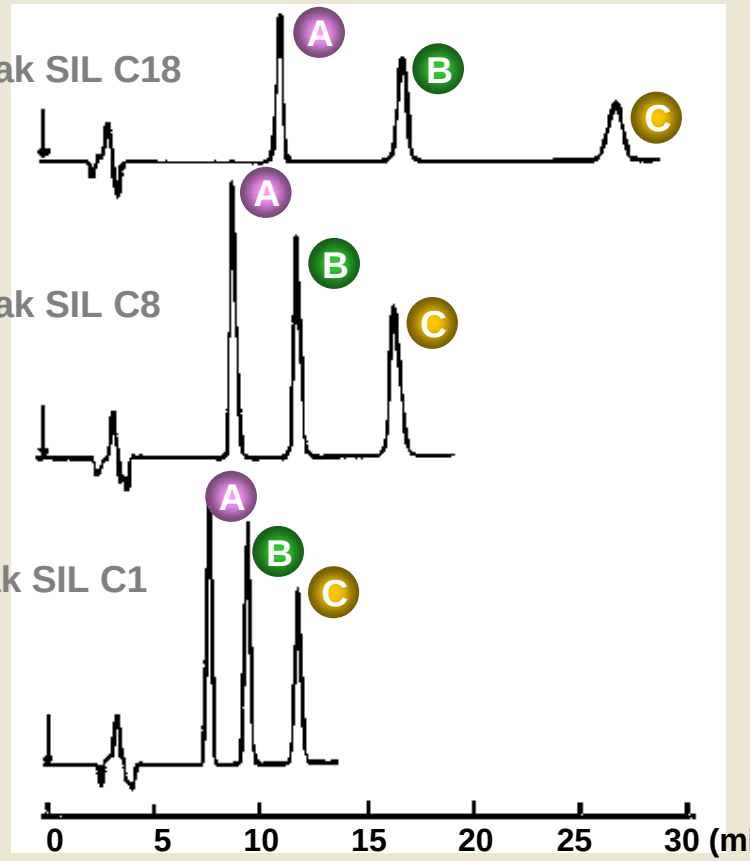
B : p-Hydroxy propyl benzoate

C : p-Hydroxy butyl benzoate

3.Separation mode

Reversed Phase Chromatography

Length of packing materials carbon chains and retention time



Mobile phase : CH_3CN/H_2O(40/60)

- A : p-Hydroxy ethyl benzoate
- B : p-Hydroxy propyl benzoate
- C : p-Hydroxy butyl benzoate

3.Separation mode

Reversed Phase and Normal Phase Chromatography

Comparison of Reversed Phase and Normal Phase

	Normal phase	Reversed phase
Stationary phase	High polarity	Low polarity
Mobile phase	Low polarity	High polarity
Interaction	Adsorption	Hydrophobic
Elution order	Low to High (Polarity)	Short to Long (Length of Carbon chain)

3.Separation mode

Reversed Phase and Normal Phase Chromatography

Comparison of
Reversed Phase and Normal Phase

Reversed Phase Chromatography

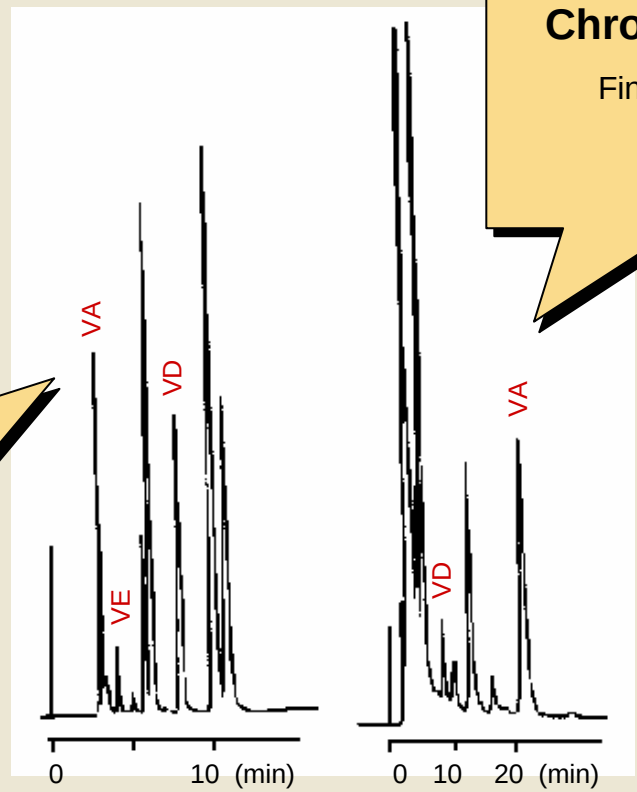
Finepak SIL C18

MeOH

Normal Phase Chromatography

Finepak SIL

n-Hexane/IPA(96/4)



3.Separation mode

Ion-exchange Chromatography

Ion-exchange Chromatography

Interaction : Ion-exchange

Stationary phase: Anion exchange gel
Cation exchange gel

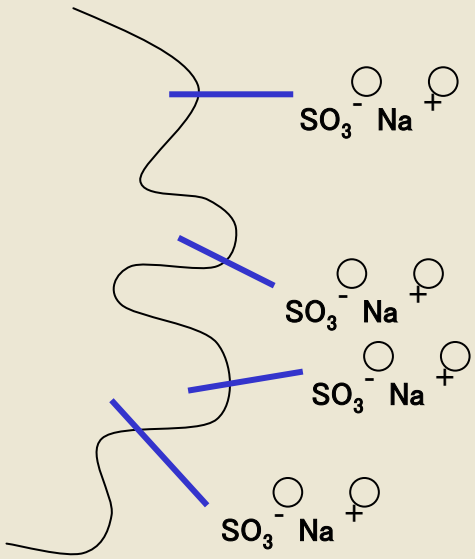
Mobile phase : Buffer solution

Sample : Ionic substances (Cations or Anions)

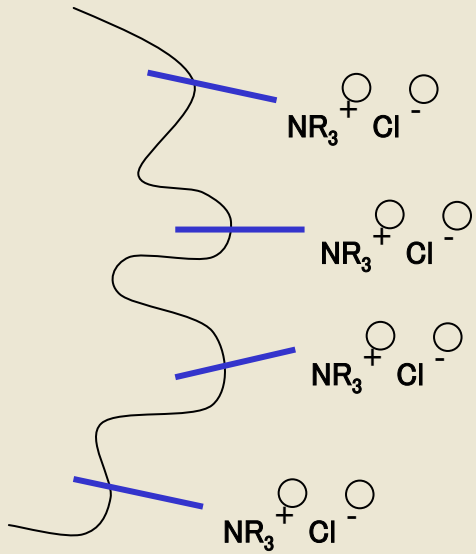
3.Separation mode

Ion-exchange Chromatography

Ion-exchange Gel



Cation exchange gel



Anion exchange gel

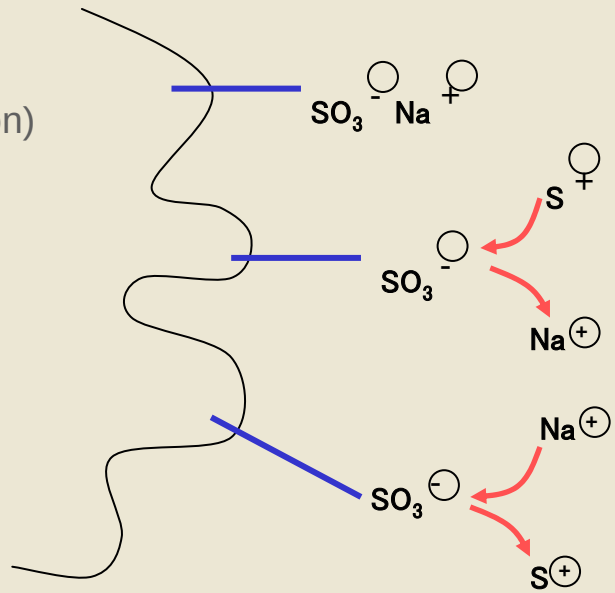
3.Separation mode

Ion-exchange Chromatography

Mobile phase solvents used for Ion-exchange

Buffer solution

- Salt concentration
- pH (Hydrogen ion concentration)
- Type of salt
- Additive (Organic solvent)



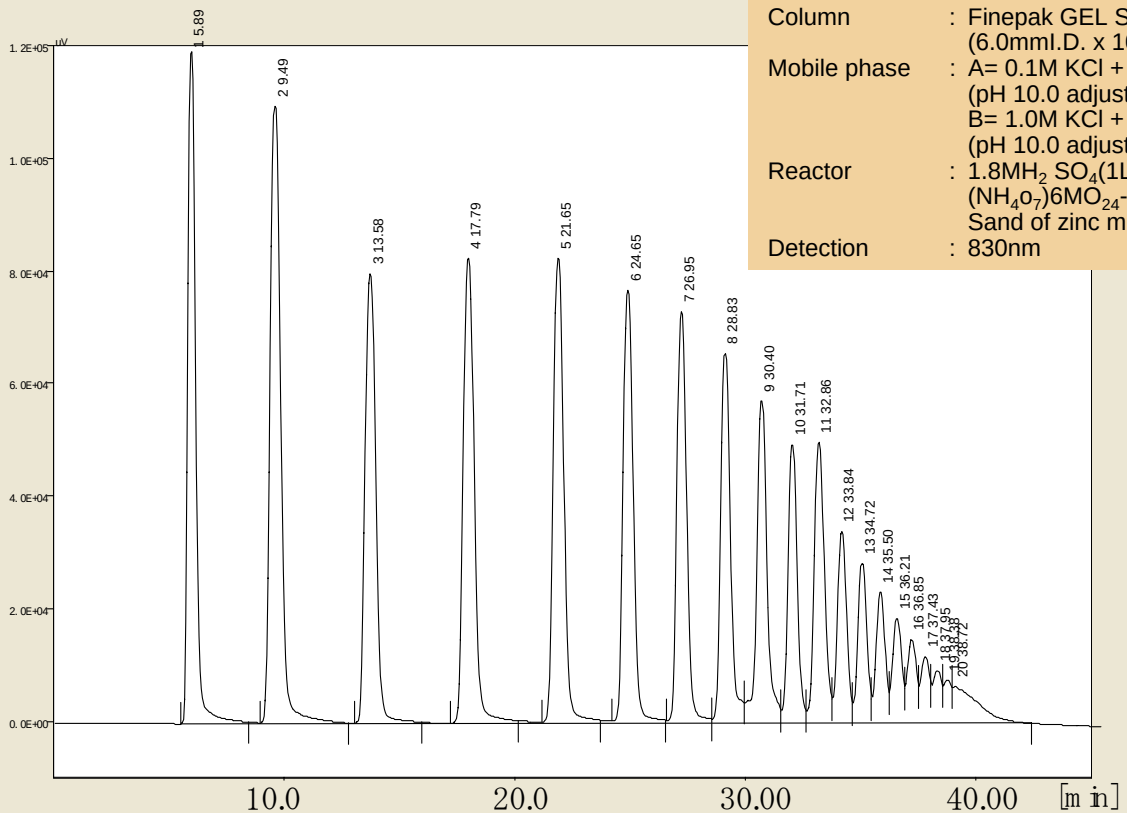
3.Separation mode

Ion-exchange Chromatography

Application data of Ion-exchange chromatography

Separation of polyphosphoric acid

Column	:	Finepak GEL SA-121 (6.0mmI.D. x 100mmL)
Mobile phase	:	A= 0.1M KCl + 1% EDTA-4Na (pH 10.0 adjusted HCl) B= 1.0M KCl + 1% EDTA-4Na (pH 10.0 adjusted HCl) gradient
Reactor	:	1.8MH_2SO_4(1L), $(NH_4O_7)6MO_{24}$-$4H_2O$ (5g), Sand of zinc metal(0.6g)
Detection	:	830nm



