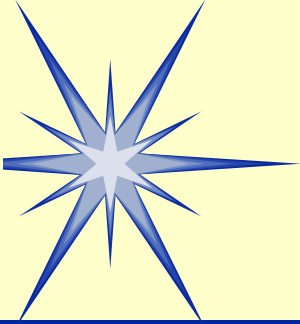
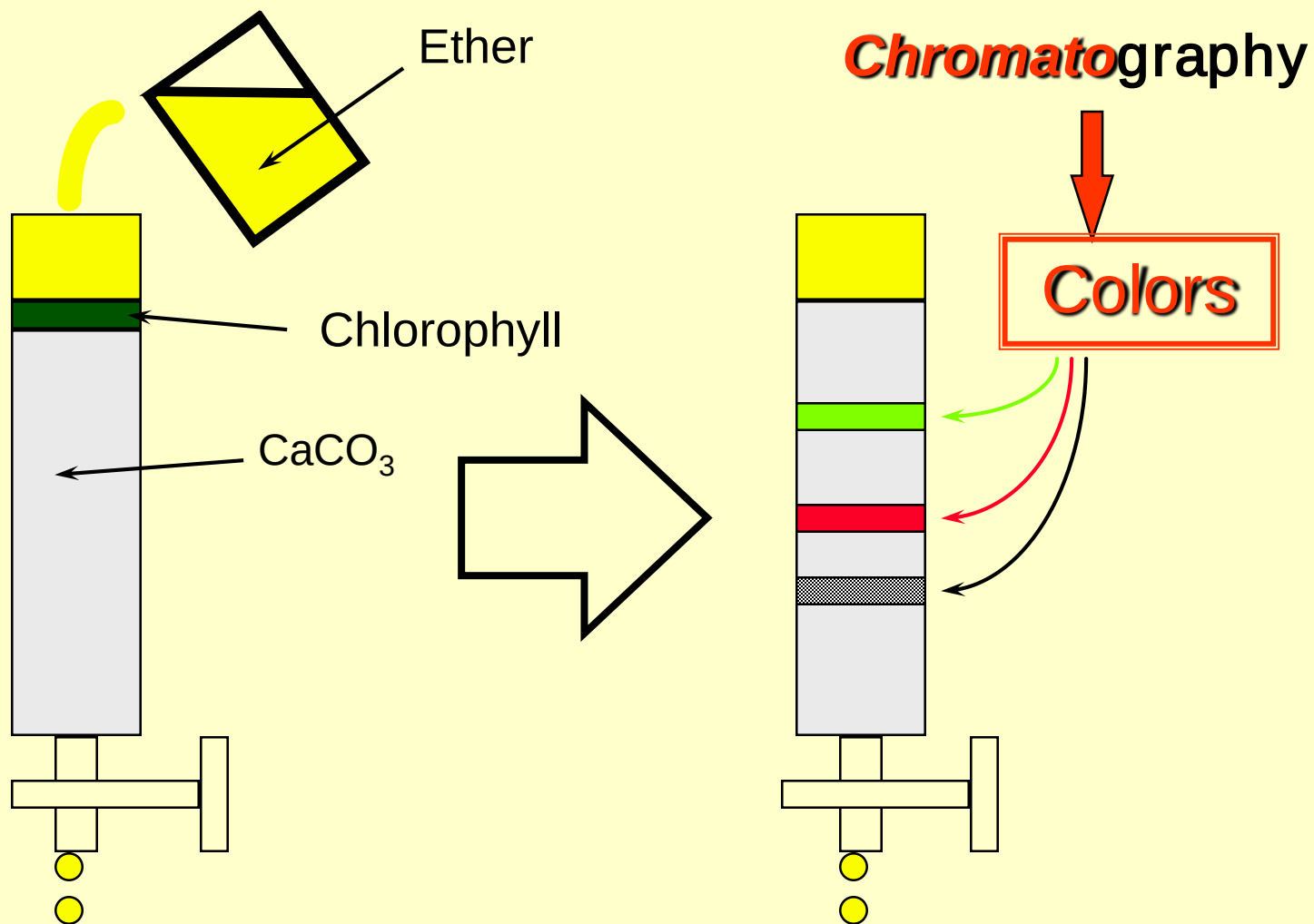


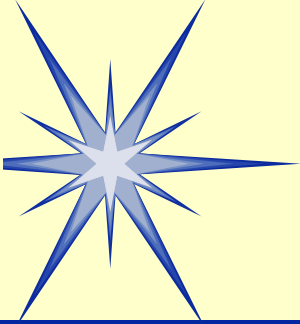
What Is HPLC?

Basic Principles



Invention of Chromatography by M. Tswett



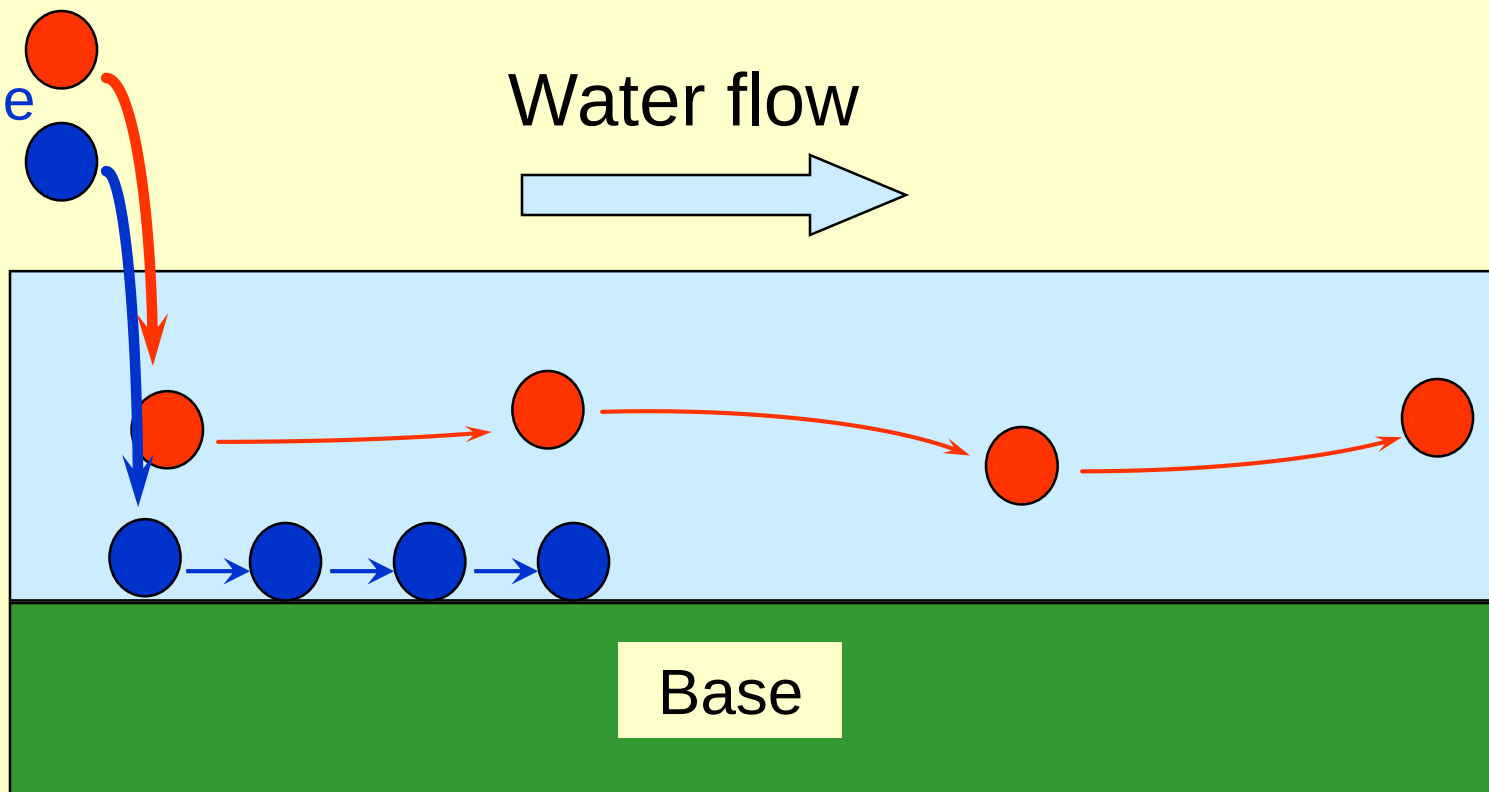


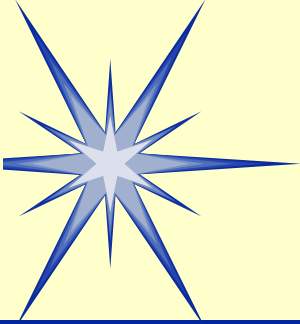
Comparing Chromatography to the Flow of a River...

Light leaf

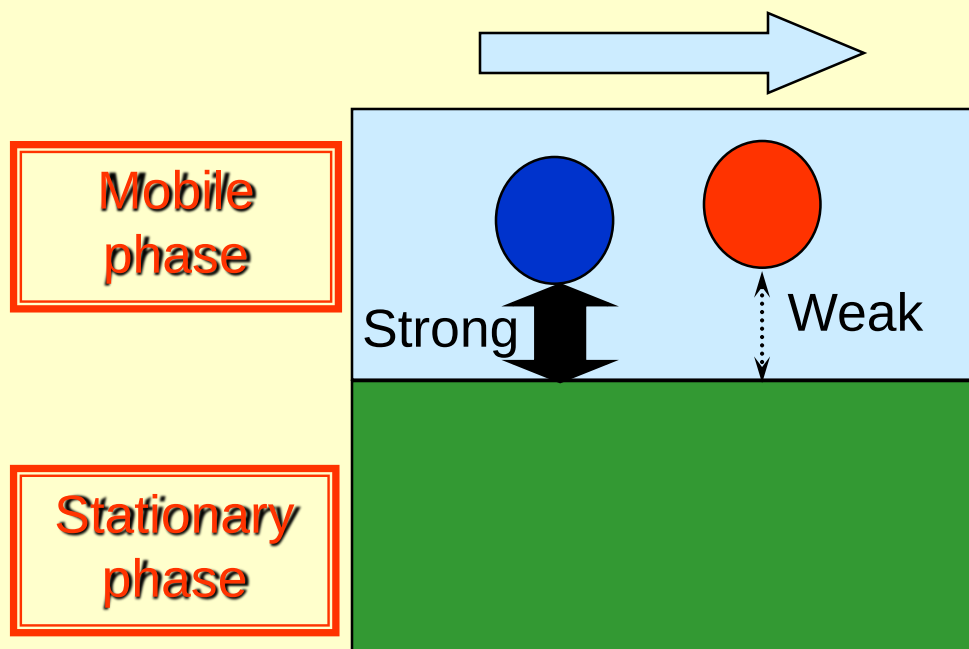
Heavy stone

Water flow

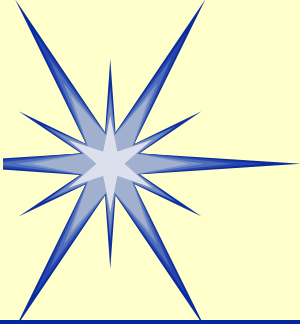




Mobile Phase / Stationary Phase

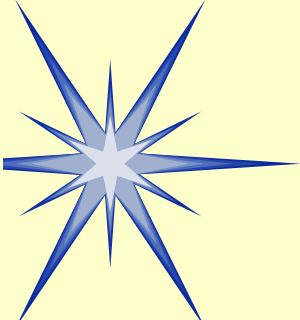


- A site in which a moving phase (**mobile phase**) and a non-moving phase (**stationary phase**) make contact via an interface that is set up.
- The affinity with the mobile phase and stationary phase varies with the solute. → **Separation** occurs due to differences in the speed of motion.



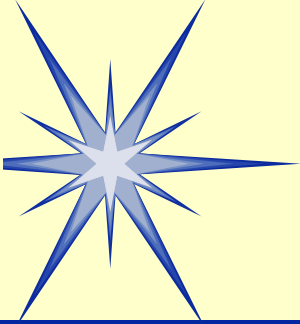
Chromato-graphy / -graph / -gram / -grapher

- Chromatography: Analytical technique
- Chromatograph: Instrument
- Chromatogram: Obtained “picture”
- Chromatographer: Person



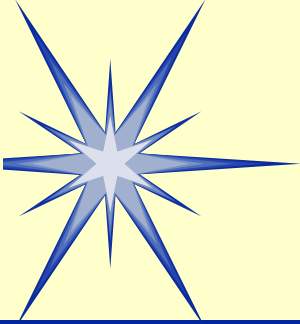
Three States of Matter and Chromatography Types

		Mobile phase		
		Gas	Liquid	Solid
Stationary phase	Gas			
	Liquid	Gas chromatography	Liquid chromatography	
	Solid			



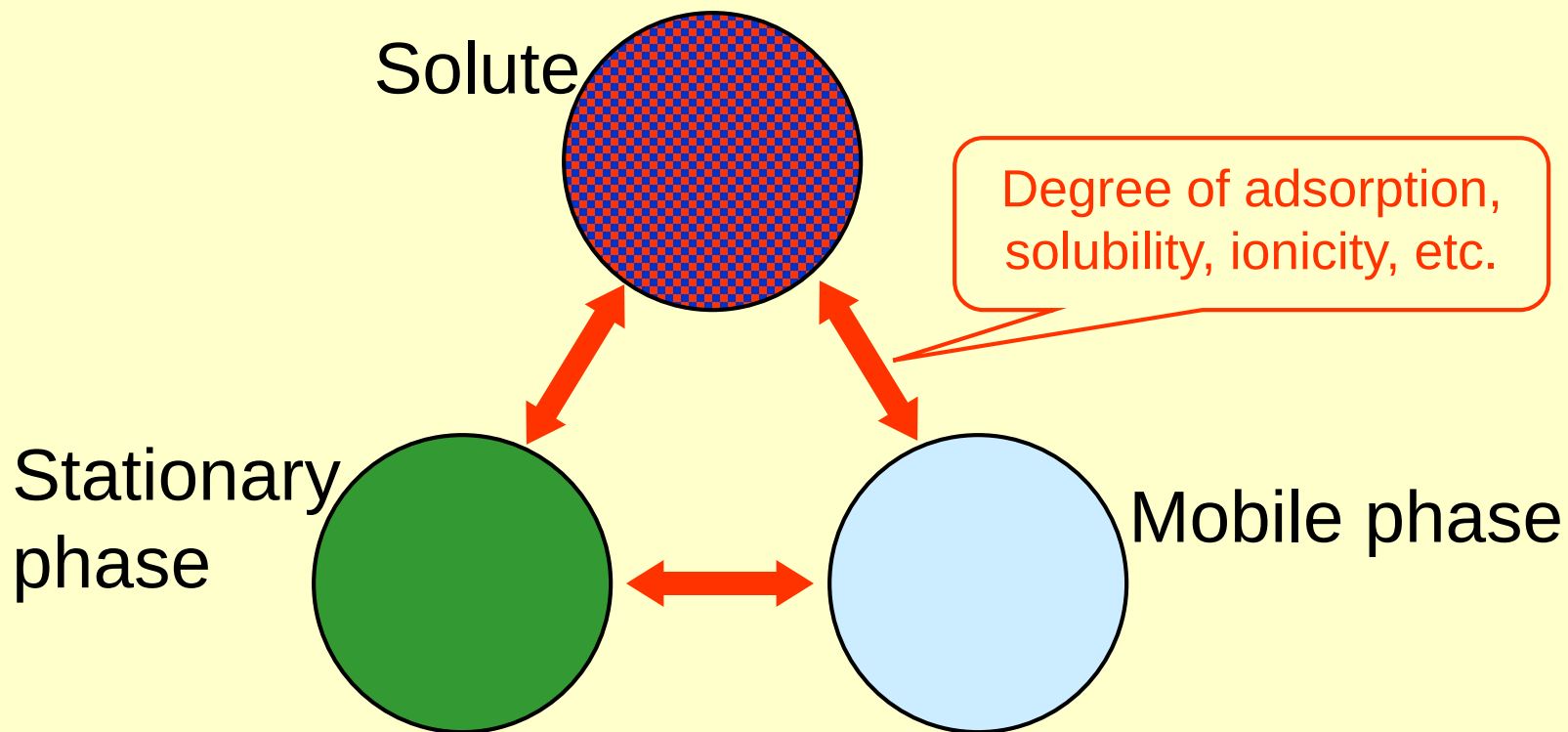
Liquid Chromatography

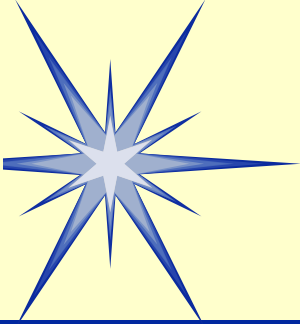
- Chromatography in which the mobile phase is a **liquid**.
 - ❖ The liquid used as the mobile phase is called the **“eluent”**.
- The stationary phase is usually a solid or a liquid.
- In general, it is possible to analyze any substance that can be stably dissolved in the mobile phase.



Interaction Between Solutes, Stationary Phase, and Mobile Phase

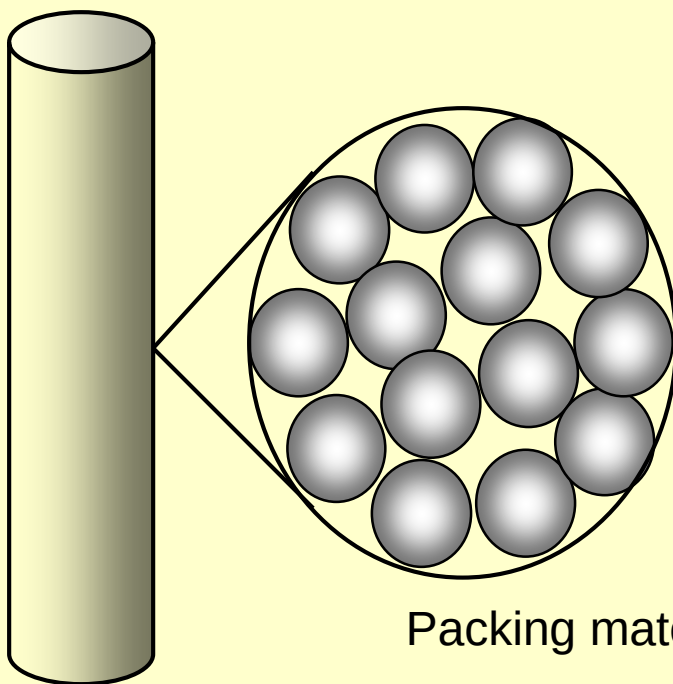
- Differences in the interactions between the solutes and stationary and mobile phases enable separation.





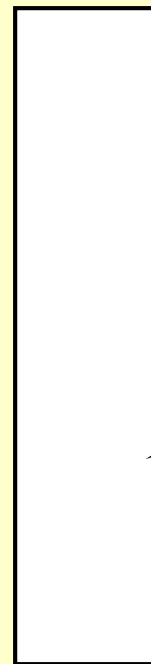
Column Chromatography and Planar Chromatography

Separation column



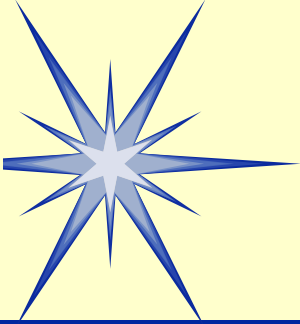
Packing material

Column Chromatography

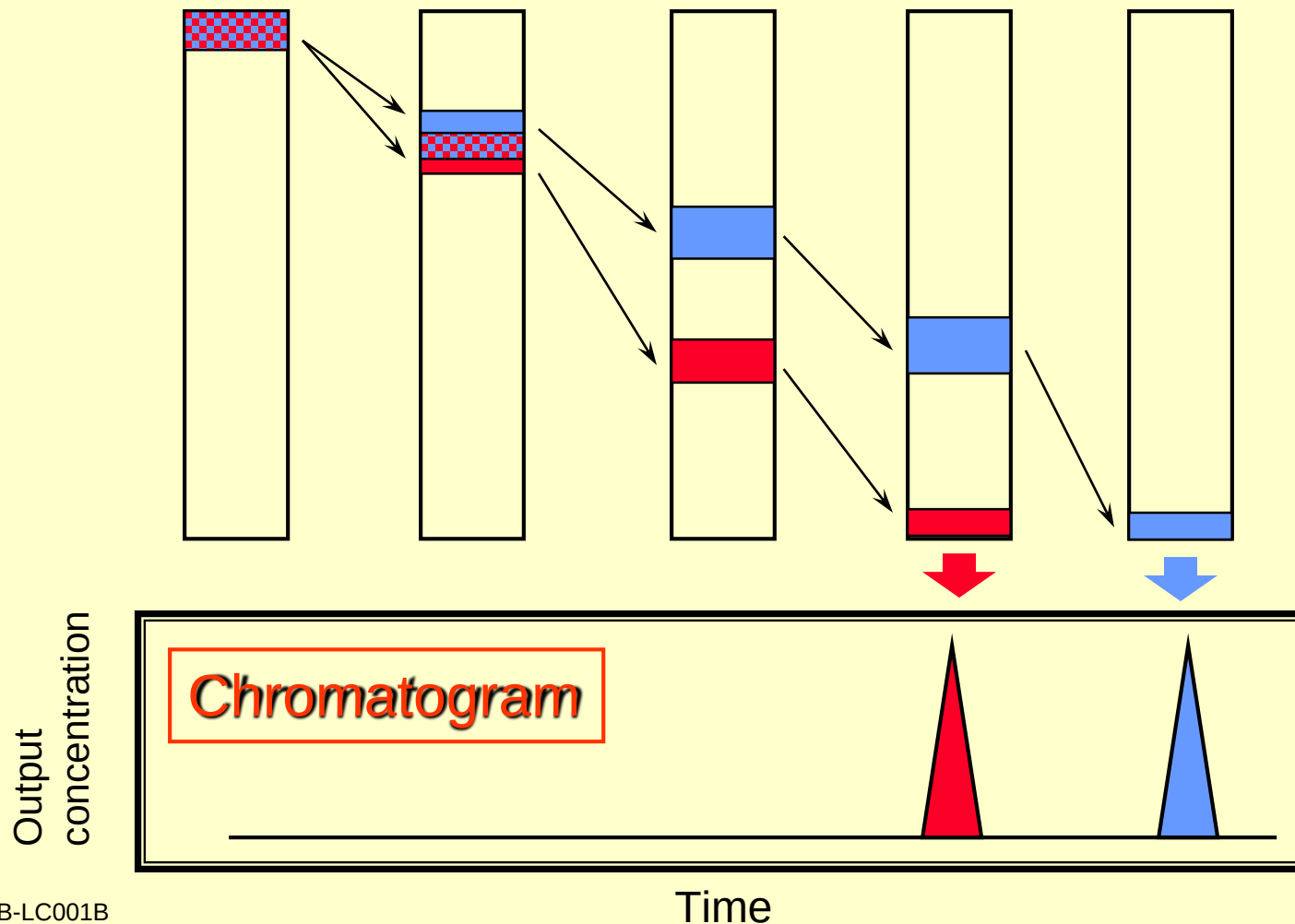


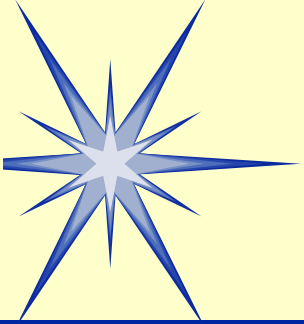
Paper or a
substrate coated
with particles

Paper Chromatography
Thin Layer Chromatography (TLC)

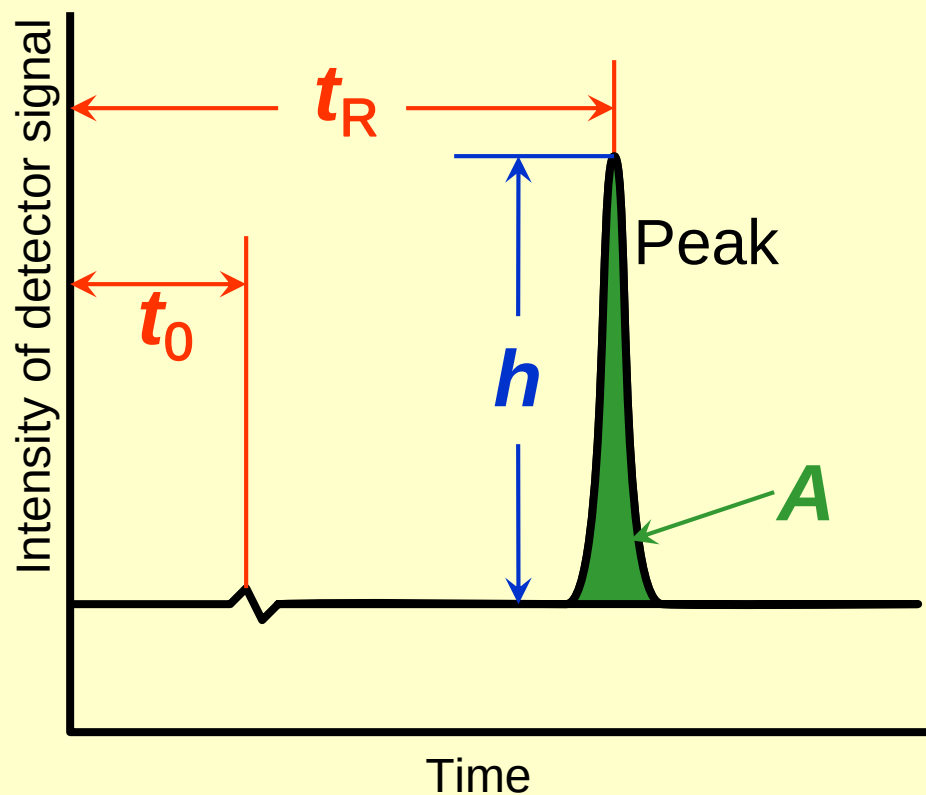


Separation Process and Chromatogram for Column Chromatography





Chromatogram

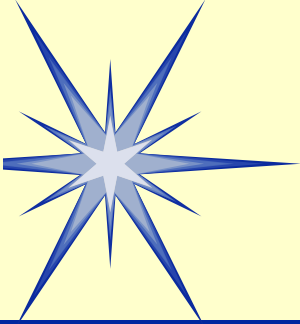


t_R : Retention time

t_0 : Non-retention time

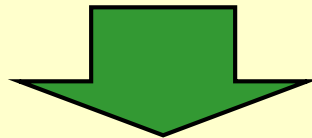
A : Peak area

h : Peak height

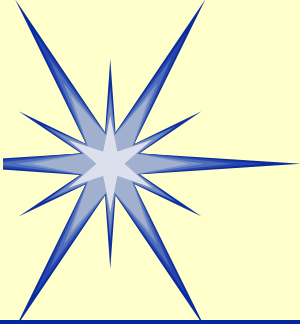


From Liquid Chromatography to High Performance Liquid Chromatography

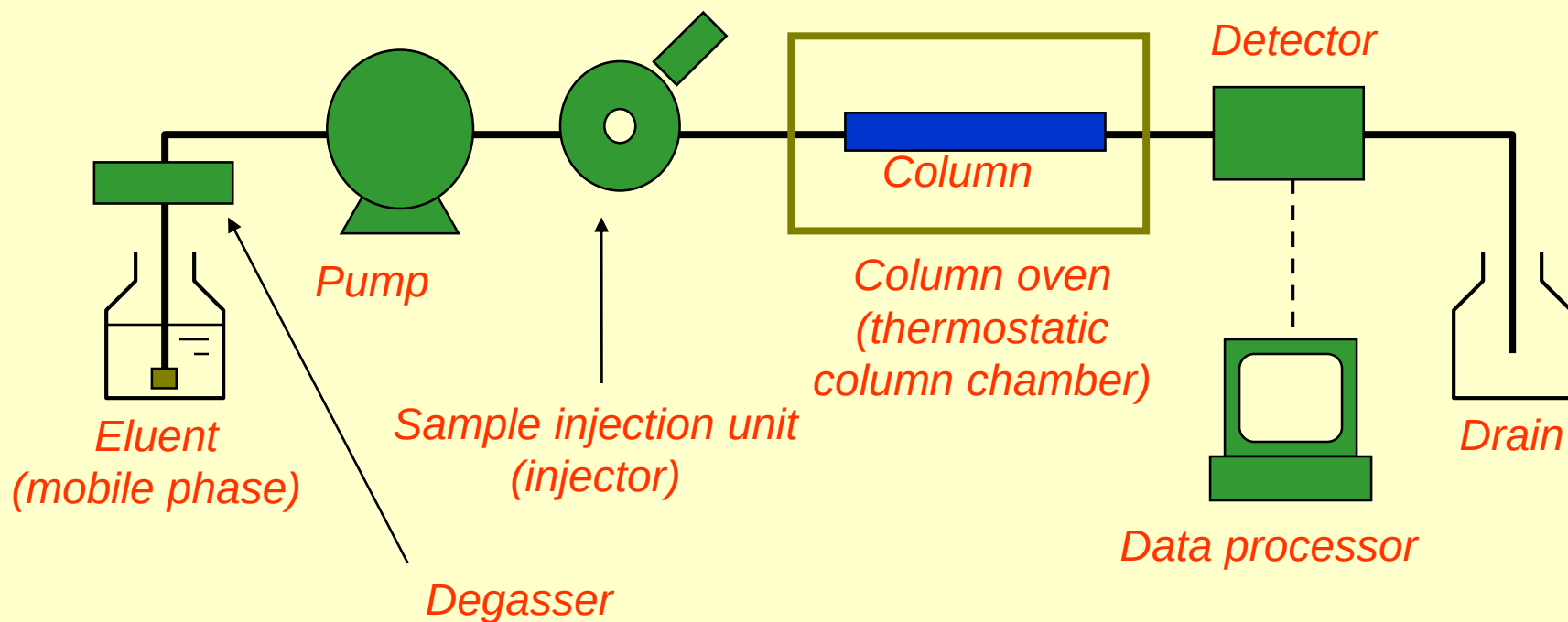
- Higher degree of separation!
 - Refinement of packing material (3 to 10 μm)
- Reduction of analysis time!
 - Delivery of eluent by pump
 - Demand for special equipment that can withstand high pressures

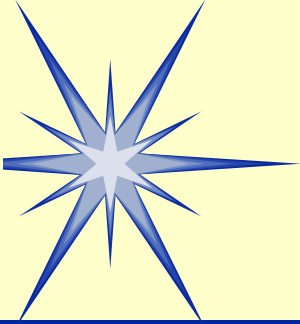


The arrival of high performance liquid chromatography!