3.Separation mode

Ion Chromatography

Summary of Ion Chromatography

Purpose : Separation of inorganic ions, organic acids

Stationary phase: Anion exchange gel
Cation exchange gel

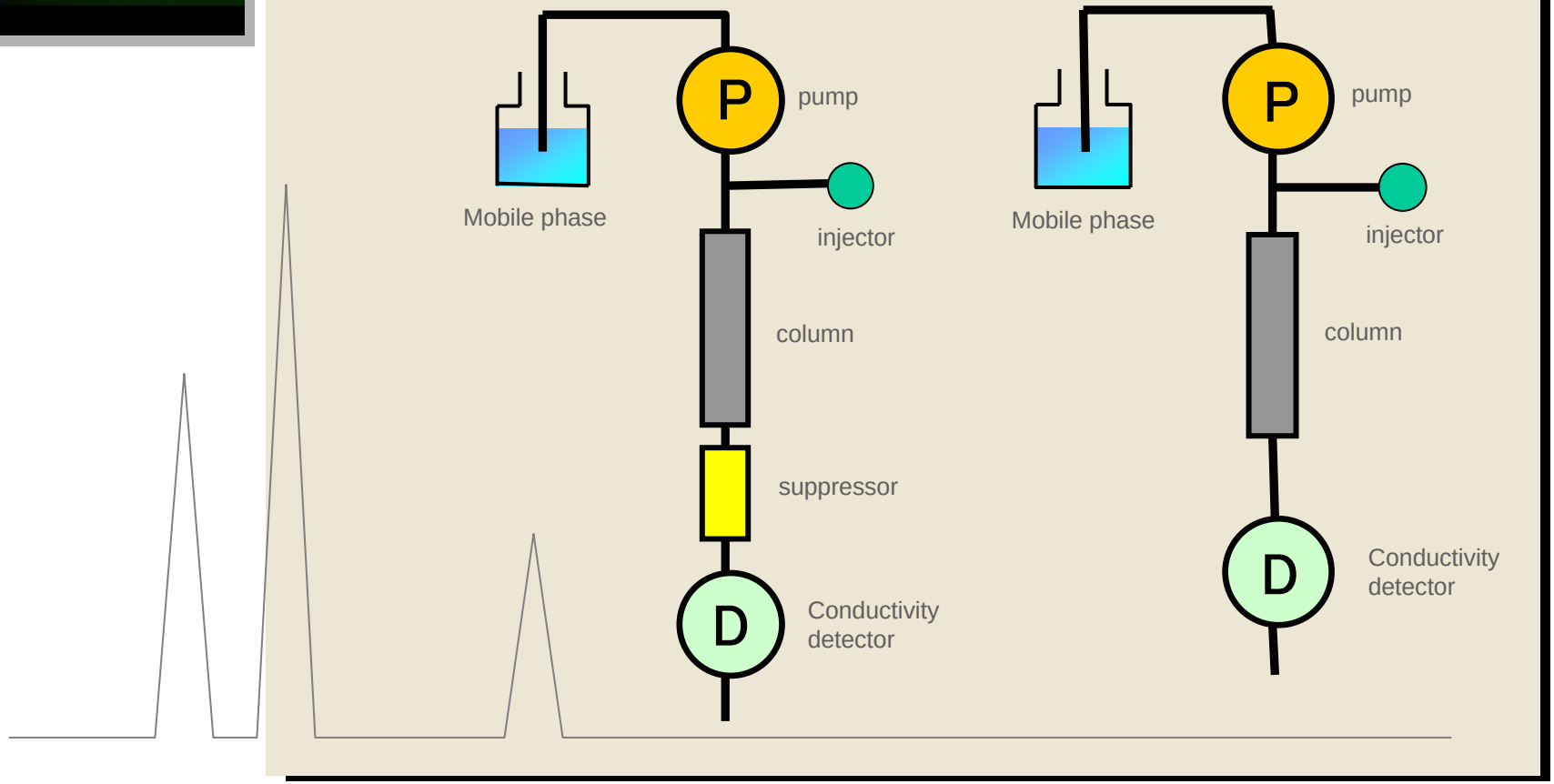
Mobile phase : Buffer solution

Detection : Conductivity detector

3.Separation mode

Ion Chromatography

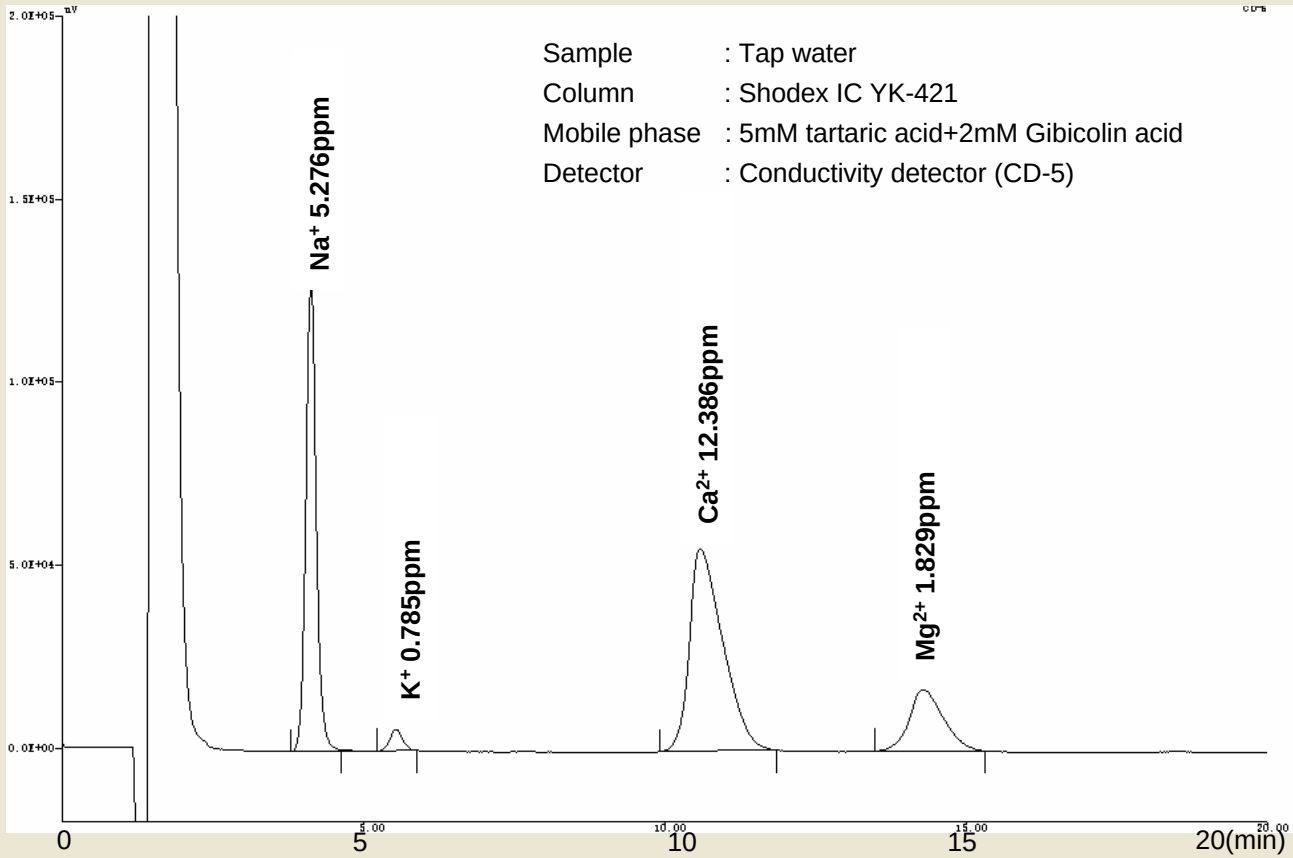
Suppressor and Non-suppressor



3.Separation mode

Ion Chromatography

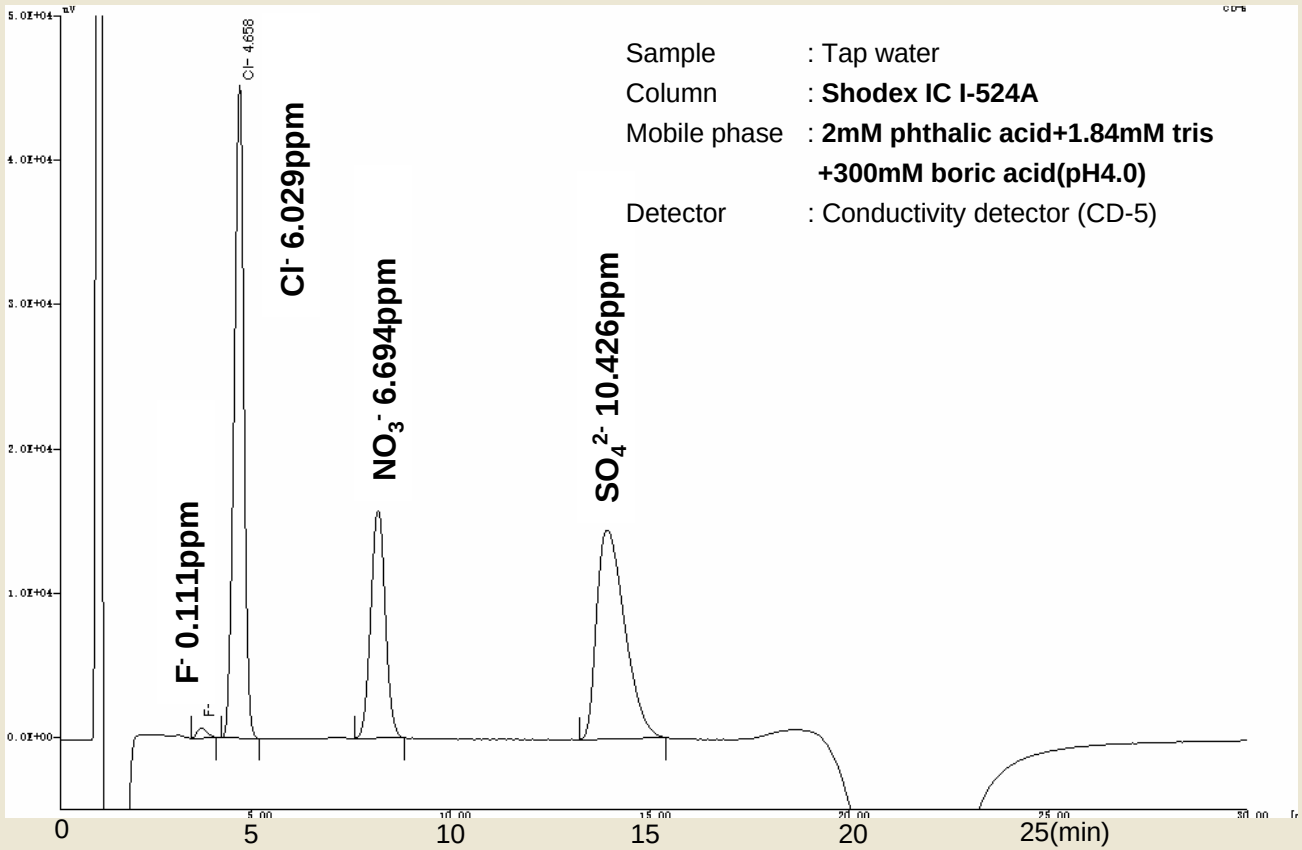
Cation measurement data



3.Separation mode

Ion Chromatography

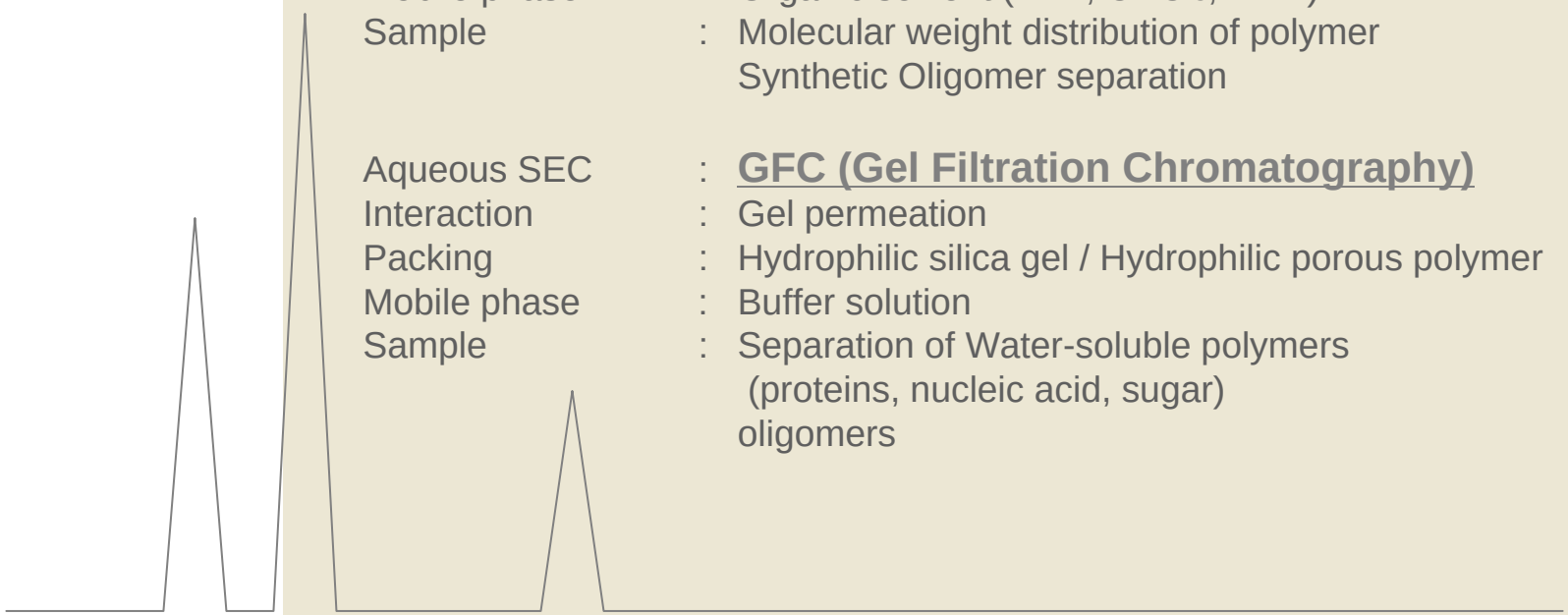
Anion measurement data



3.Separation mode
Size Exclusion Chromatography (SEC)

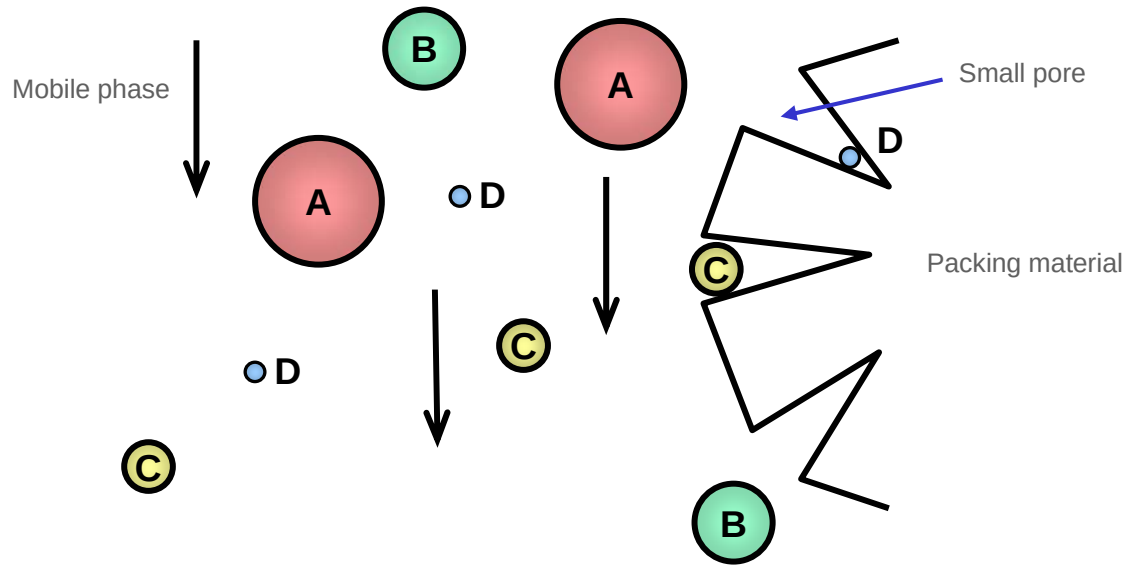
GPC and GFC

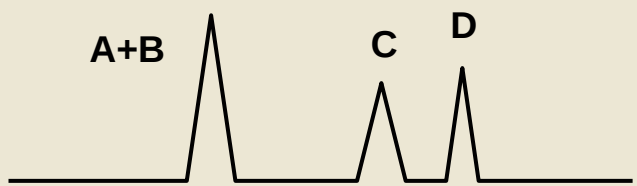
Non-aqueous SEC	:	**GPC (Gel Permeation Chromatography)**
Interaction	:	Gel permeation
Packing	:	Cross-Linked porous Polystyrene
Mobile phase	:	Organic solvent (THF, $CHCl_3$, DMF)
Sample	:	Molecular weight distribution of polymer Synthetic Oligomer separation
Aqueous SEC	:	**GFC (Gel Filtration Chromatography)**
Interaction	:	Gel permeation
Packing	:	Hydrophilic silica gel / Hydrophilic porous polymer
Mobile phase	:	Buffer solution
Sample	:	Separation of Water-soluble polymers (proteins, nucleic acid, sugar) oligomers



3.Separation mode
Size Exclusion Chromatography (SEC)

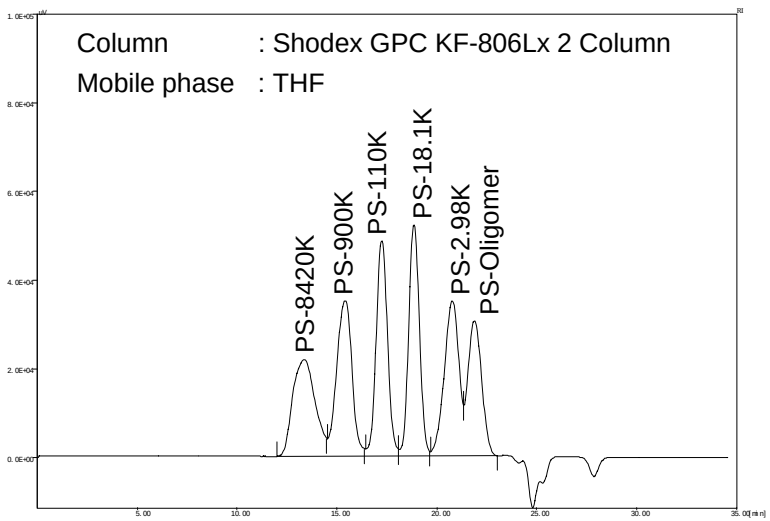
SEC Separation mechanism

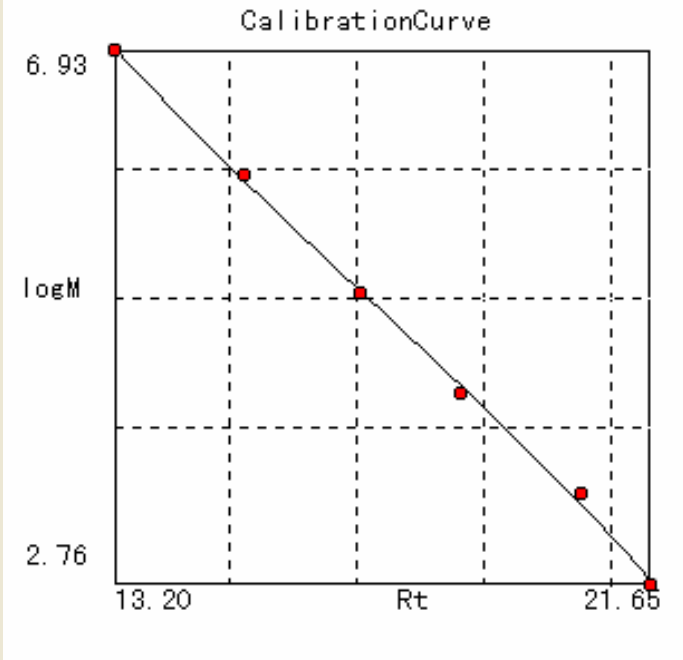




3.Separation mode
Size Exclusion Chromatography (SEC)

Gel permeation chromatography and calibration curve

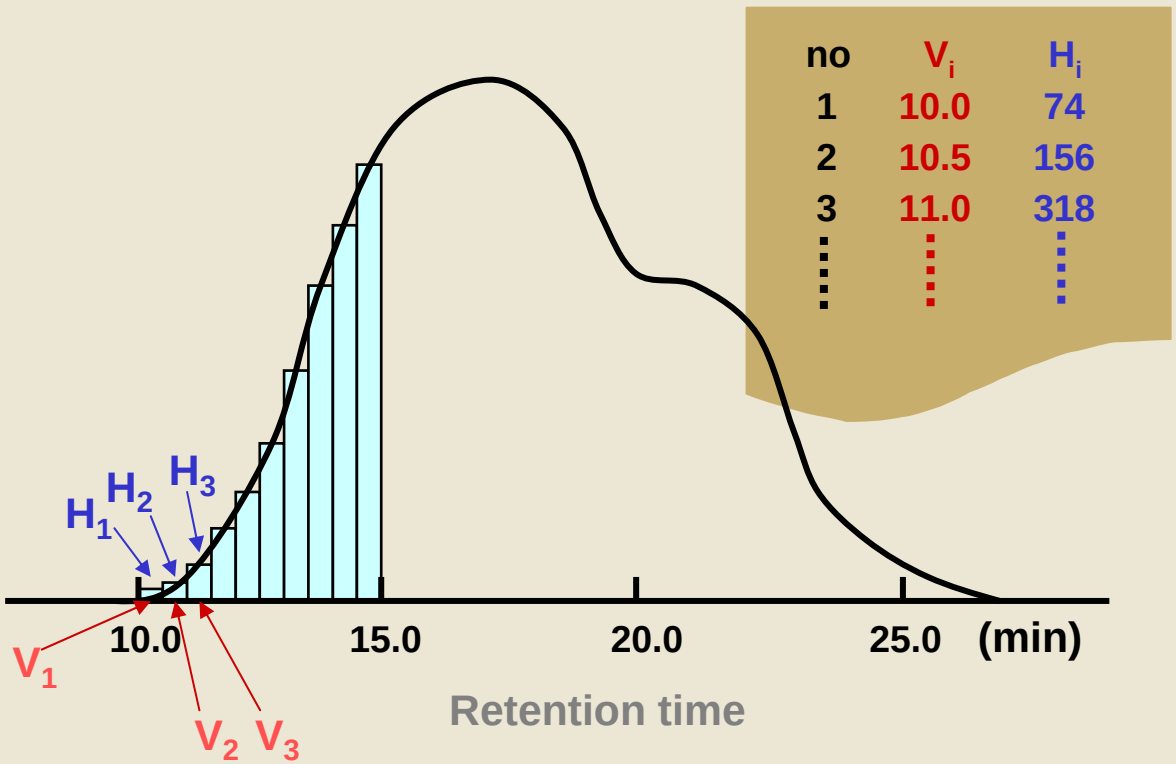




3.Separation mode

Size Exclusion Chromatography (SEC)

Peak analysis of polymer
to calculate molecular weight distribution



3.Separation mode

Size Exclusion Chromatography (SEC)

Molecular weight calculation

N	V_i	H_i	M_i	H_i/M_i	H_iM_i	$H_iM_i^2$
1	11.12	74	2094050	-	-	-
2	11.37	387	1734413	-	-	-
3	11.62	1539	1432619	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
		Σh_i	Σm_i	$\Sigma H_i/M_i$	ΣH_iM_i	$\Sigma H_iM_i^2$

$\overline{Mn} = \Sigma H_i / \Sigma H_i/M_i = 15.5 \times 10^4$

$\overline{Mw} = \Sigma H_iM_i / \Sigma H_iM_i = 28.6 \times 10^4$

$\overline{Mz} = \Sigma H_iM_i^2 / \Sigma H_iM_i = 46.5 \times 10^4$

$D = Mw/Mn = 1.84$

3.Separation mode

Size Exclusion Chromatography (SEC)

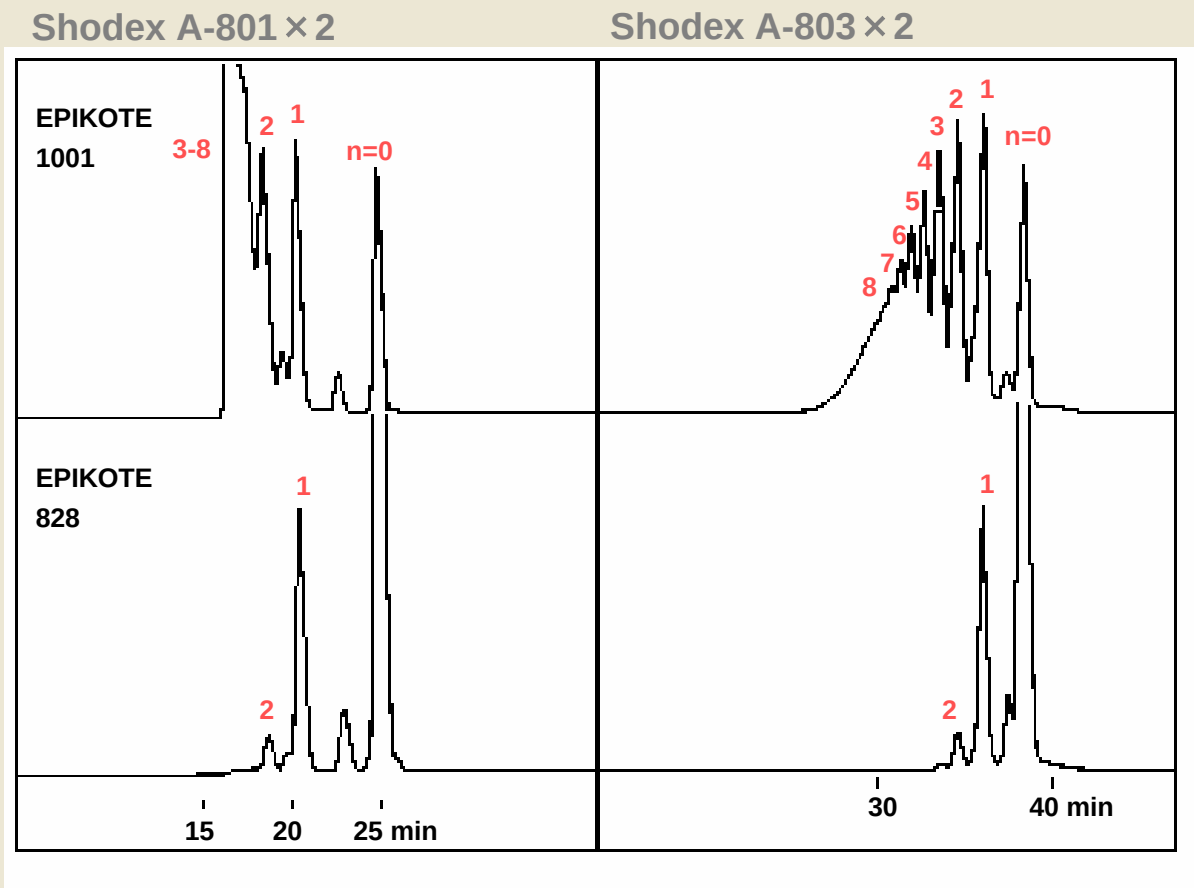
Column selection

Molecular weight of the sample	:	Exclusion limit molecular weight
Ability to dissolve the sample	:	Applicable to packing materials
Molecular weight distribution	:	Range of calibration curve

3.Separation mode

Size Exclusion Chromatography (SEC)

Column suited to the sample in terms of molecular weight



Eluent : THF Flow rate : 1.0ml/min

3.Separation mode

Size Exclusion Chromatography (SEC)

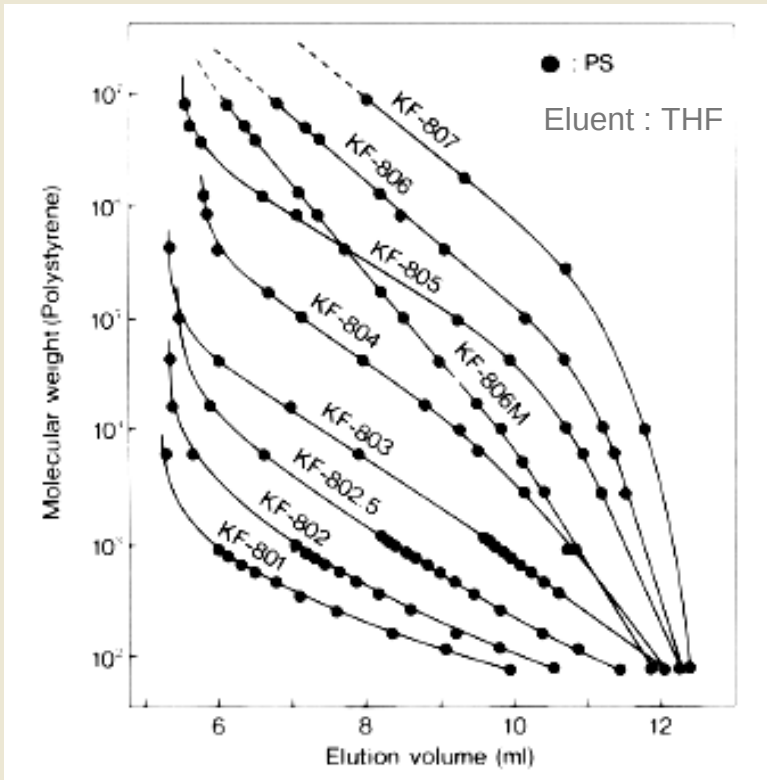
Solvent and column

Solvent	Column
THF	Finepak GEL 101F Shodex KF series
$CHCl_3$	Finepak GEL 101C Shodex K series
DMF	Shodex KD series
H_2O, Buffer solution	Shodex SB series Shodex KS series

3.Separation mode

Size Exclusion Chromatography (SEC)

Calibration curves for columns



3.Separation mode

Columns for exclusive use

Columns for Exclusive use

Amino acids : Aapak (Cation exchange)

Organic acid : Shodex Ionpak KC-811
(ion exclusion and partition & adsorption)

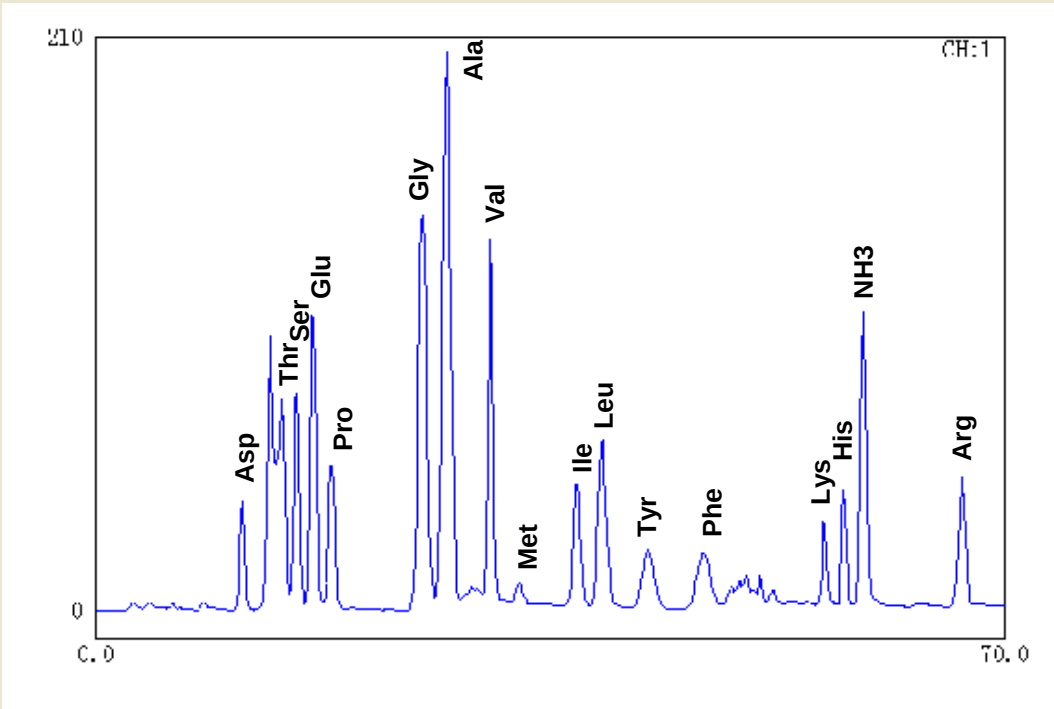
Sugar : Shodex Ionpak KS series (aqueous SEC)
Shodex Sugar series (ligand exchange)
Finepak SIL NH2-5 (Normal phase)
Finepak GEL SA-121 (Strong anion exchange)

N-methyl carbamate : Carbamatepak (Reversed phase)

3.Separation mode

Columns for exclusive use

Amino Acid Analysis

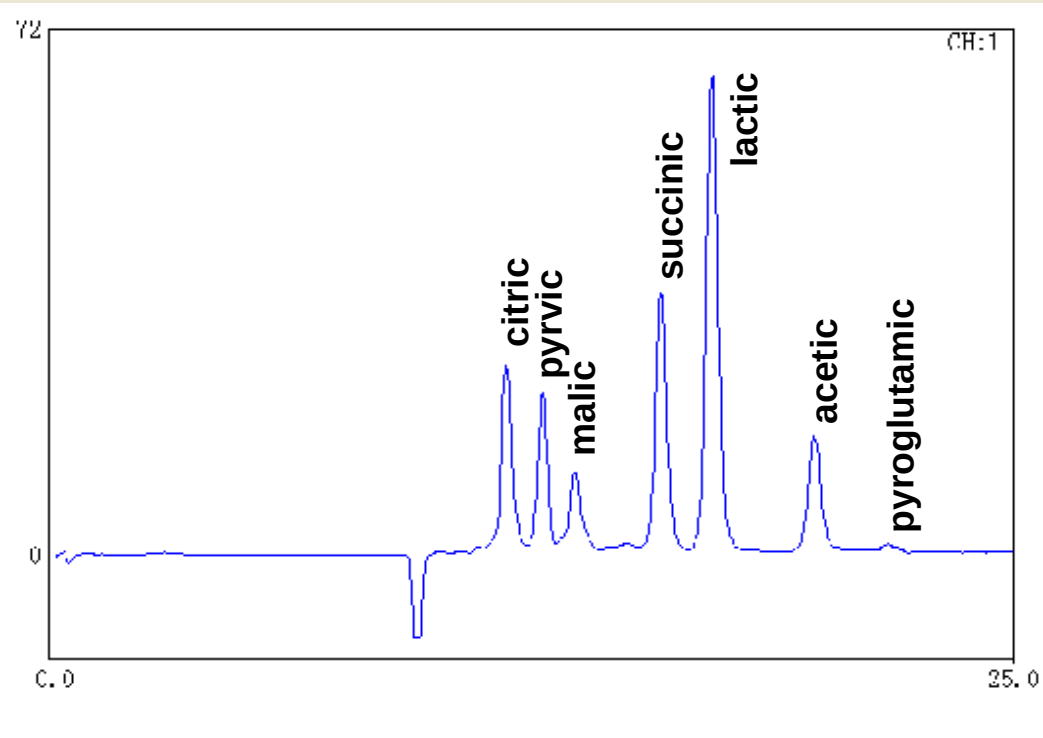


Column	:	AApak Na II-H
Mobile phase	:	Sodium citrate buffer Stepwise gradient
Detection	:	OPA post label Ex 345nm Em 445nm
Sample	:	Sake

3.Separation mode

Columns for exclusive use

Organic Acid Analysis



Column	:	Shodex Ionpak KC811x2
Mobile phase	:	
Detection	:	BTB post label UV 445nm
Sample	:	Sake

3.Separation mode

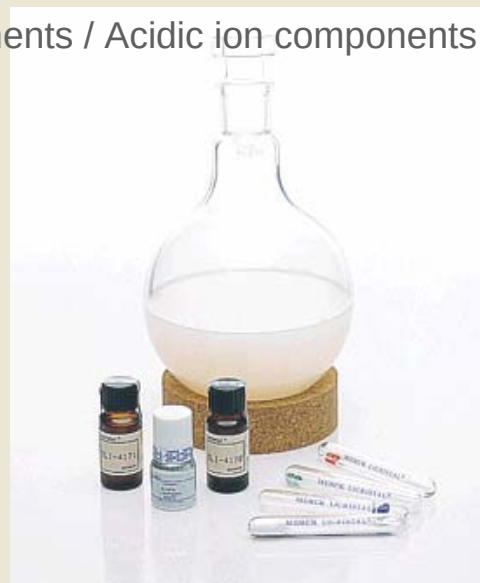
Ion suppression method & Ion-pair chromatography

Ion suppression method & Ion-pair chromatography

Separation method to analyze ionic compounds by reversed-phase chromatography

Ion suppression method : Acidic ion components

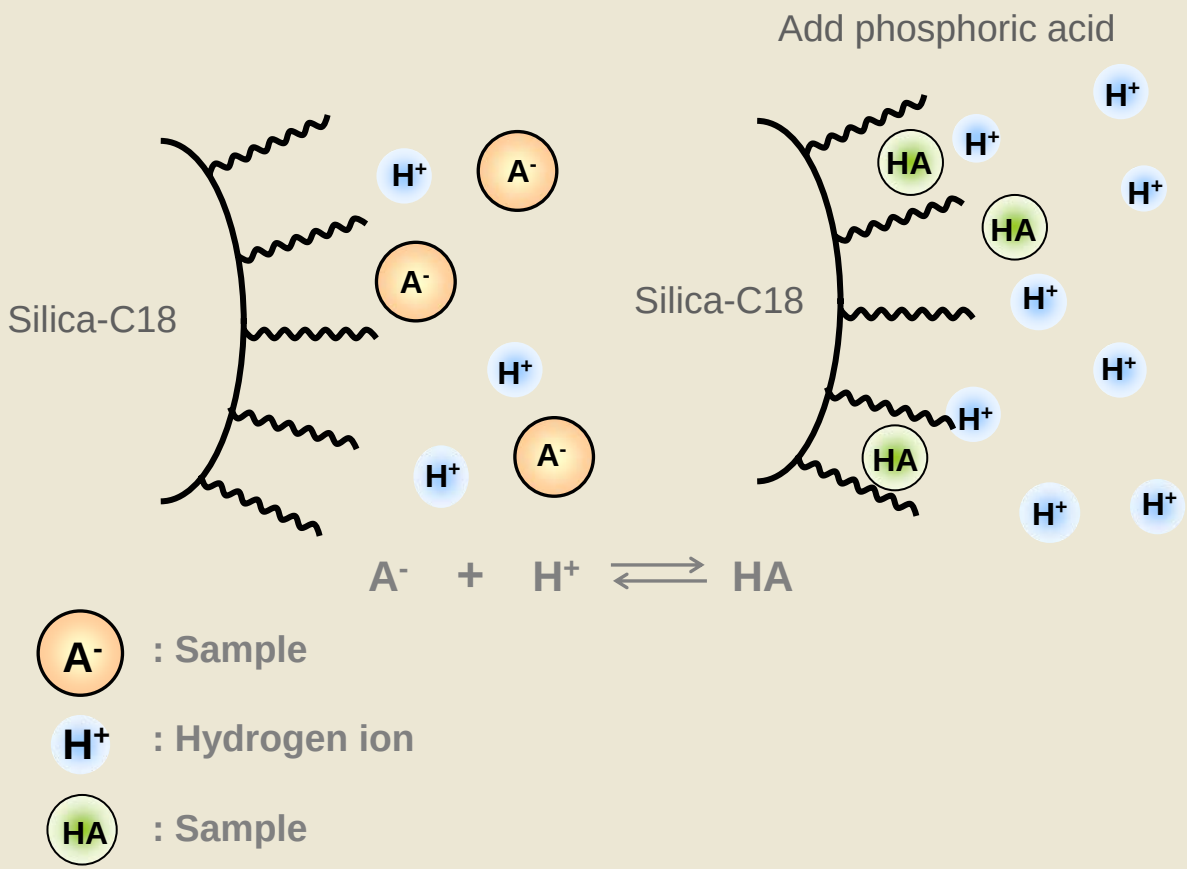
Ion pair chromatography : Basic ion components / Acidic ion components



3.Separation mode

Ion suppression method & Ion-pair chromatography

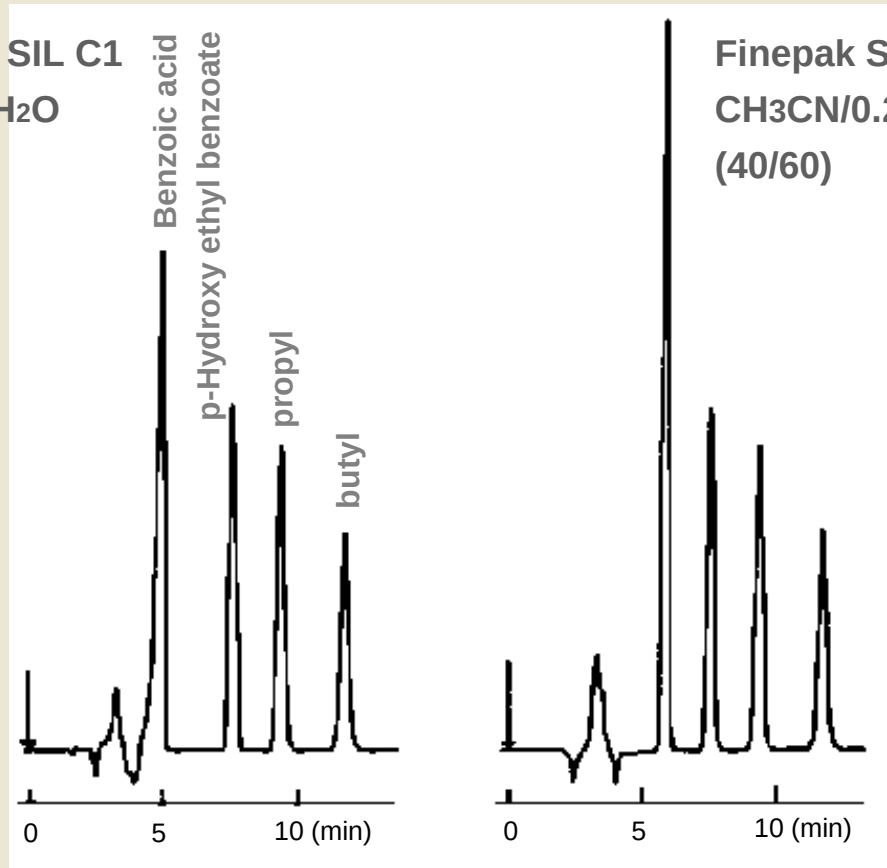
Diagram of Ion suppression method



3.Separation mode

Ion suppression method & Ion-pair chromatography

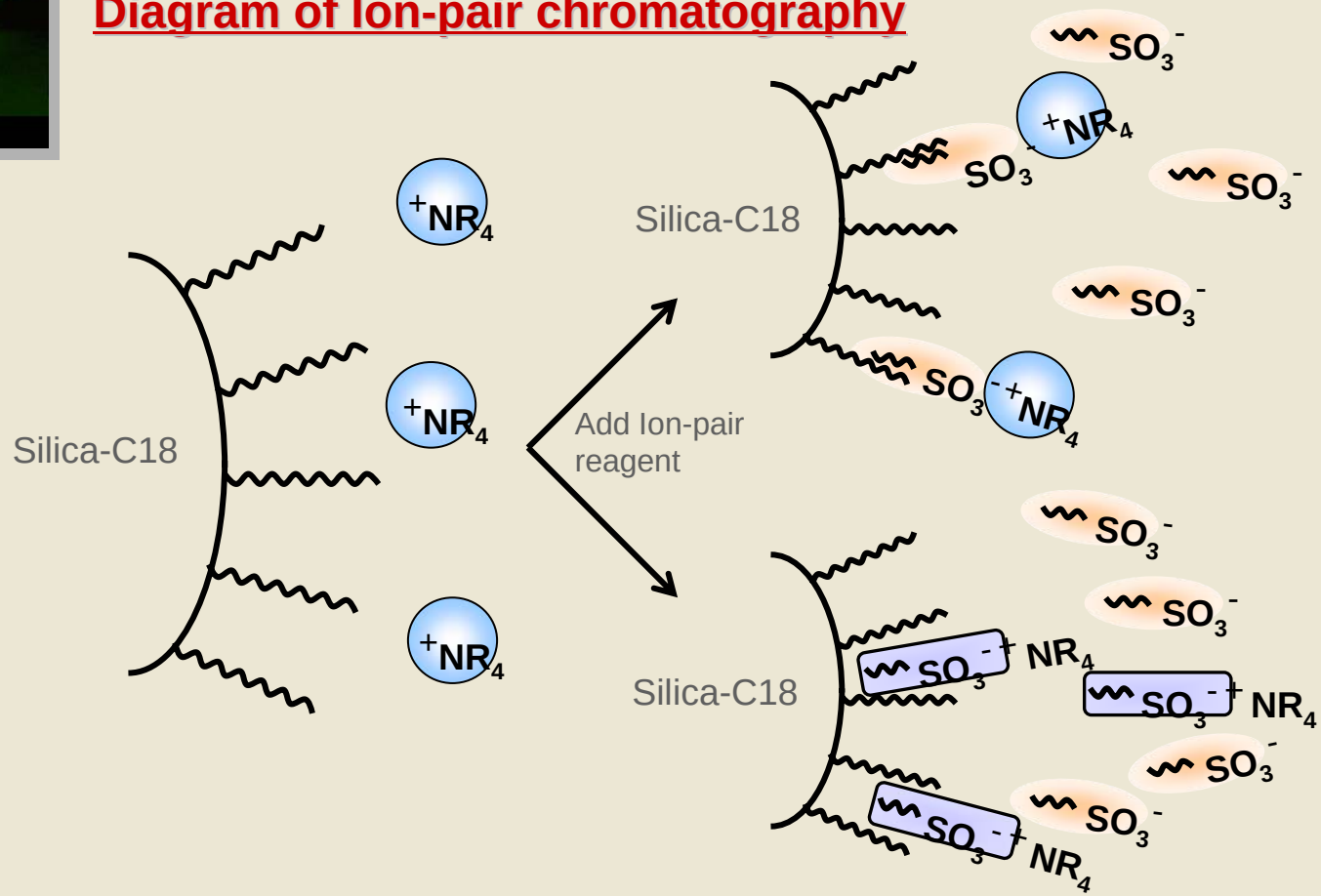
Chromatogram when Ion suppression method is used



3.Separation mode

Ion suppression method & Ion-pair chromatography

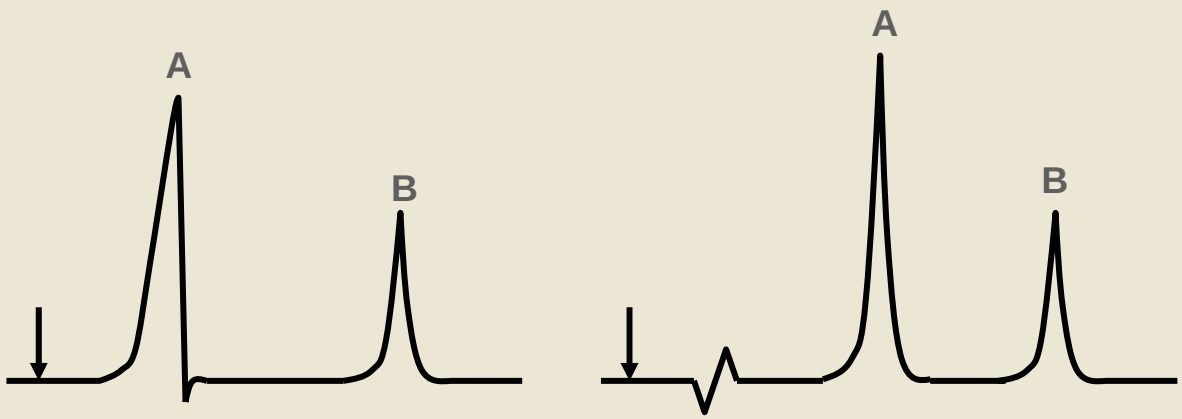
Diagram of Ion-pair chromatography



3.Separation mode

Ion suppression method & Ion-pair chromatography

Chromatogram when Ion-pair chromatography is used



Without Ion-pair reagent

With Ion-pair reagent

Typical ion reagents

Acidic ions : Tetra alkyl ammonium halide

Basic ions : l-Alkyl sulfonate

3.Separation mode

Ion suppression method & Ion-pair chromatography

Ion-pair chromatography

Effects of basic additives

- Stable pH
- Longer retention time
- Ion pair reagent effect

3.Separation mode

Ion suppression method & Ion-pair chromatography

Acid and basic sample for Reversed phase LC

Method	Sample	Reversed phase
Ion suppression	Weak acidic sample	phosphoric acid acetic acid perchloric acid trifluoroacetic acid
Ion pair	Acidic sample Basic sample	Tetra alkyl ammonium halide l-Alkykl surfonate (acetic acid) (trifluoroacetic acid)
Addition of salt		phosphate citrate

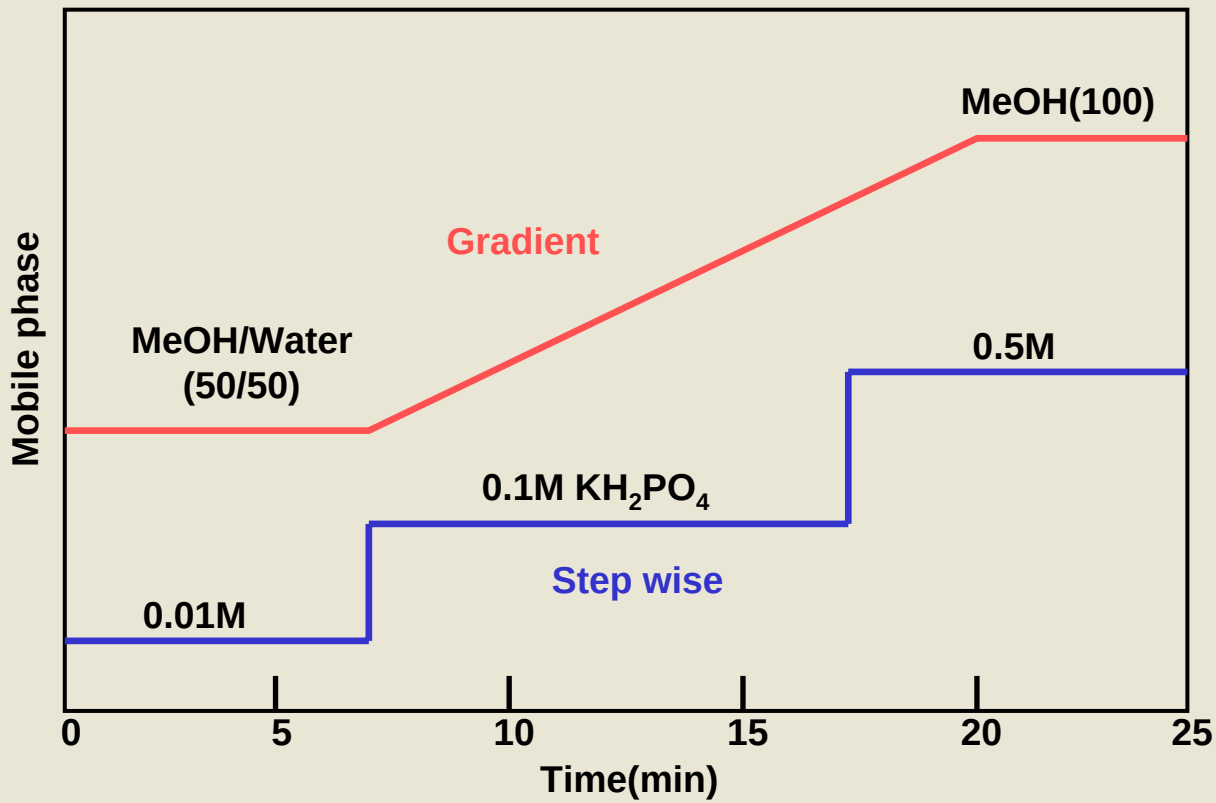
Review of Section 3

4 separation modes
Polarity of packing material and solvent
Change of mobile phase and elution
Ion suppression method and Ion pair method
Salt effect