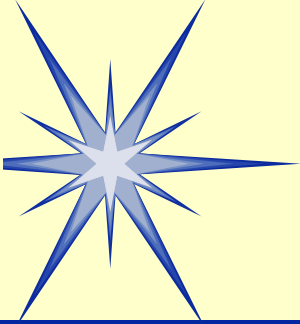
Flow Channel Diagram for High Performance Liquid Chromatograph





Advantages of High Performance Liquid Chromatography

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

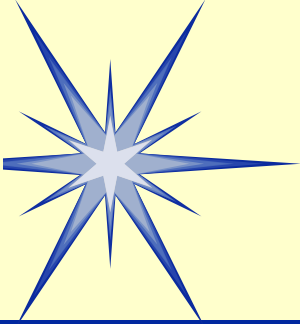


Fields in Which High Performance Liquid Chromatography Is Used

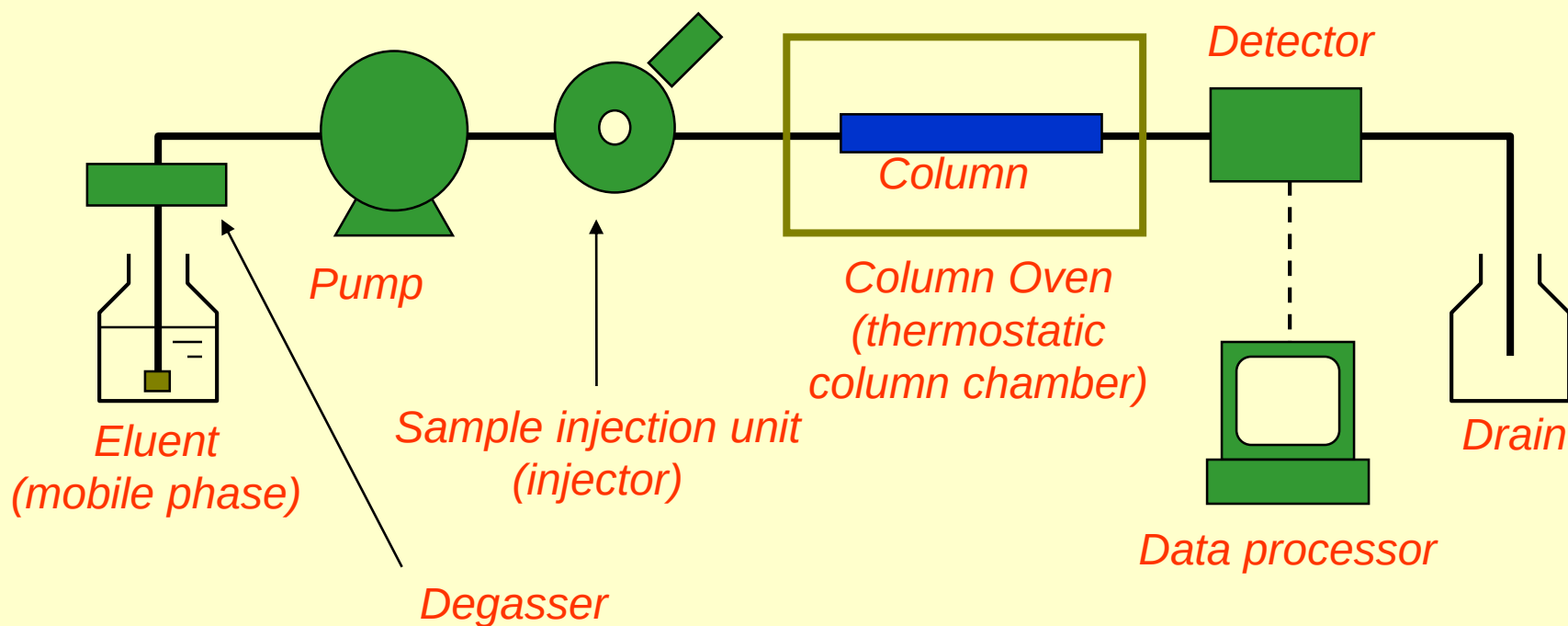
- Biogenic substances
 - ❖ Sugars, lipids, nucleic acids, amino acids, proteins, peptides, steroids, amines, etc.
- Medical products
 - ❖ Drugs, antibiotics, etc.
- Food products
 - ❖ Vitamins, food additives, sugars, organic acids, amino acids, etc.
- Environmental samples
 - ❖ Inorganic ions
 - ❖ Hazardous organic substances, etc.
- Organic industrial products
 - ❖ Synthetic polymers, additives, surfactants, etc.

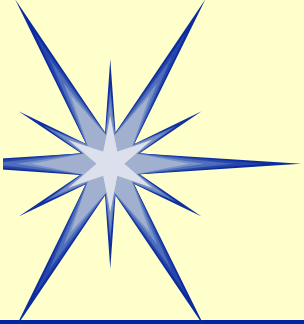
HPLC Hardware: Part 1

Solvent Delivery System,
Degasser, Sample Injection Unit,
Column Oven



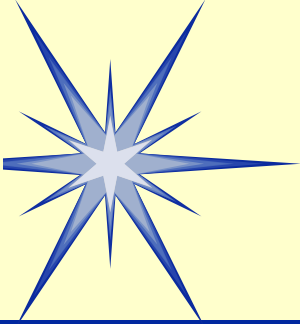
Flow Channel Diagram for HPLC





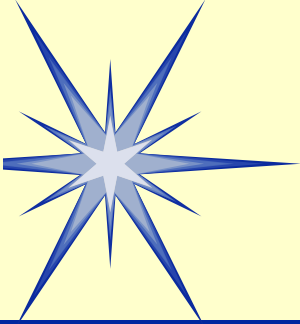
Solvent Delivery Pump

- Performance Requirements
 - ❖ Capacity to withstand high load pressures.
 - ❖ Pulsations that accompany pressure fluctuations are small.
 - ❖ Flow rate does not fluctuate.
 - ❖ Solvent replacement is easy.
 - ❖ The flow rate setting range is wide and the flow rate is accurate.

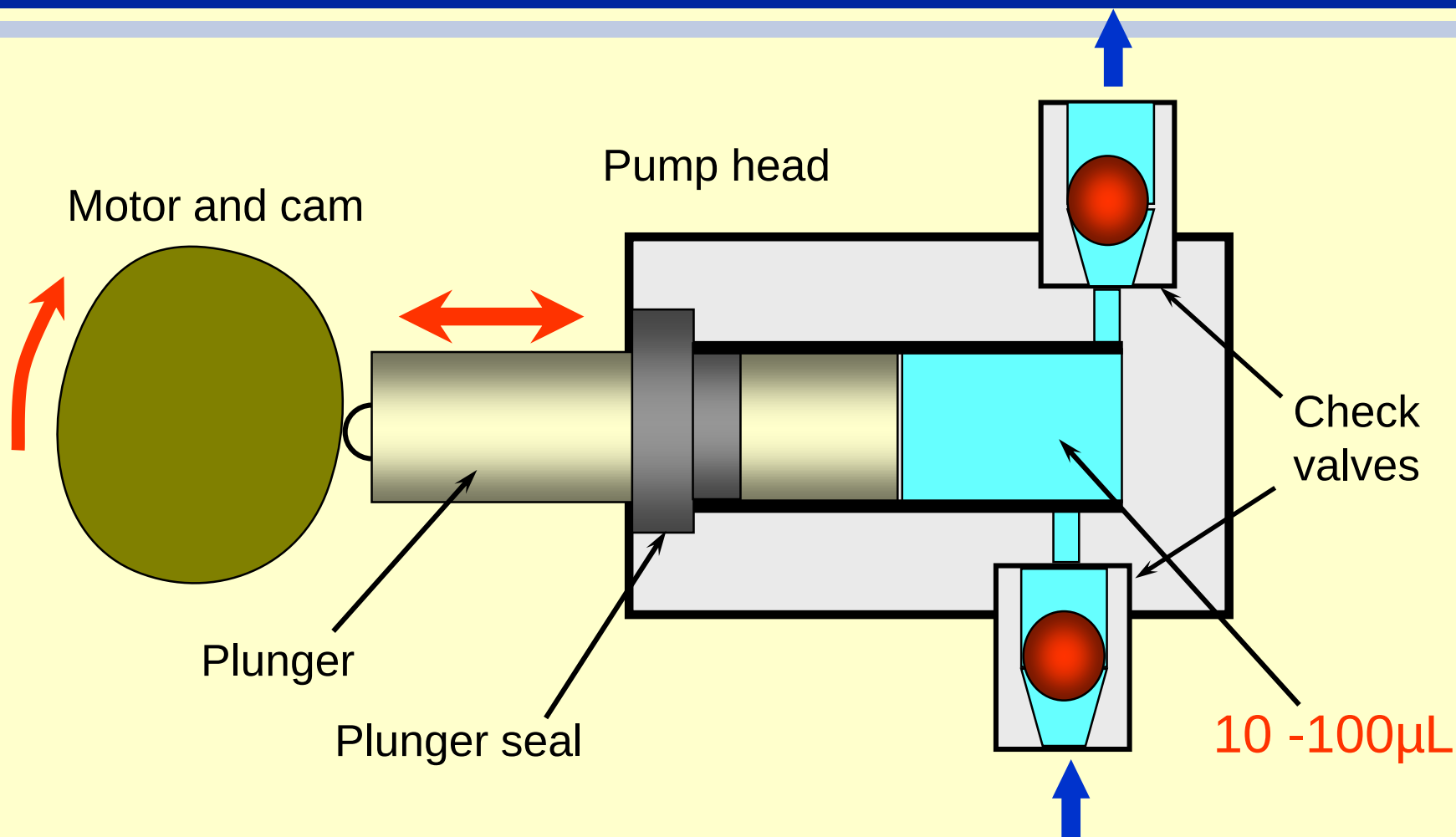


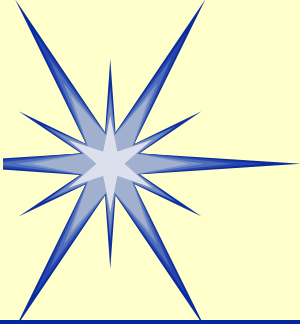
Solvent Delivery Pump: Representative Pumping Methods

- Syringe pump
- Plunger pump
- Diaphragm pump

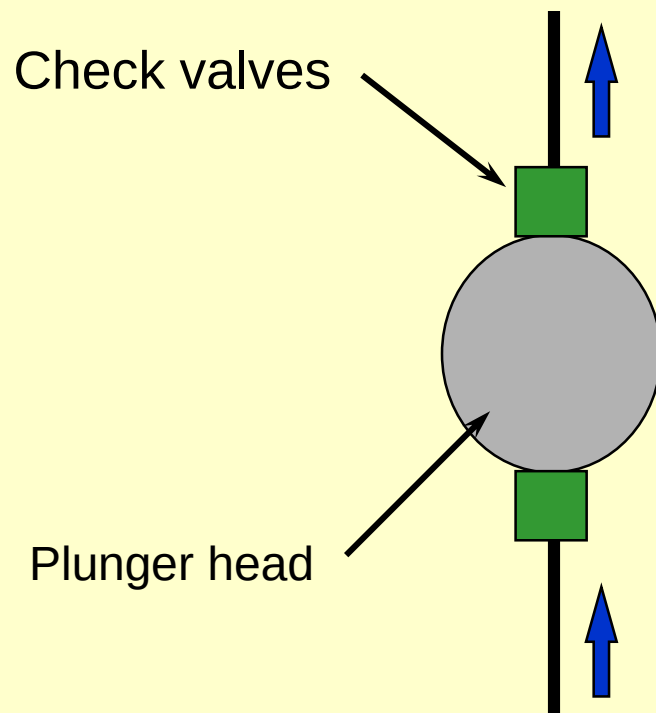


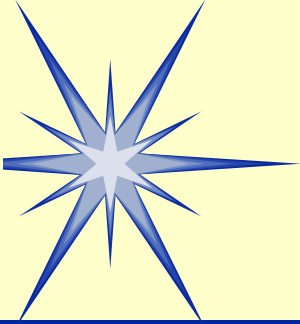
Solvent Delivery Pump: Schematic Diagram of Plunger Pump



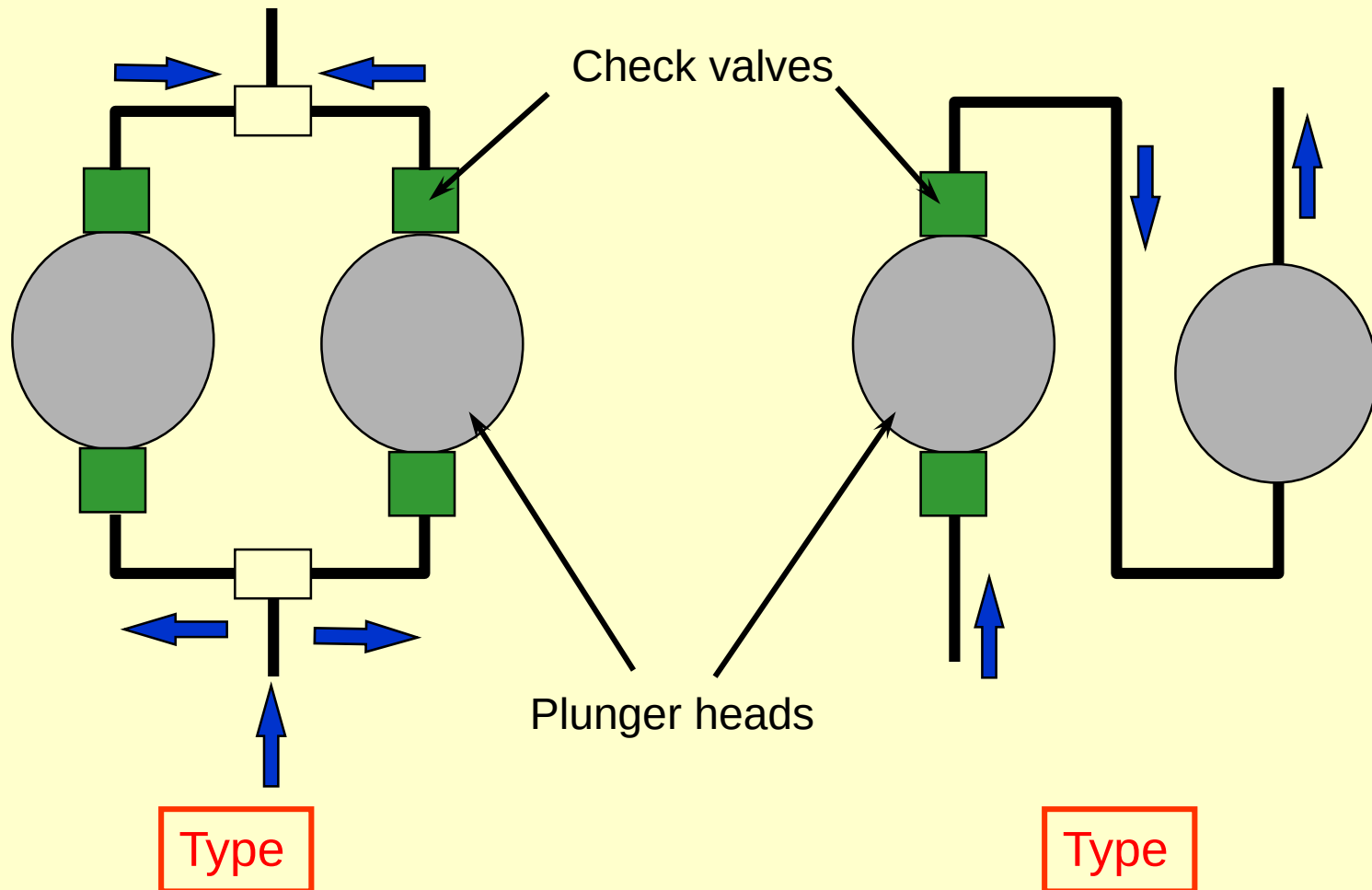


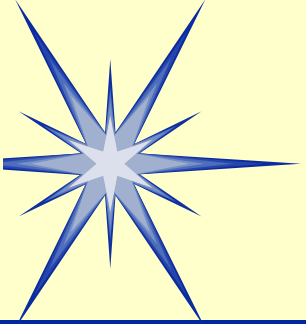
Solvent Delivery Pump: Single Plunger Type





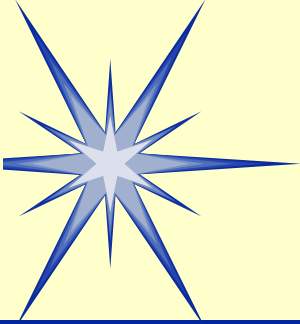
Solvent Delivery Pump: Dual Plunger Type





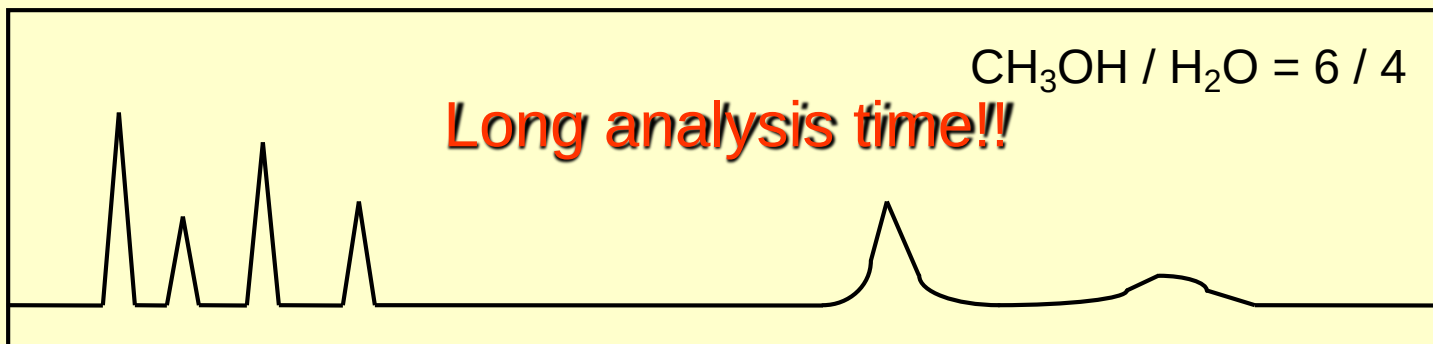
Gradient System

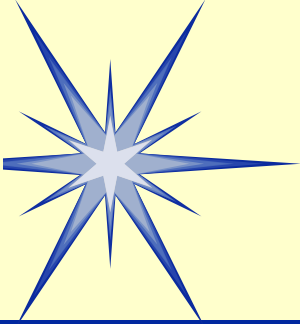
- Isocratic system
 - ❖ Constant eluent composition
- Gradient system
 - ❖ Varying eluent composition
 - ★ HPGE (High Pressure Gradient)
 - ★ LPGE (Low Pressure Gradient)



Aim of Gradient System (1)

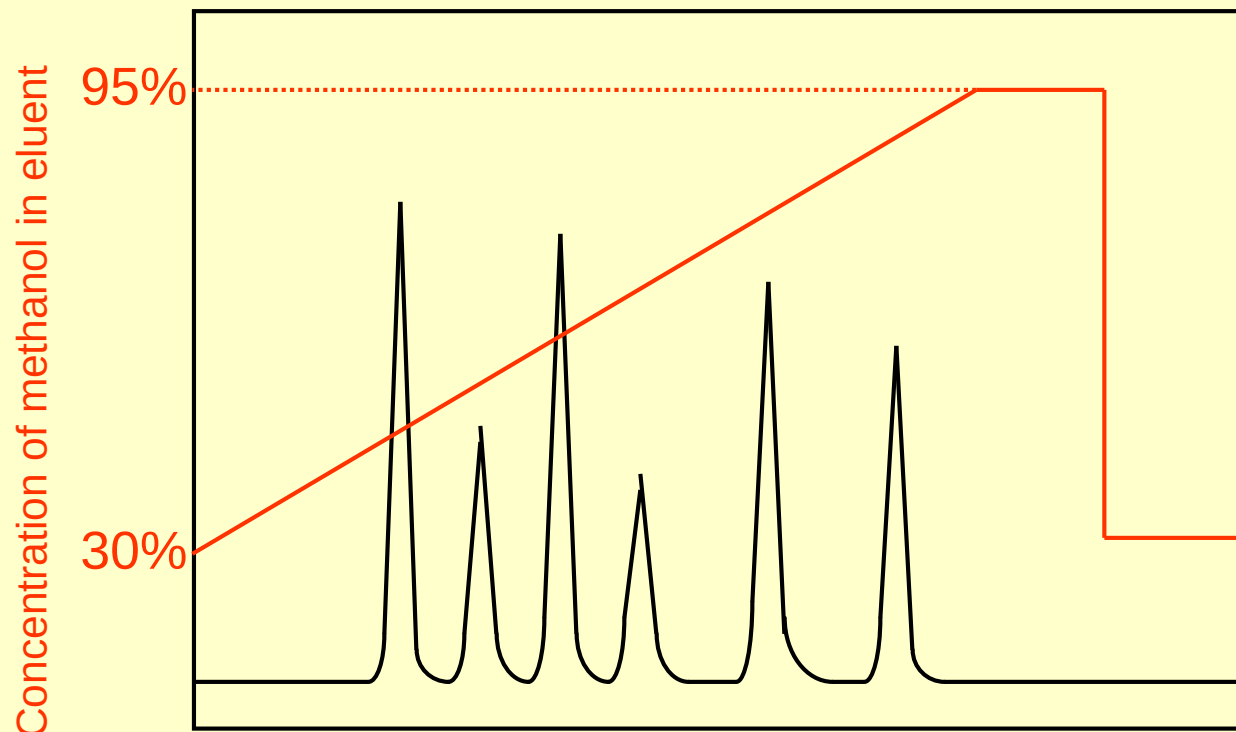
- In isocratic mode

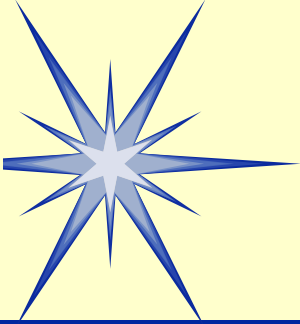




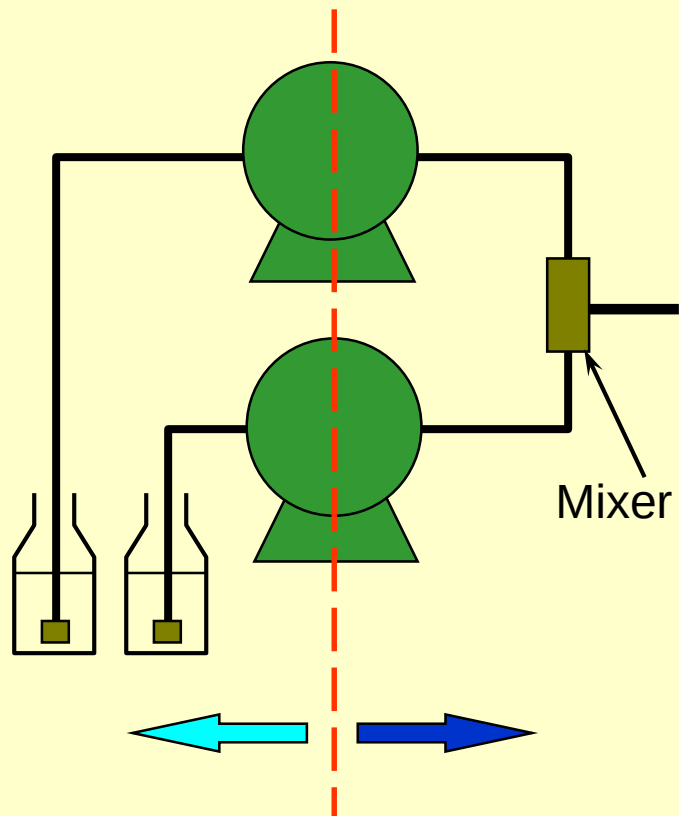
Aim of Gradient System (2)