4. Gradient elution method

4. Gradient elution method

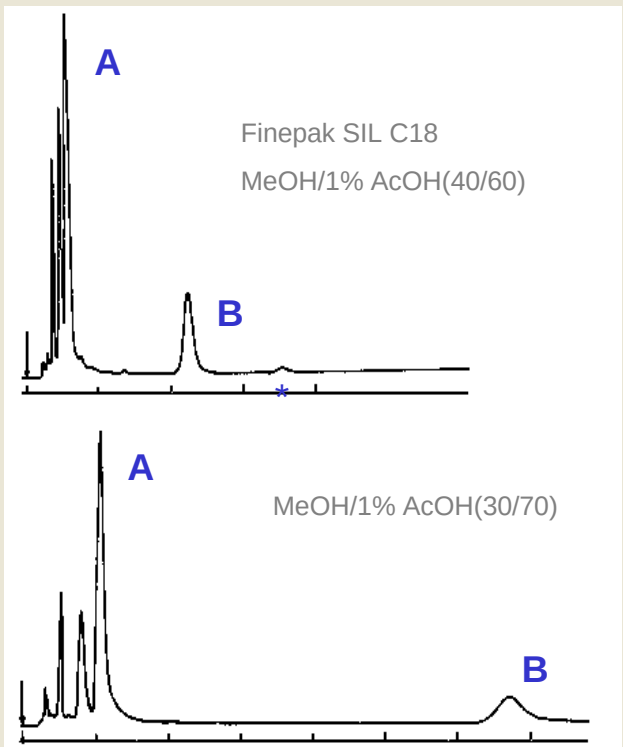
For separation of a sample containing many components



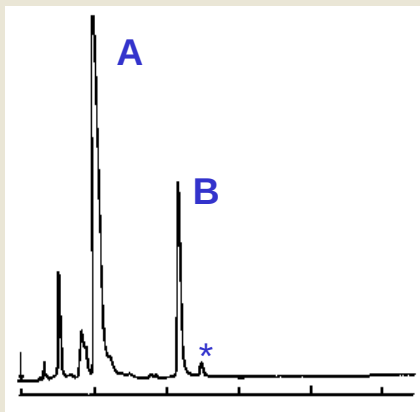
4. Gradient elution method

Advantage of gradient elution method

Isocratic elution method



Gradient elution method



MeOH/1% AcOH
30/70→45/55
Linear Gradient、16min

A : Chlorogenic acid
B : Rutin
*** : Impurity**

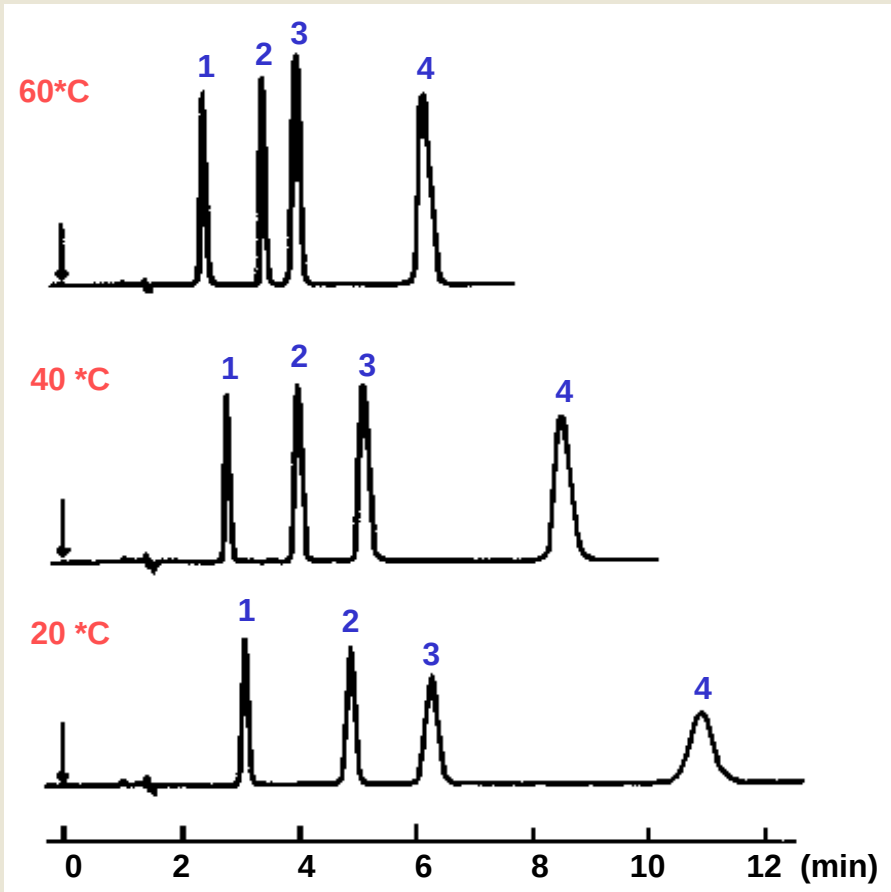
4. Gradient elution method

Precautions in gradient elution method

- Can the gradient save time ?
- Reproducibility
- Baseline
- Ghost peak
- Salt

4. Gradient elution method

Effect of temperature on retention time



Finepak SIL C18

CH_3CN/H_2O(90/10)

Sample :

1. Benzene
2. Anthracene
3. Pyrene
4. Benz(a)pyrene

Review of Section 4

Gradient elution method
Temperature effect

5. Detector

5. HPLC detectors

HPLC detectors

UV-VIS(Absorption)

PDA (Absorption)

Differential refractometer(Refractive index)

Fluorometric (Florescence)

Electrochemical (ECD) (Oxidation -reduction)

Conductivity

Mass

Chiral (OR)

Circular dichroism (CD)

5. HPLC detectors

UV/Vis detector

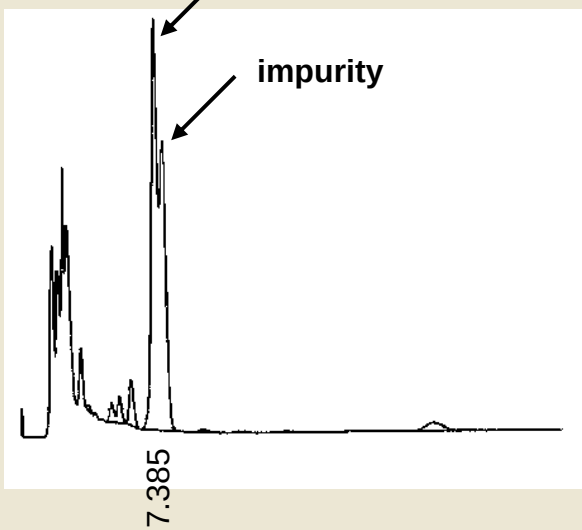
- Selective detection minimizing effects from other components

- High sensitivity detection at maximum absorption wavelength

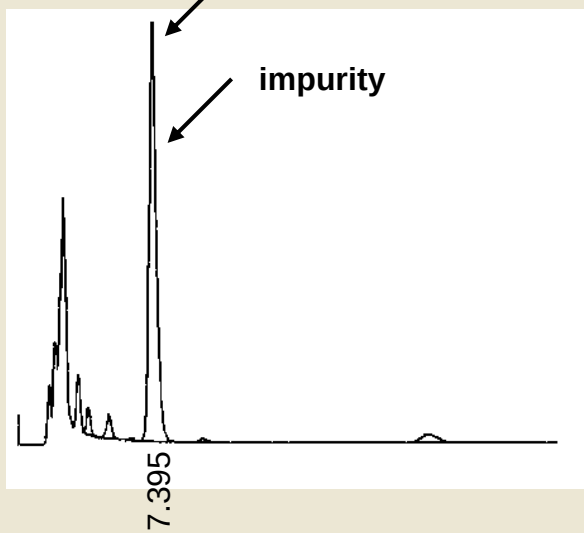
5. HPLC detectors

Improved selectivity

Traditional medicine



Wavelength=260nm

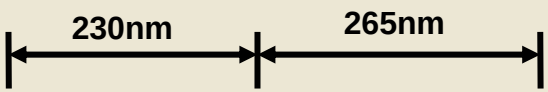


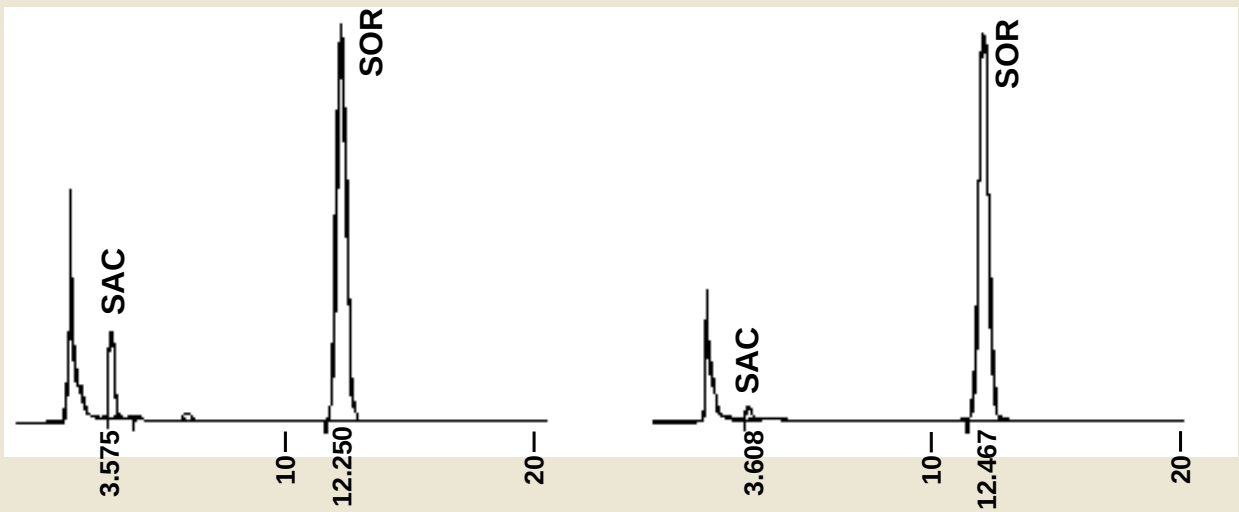
Wavelength=340nm

5. HPLC detectors

Improved sensitivity

Saccharin (SAC) and sorbin acid (SOR)



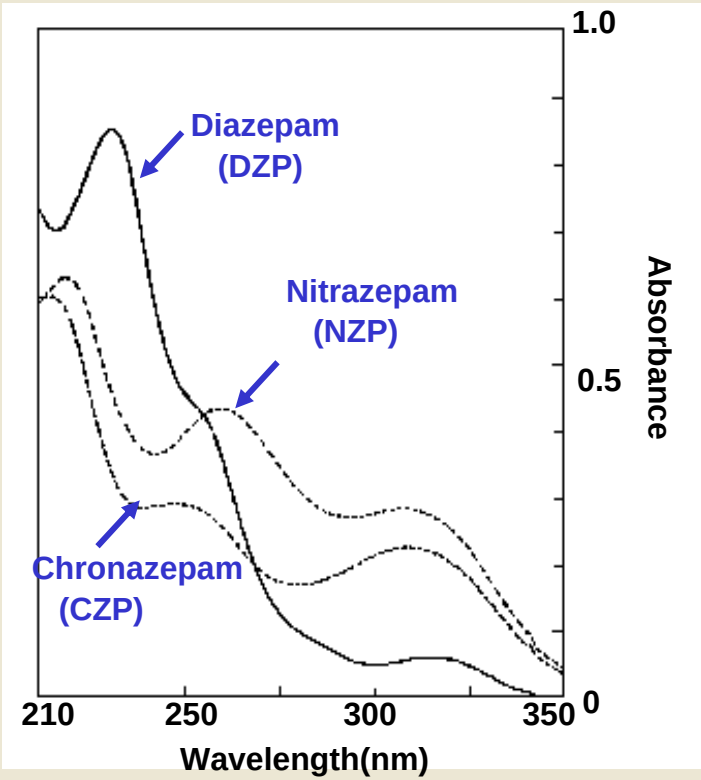


Wavelength programming

Fixed wavelength at 265nm

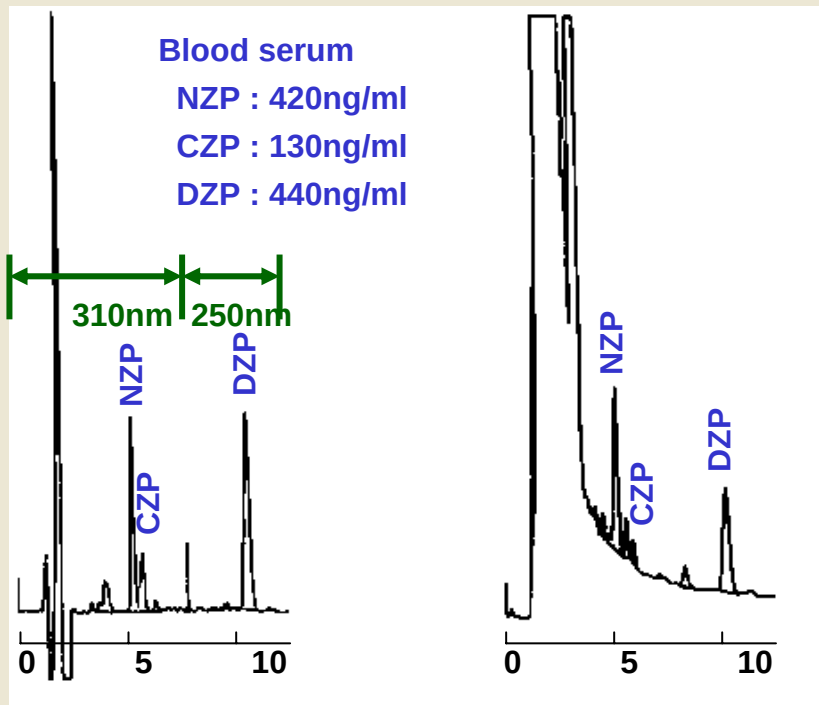
5. HPLC detectors

UV spectrum measurement
to find wavelength effective for wavelength programming



5. HPLC detectors

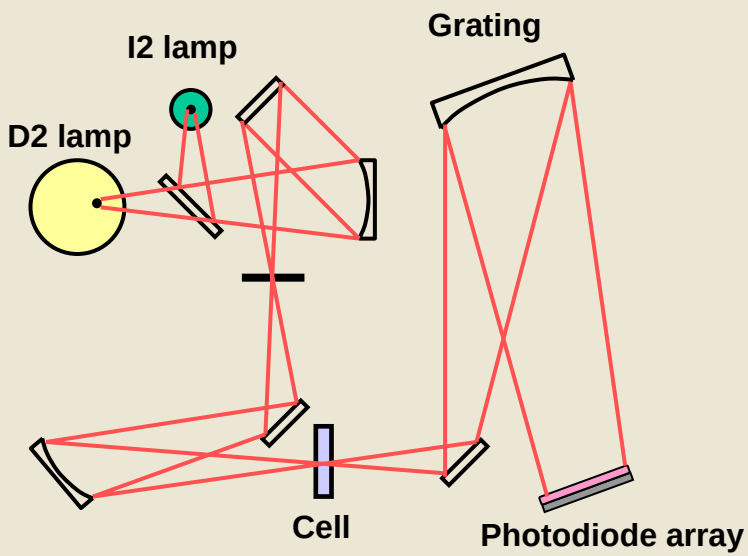
Wavelength programming and fixed wavelength