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



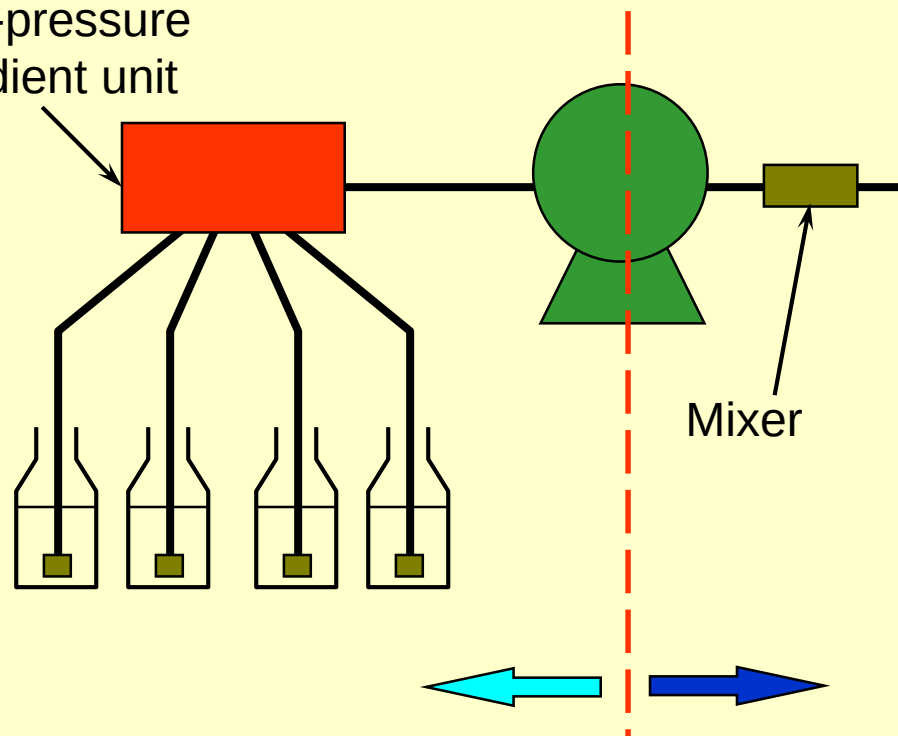


High- / Low-Pressure Gradient System

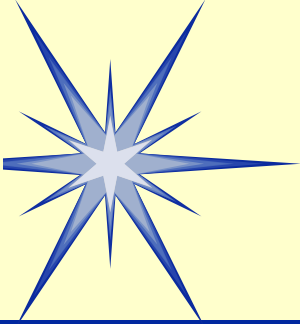


High-pressure gradient

Low-pressure
gradient unit

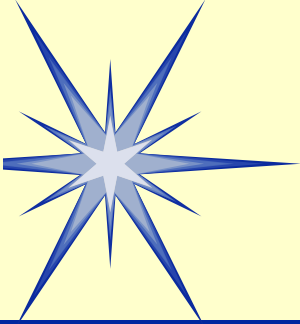


Low-pressure gradient



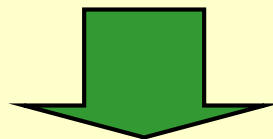
Advantages and Disadvantages of High- / Low-Pressure Gradient Systems

- High-pressure gradient system
 - ❖ High gradient accuracy
 - ❖ Complex system configuration (multiple pumps required)
- Low-pressure gradient system
 - ❖ Simple system configuration
 - ❖ Degasser required

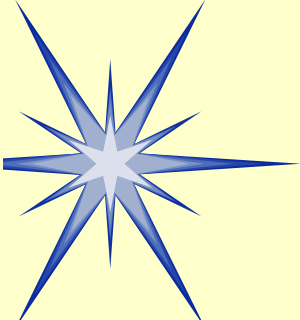


Degasser

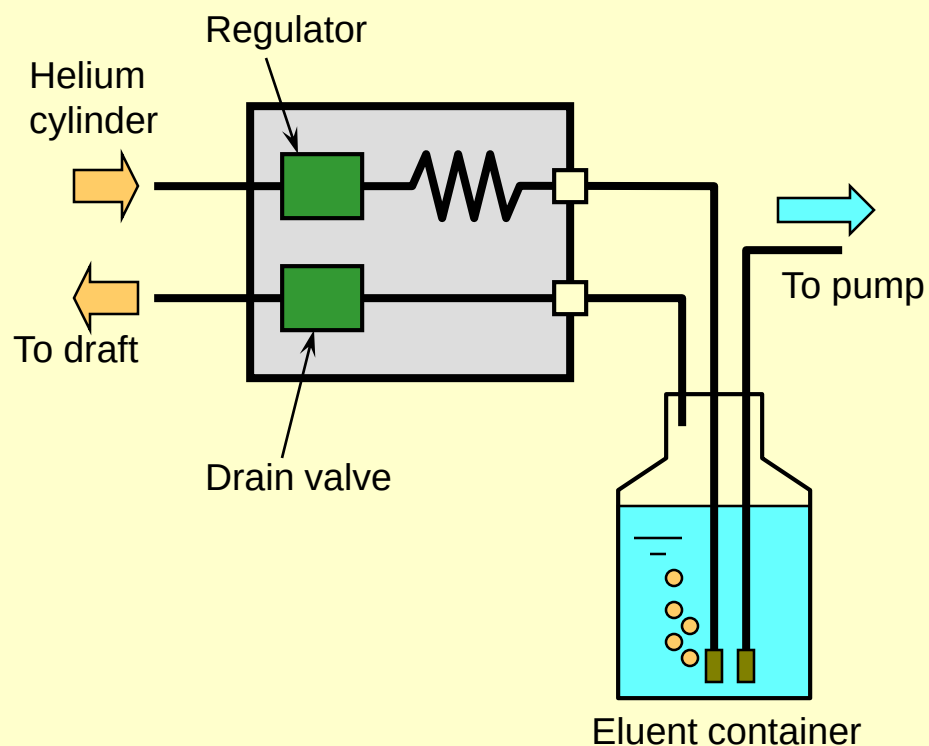
- Problems caused by dissolved air in the eluent
 - ❖ Unstable delivery by pump
 - ❖ More noise and large baseline drift in detector cell



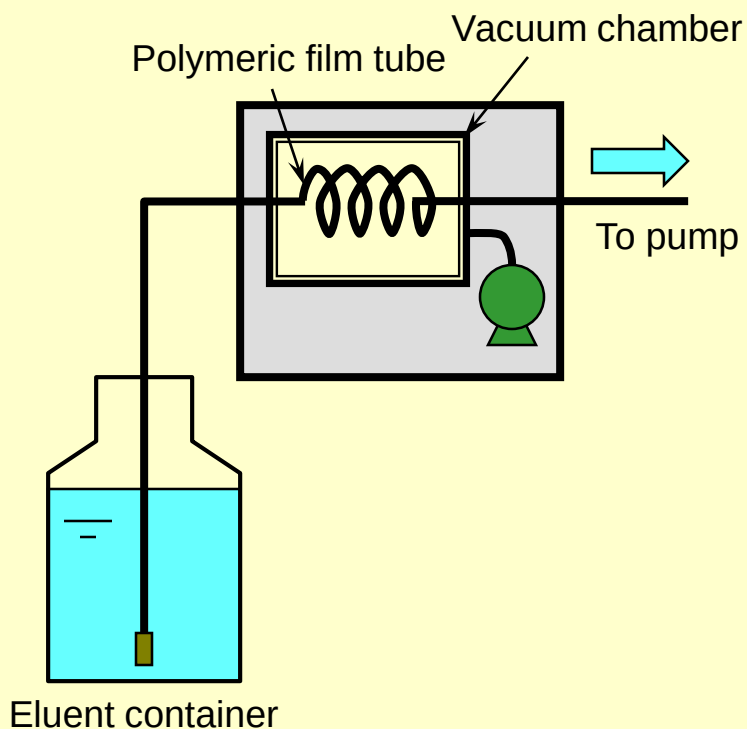
In order to avoid these problems, the eluent must be degassed.



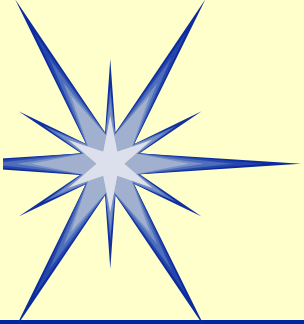
Online Degasser



Helium purge method

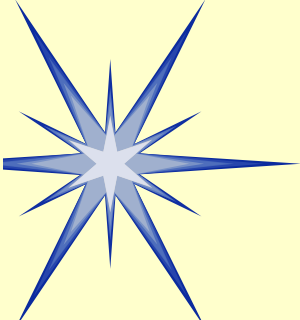


Gas-liquid separation membrane method

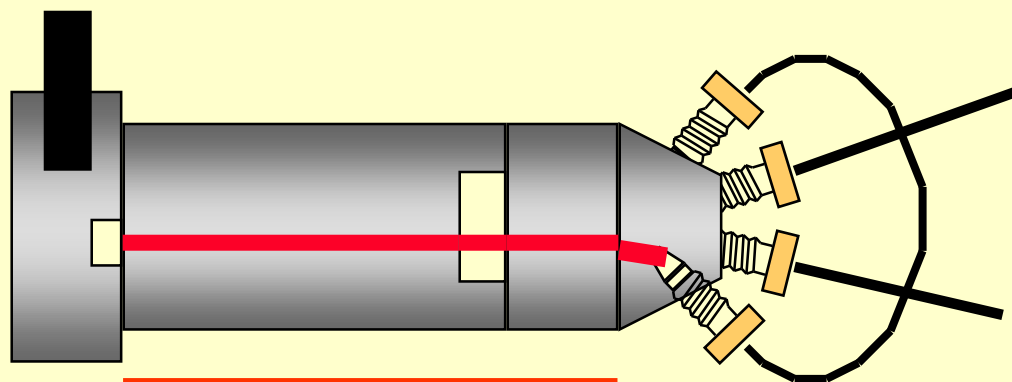


Sample Injection Unit (Injector)

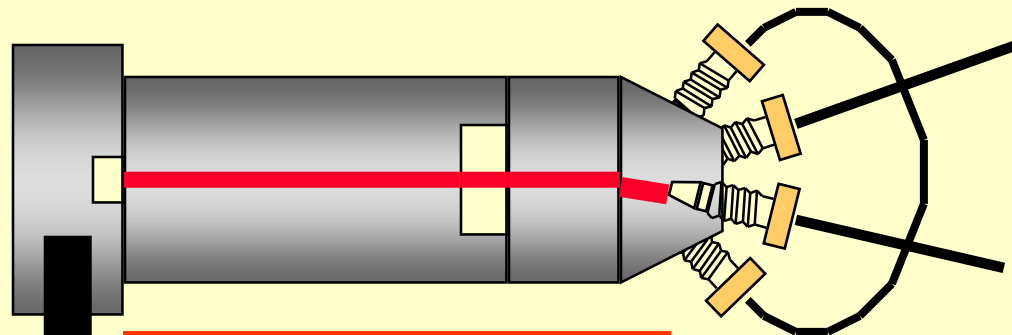
- Performance Requirements
 - ❖ No sample remaining in unit
 - ❖ Minimal broadening of sample band
 - ❖ Free adjustment of injection volume
 - ❖ Minimal loss
 - ❖ Superior durability and pressure resistance



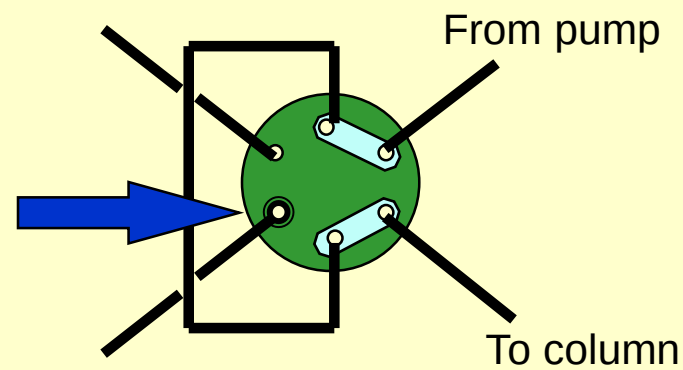
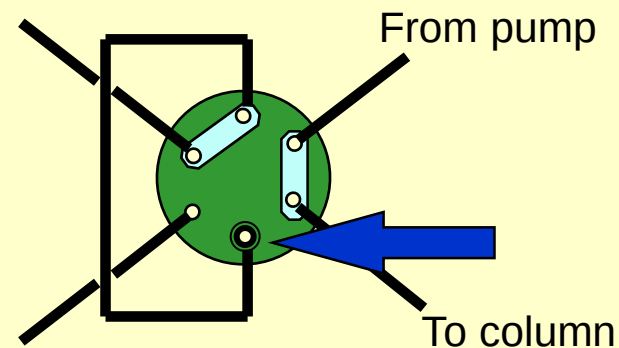
Manual Injector

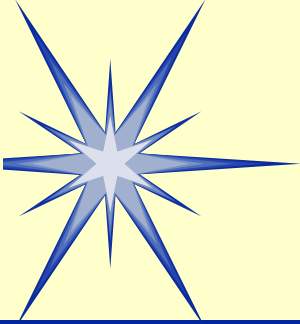


LOAD position

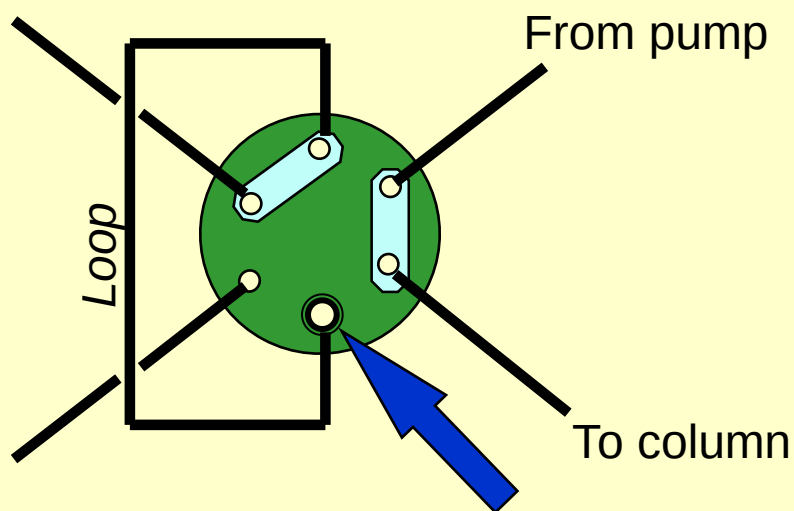


INJECT position

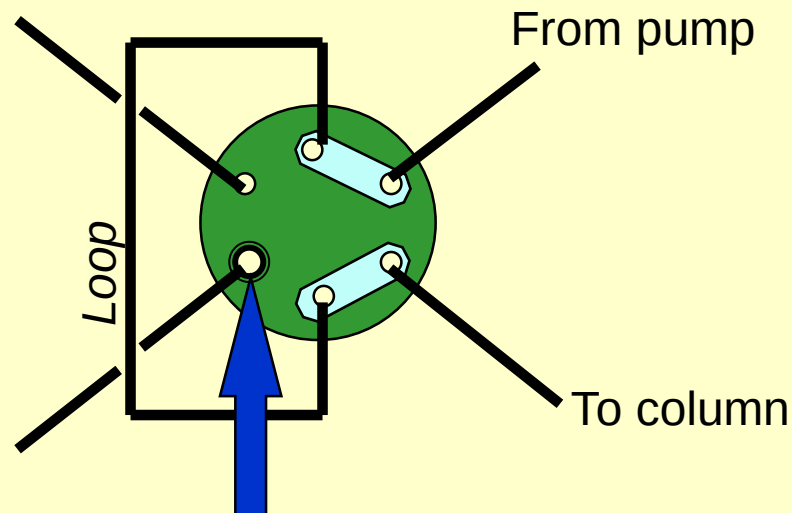




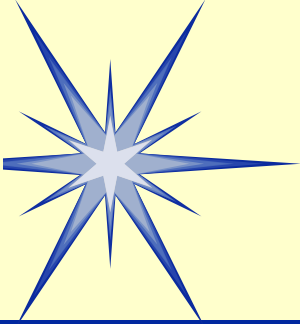
Manual Injector: Operating Principle of Sample Injection



LOAD

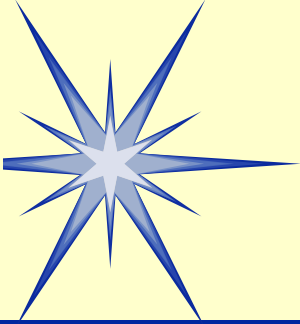


INJECT

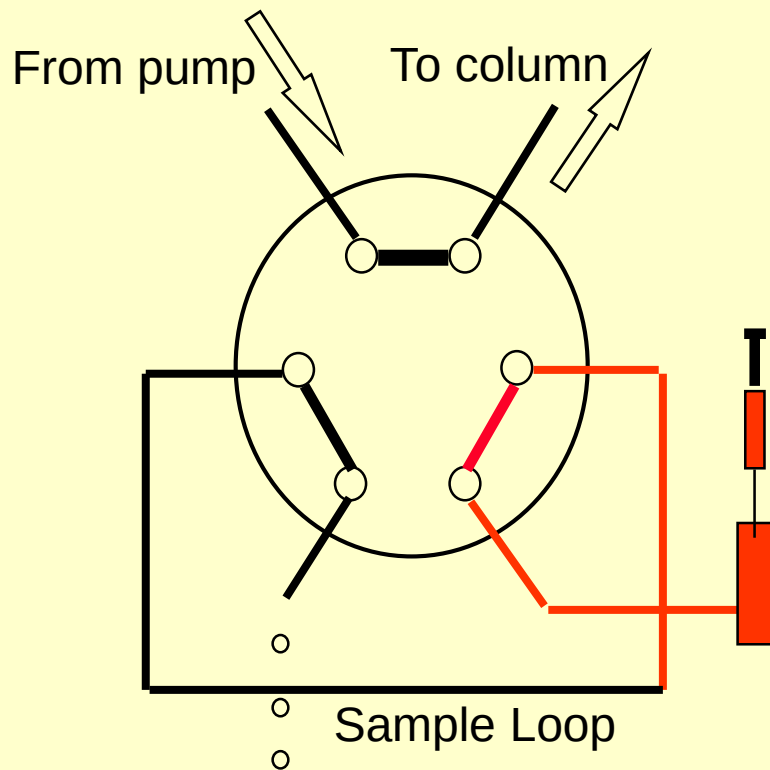


Manual Injector: Injection Method

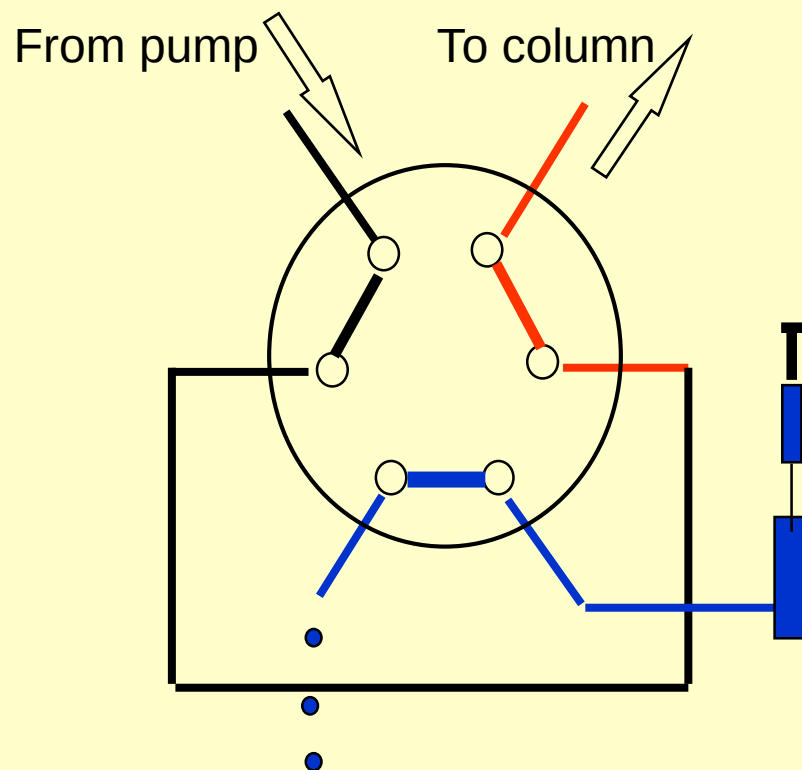
- Syringe measurement method
 - ❖ It is desirable that no more than half the loop volume is injected.
- Loop measurement method
 - ❖ It is desirable that at least 3 times the loop volume is injected.



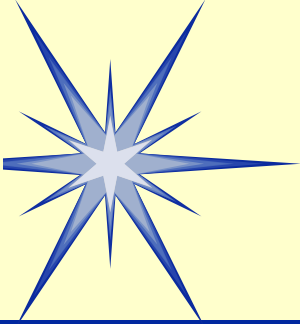
Autosampler (Pressure Injection Method)



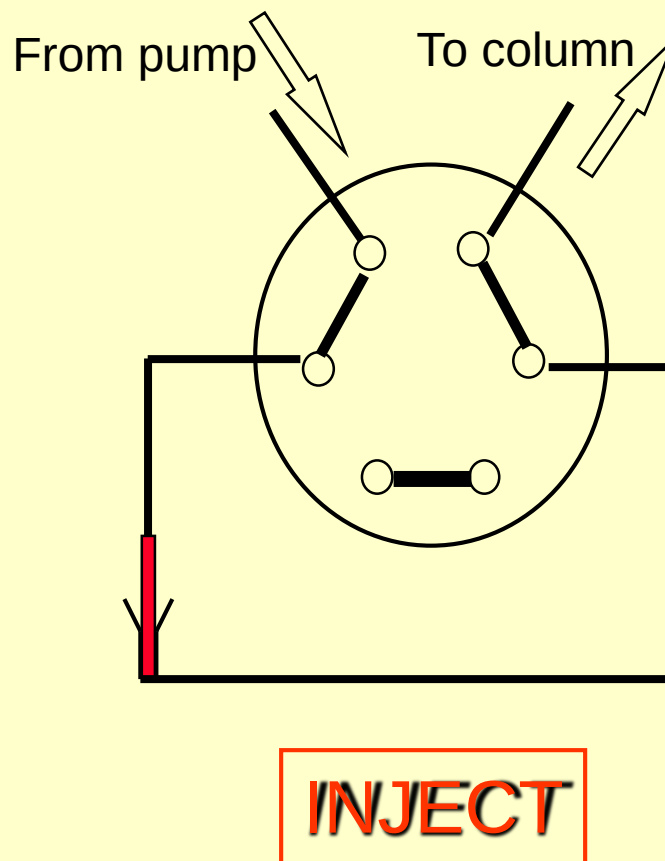
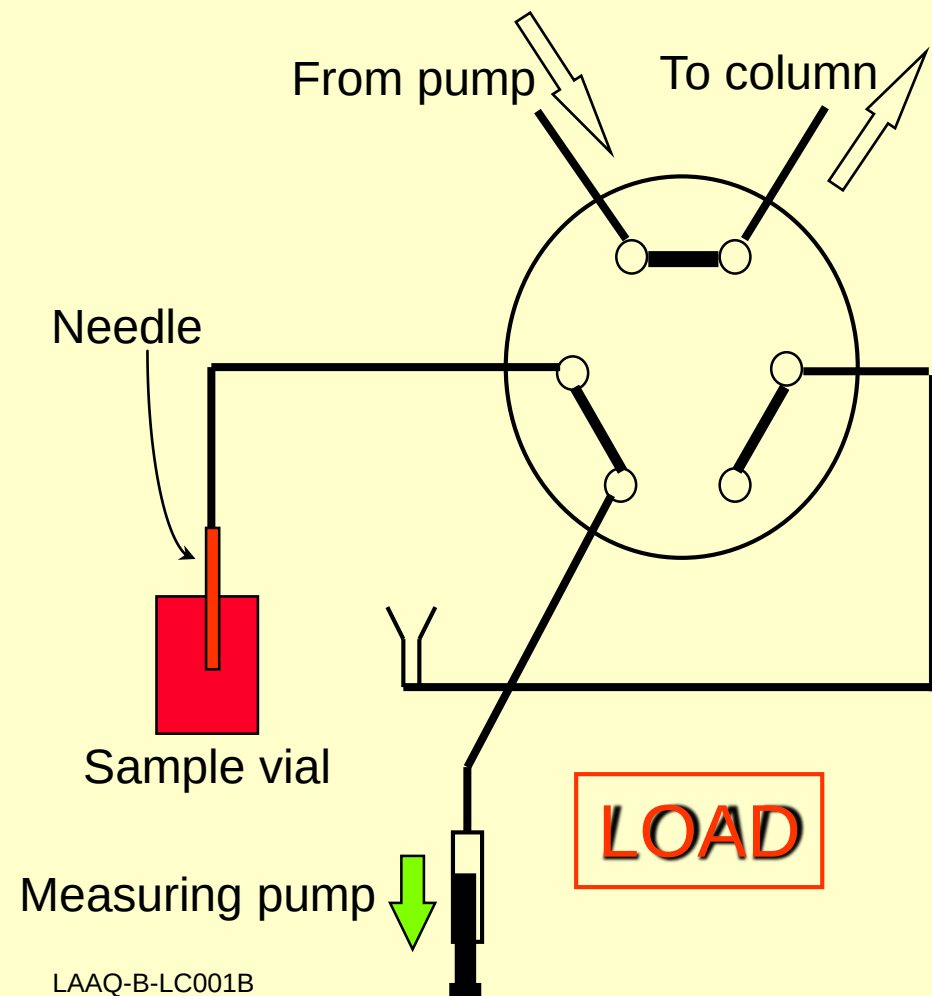
LOAD

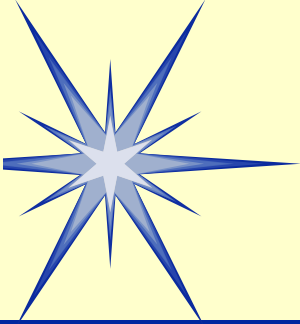


INJECT



Autosampler (Total-Volume Injection Method)



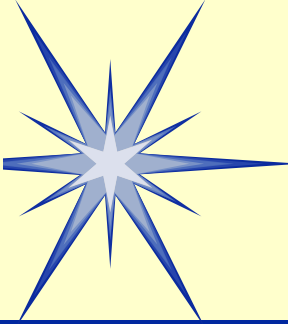


Column Oven

- Air circulation heating type
- Block heating type
 - ❖ Aluminum block heater
- Insulated column jacket type
 - ❖ Water bath

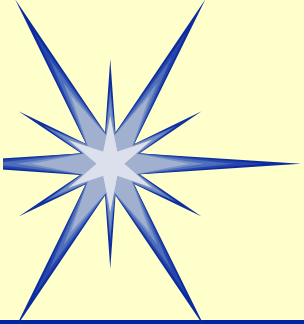
Tubing and Preparation for Solvent Delivery

Prior to Analysis



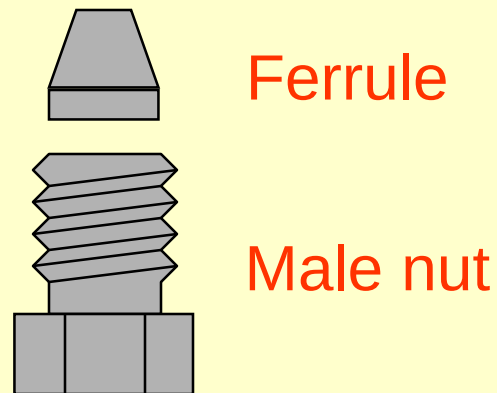
Tubing

- Material
 - ❖ Stainless steel (SUS)
 - ❖ PEEK (polyether ether ketone)
 - ❖ Fluororesin
- O.D. (outer diameter)
 - ❖ 1.6 mm
- I.D. (inner diameter)
 - ❖ 0.1 mm
 - ❖ 0.3 mm
 - ❖ 0.5 mm
 - ❖ 0.8 mm etc.



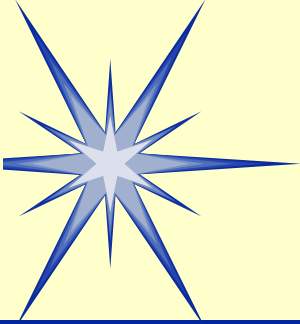
Connectors

- Male nut (SUS)
Ferrule (SUS)
 - ❖ Sealing possible up to 40 MPa



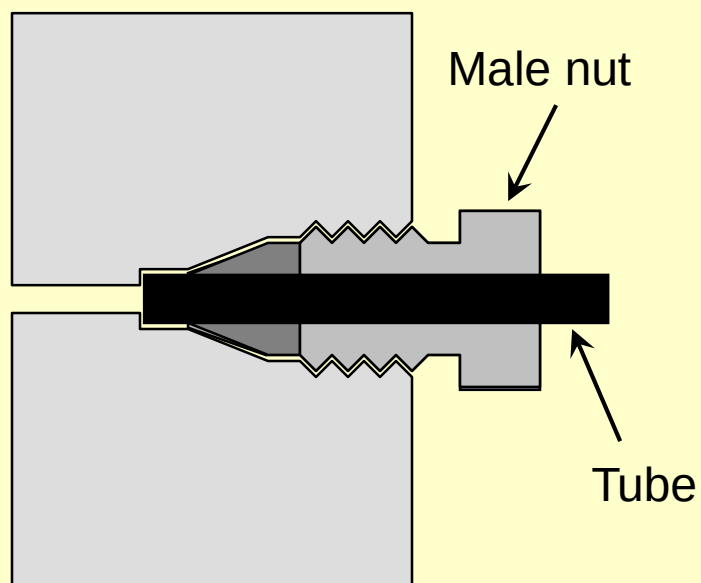
- Male nut (PEEK)
 - ❖ Can be connected without any tools
 - ❖ Resists pressures of up to approx. 25 MPa



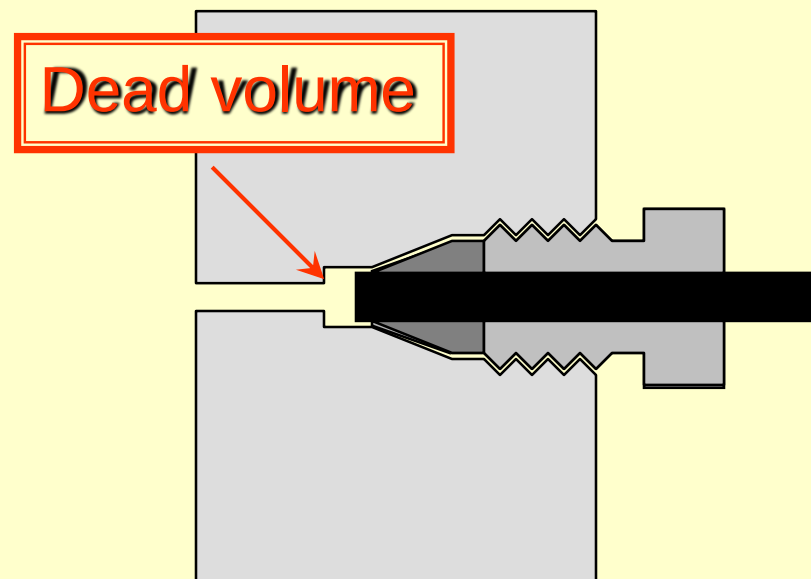


Dead Volume (Extra-column volume)

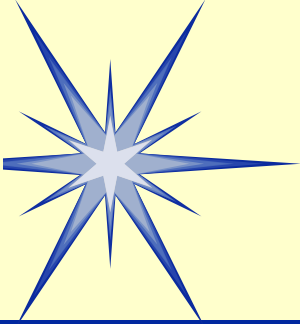
- Dead volume can cause peaks broadening.



Excellent connection



Poor connection



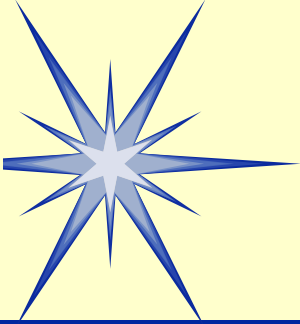
Mobile Phase

● Water

- ❖ “Ultrapure water” can be used with confidence.
- ❖ Commercial “distilled water for HPLC” is also acceptable.

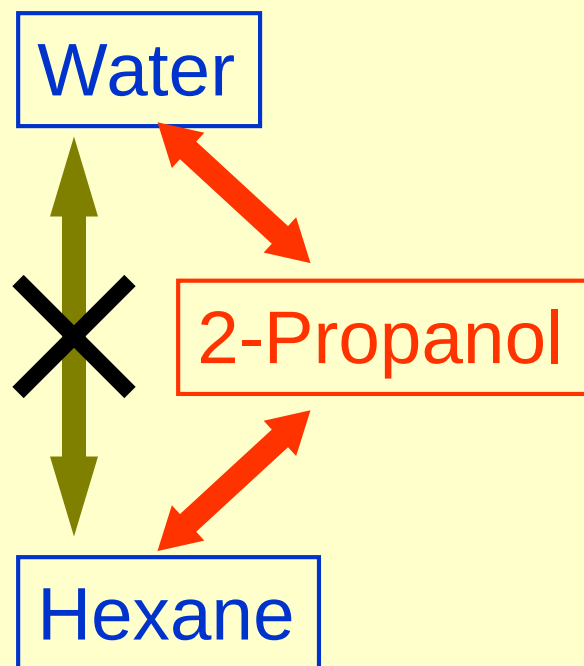
● Organic Solvent

- ❖ HPLC-grade solvent can be used with confidence.
- ❖ Special-grade solvent is acceptable depending on the detection conditions.
- ❖ Care is required regarding solvents containing stabilizers (e.g., tetrahydrofuran and chloroform)

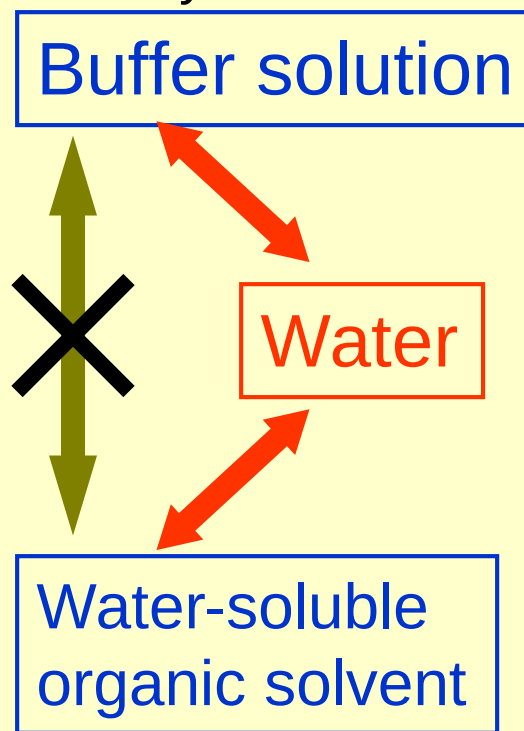


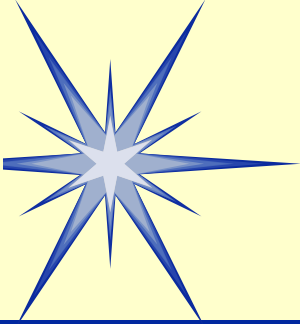
Replacement of Eluent

- Mutually insoluble solvents must not be exchanged directly.

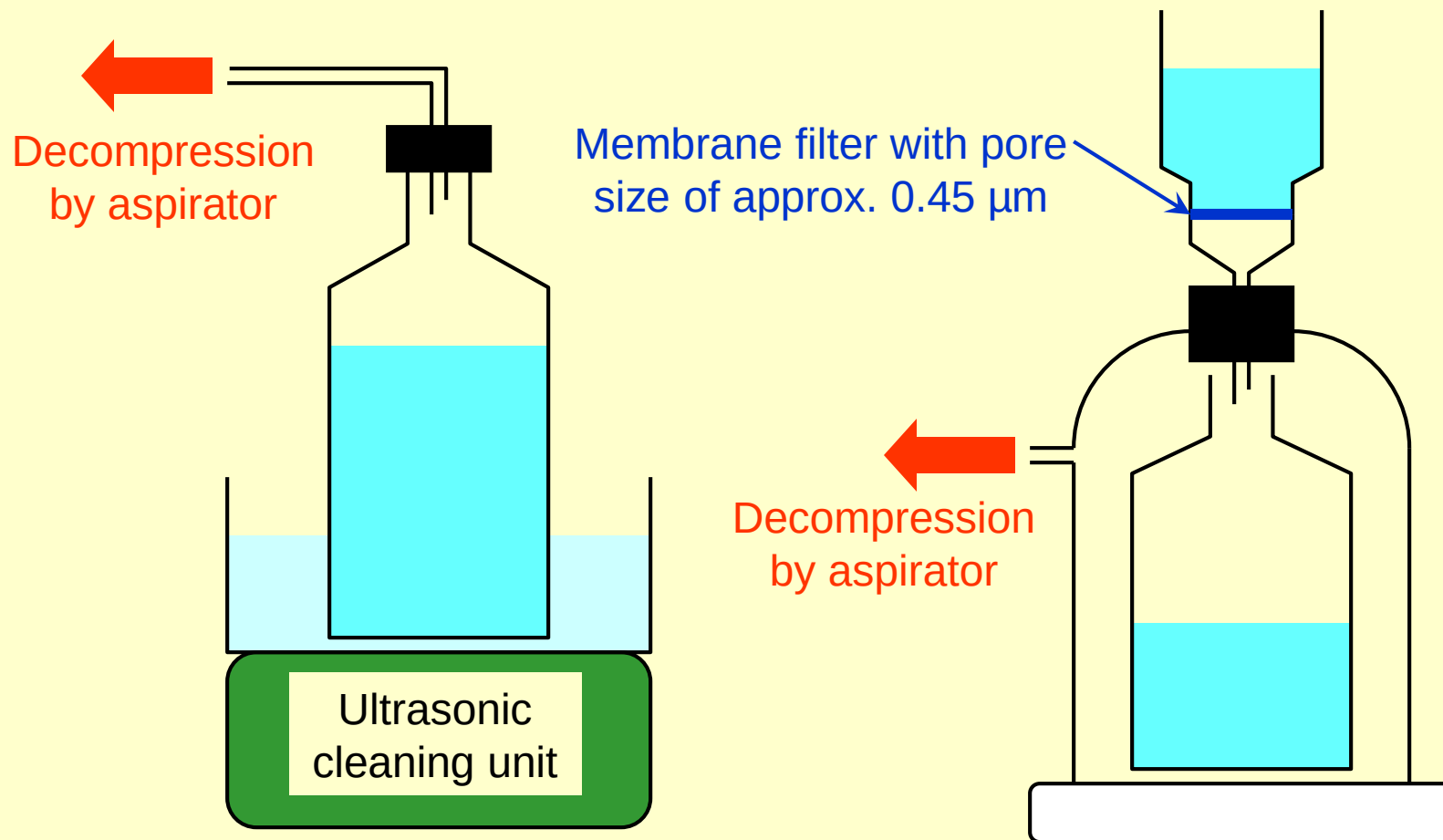


- Aqueous solutions containing salt and organic solvents must not be exchanged directly.



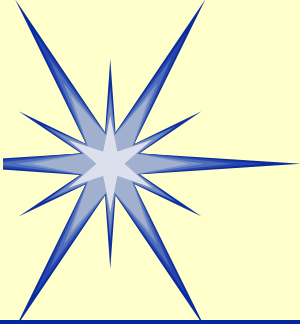


Mixing, Filtration, and Offline Degassing of the Eluent



Reversed Phase Chromatography Part 1

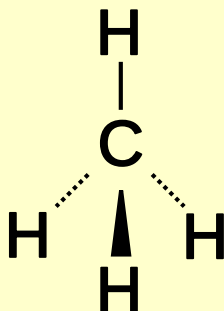
Basic Principles



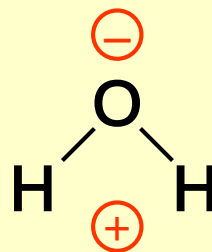
Polarity of Substances

● Polarity

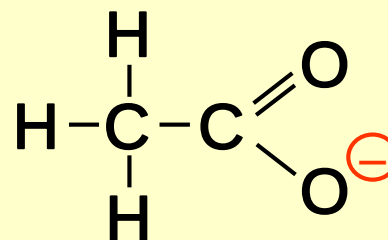
- ❖ Property of a substance whereby the positions of the electrons give rise to positive and negative poles
- ❖ Water: Polar
Methane: Nonpolar



Methane



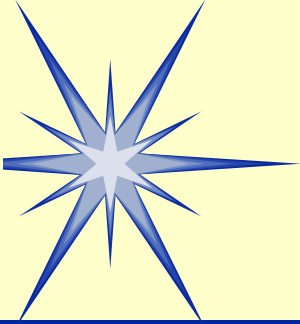
Water



Acetic acid

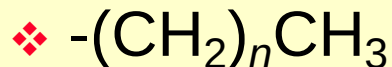
● Miscibility of solvents

- ❖ Solvents of similar polarities can be easily dissolved together.
- ❖ Polar and nonpolar molecules have a similar relationship to that of water and oil.

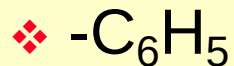


Nonpolar (Hydrophobic) Functional Groups and Polar (Hydrophilic) Functional Groups

- Nonpolar Functional Groups



- ★ Alkyl groups

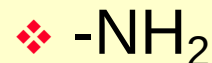


- ★ Phenyl groups

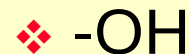
- Polar Functional Groups



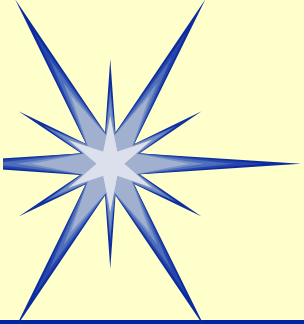
- ★ Carboxyl groups



- ★ Amino groups

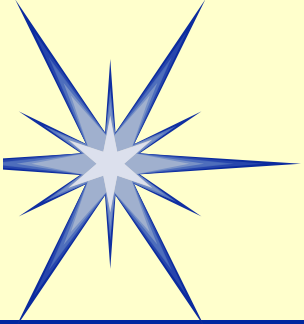


- ★ Hydroxyl groups



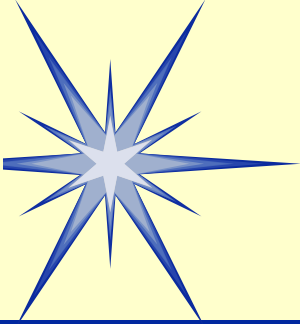
Partition Chromatography

- A liquid (or a substance regarded as a liquid) is used as the stationary phase, and the solute is separated according to whether it dissolves more readily in the stationary or mobile phase.
- Liquid-liquid chromatography



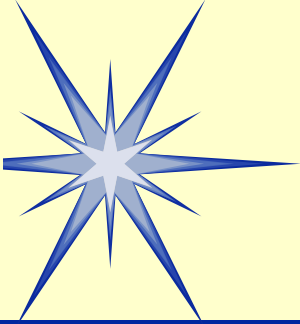
Normal Phase / Reversed Phase

	Stationary phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)



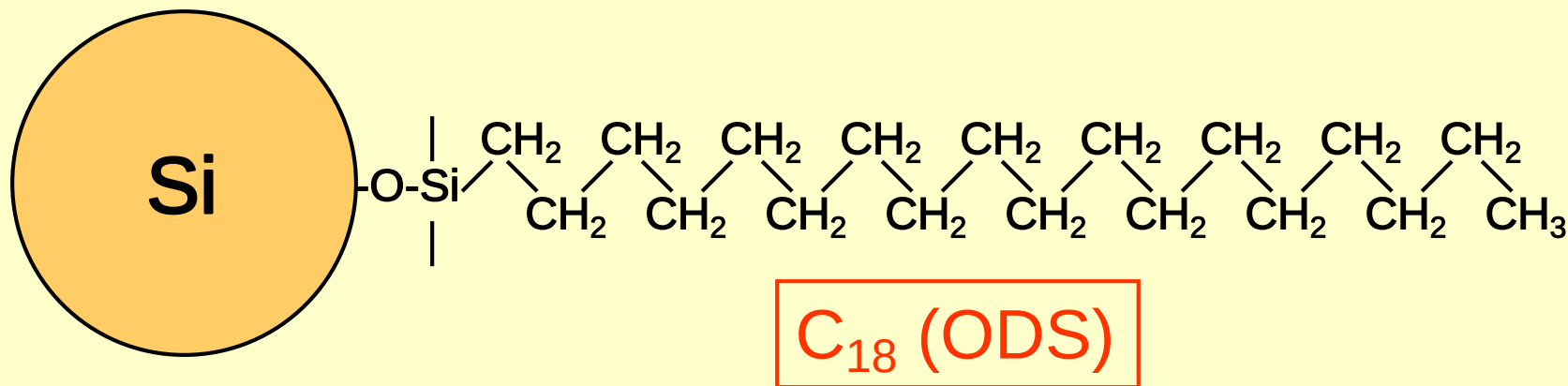
Reversed Phase Chromatography

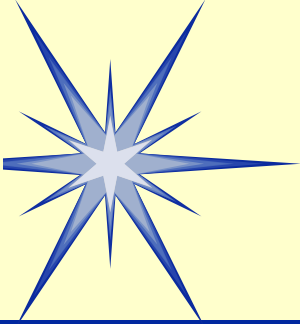
- Stationary phase: Low polarity
 - ❖ Octadecyl group-bonded silical gel (ODS)
- Mobile phase: High polarity
 - ❖ Water, methanol, acetonitrile
 - ❖ Salt is sometimes added.



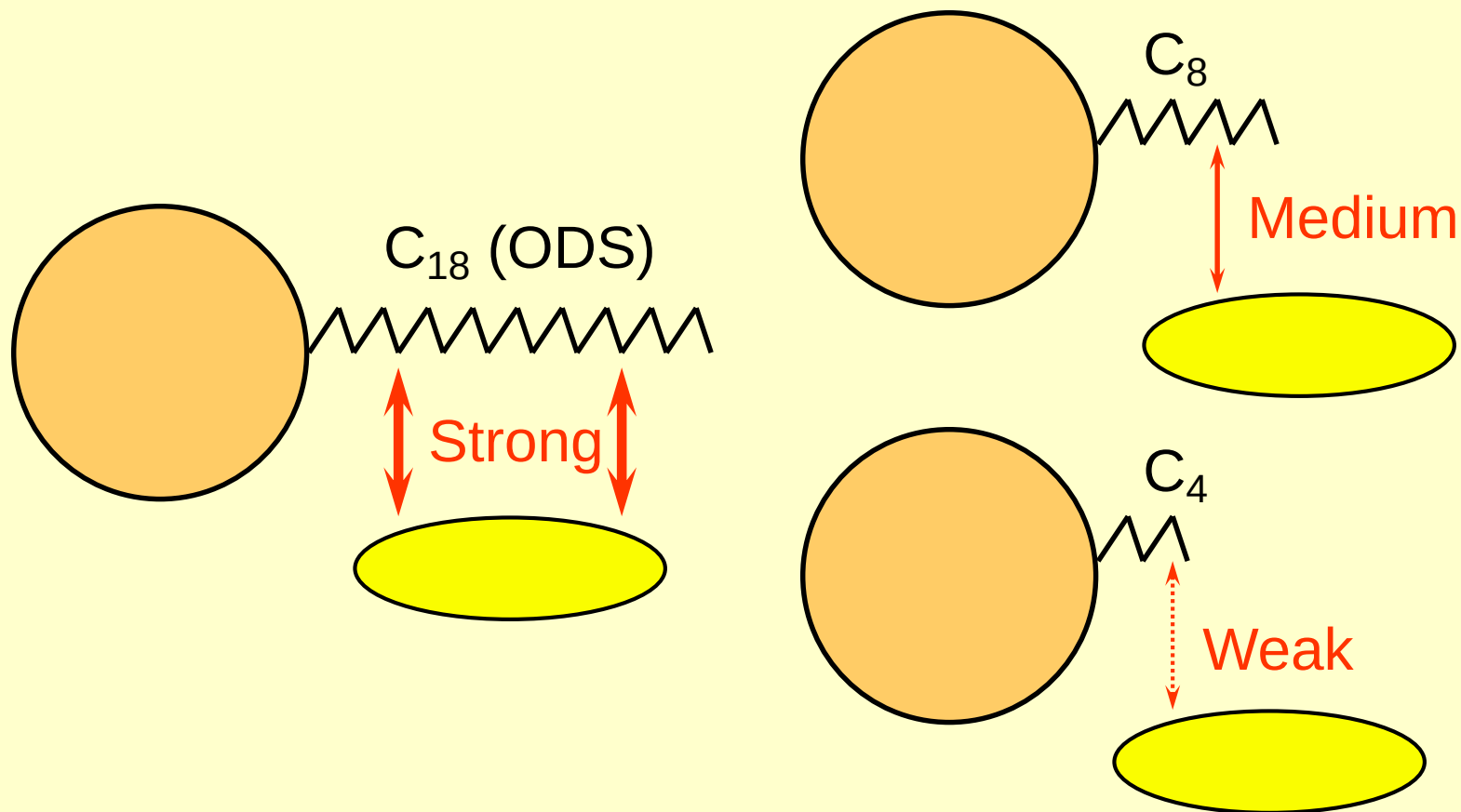
Separation Column for Reversed Phase Chromatography

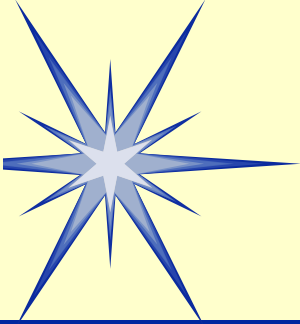
- C₁₈ (ODS) type
- C₈ (octyl) type
- C₄ (butyl) type
- Phenyl type
- TMS type
- Cyano type



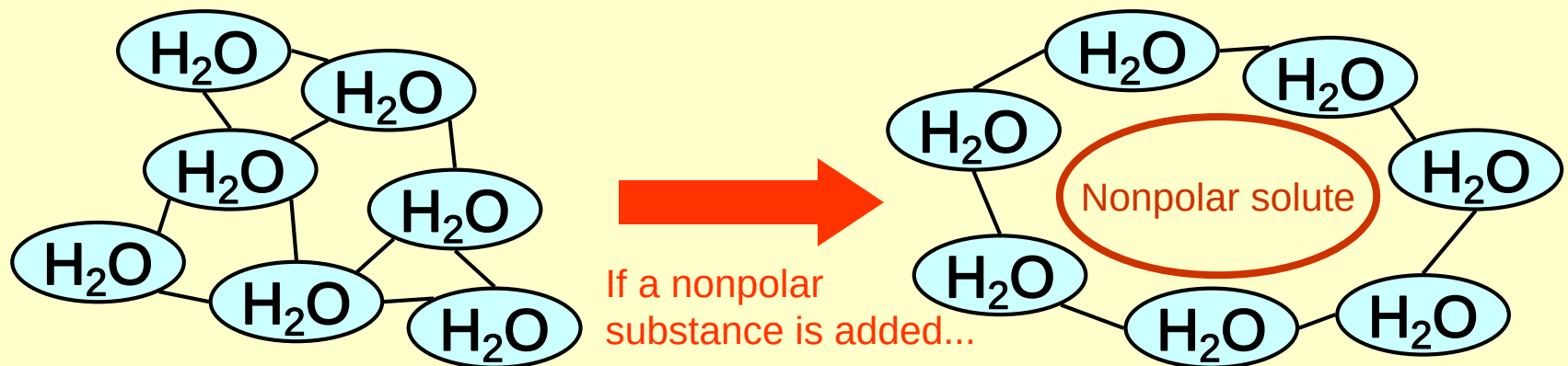


Effect of Chain Length of Stationary Phase





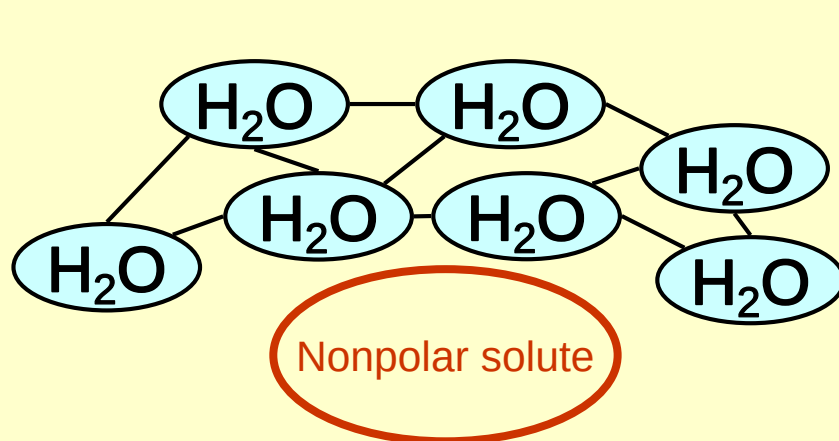
Hydrophobic Interaction



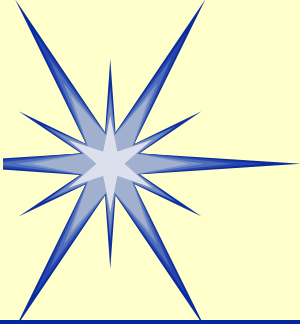
Network of hydrogen bonds

If a nonpolar substance is added...