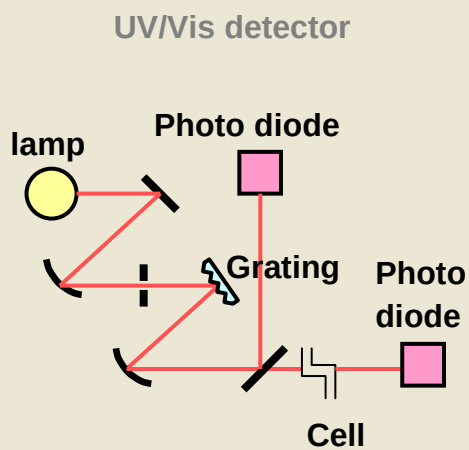


Wavelength programming Fixed wavelength

5. HPLC detectors

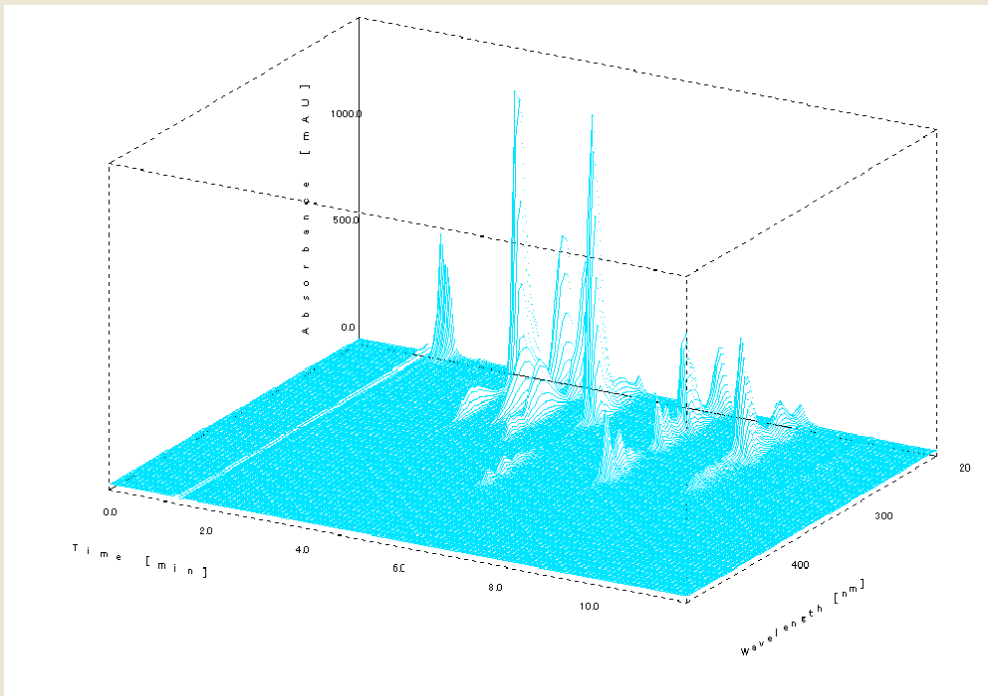
Optics of Multi-wavelength detector





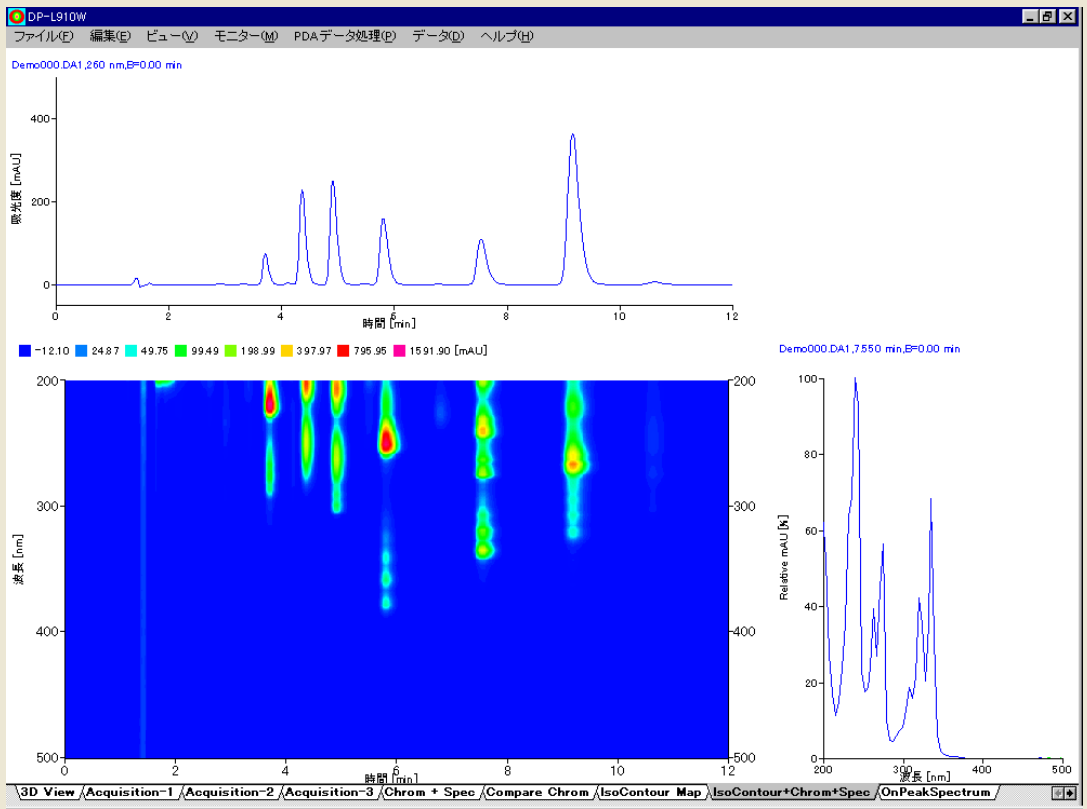
5. HPLC detectors

Multi-wavelength detector
3D chromatogram



5. HPLC detectors

Multi-wavelength detector
Cont. data



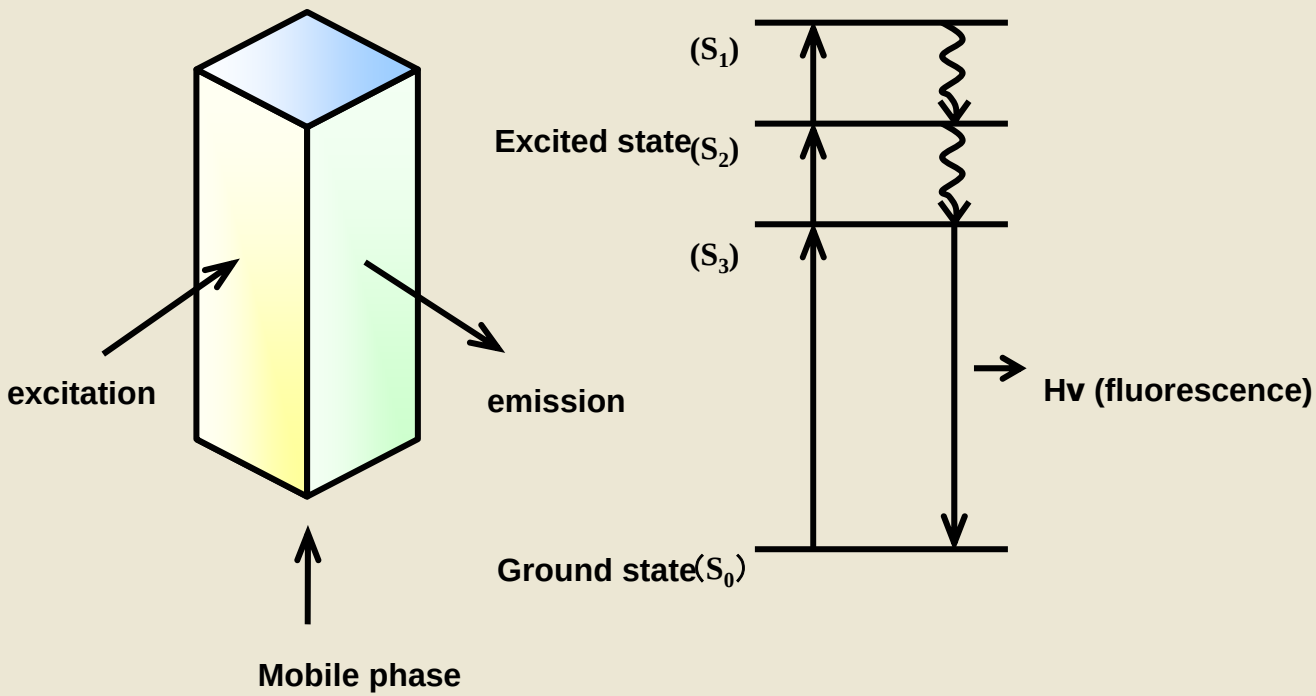
5. HPLC detectors

Features of Multi-wavelength detector

1. Spectrum collection at any time
2. Library search
3. Purity check
4. Quantitative analysis at 6 wavelengths

5. HPLC detectors

Principle of Fluorescence detector



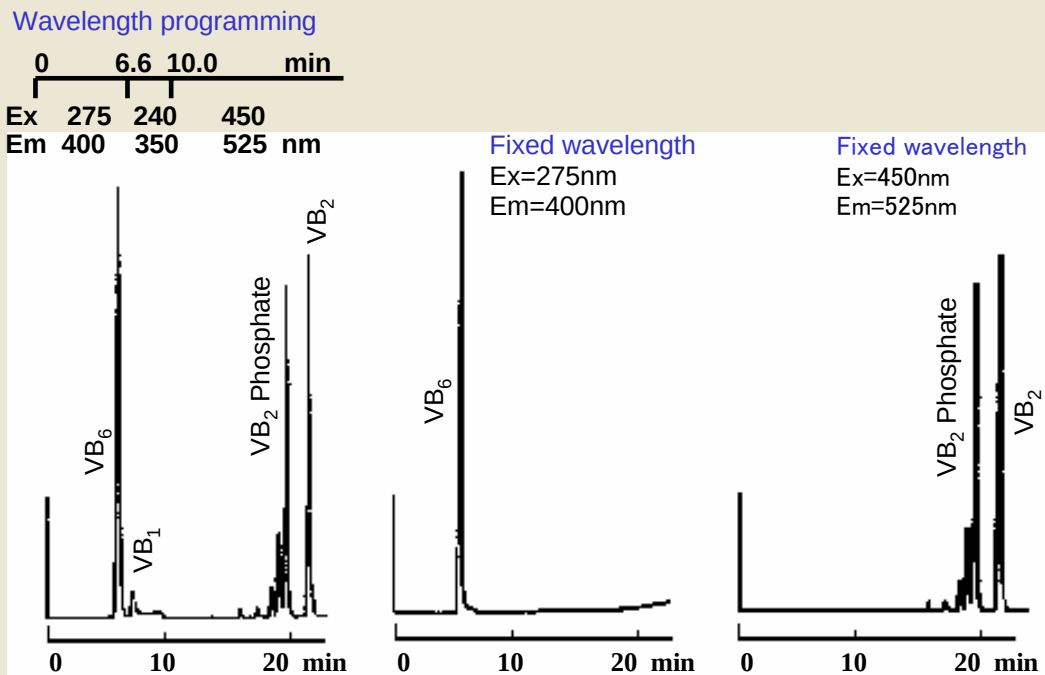
5. HPLC detectors

Features of Fluorescence detector

1. Selective detection
2. Detection at any excitation or emission wavelength
3. High sensitivity

5. HPLC detectors

Wavelength programming by Fluorescence detector

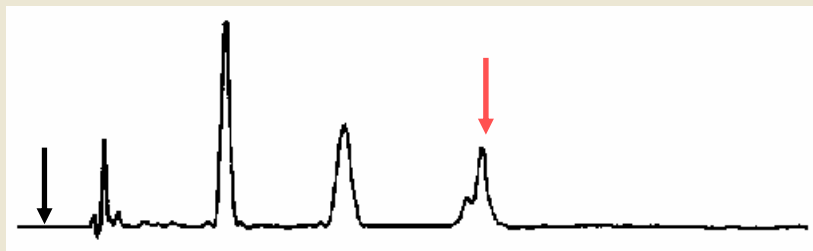


Column : Finepak SIL C18S
Mobil phase : MeOH/Phosphate Buffer Gradient

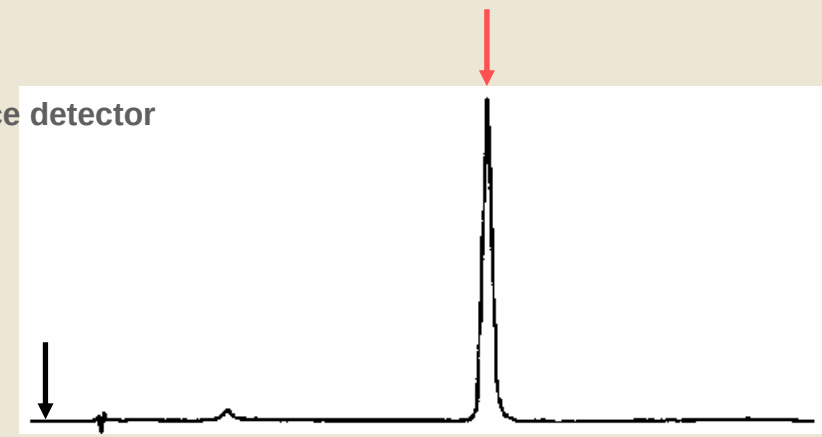
5. HPLC detectors

Selectivity of UV detector and Fluorescence detector

UV detector

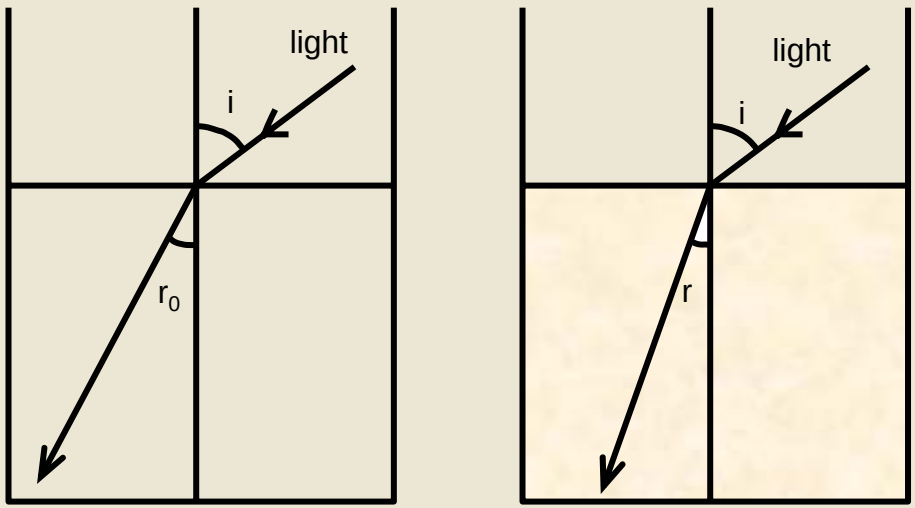


Fluorescence detector



5. HPLC detectors

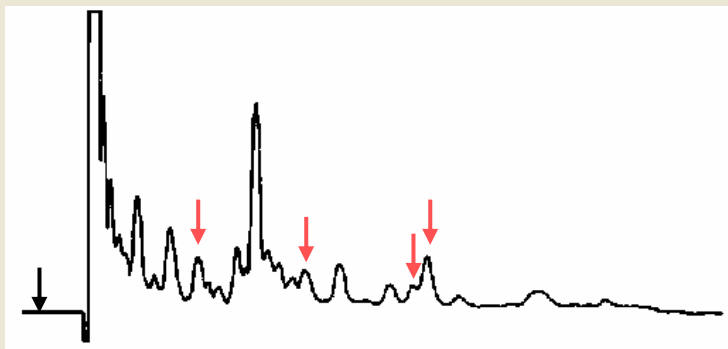
Principle of RI detection



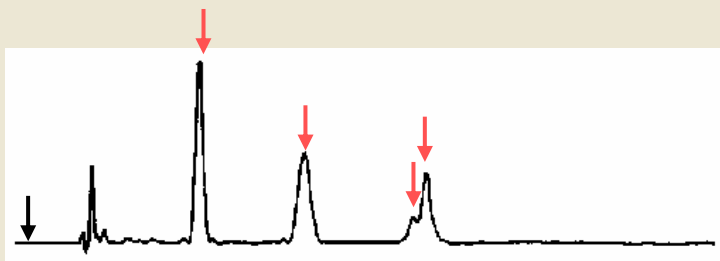
$r_0 > r$

5. HPLC detectors

UV detector and RI detector



RI detector



UV detector

5. HPLC detectors

Considerations for IR detection

1. Temperature change
2. Replacement of solvent (reference cell and sample cell)
3. Unstable when solvent mixed
4. Replacement of solvent inside column

5. HPLC detectors

Detectors

	UV	Fluorescence	RI
Sensitivity	ng	pg	μg
Detection selectivity	selective	highly selective	universal
Temperature Influence	small	small	large
Gradient elution	possible	possible	impossible

5. HPLC detectors

Label method

Samples absorb less UV/Vis light .
Samples do not fluoresce.

↓

Improved sensitivity and selectivity required

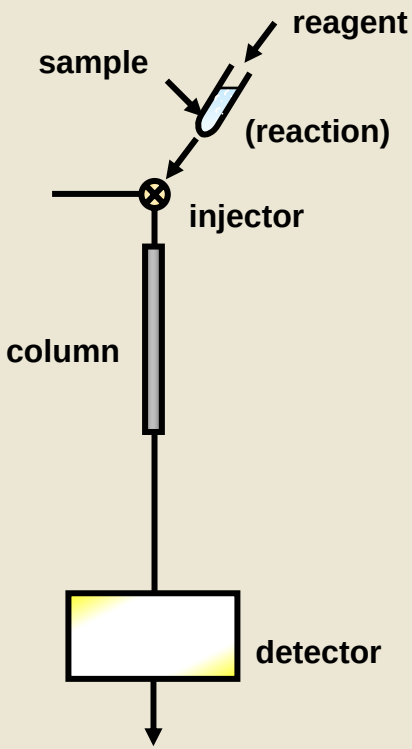
↓

Label method

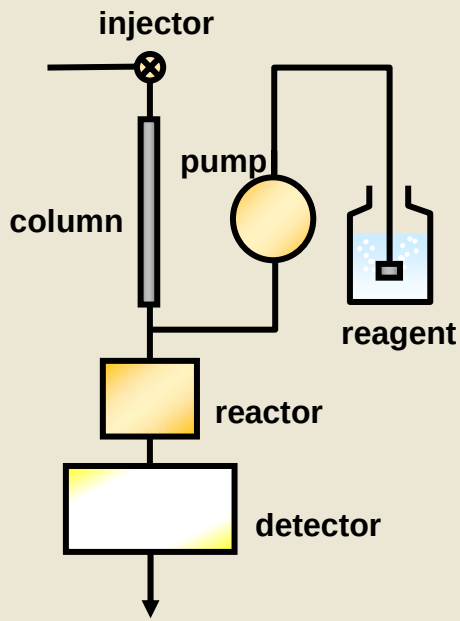
5. HPLC detectors

Label method

Pre-label method



Post-label method



5. HPLC detectors

Label method

Post-label method

Aminoacid	0PA ninhydrine	Fluorescence Absorption in Visible range
Sugar	guanidine	Fluorescence
Organic acid	brom thymol blue	Absorption in Visible range
Catecholamine	ethylenediamine THI	Fluorescence Fluorescence
Bile acid	NAD HSD	Fluorescence Fluorescence

5. HPLC detectors

Pre and Post column derivatization method

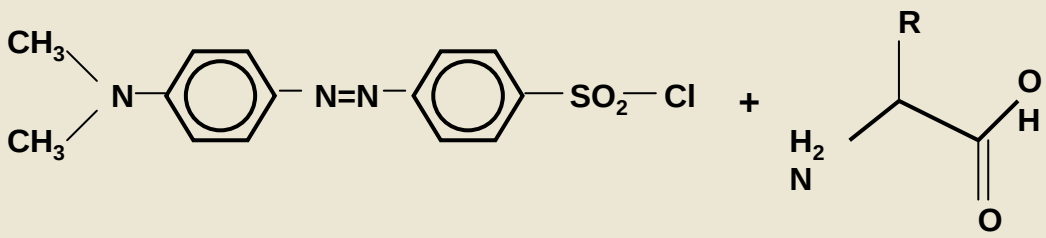
	Pre-column	Post-column
LC system required	Standard system	Reaction system is required
Reproducibility	less than post-column	good
Operation	for all samples	only reagents
Reagents	wide range	limited
Applicability	spot	routine

5. HPLC detectors

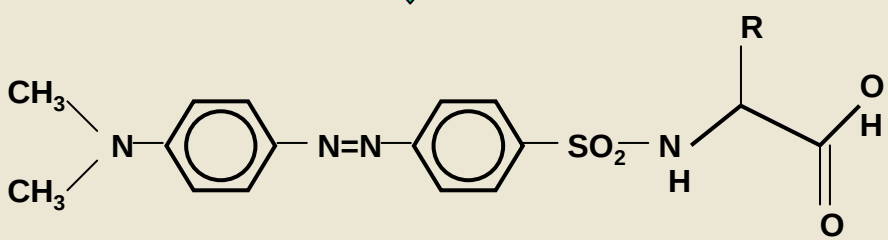
Pre-column derivatization method

Dabcyl-Cl

Amino acid



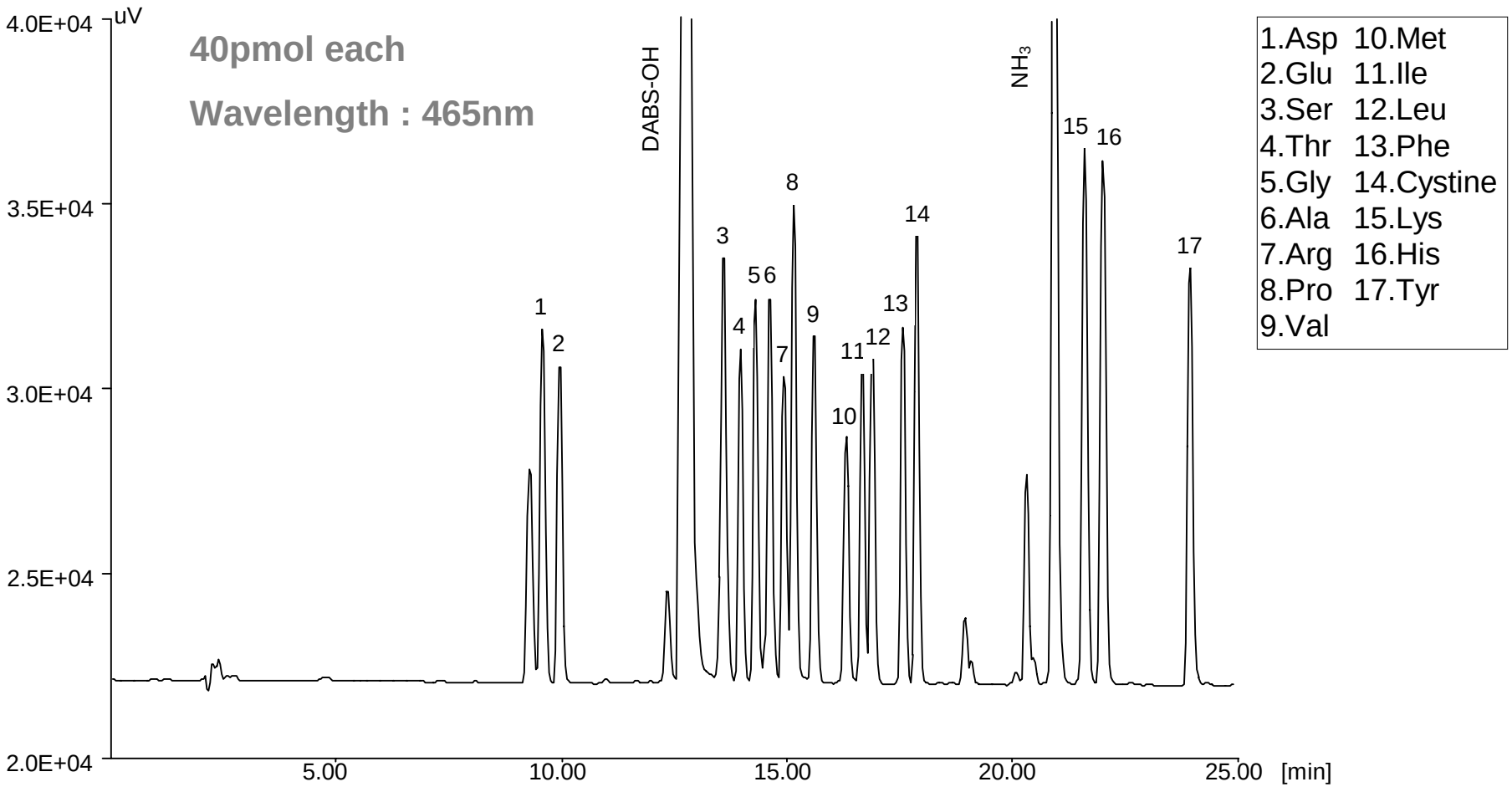
pH8.1、70℃、
12min



Dabcyl - Amino acid

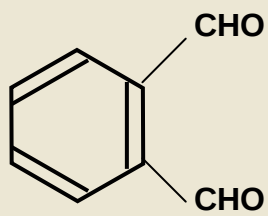
5. HPLC detectors

Separation of Dabcyl - Amino acid



5. HPLC detectors

Post-column derivatization method

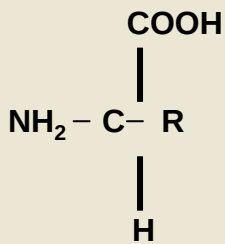


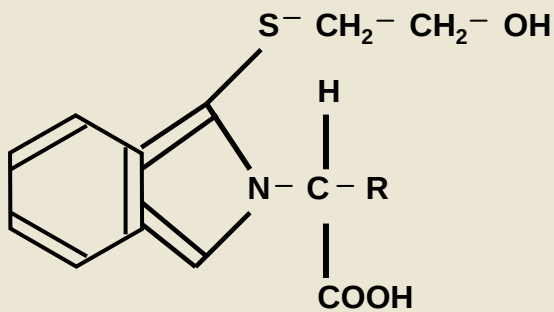
orthophthalaldehyde (OPA)