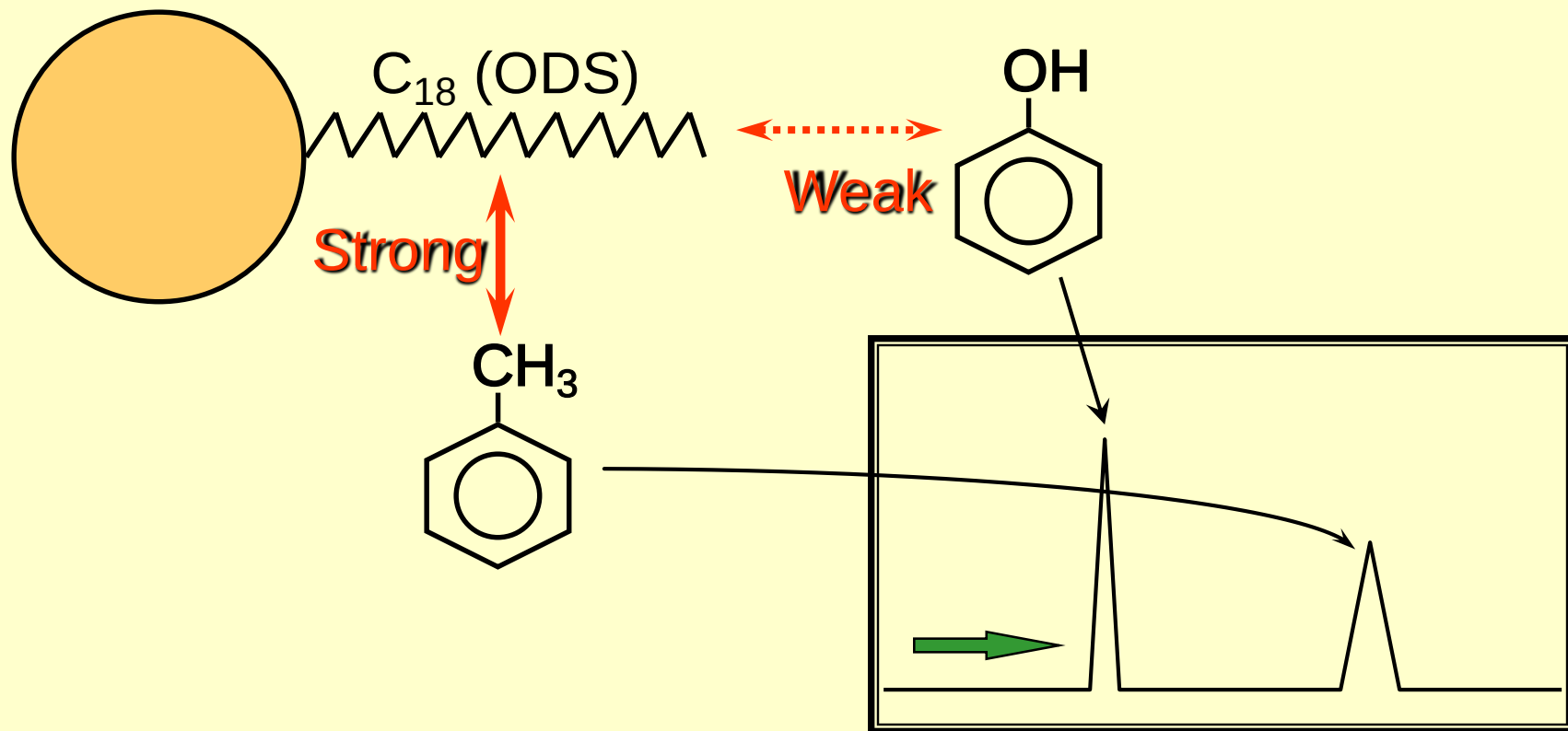
...the network is broken and...

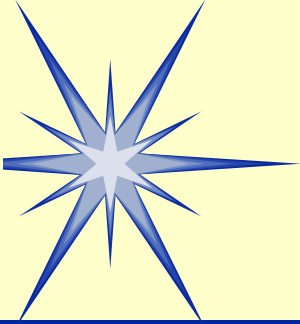


...the nonpolar substance is pushed to a nonpolar location.



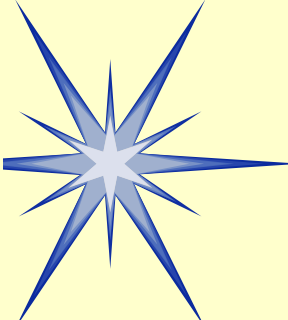
Relationship Between Retention Time and Polarity





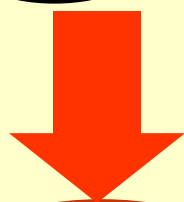
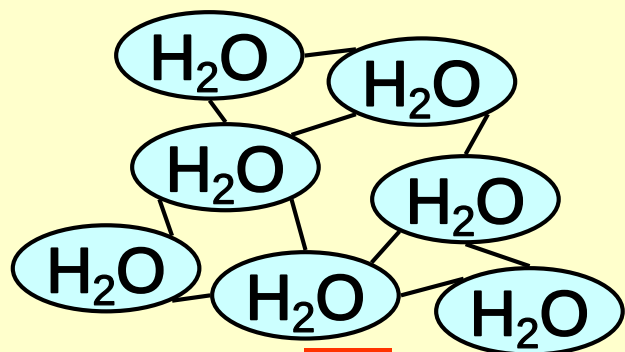
Basic Settings for Eluent Used in Reversed Phase Mode

- Water (buffer solution) + water-soluble organic solvent
 - ❖ Water-soluble organic solvent: Methanol
Acetonitrile
Tetrahydrofuran etc.
 - ❖ The **mixing ratio** of the water (buffer solution) and organic solvent has the greatest influence on separation.
 - ❖ If a buffer solution is used, its **pH** value is an important separation parameter.



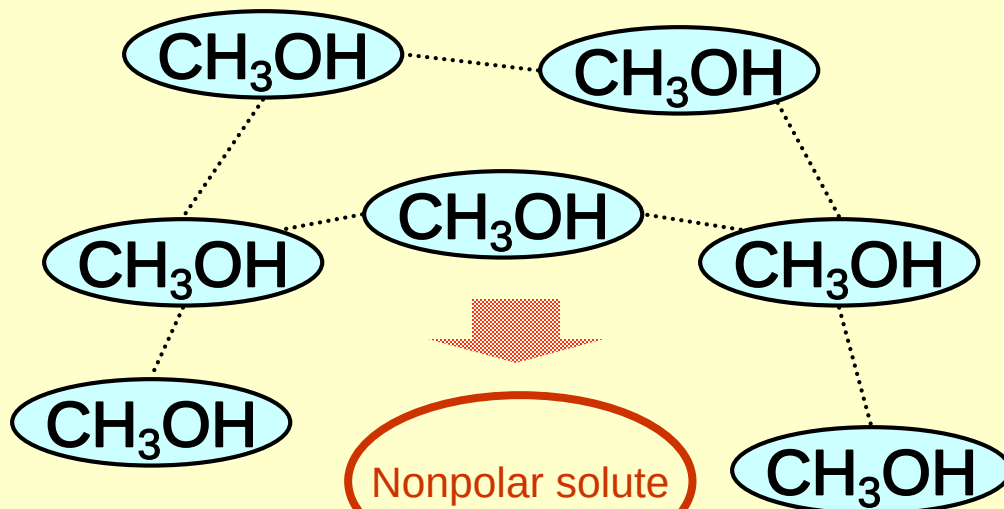
Difference in Solute Retention Strengths for Water and Water-Soluble Organic Solvents

Tightly packed network



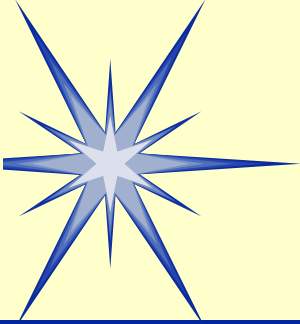
Nonpolar solute

Loose network

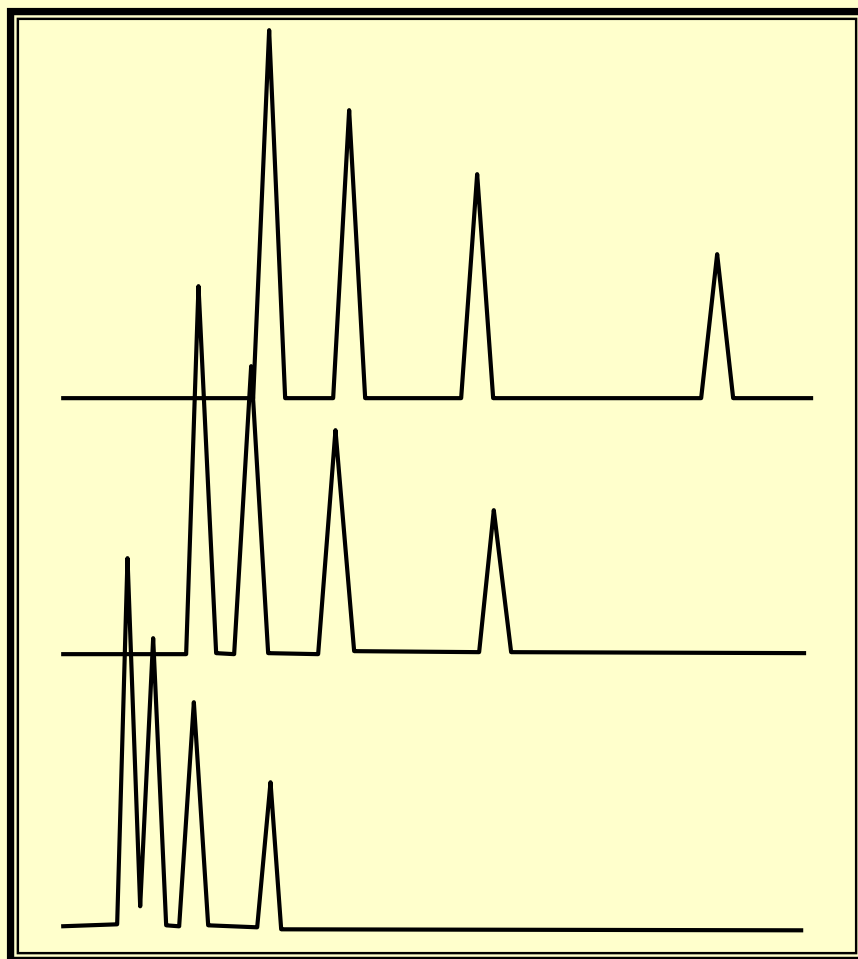


Nonpolar solute

Nonpolar stationary phase



Relationship between Polarity of Eluent and Retention Time in Reversed Phase Mode



Eluent: Methanol / Water

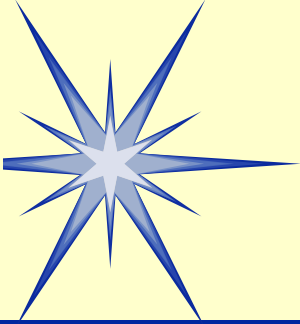
60/40

70/30

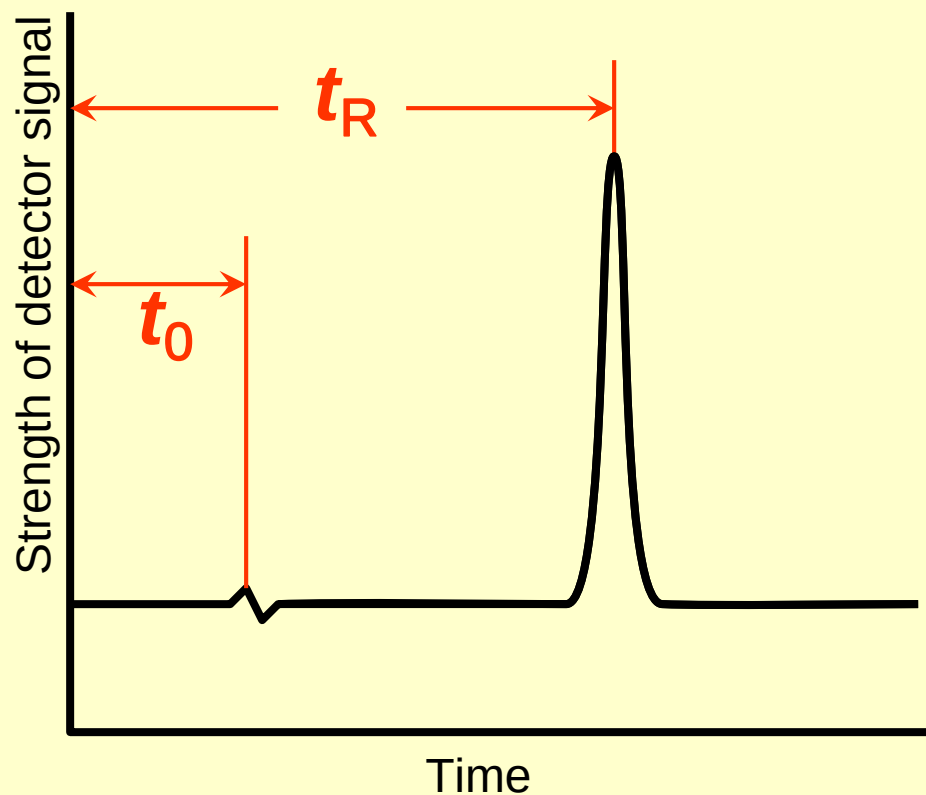
80/20

Chromatogram Parameters

Methods for Expressing Separation
and Column Performance



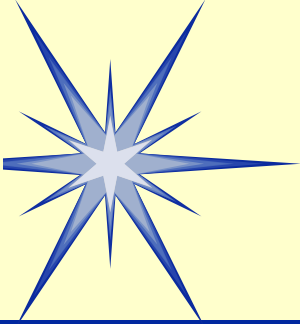
Retention Factor, k



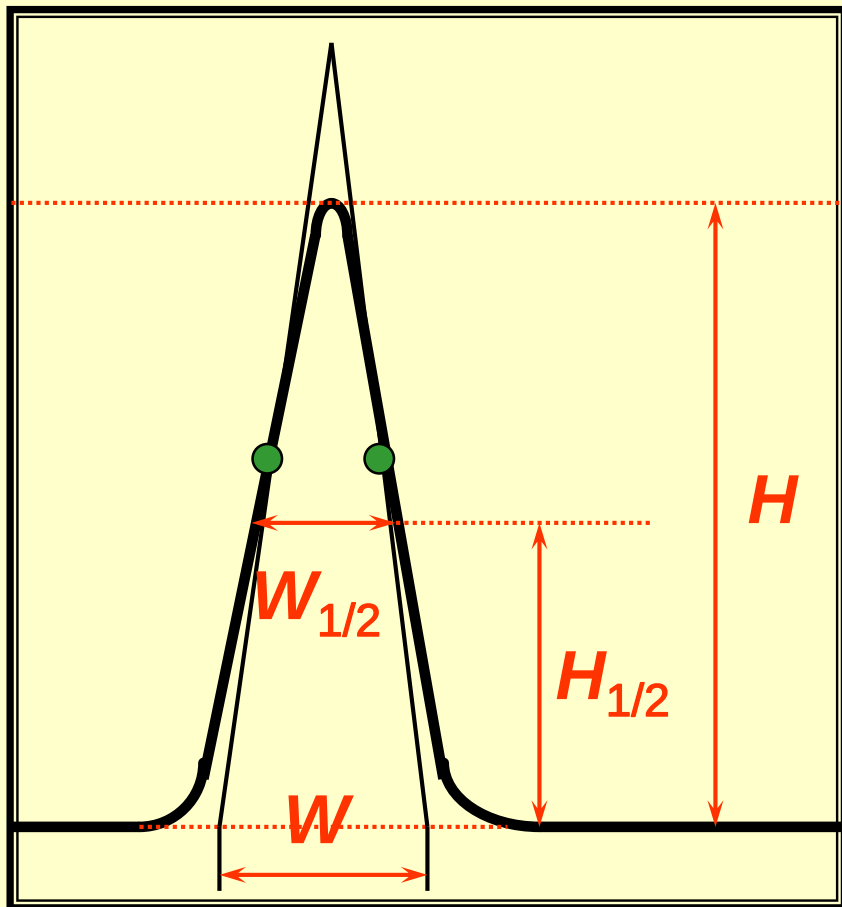
$$k = \frac{t_R - t_0}{t_0}$$

t_R : Retention time

t_0 : Non-retention time



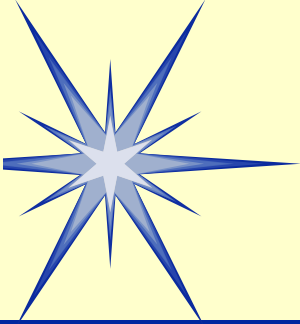
Theoretical Plate Number, N



$$N = 16 \left(\frac{t_R}{W} \right)^2$$

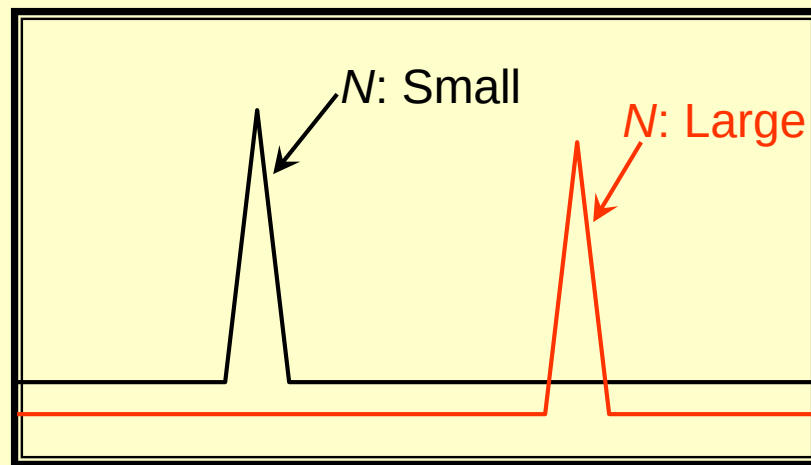
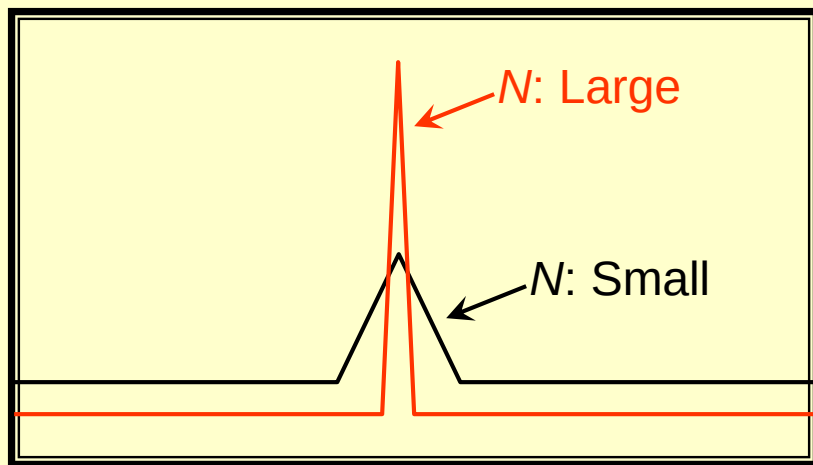
$$= 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2$$

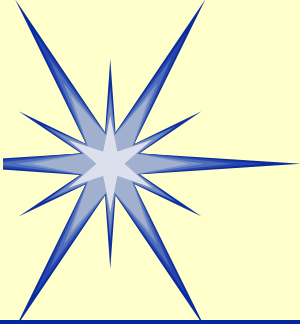
$$= 2\pi \left(\frac{t_R \bullet H}{Area} \right)^2$$



Evaluation of Column Efficiency Based on Theoretical Plate Number

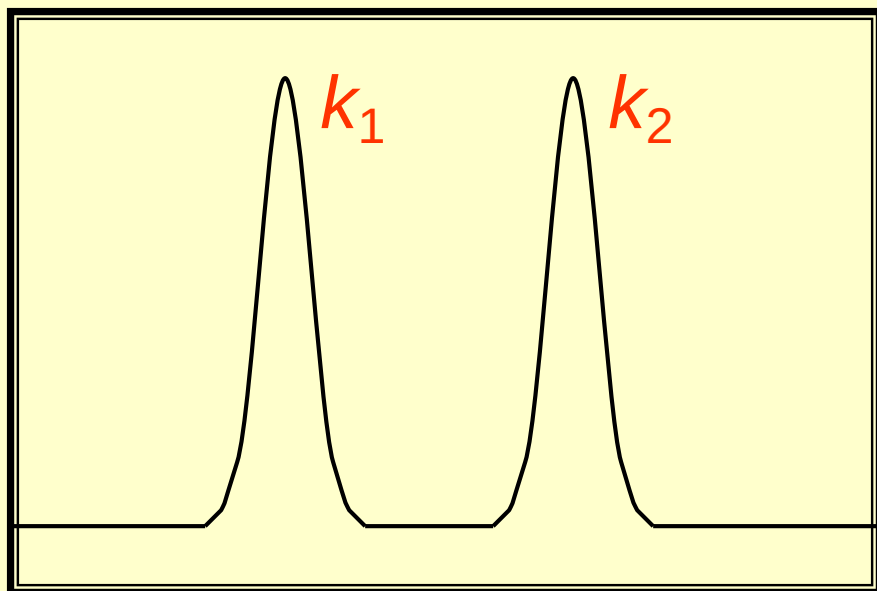
- If the retention times are the same, the peak width is smaller for the one with the larger theoretical plate number.
- If the peak width is the same, the retention time is longer for the one with the larger theoretical plate number.



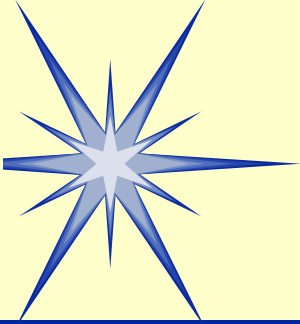


Separation Factor, α

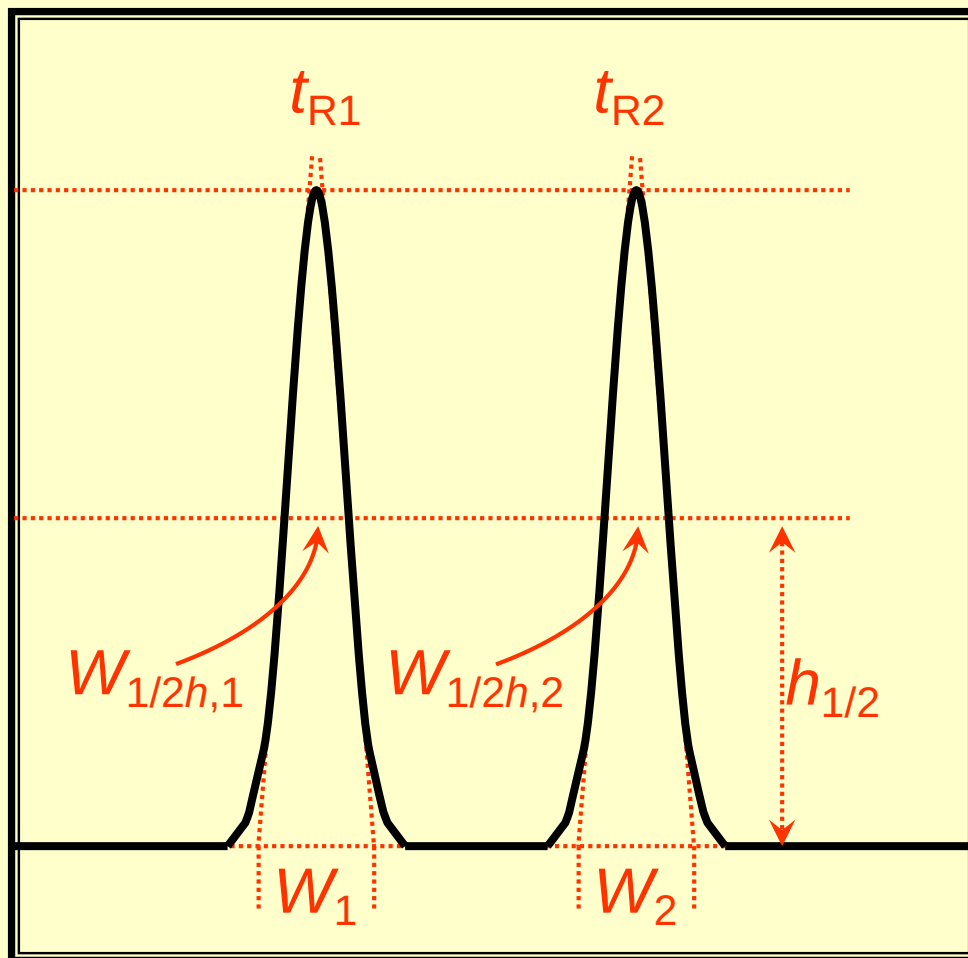
- Separation factor: Ratio of k 's of two peaks



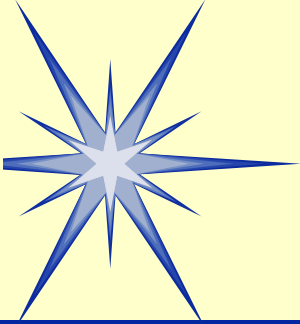
$$\alpha = \frac{k_2}{k_1}$$
$$(k_2 > k_1)$$



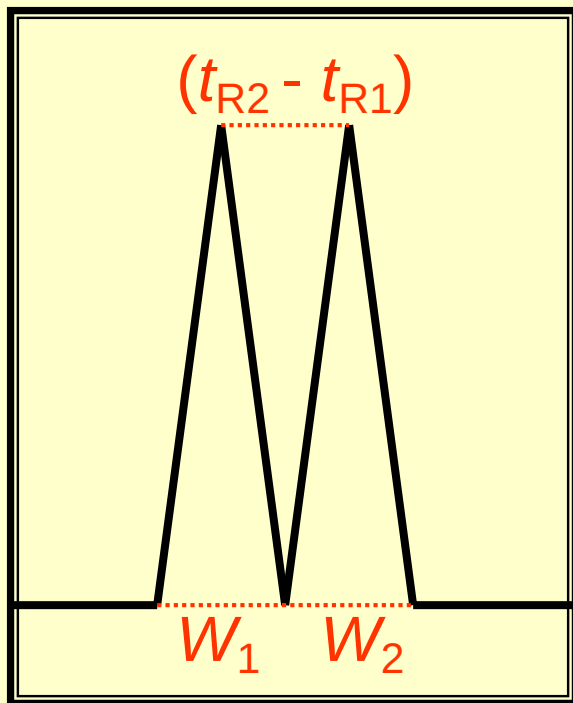
Resolution, R_S



$$R_S = \frac{t_{R_2} - t_{R_1}}{\frac{1}{2}(W_1 + W_2)}$$
$$= 1.18 \times \frac{t_{R_2} - t_{R_1}}{W_{1/2h,1} + W_{1/2h,2}}$$



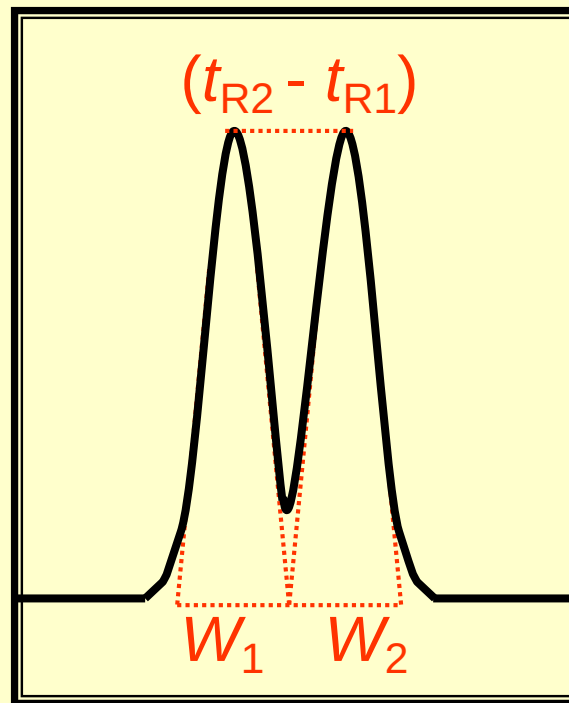
Resolution Required for Complete Separation



$$t_{R2} - t_{R1} = W_1 = W_2$$

$$R_S = 1$$

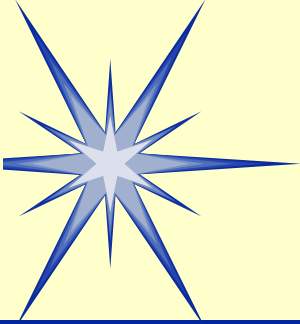
If the peaks are isosceles triangles, they are completely separated.



$$t_{R2} - t_{R1} = W_1 = W_2$$

$$R_S = 1$$

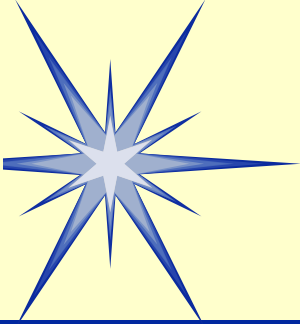
If the peaks are Gaussian distributions, $R_S > 1.5$ is necessary for complete separation.