2-mercapto ethanol

+ HS– CH_2– CH_2 – OH +

Amino acid

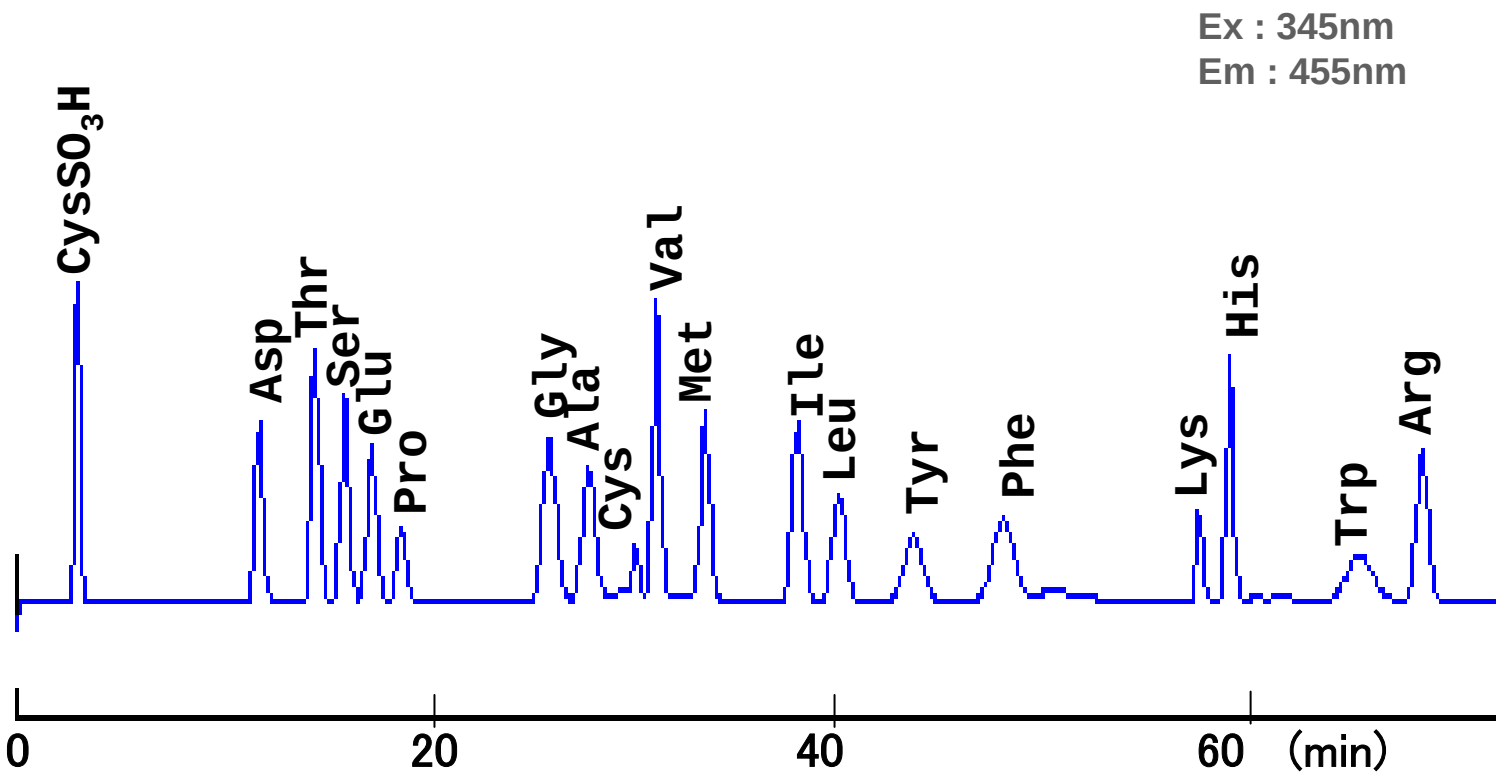




Derivative compound

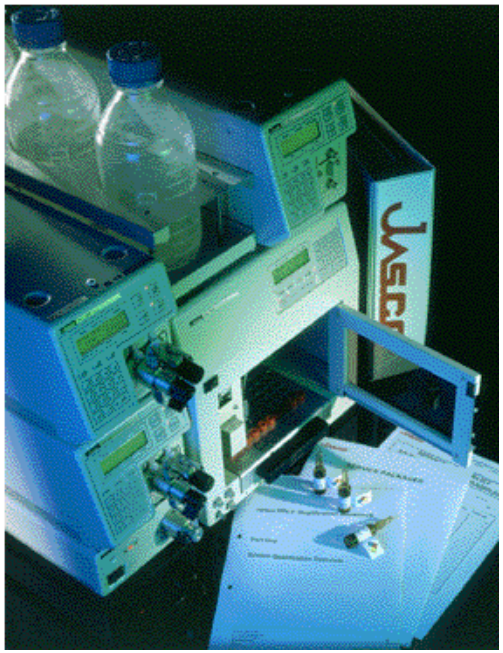
5. HPLC detectors

Post column derivatization method



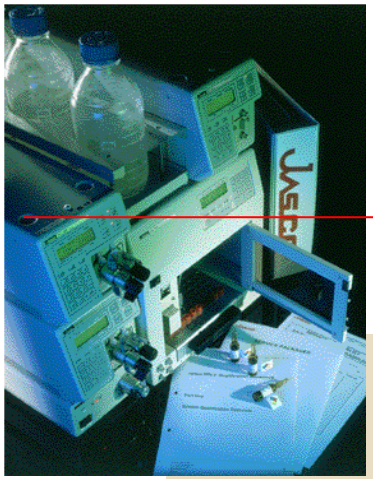
Review of Section 5

Detectors
Selectivity and sensitivity
Pre-/Post- column derivatization methods



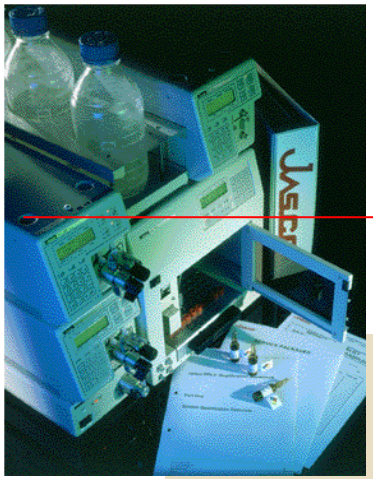
The JASCO advanced technology team has again met the challenge and designed a new line of HPLC instruments, The LC-1500series more than satisfies in response to the growing demand for greatly expanded HPLC analyses in the fields of not only biochemistry, pharmaceutical and medical science, but also in the areas of among other organic and inorganic compounds, foods, agricultural sciences, polymeric and natural substances and pollution. The LC-1500 series comprises pumps, detectors, autosamplers, its own column oven and other units each having built-in intelligence and incorporating many features with much higher levels of operability and reliability in addition to multiple functions, higher performance and higher accuracy than before, making them the most advanced instruments available.

6. Data processing



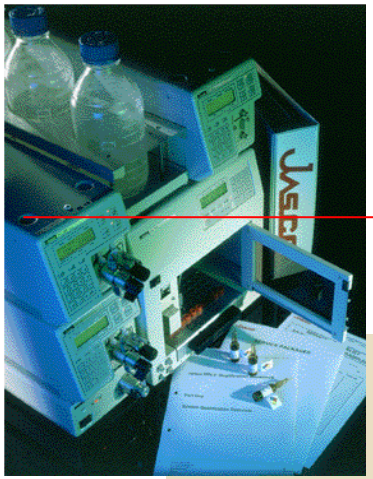
Data processing in HPLC

1. Qualitative analysis
2. Quantitative analysis
3. Molecular weight distribution

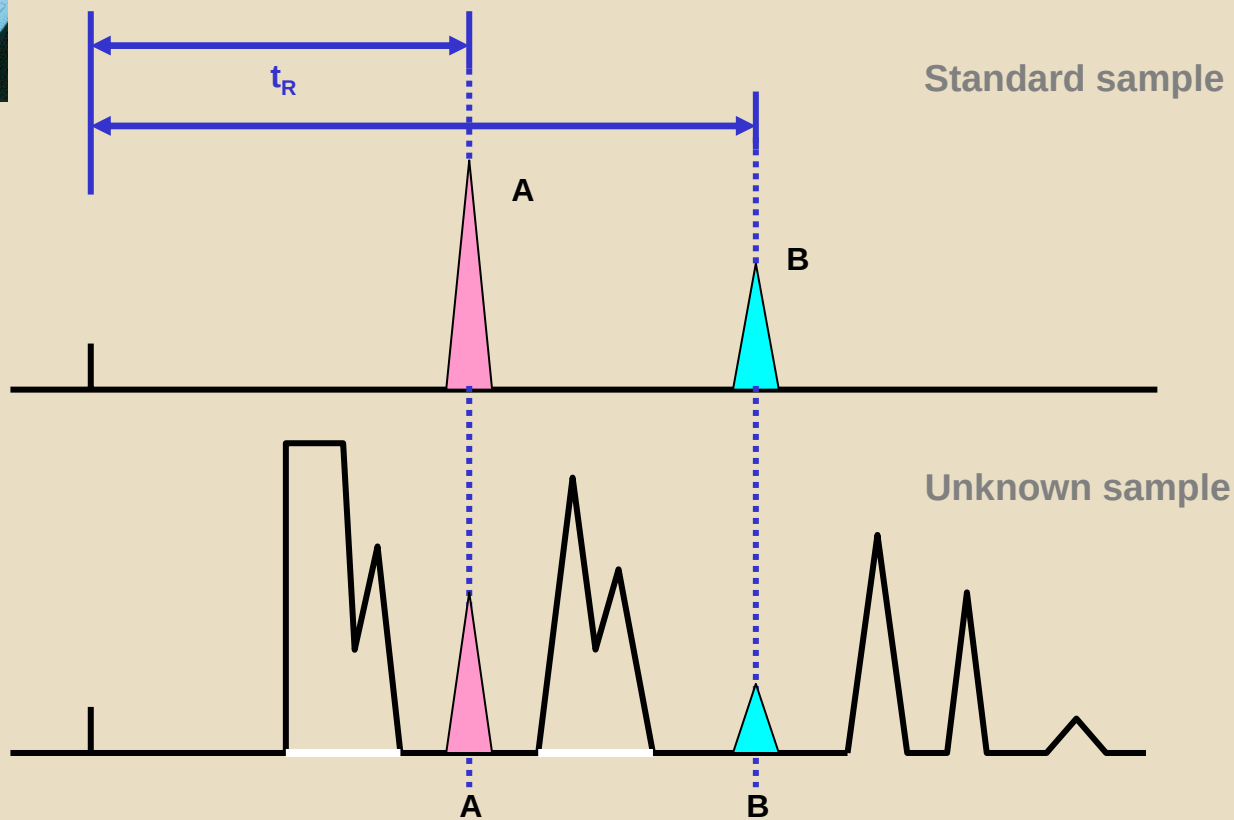


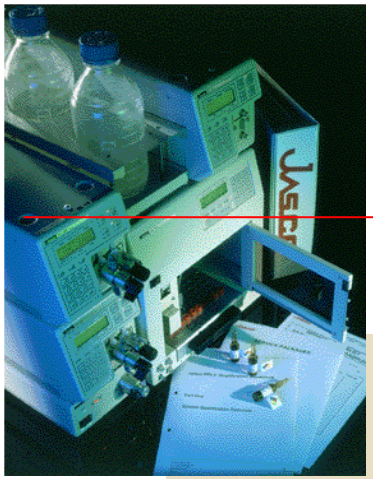
Qualitative analysis

1. Retention time
2. Retention volume of the standard sample
3. Sample components are collected after separation, and subjected to spectrometric analysis such as IR, NMR and MS.

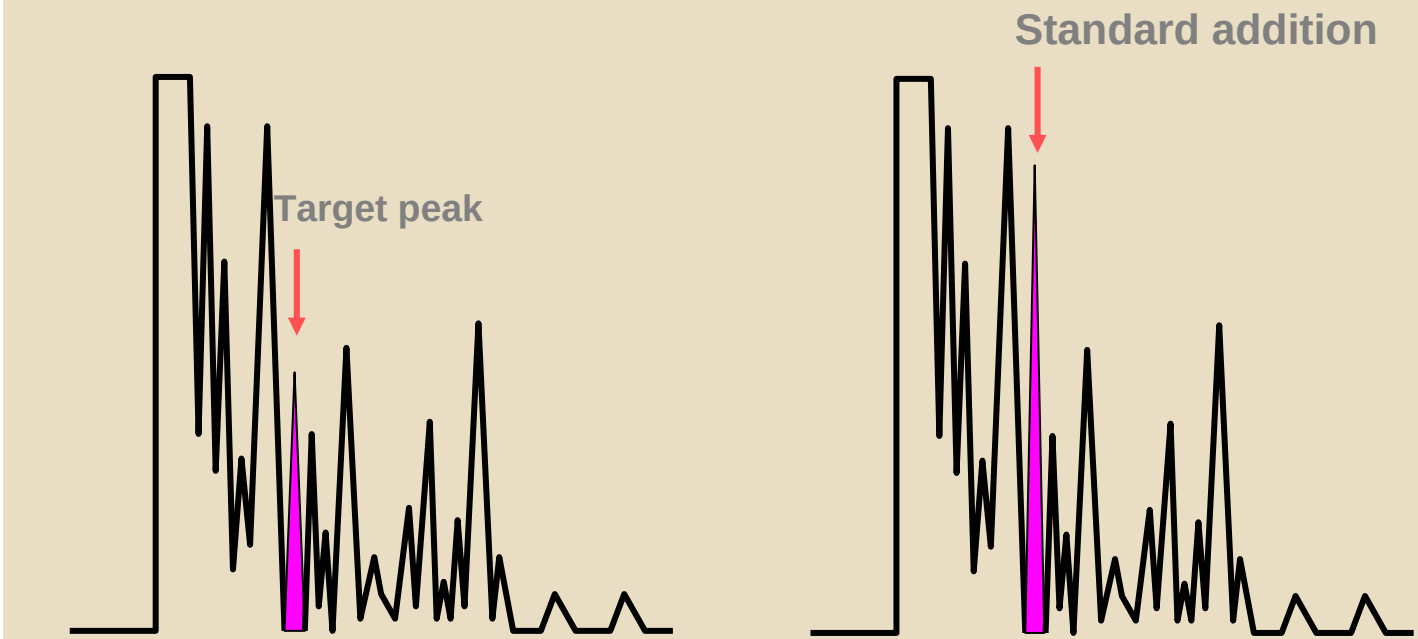


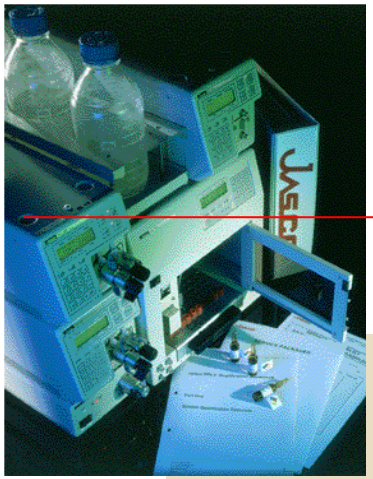
Identification from retention time





Standard addition method





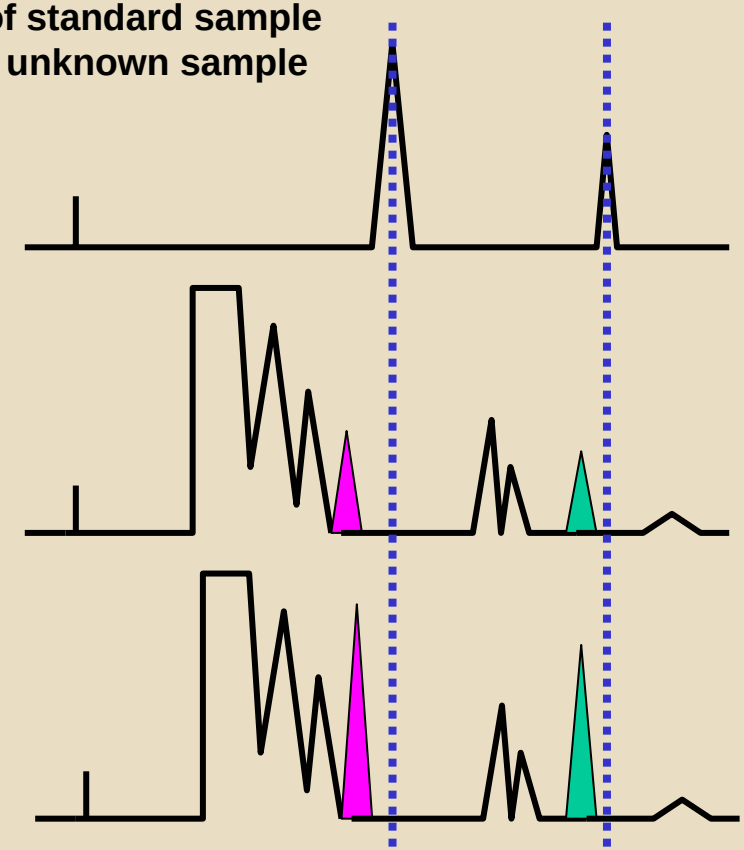
Standard addition method

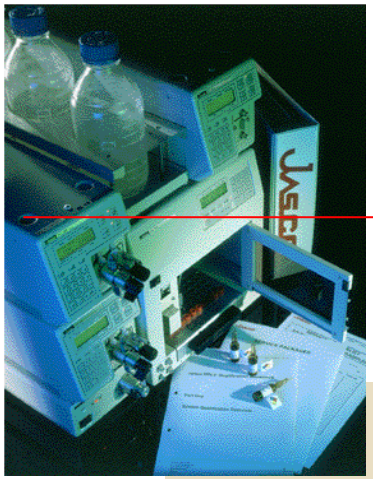
Retention time of standard sample is different from unknown sample

Standard sample

Unknown sample

Unknown sample and Standard sample





Identification using a different instruments after preparative analysis

Identification from retention time

Limitation:

On flow UV spectrum

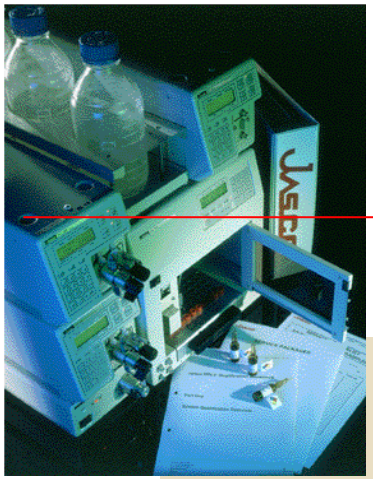
On flow emission spectrum

Multi-channel detector

Preparative analysis

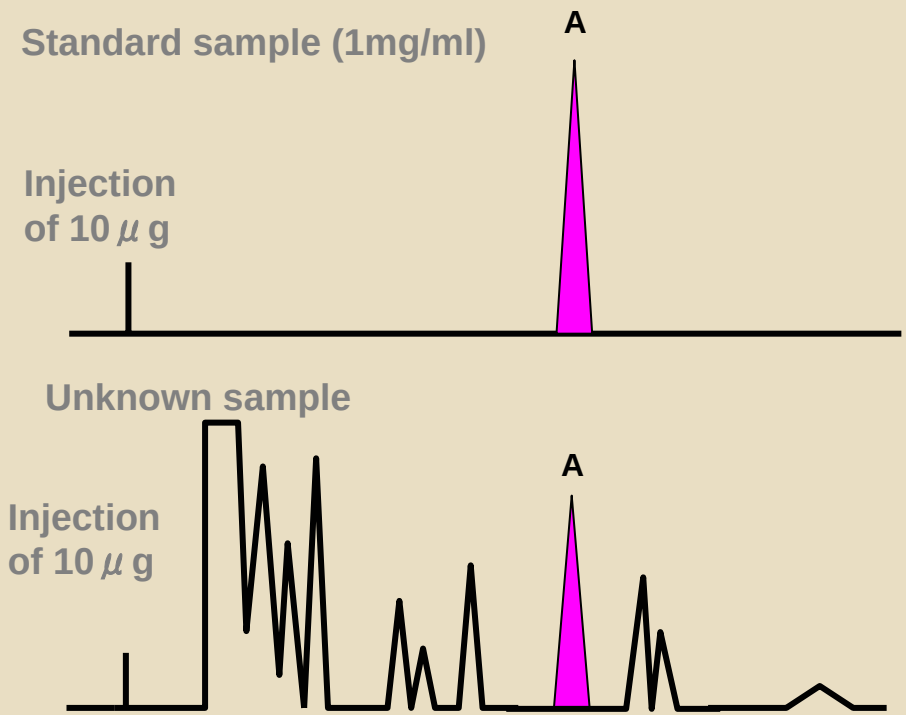
Spectrum measurement using a different instrument

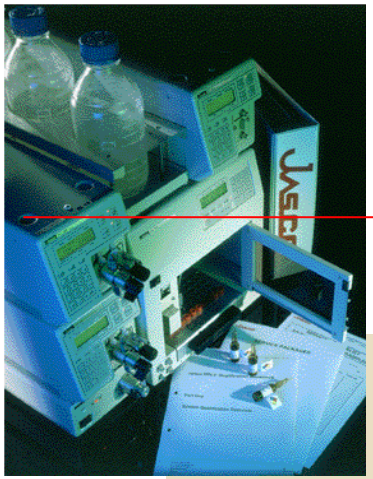
Quantitative analysis



The amount of a component can be calculated from the peak height and peak area of the chromatogram.

How much component A ?

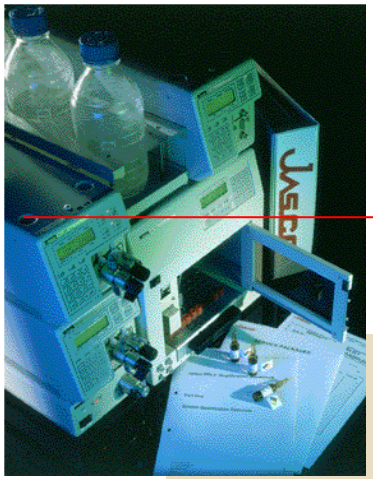




Calibration method

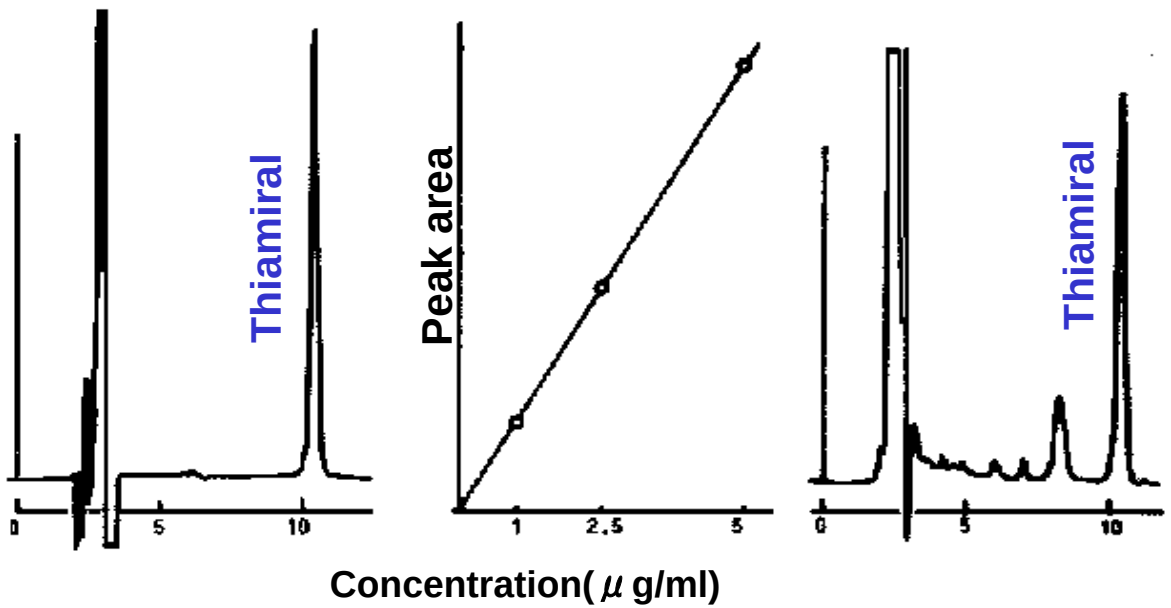
External standard sample

Internal standard sample

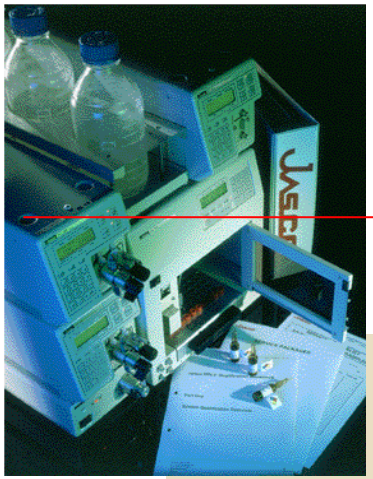


External standard sample

Thiamiral in serum



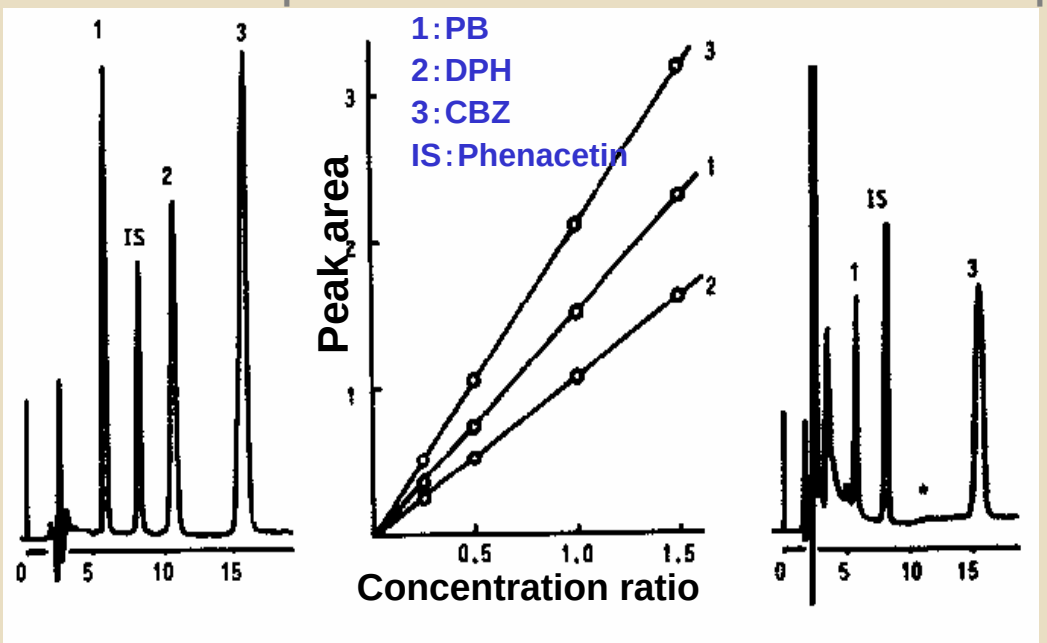
Finepak SIL C18T-5、CH_3CN/10mM KH_2PO_4 aq. (50:50)
UV 288nm



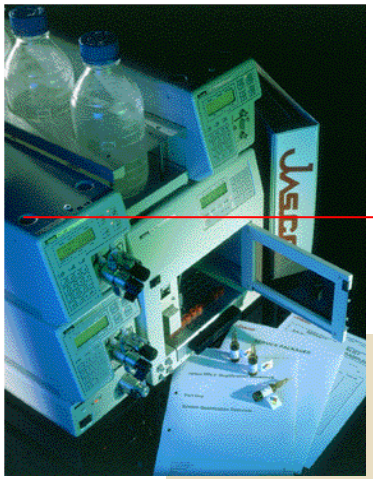
Internal standard sample

Anticonvulsants in serum

Standard sample　　Calibration curve　　Unknown sample



Finepak SIL C18T、CH_3CN/5mM KH_2PO_4 aq.



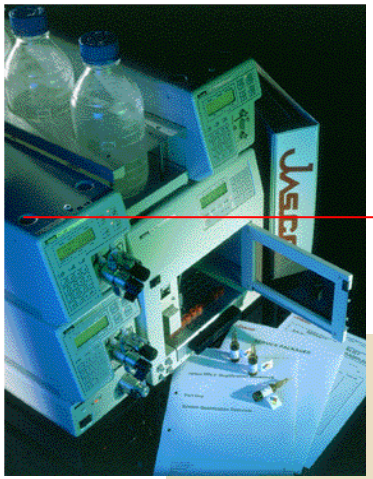
Guide for selecting the internal standard sample

No overlapping peaks

No Components included in unknown sample

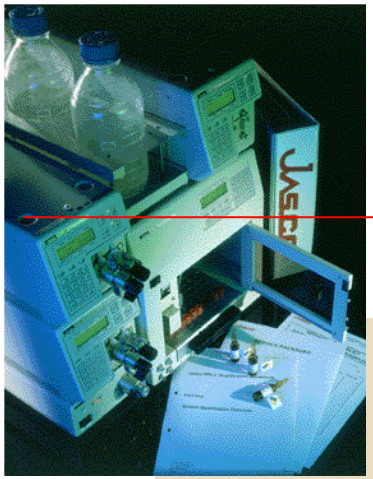
Chemical and physical stability

High purity

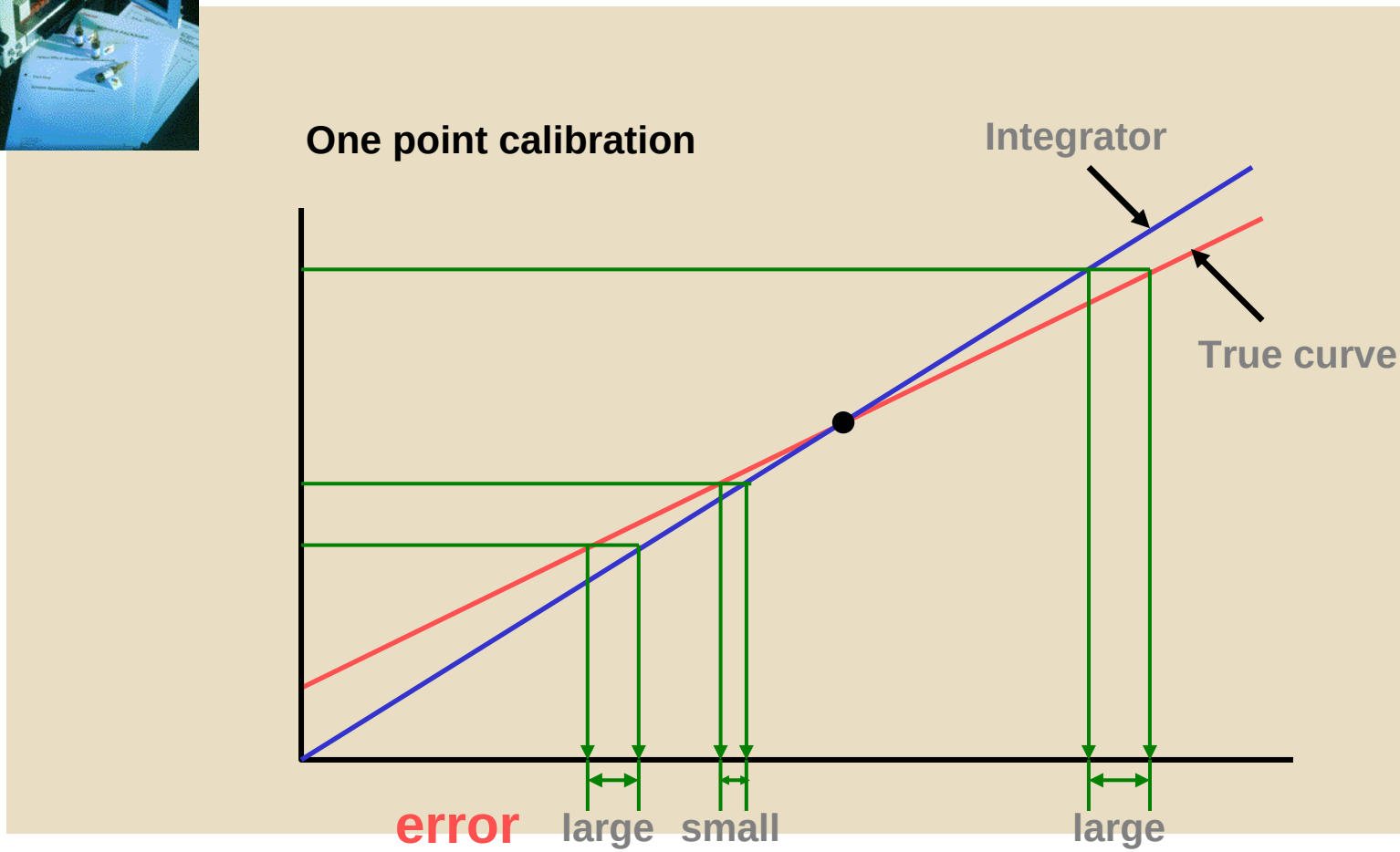


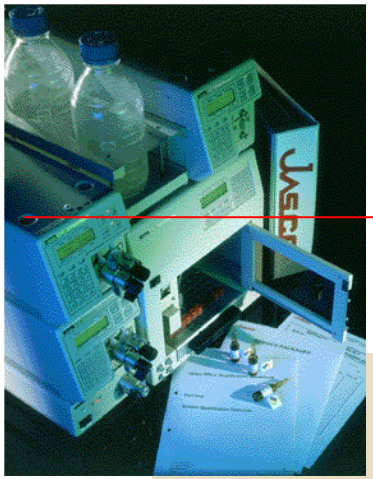
External standard and Internal standard samples

	External standard	Internal standard
Error	injection volume	volume to be added
Correction of Pre-treatment loss	impossible	possible



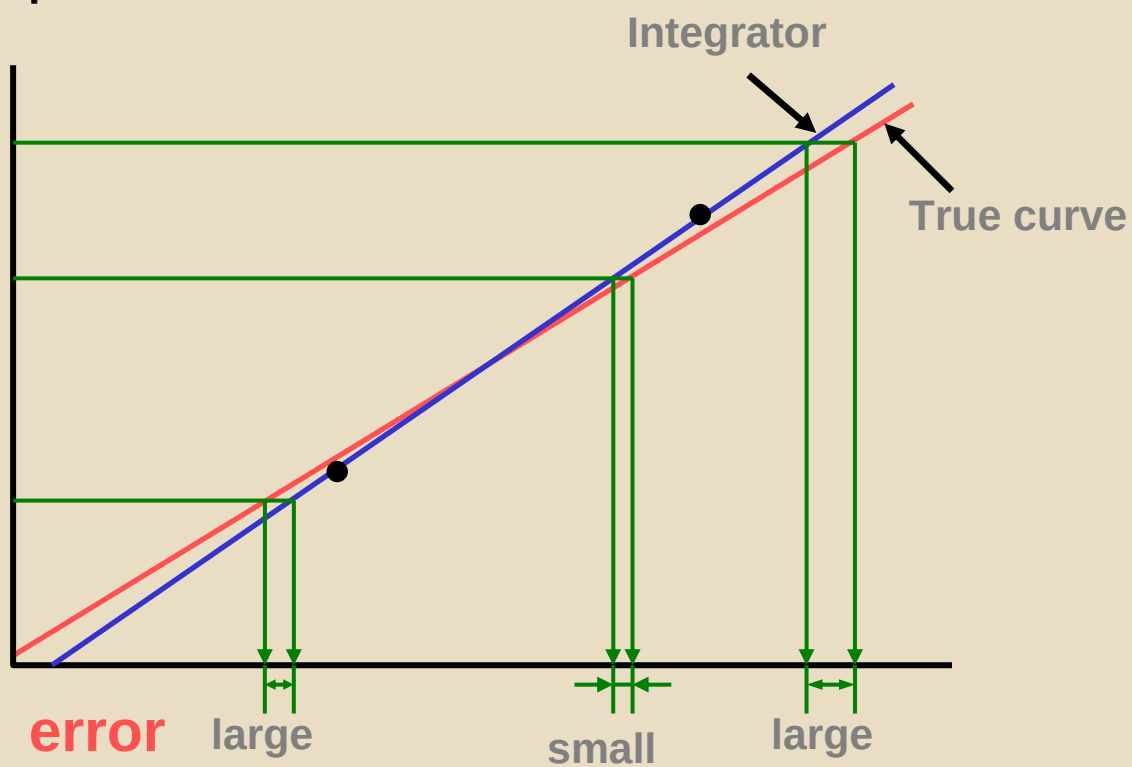
Caution when using an Integrator

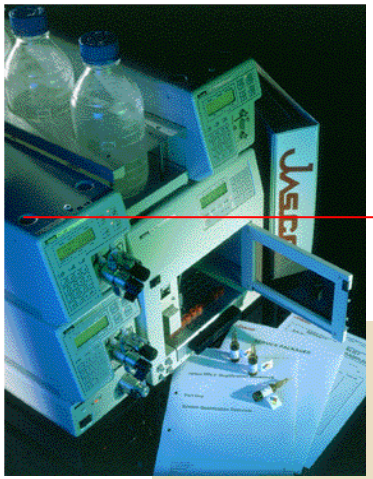




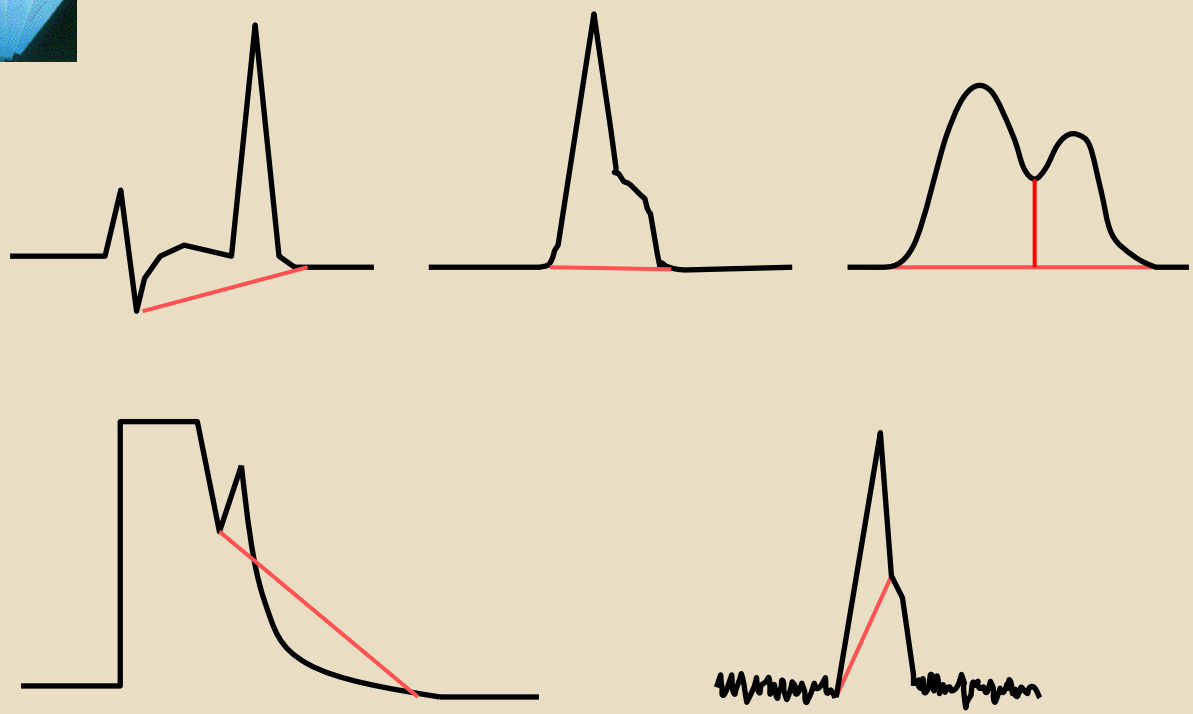
Caution when using an Integrator

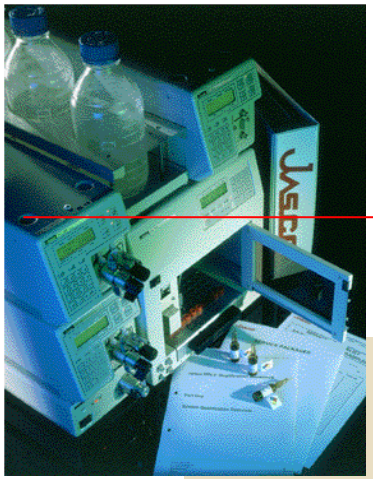
Two point calibration





Baseline





Considerations when performing quantitative analysis

Standard sample

Integrator

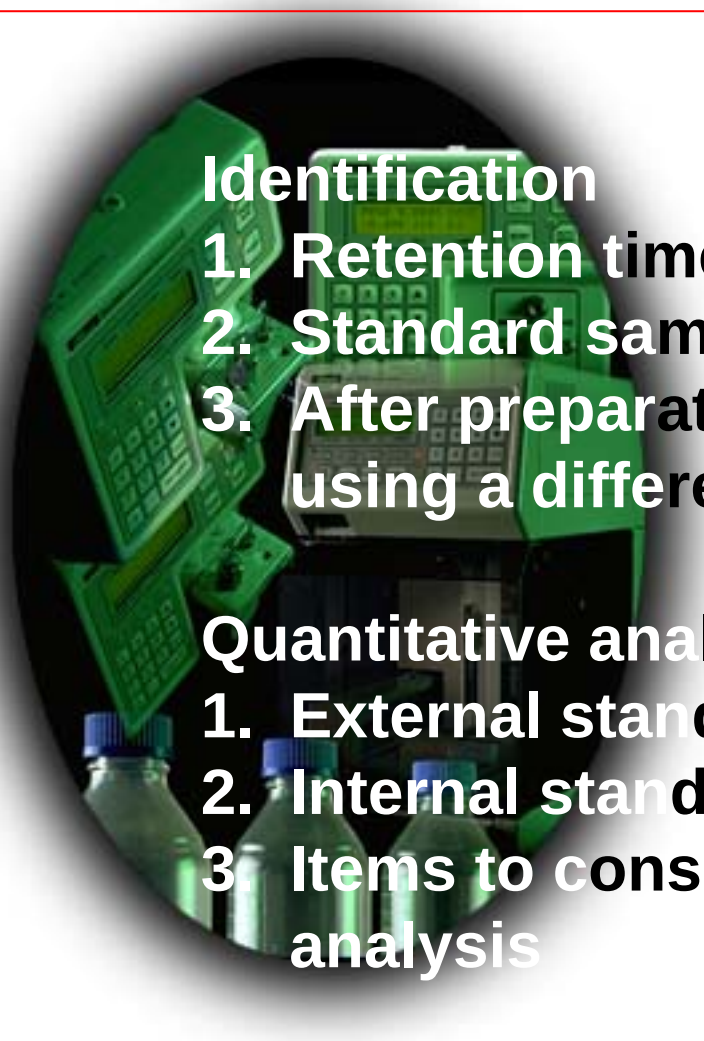
Micro syringe

Sample preparation

Concentration change of standard sample

Contamination

Review of Section 6

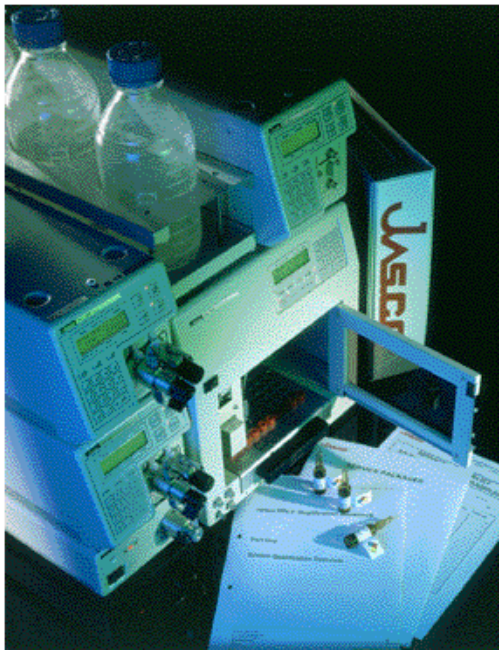


Identification

1. **Retention time**
2. **Standard sample**
3. **After preparative analysis, measure spectrum using a different method**

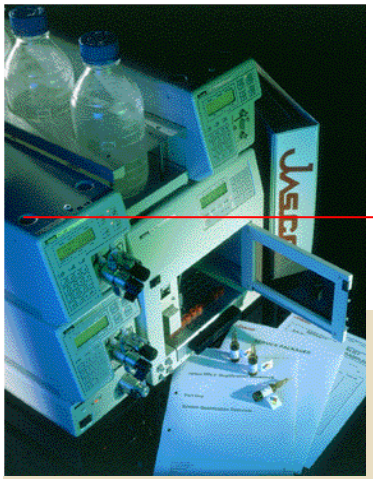
Quantitative analysis

1. **External standard sample**
2. **Internal standard sample**
3. **Items to consider when performing quantitative analysis**



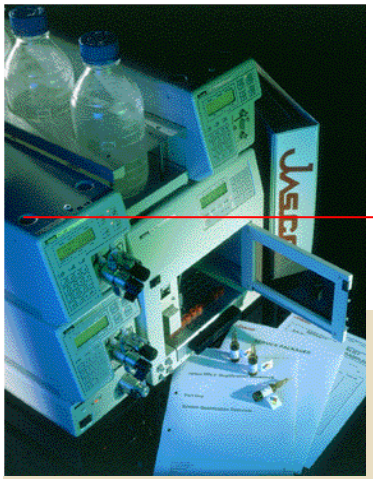
The JASCO advanced technology team has again met the challenge and designed a new line of HPLC instruments, The LC-1500series more than satisfies in response to the growing demand for greatly expanded HPLC analyses in the fields of not only biochemistry, pharmaceutical and medical science, but also in the areas of among other organic and inorganic compounds, foods, agricultural sciences, polymeric and natural substances and pollution. The LC-1500 series comprises pumps, detectors, autosamplers, its own column oven and other units each having built-in intelligence and incorporating many features with much higher levels of operability and reliability in addition to multiple functions, higher performance and higher accuracy than before, making them the most advanced instruments available.