Relationship Between Resolution and Other Parameters

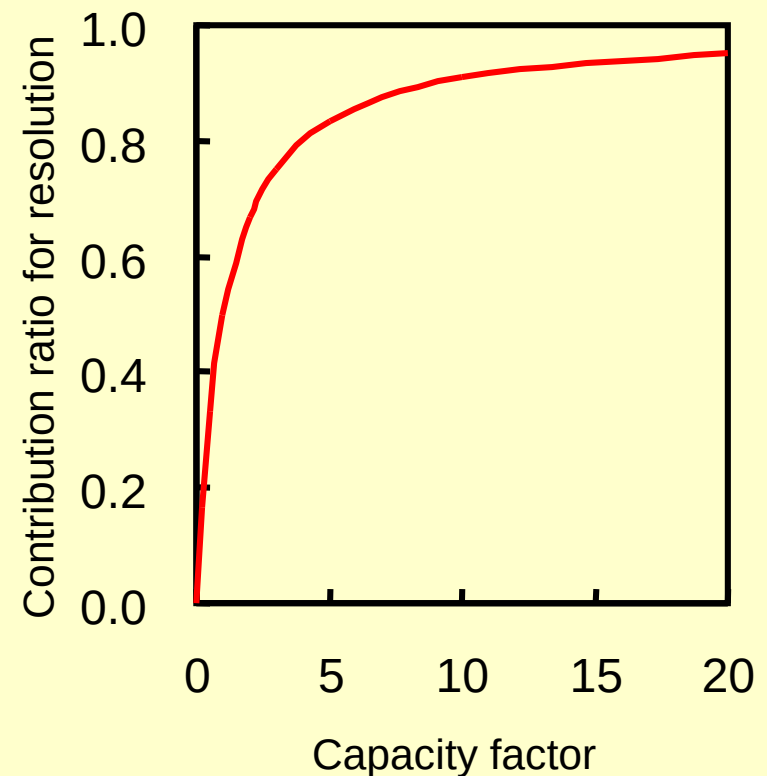
- The resolution is a function of the separation factor, the theoretical plate number, and the retention factor.
- The separation can be improved by improving these 3 parameters!

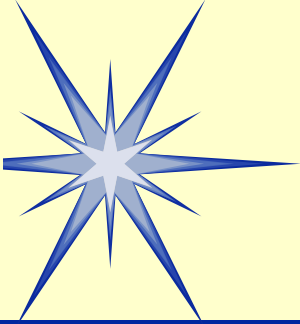
$$R_s = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)}$$
$$= \frac{1}{4} \sqrt{N} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k_2'}{k_2' + 1} \right)$$



Contribution of Capacity Factor to Resolution

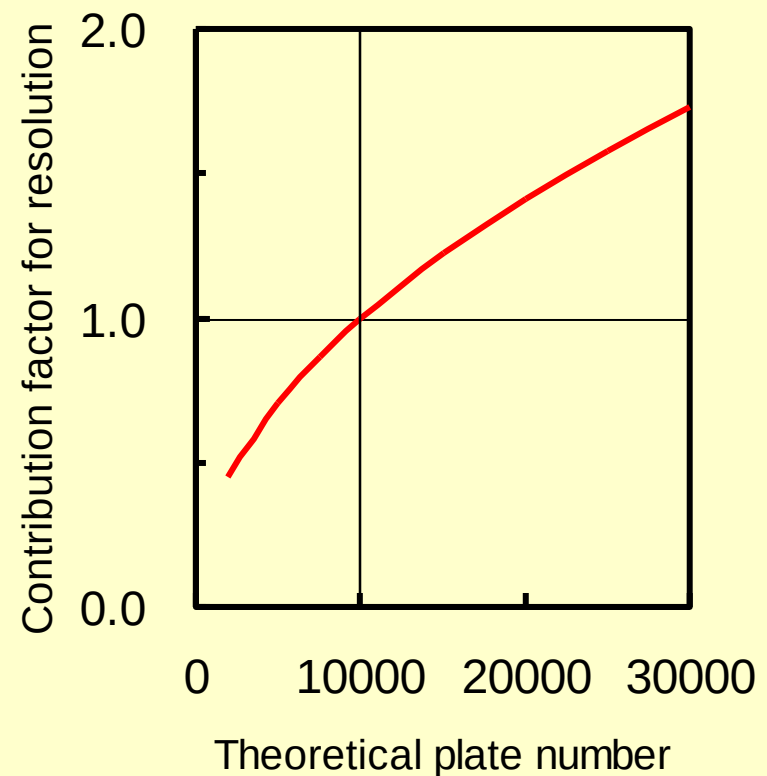
- Increasing the capacity factor improves separation!
- A capacity factor of around 3 to 10 is appropriate. Exceeding this just increases the analysis time.

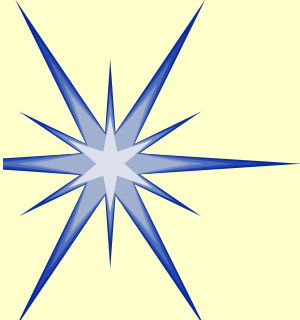




Contribution of Theoretical Plate Number to Resolution

- The resolution increases in proportion to the square root of the theoretical plate number.





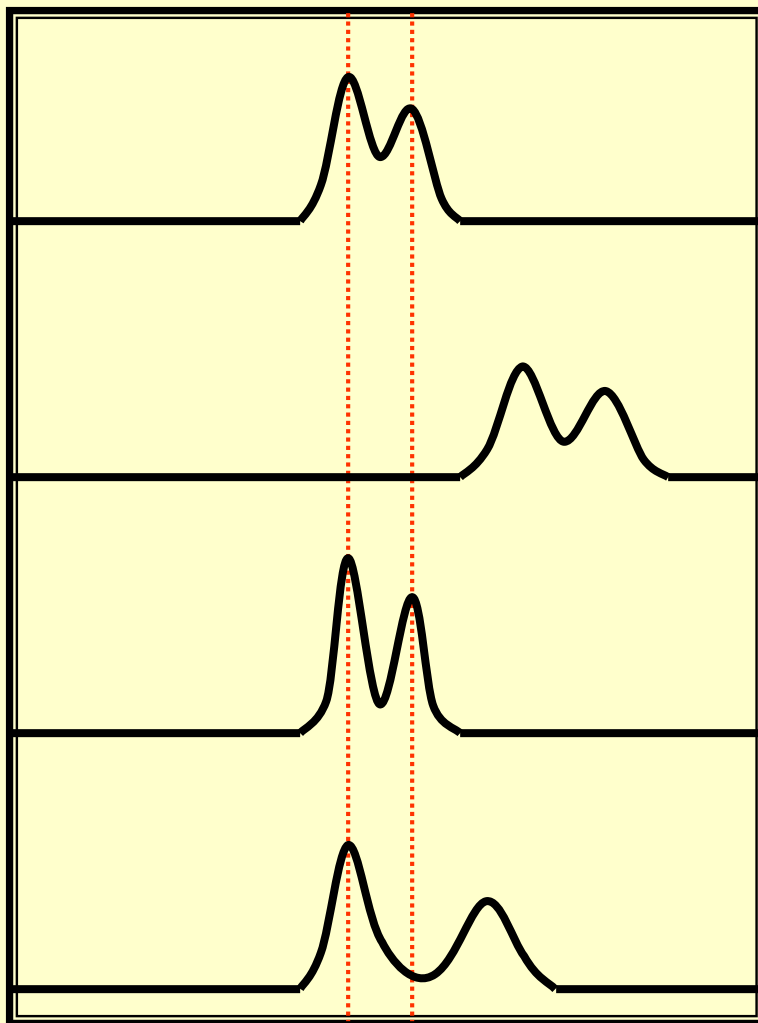
To Improve Separation...

Before
adjustment

k' increased

N increased

α increased



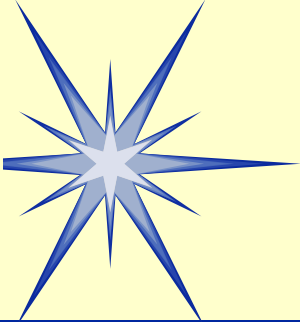
Eluent replaced with one
of lower elution strength.

Column replaced with one of
superior performance.
Column lengthened.

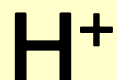
Column (packing material) replaced.
Eluent composition changed.
Column temperature changed.

pH Buffer Solution Used for Eluent

Selection and Preparation of Buffer Solution



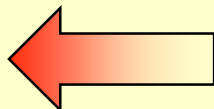
Acid Dissociation Equilibrium



If an acid is added...

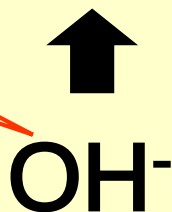
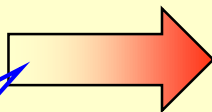


...the equilibrium shifts to the left to offset the increase in H^+ .

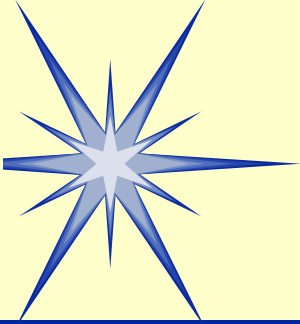


If an alkali is added...

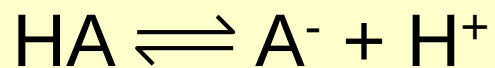
...the equilibrium shifts to the right to offset the decrease in H^+ .



The equilibrium always shifts in a way that offsets changes.



Acid Dissociation Constant and pH-Based Abundance Ratio

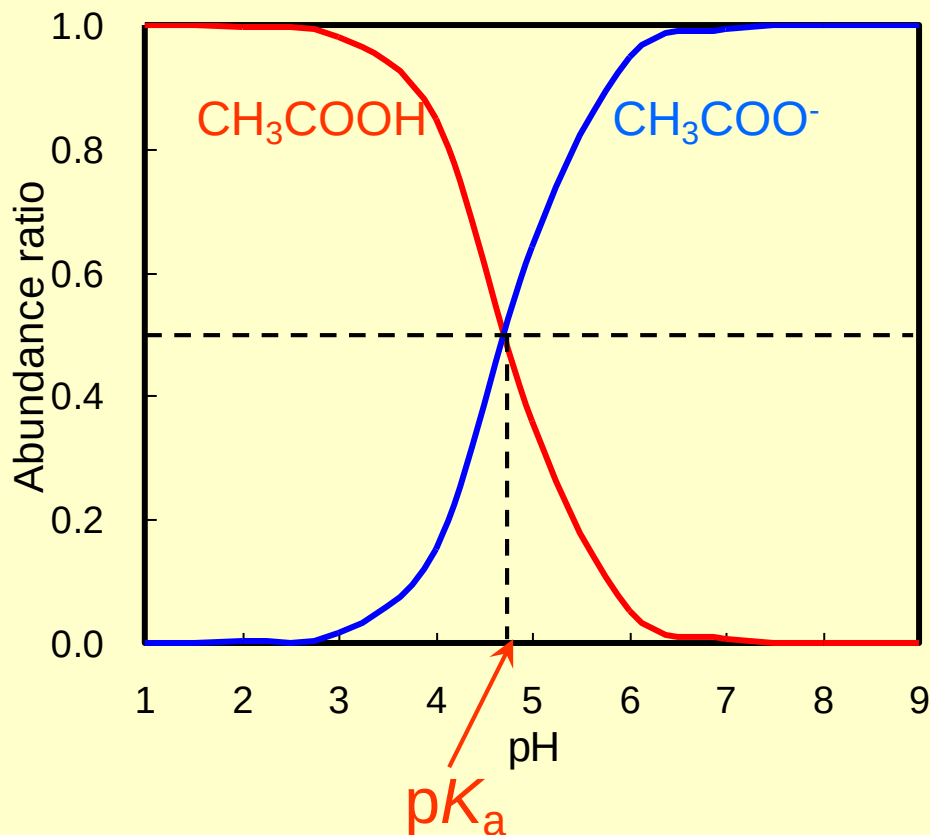


The acid dissociation constant, K_a , is defined as follows:

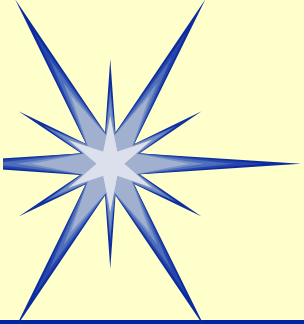
$$K_a = \frac{[\text{A}^-][\text{H}^+]}{[\text{HA}]}$$

$$\text{pH} = \text{p}K_a + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$\left(\begin{array}{l} \text{pH} = -\log[\text{H}^+] \\ \text{p}K_a = -\log K_a \end{array} \right)$$

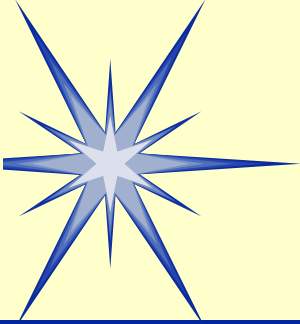


Relationship Between Abundance Ratio and pH Value of Acetic Acid and Acetic Acid Ions



Preparing pH Buffer Solution

- Use a weak acid with a pK_a value close to the desired pH value.
 - ❖ Example: Preparing a buffer solution for a pH value of around 4.8.
 - Use acetic acid, which has a pK_a value of 4.8.
- Make the concentrations of HA and A^- roughly equal.
 - Mix an acid with its salt.
 - ❖ Example: Mix acetic acid and sodium acetate so that they have the same molar concentration.



Buffer Solutions Used for HPLC Eluent

- Requirements

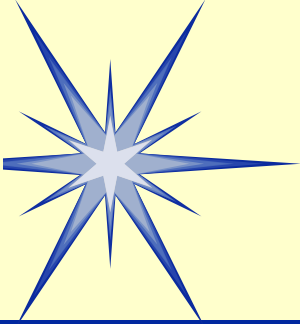
- ❖ High buffering power at prescribed pH.
- ❖ Does not adversely affect detection.
- ❖ Does not damage column or equipment.
- ❖ Inexpensive.

- Commonly Used Acids

- ❖ Phosphoric acid
 - ★ pK_a 2.1, 7.2, 12.3
- ❖ Acetic acid
 - ★ pK_a 4.8
- ❖ Citric acid
 - ★ pK_a 3.1, 4.8, 6.4

- Concentration

- ❖ If only to adjust pH, 10 mmol/L is sufficient.



Characteristics of Phosphate Buffer Solution

- Advantages

- ❖ Three dissociation states
(pK_a 2.1, 7.2, 12.3)
 - ★ Possible to prepare buffer solutions of various pH values.
- ❖ No UV absorption
- ❖ Inexpensive

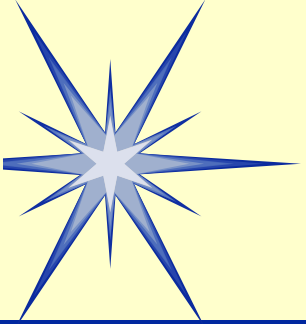
- Disadvantages

- ❖ No volatility
 - ★ Difficult to use for LCMS or evaporative light scattering detection.

Reversed Phase Chromatography

Part 2

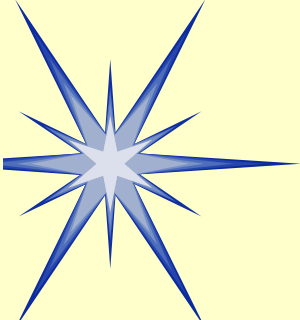
Consideration of Analytical Conditions



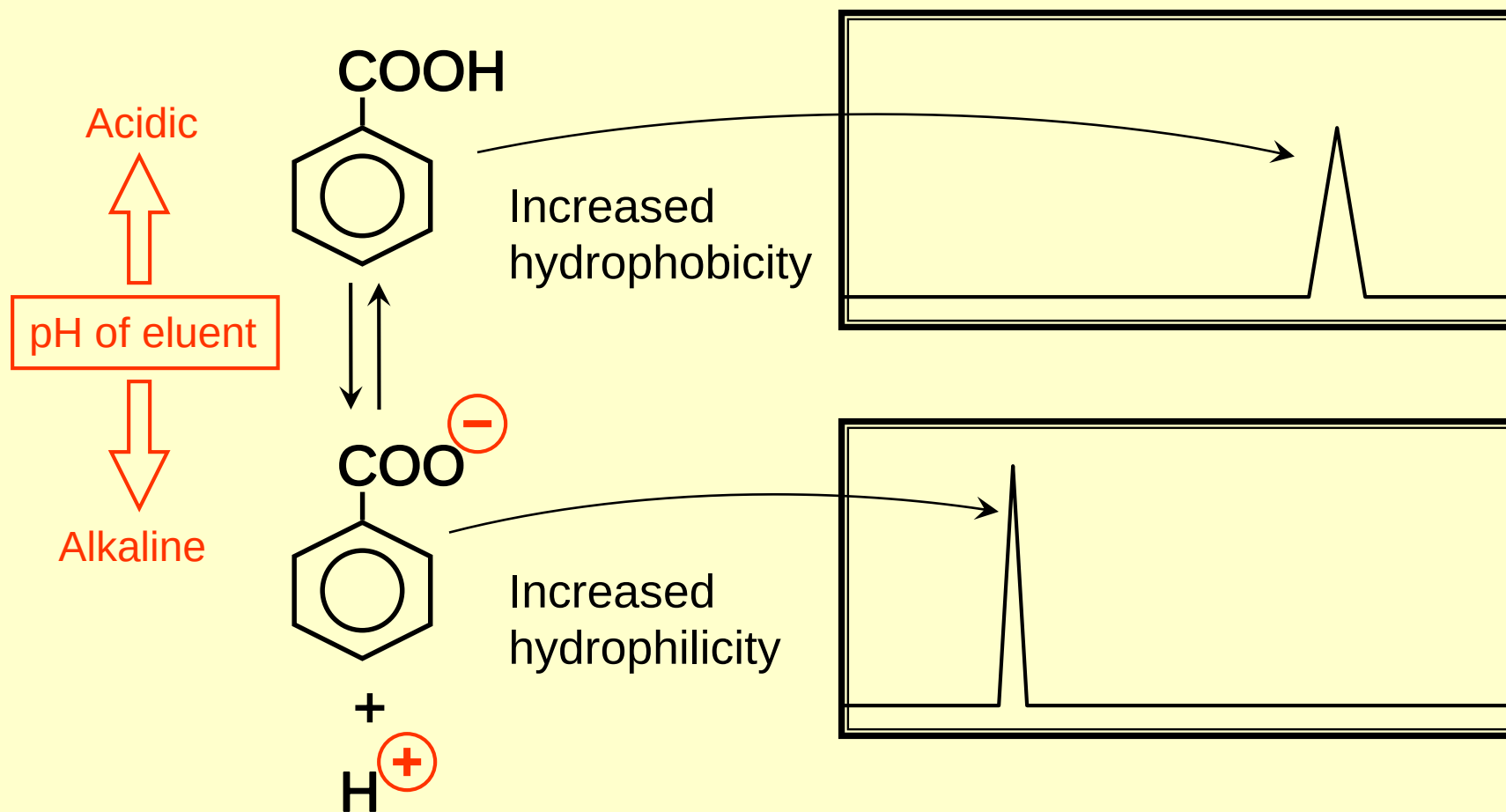
Guidelines for Setting Mobile Phase Conditions (1)

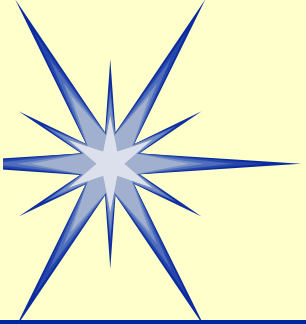
Neutral (Nonionic) Substances

- Eluent Composition
 - ❖ Water / acetonitrile
 - ❖ Water / methanol
- Separation Adjustment
 - ❖ Changing the mixing ratio of the water and organic solvent
 - ❖ Changing the type of organic solvent



pH of Eluent and Retention of Ionic Solutes



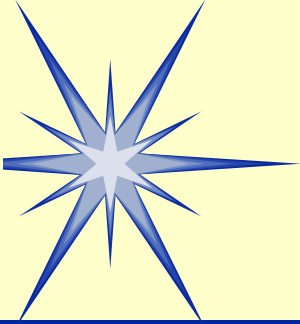


Guidelines for Setting Mobile Phase Conditions (2)

Acidic (Anionic) Substances

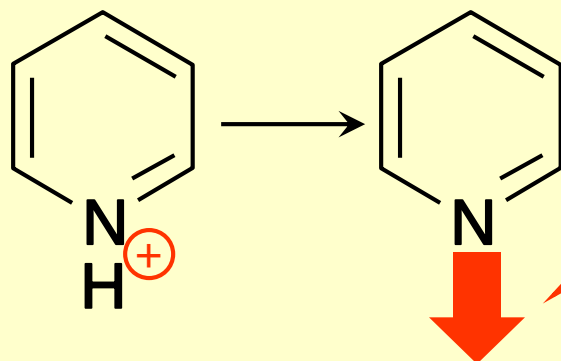
- Eluent Composition
 - ❖ Acidic buffer solution / acetonitrile
 - ❖ Acidic buffer solution / methanol

Increase retention strength by making the eluent acidic and suppressing ionization!

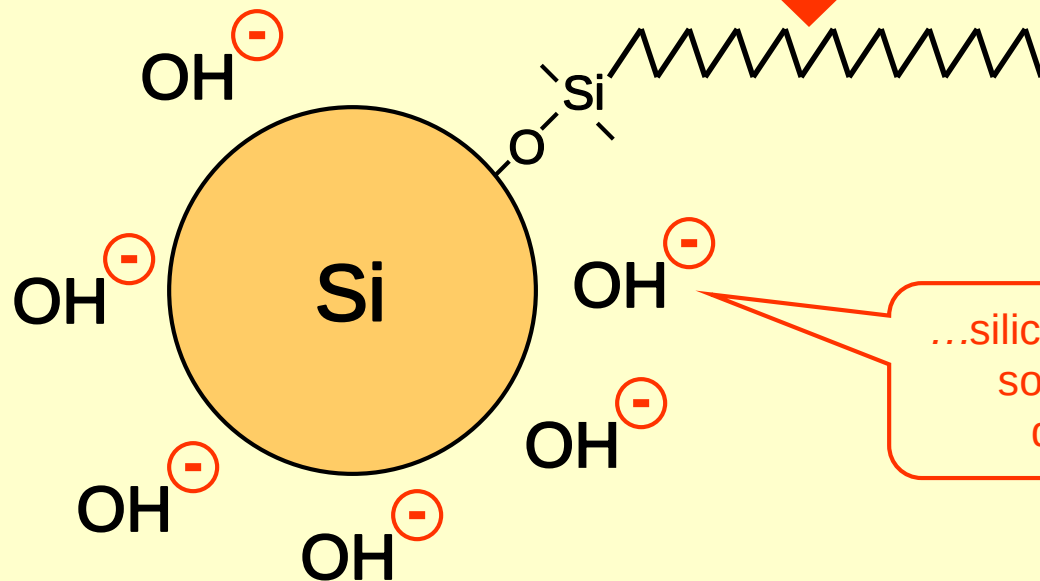


Analysis of Basic Substances (1)

Problems Encountered with Alkaline Eluents



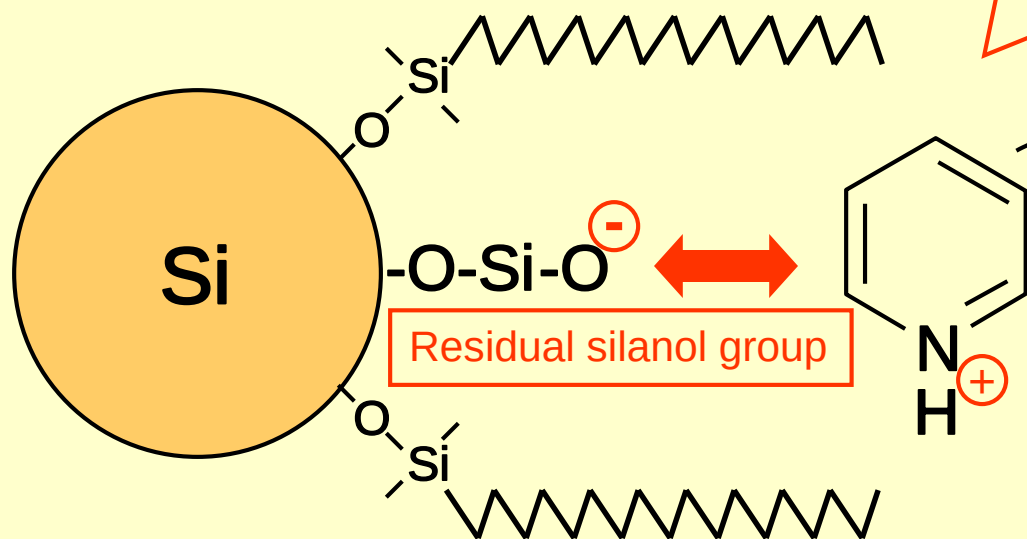
With alkaline eluents, although the ionization of basic substances is suppressed, and the retention strength increases...



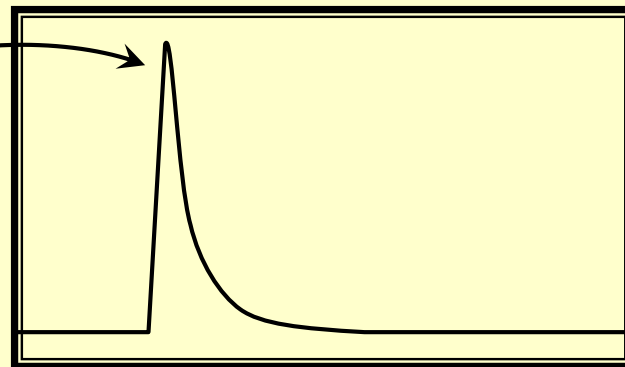
...silica gel dissolves in alkalis, so the packing material deteriorates rapidly.

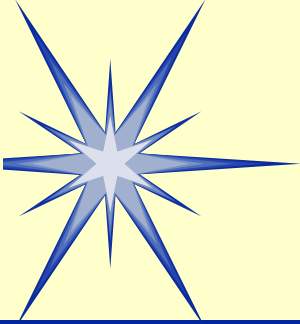
Analysis of Basic Substances (2)

Influence of Residual Silanol Groups



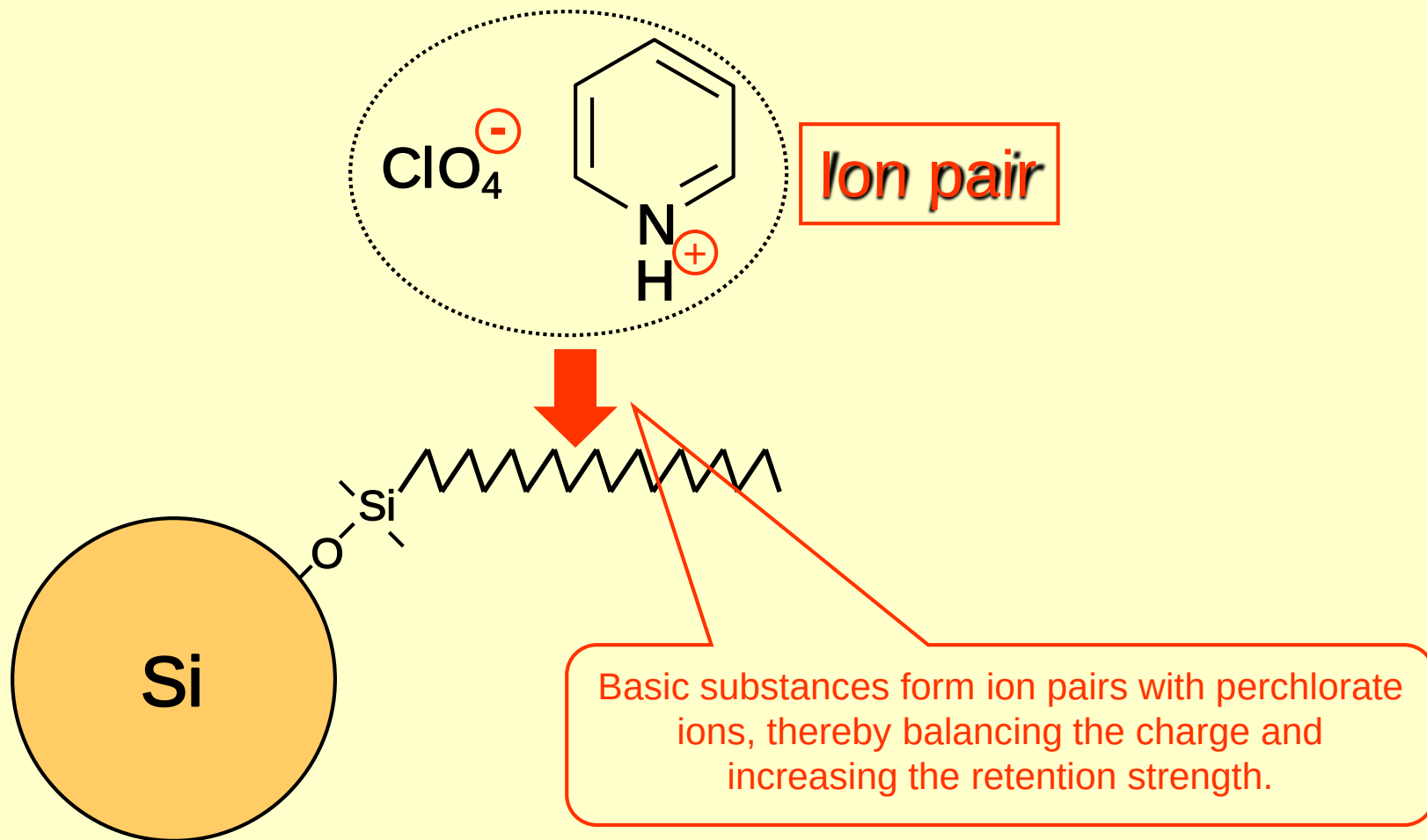
Basic substances interact with the residual silanol groups, causing delayed elution and tailing.

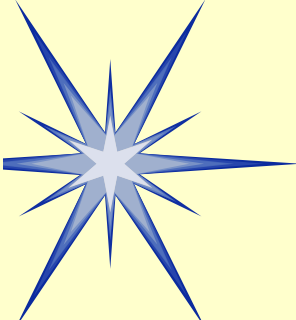




Analysis of Basic Substances (3)

Addition of Sodium Perchlorate





Guidelines for Setting Mobile Phase Conditions (3)

Basic Substances (Cationic Substances)

- Eluent Composition

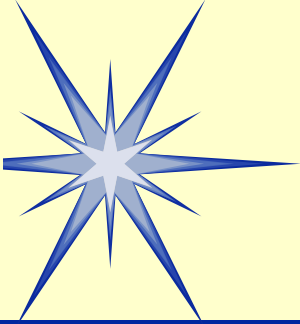
- ❖ Acidic buffer solution containing anions with a low charge density (e.g., perchlorate ions) / acetonitrile
- ❖ As above / methanol

Making eluent acidic

- Suppresses dissociation of residual silanol groups
- Prevents tailing!

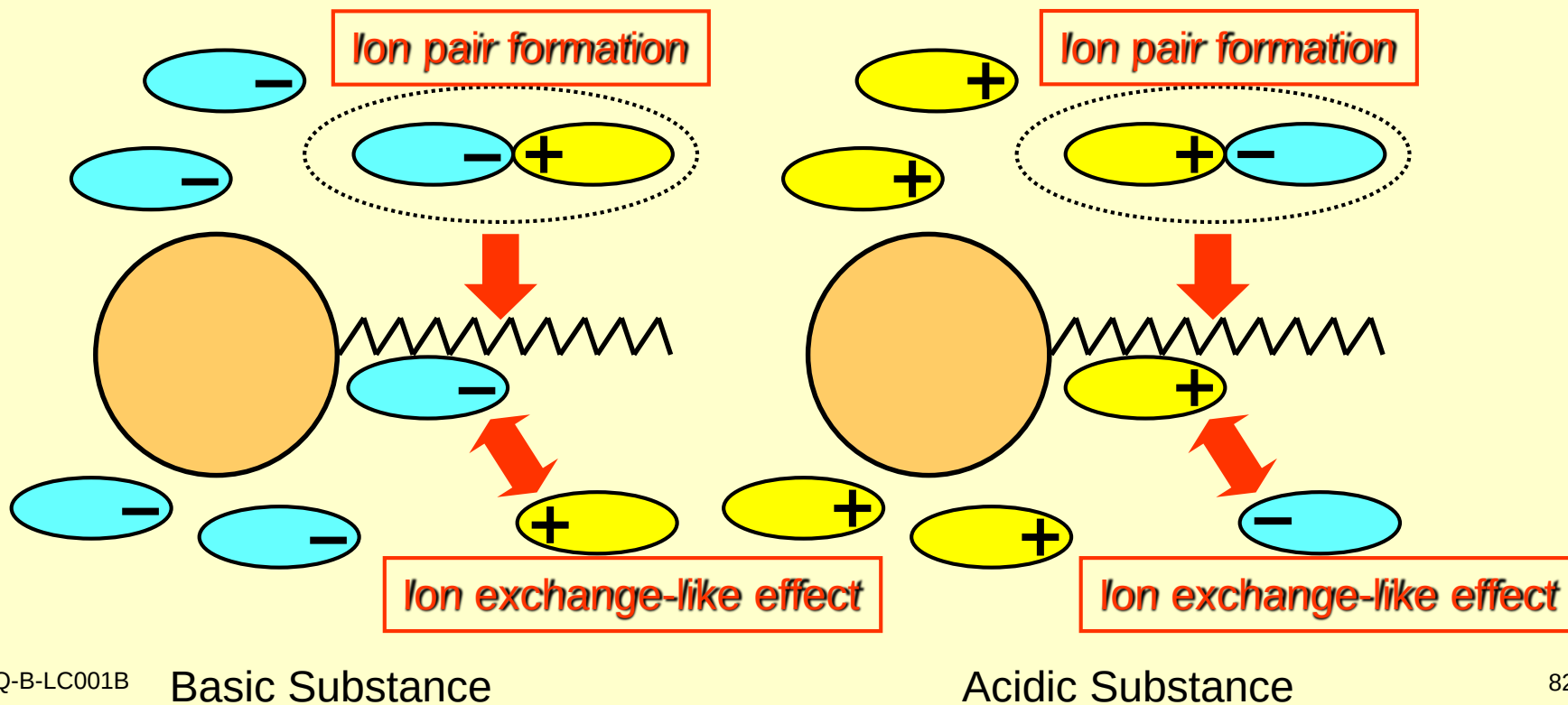
Adding perchlorate ions

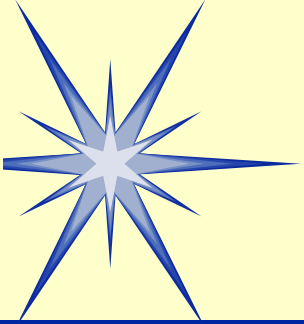
- Forms ion pairs → Increases retention strength!
- Suppresses tailing!



Reversed Phase Ion Pair Chromatography

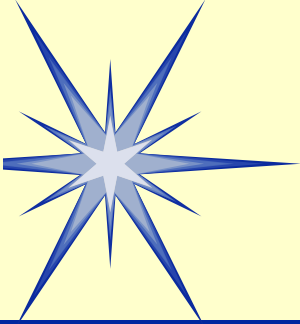
- Increase the retention strength by adding an ion pair reagent with the opposite charge to the target substance into the eluent.





Representative Ion Pair Reagents

- Anionic Compounds
 - ❖ Tetra-*n*-butylammonium hydroxide (TBA)
- Cationic Compounds
 - ❖ Pentanesulfonic acid sodium salt (C5)
 - ❖ Hexanesulfonic acid sodium salt (C6)
 - ❖ Heptanesulfonic acid sodium salt (C7)
 - ❖ Octanesulfonic acid sodium salt (C8)

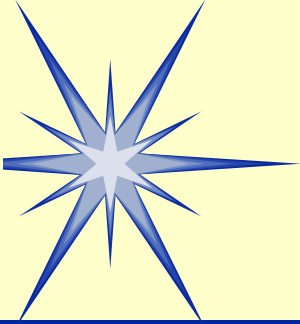


Points to Note Concerning the Use of Ion Pairs

- Selection of Ion Pair Reagent
 - ❖ In general, the retention strength increases with the length of the alkyl chain.
- pH of Eluent
 - ❖ The retention strength changes according to whether or not ionization takes place.
- Concentration of Ion Pair Reagent
 - ❖ In general, the retention strength increases with the ion pair concentration, but there is an upper limit.
- Proportion of Organic Solvent in Eluent
 - ❖ Optimize the separation conditions by considering the type and concentration of the ion pair reagent.

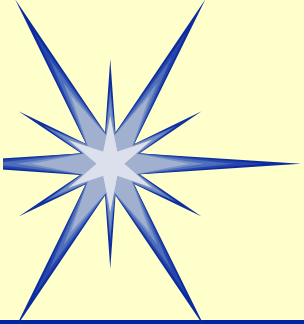
HPLC Separation Modes

Separation Modes Other Than
Reversed Phase Chromatography



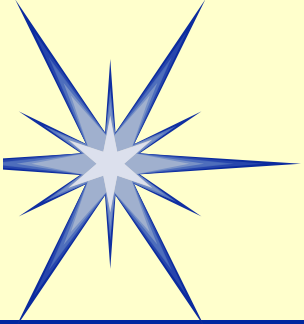
HPLC Separation Modes

- Adsorption (liquid-solid) chromatography
- Partition (liquid-liquid) chromatography
 - ❖ Normal phase partition chromatography
 - ❖ Reversed phase partition chromatography
- Ion exchange chromatography
- Size exclusion chromatography



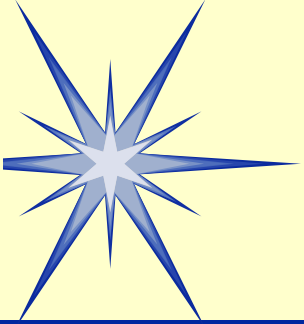
Adsorption Chromatography

- A solid such as silica gel is used as the stationary phase, and differences, mainly in the degree of adsorption to its surface, are used to separate the solutes.
- Liquid-solid chromatography
- The retention strength increases with the hydrophilicity of the solute.



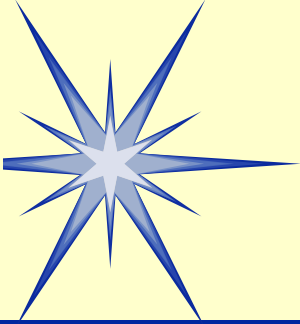
Partition Chromatography

- A liquid (or a substance regarded as a liquid) is used as the stationary phase, and the solute is separated according to whether it dissolves more readily in the stationary or mobile phase.
- Liquid-liquid chromatography



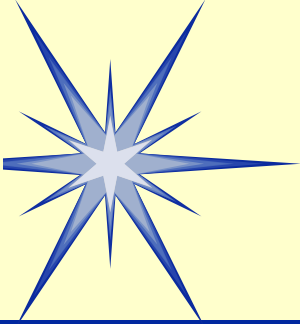
Normal Phase and Reversed Phase

	Solid phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)

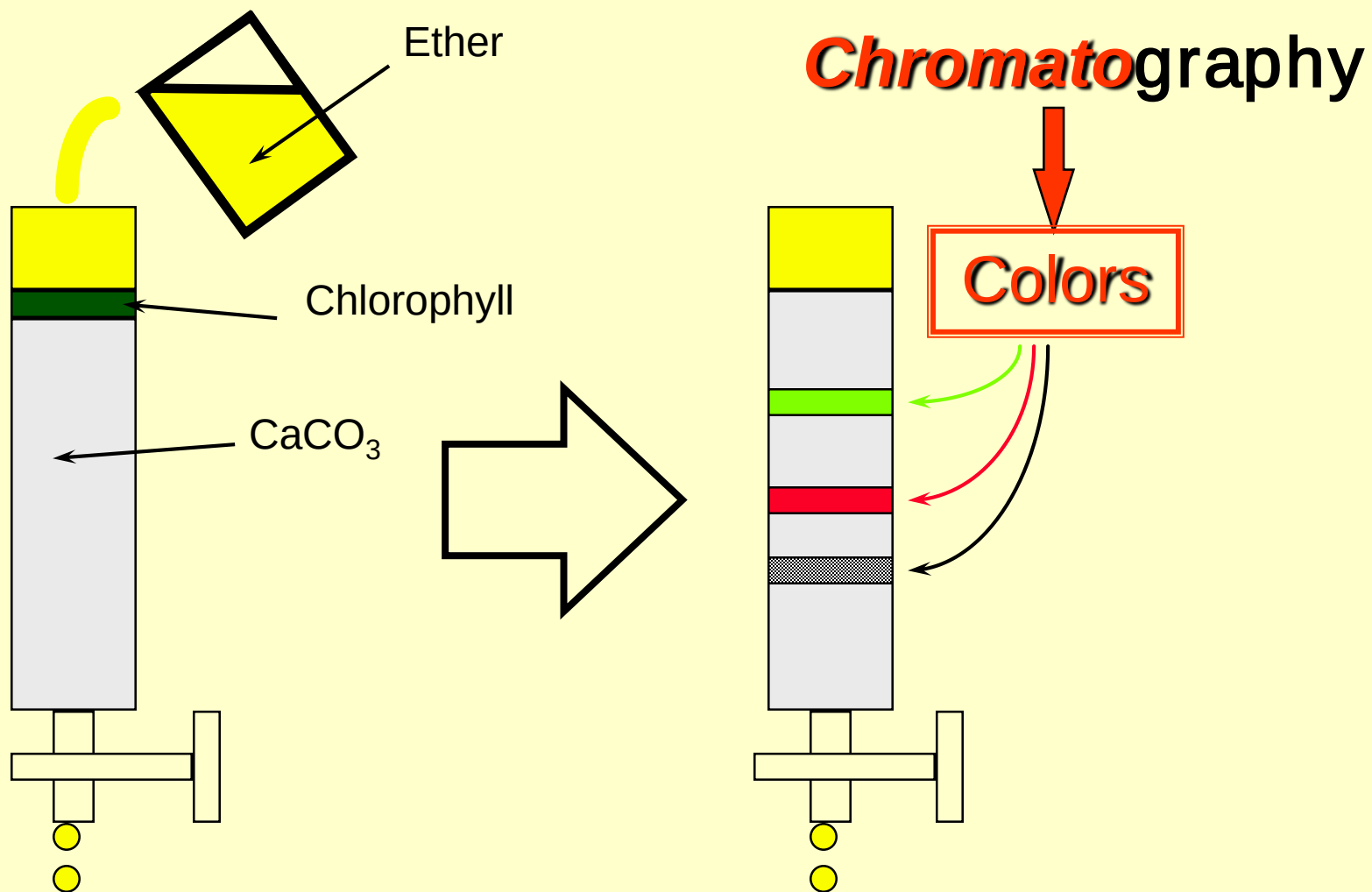


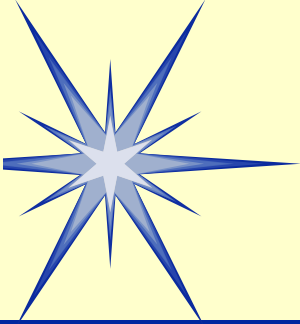
Normal Phase (Partition) Chromatography

- Partition chromatography in which the stationary phase has a high polarity (hydrophilic) and the mobile phase has a low polarity (hydrophobic)
- Essentially based on the same separation mechanism as adsorption chromatography in which the stationary phase has a hydrophilic base, such as silica gel



Invention of Chromatography by M. Tswett





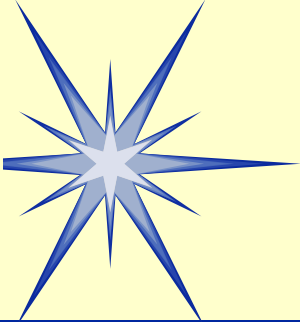
Stationary Phase and Mobile Phase Used in Normal Phase Mode

- Stationary Phase

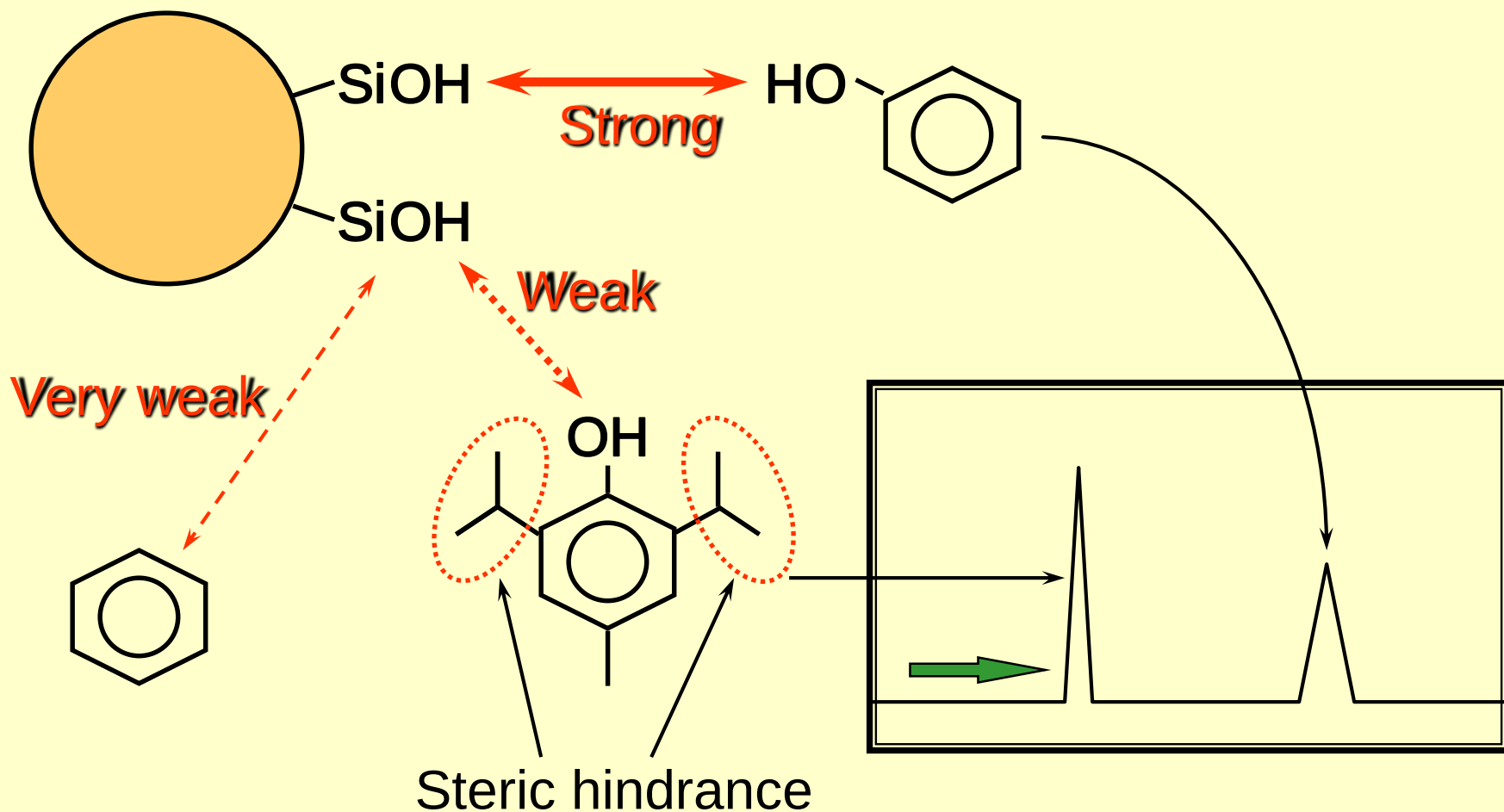
- ❖ Silica gel: $-\text{Si}-\text{OH}$
- ❖ Cyano type: $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CN}$
- ❖ Amino type: $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$
- ❖ Diol type: $-\text{Si}-\text{CH}_2\text{CH}_2\text{CH}_2\text{OCH}(\text{OH})-\text{CH}_2\text{OH}$

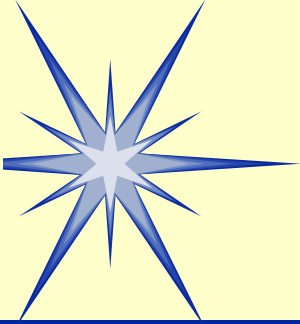
- Mobile Phase

- ❖ Basic solvents: Aliphatic hydrocarbons, aromatic hydrocarbons, etc.
- ❖ Additional solvents: Alcohols, ethers, etc.