7. Sample preparation



Sample preparation

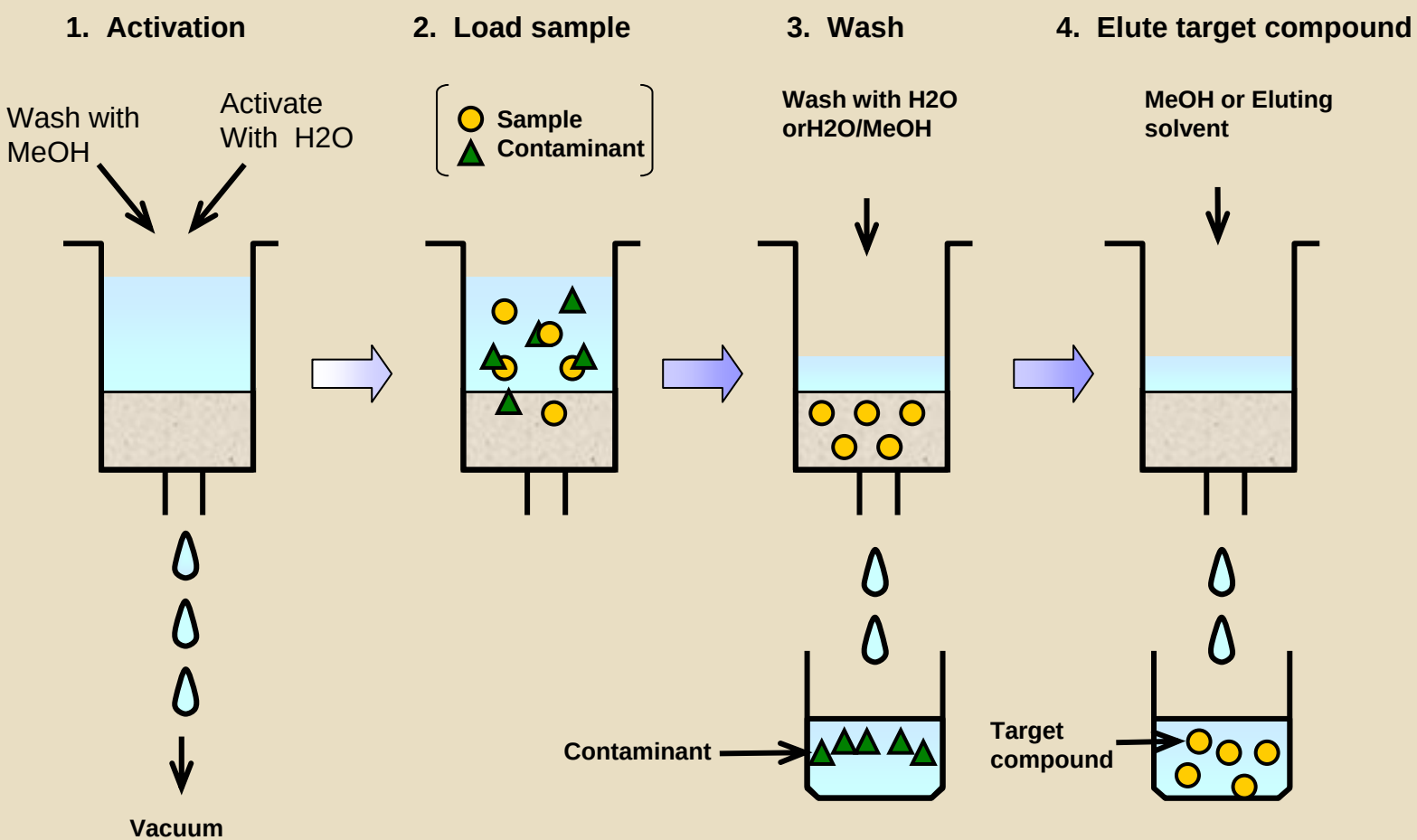
Cause	Problem	Countermeasures
Sample is not liquid.	not possible to inject	extraction / dissolving
Concentration is too high.	over load for column / out of detection range	dilution
Concentration is too low.	cannot detect	concentration / derivative
Contains foreign particles	clogged up	centrifugation / filtration
Includes components which damage column		solvent extraction /derivative
Includes interference for separation	quantitation error	solvent extraction /derivative
Solvent unsuitable	deterioration of column	pH adjustment



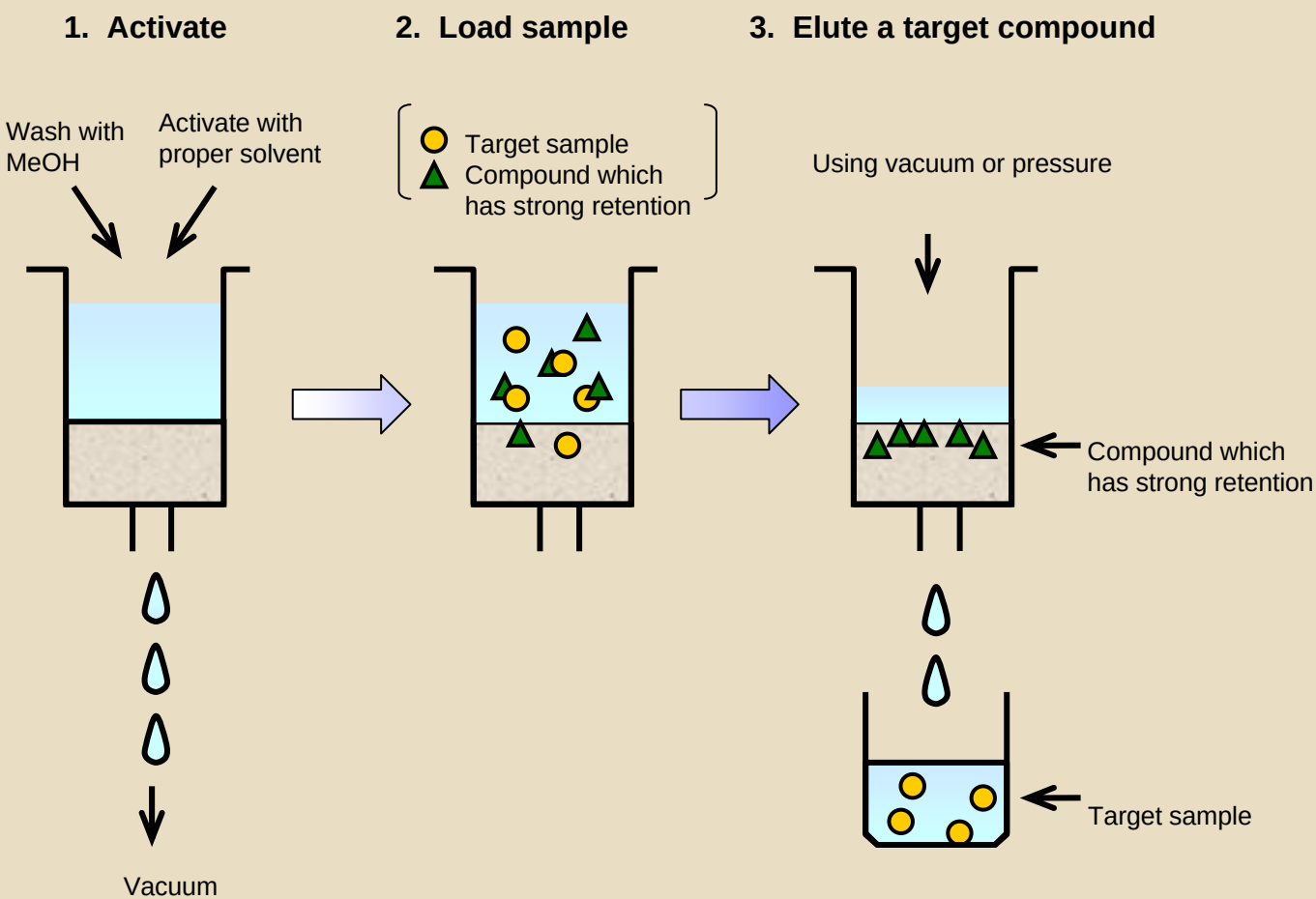
Sample preparation Method

Filtration	0.45um, 0.2um membrance filter
Extraction	Solvent extraction Solid phase extraction
Concentration	Evaporation Solid phase exraction (Bond Elut) Fused drying
Deprotaination	Organic acid
Homonization	

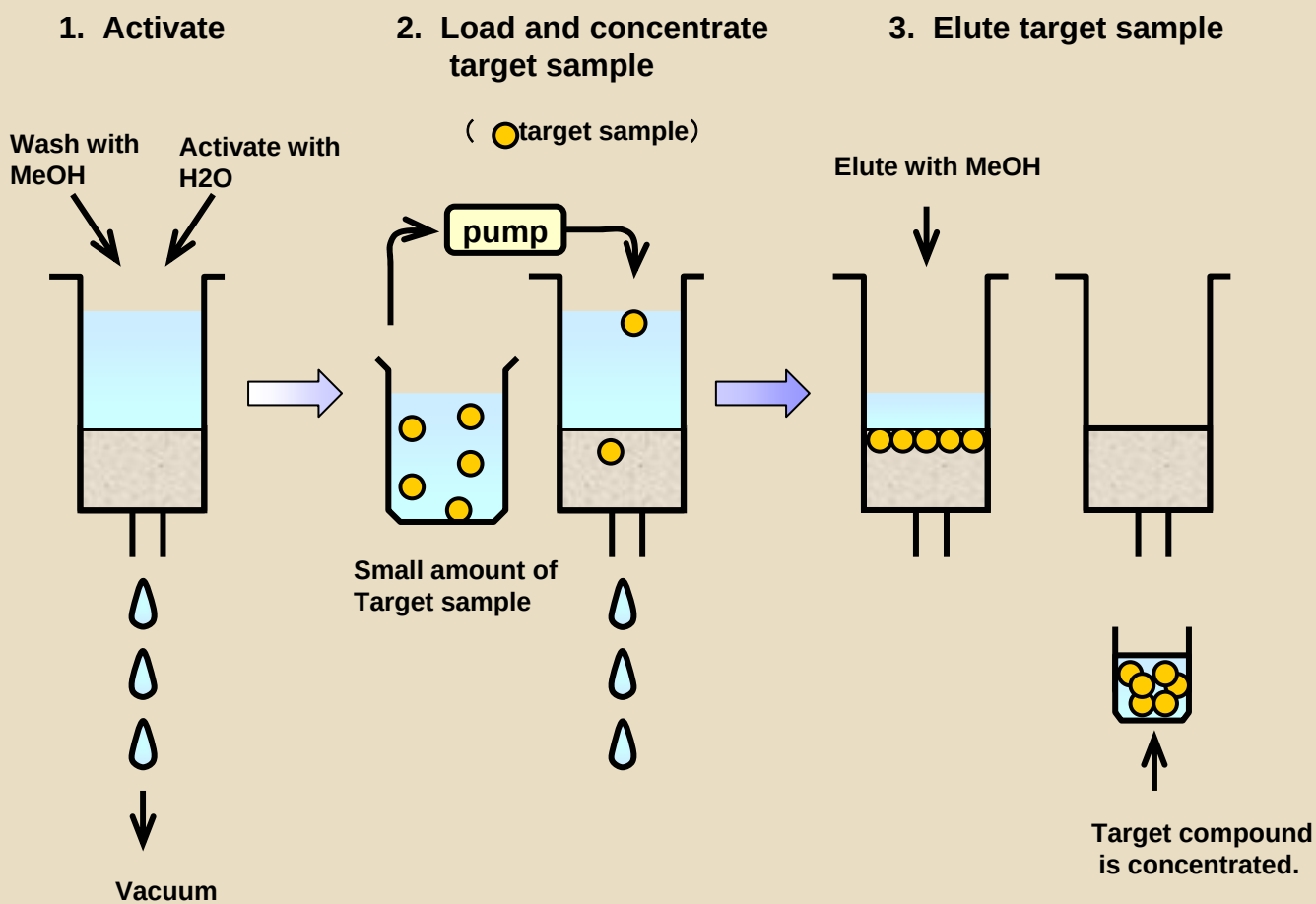
Solid phase extraction

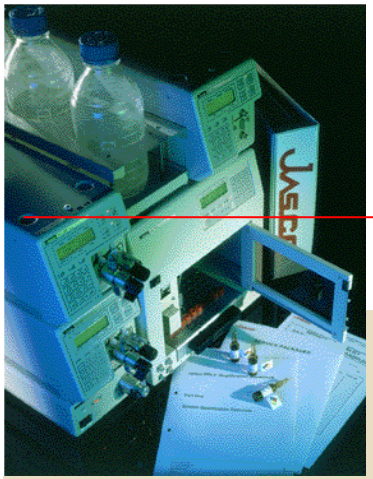


Removing contaminants which have strong retention



Concentration





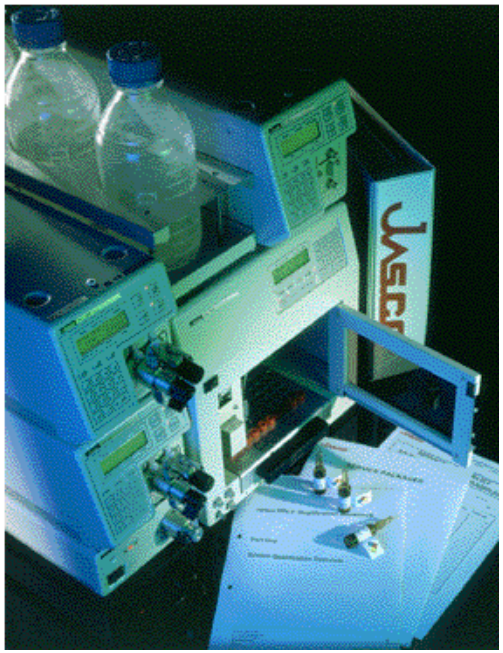
Considerations when preparing sample

- Recovery rate
- Contamination

Review of Section 7

1. The most appropriate preparation method depends on various factors including the sample(target compound), the amount of target compound in the sample, and the kinds of contaminant.

2. Consider such factors as the sample state, amount, running cost, running time, and handling.



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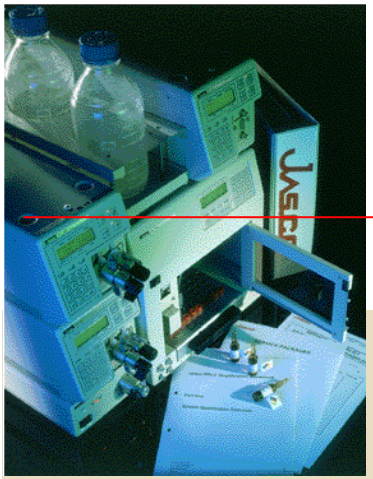
8. Procedure for developing analytical conditions



Procedure for developing analytical conditions

Step one : clear analytical purpose, and research the target compound.

(1) Molecular weight
Molecular structure
Functional group

(2) Solubility, stability
UV, FP absorption

(3) Amount of concentration, contaminant

(4) Application data
reference literature, magazines

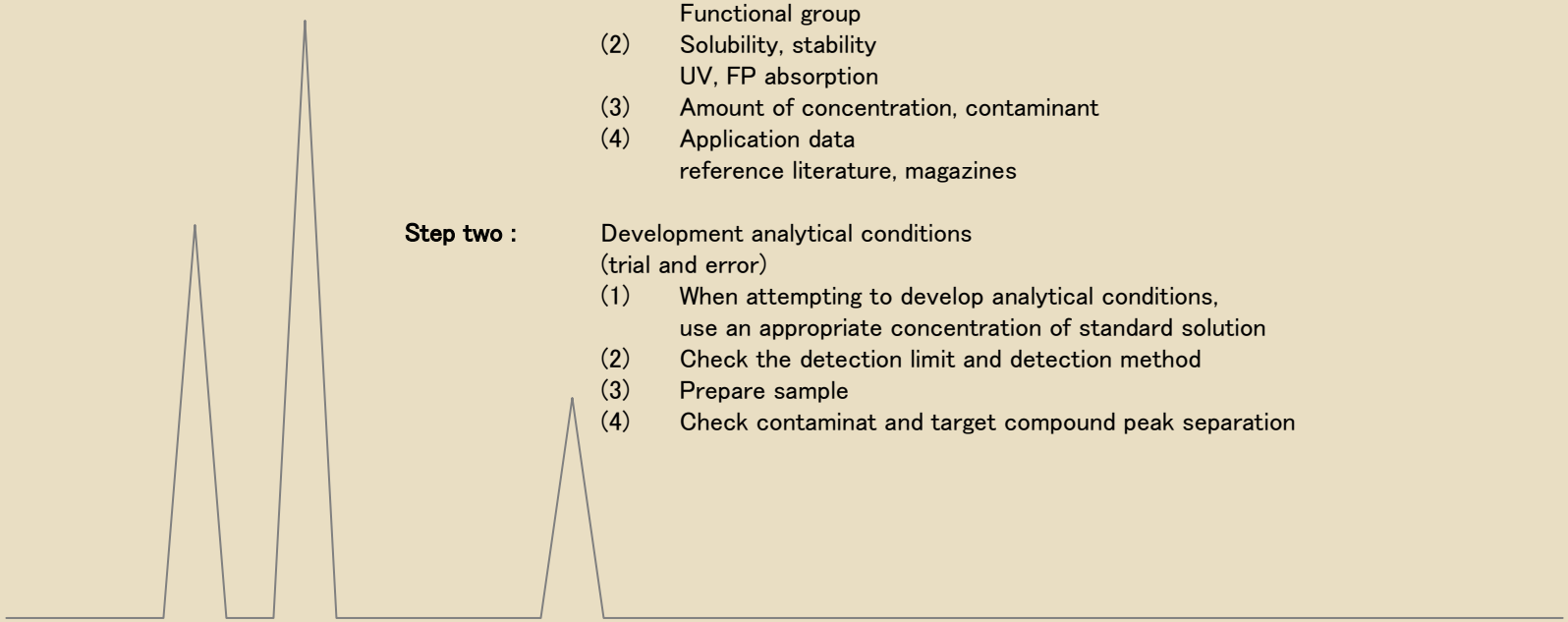
Step two : Development analytical conditions
(trial and error)

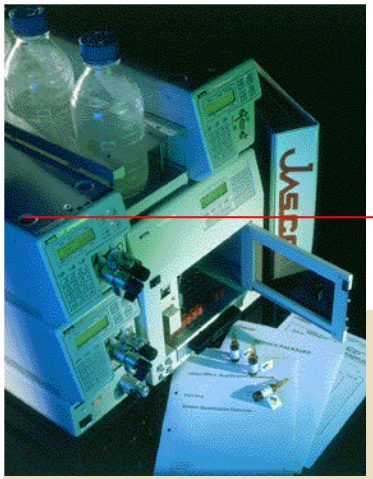
(1) When attempting to develop analytical conditions,
use an appropriate concentration of standard solution

(2) Check the detection limit and detection method

(3) Prepare sample

(4) Check contaminat and target compound peak separation





Procedure for developing analytical conditions

Step three : Establish analytical condition for routine analysis

(1) Linearity of calibration curve
(2) Reproducibility of analysis
(3) Check for contaminants that retain strongly in the column
(4) Check for Correlation with other methods.

Step Four Routine quantitative analysis

(1) Lifetime of column
(2) Running cost
(3) Develop analytical procedure (SOP)
(4) Check HPLC and column performance.