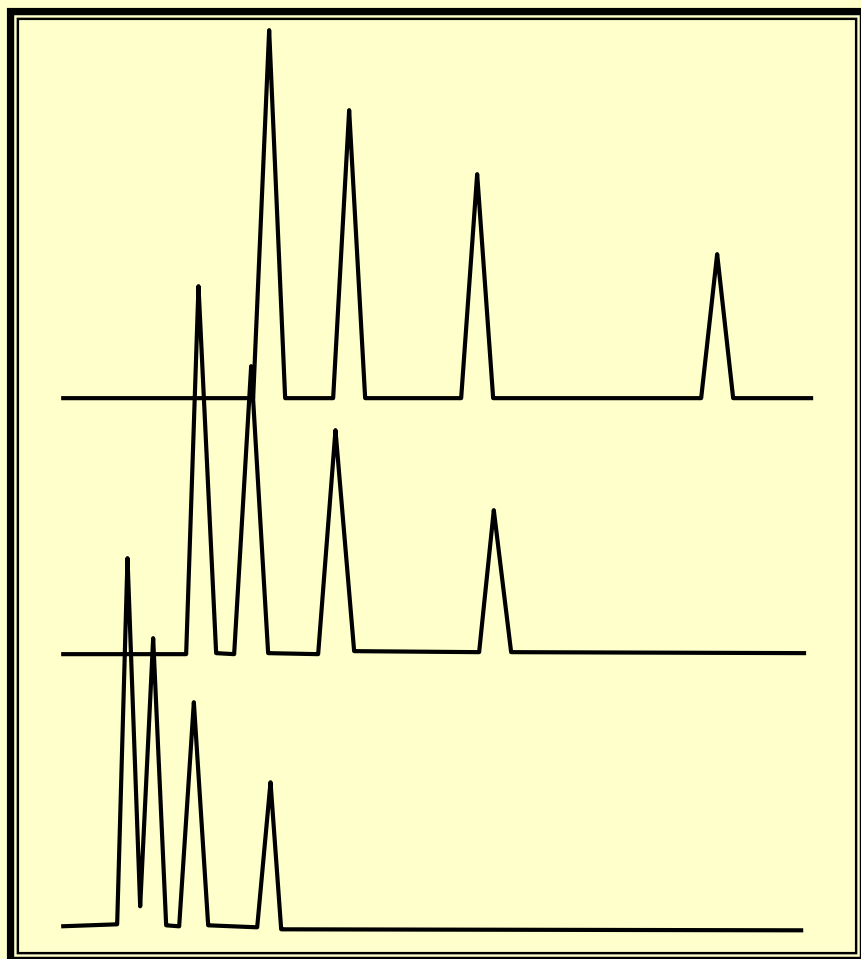


Relationship between Hydrogen Bonding and Retention Time in Normal Phase Mode





Relationship Between Eluent Polarity and Retention Time in Normal Phase Mode

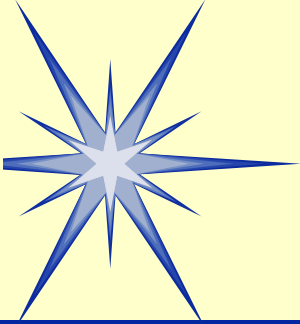


Eluent: Hexane/methanol

100/0

98/2

95/5



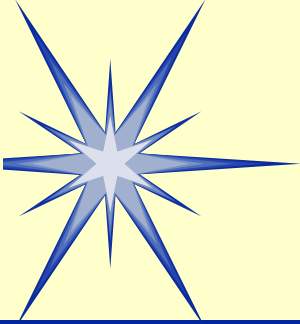
Comparison of Normal Phase and Reversed Phase

● Normal Phase

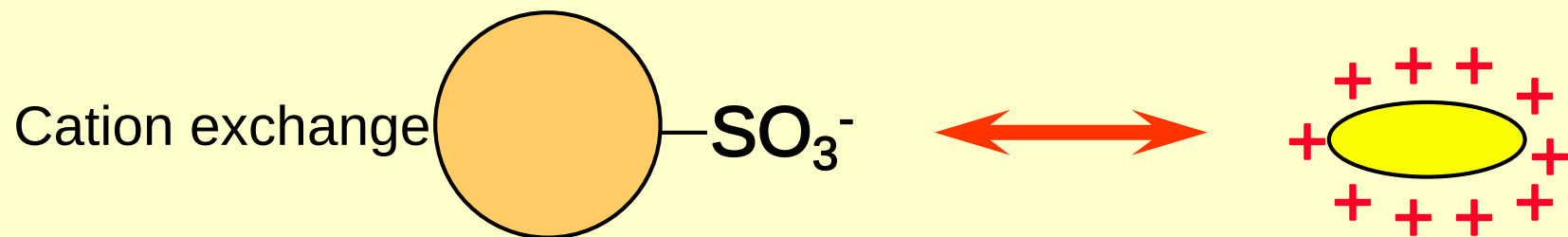
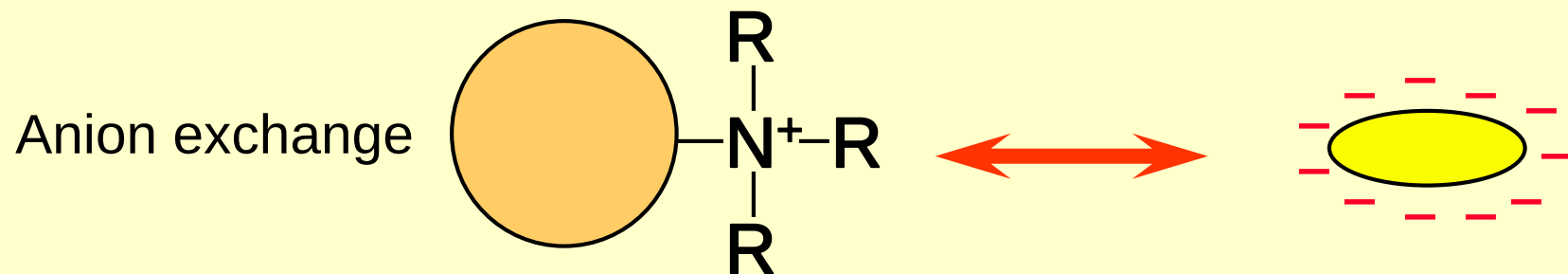
- ❖ Effective for separation of structural isomers
- ❖ Offers separation selectivity not available with reversed phase
- ❖ Stabilizes slowly and is prone to fluctuations in retention time
- ❖ Eluents are expensive

● Reversed Phase

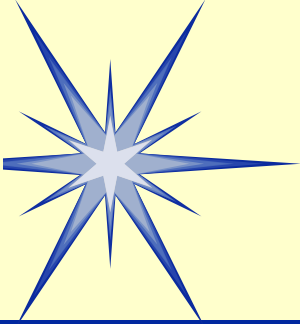
- ❖ Wide range of applications
- ❖ Effective for separation of homologs
- ❖ Stationary phase has long service life
- ❖ Stabilizes quickly
- ❖ Eluents are inexpensive and easy to use



Ion Exchange Chromatography



Electrostatic interaction
(Coulomb force)



Stationary Phase Used in Ion Exchange Mode

- Base Material

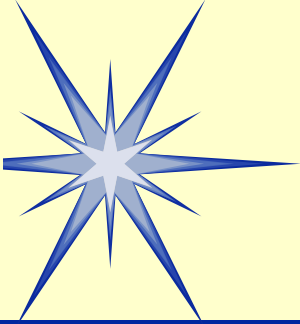
- ❖ Resin is often used.
- ❖ Silica gel is also used.

- Cation Exchange Column

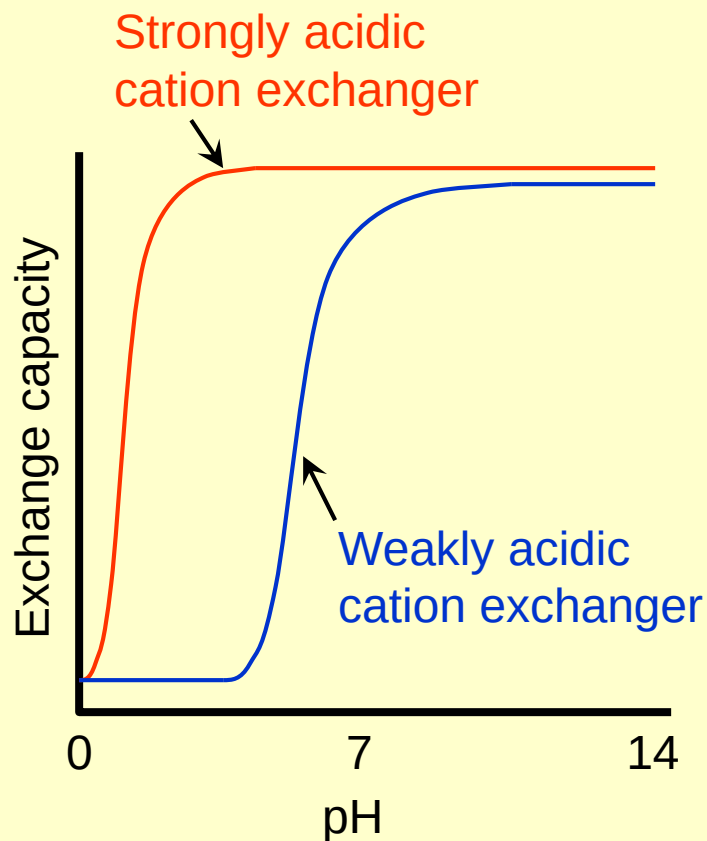
- ❖ Strong cation exchange (SCX) $-\text{SO}_3^-$
- ❖ Weak cation exchange (WCX) $-\text{COO}^-$

- Anion Exchange Column

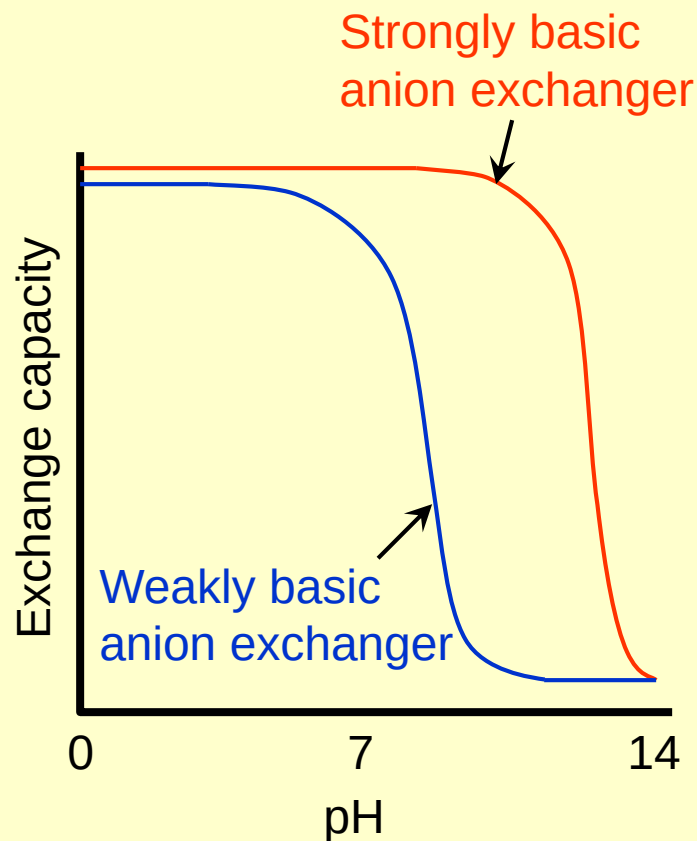
- ❖ Strong anion exchange (SAX) $-\text{NR}_3^+$
- ❖ Weak anion exchange (WAX) $-\text{NHR}_2^+$



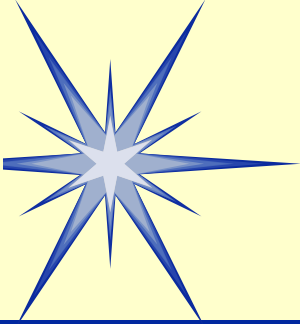
Dependence of Exchange Capacity of Ion Exchanger on pH of Eluent



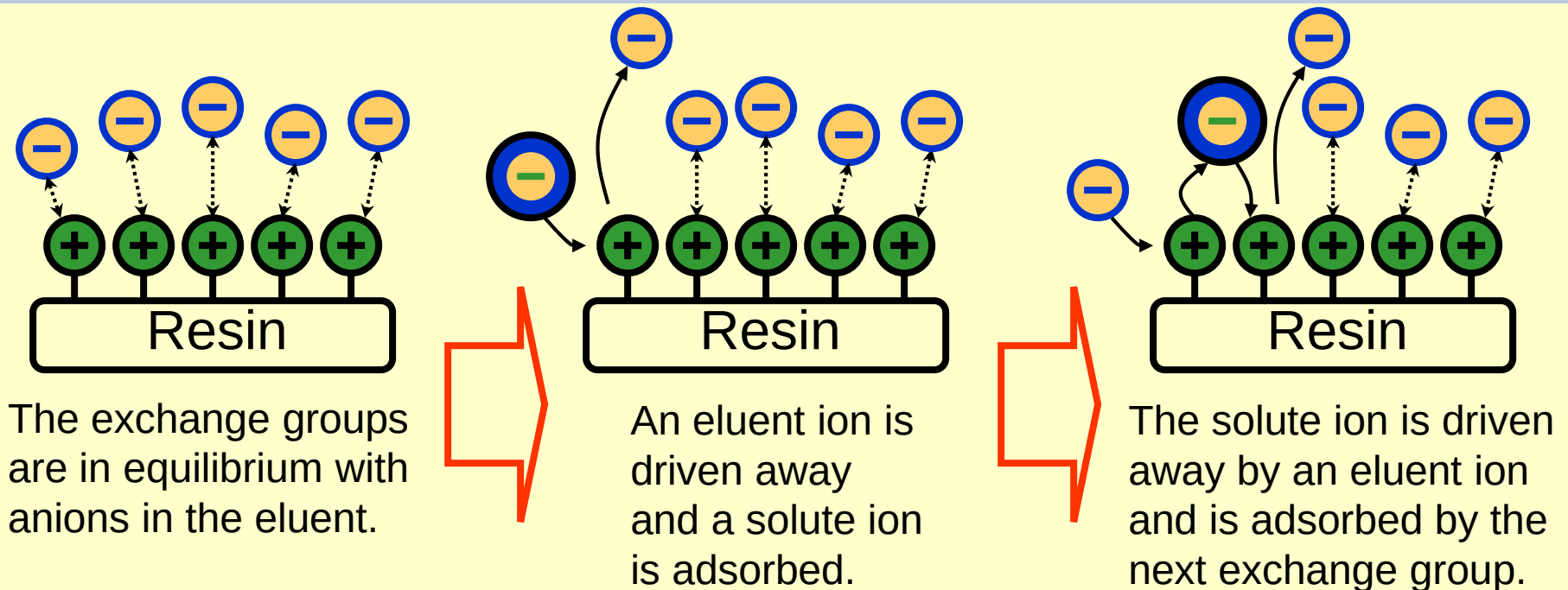
Cation exchange mode



Anion exchange mode

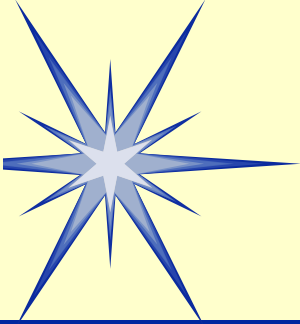


Relationship between Retention Time and Salt Concentration of Eluent in Ion Exchange Mode

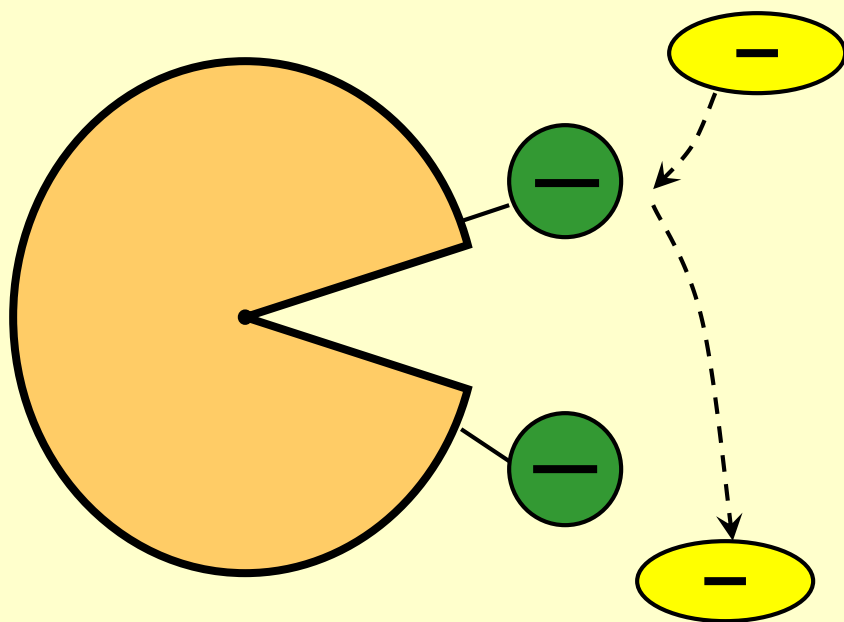


Solute ions and eluent ions compete for ion exchange groups.

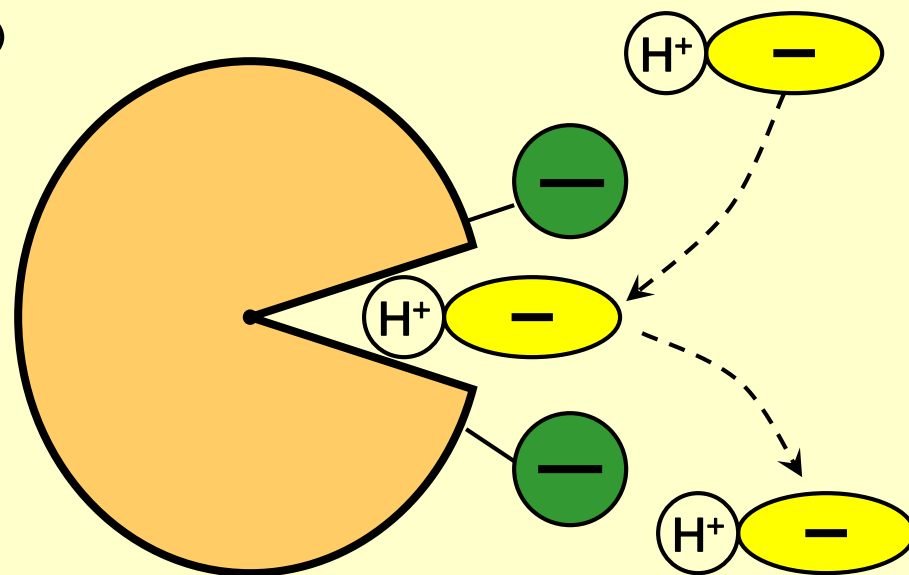
If the salt concentration of the eluent increases, the solutes are eluted sooner.



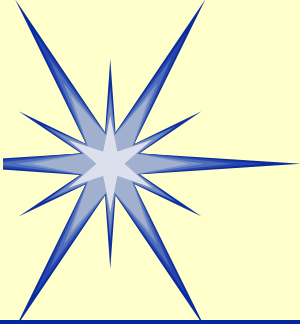
Ion Exclusion Chromatography



Strong acid ions are repelled by charge and cannot enter the pore.

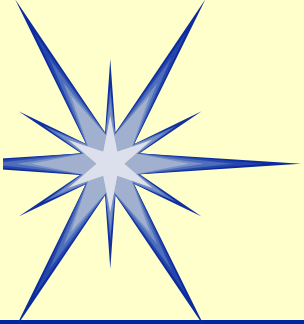


Depending on the level of dissociation, some weak acid ions can enter the pore.

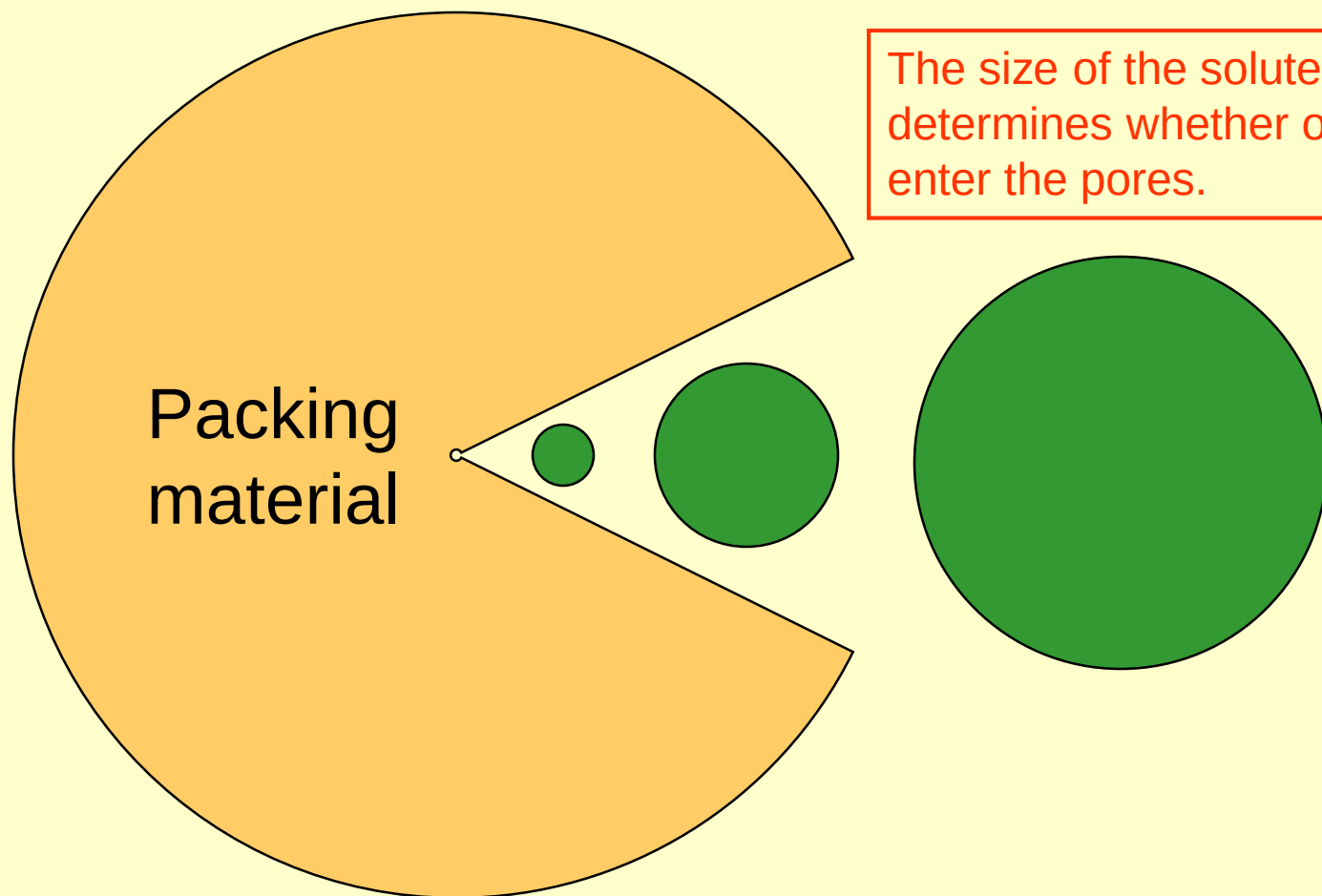


Size Exclusion Chromatography

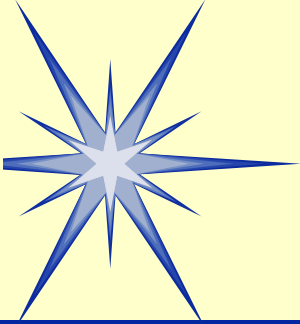
- Separation is based on the size (bulkiness) of molecules.
- The name varies with the application field!
 - ❖ Size Exclusion Chromatography (SEC)
 - ❖ Gel Permeation Chromatography (GPC)
 - ★ Chemical industry fields, synthetic polymers, nonaqueous systems
 - ❖ Gel Filtration Chromatography (GFC)
 - ★ Biochemical fields, biological macromolecules, aqueous systems



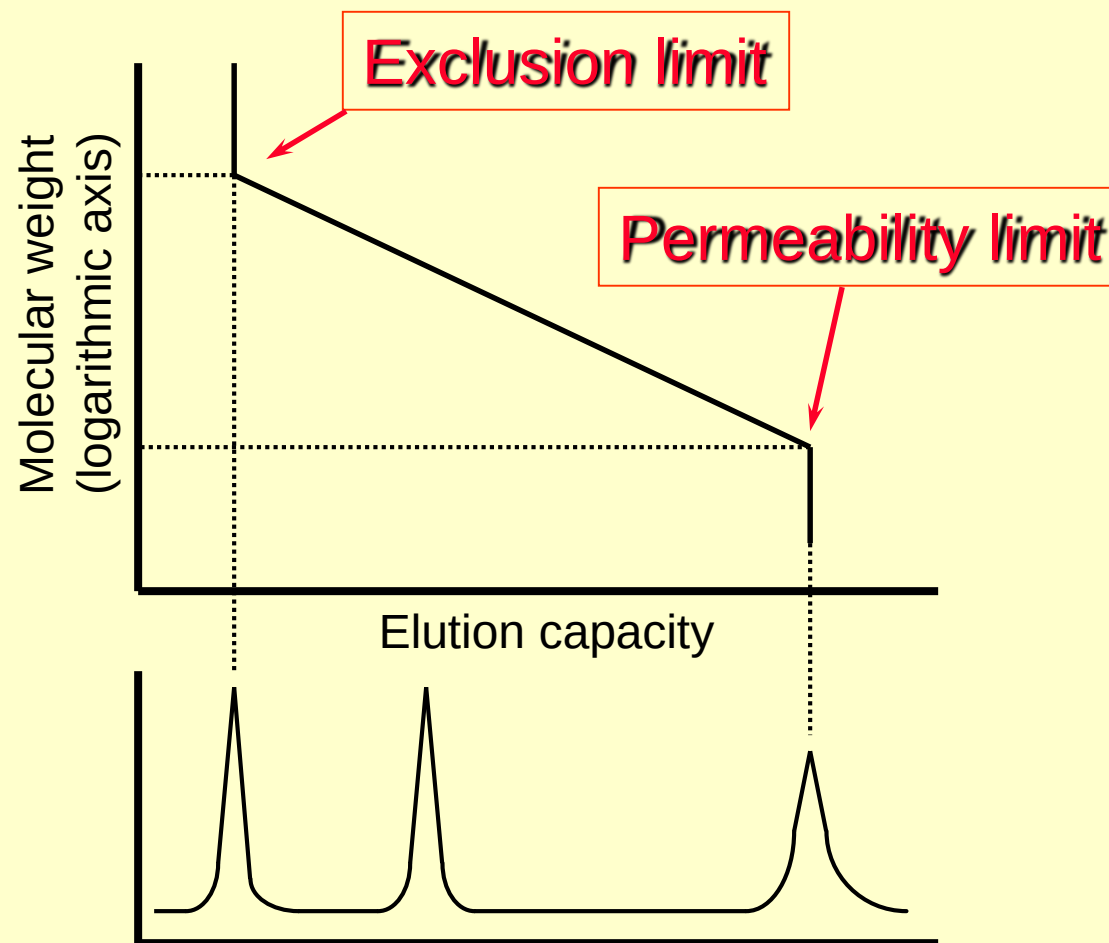
Principle of Size Exclusion Mode

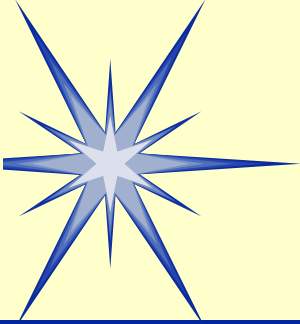


The size of the solute molecules determines whether or not they can enter the pores.

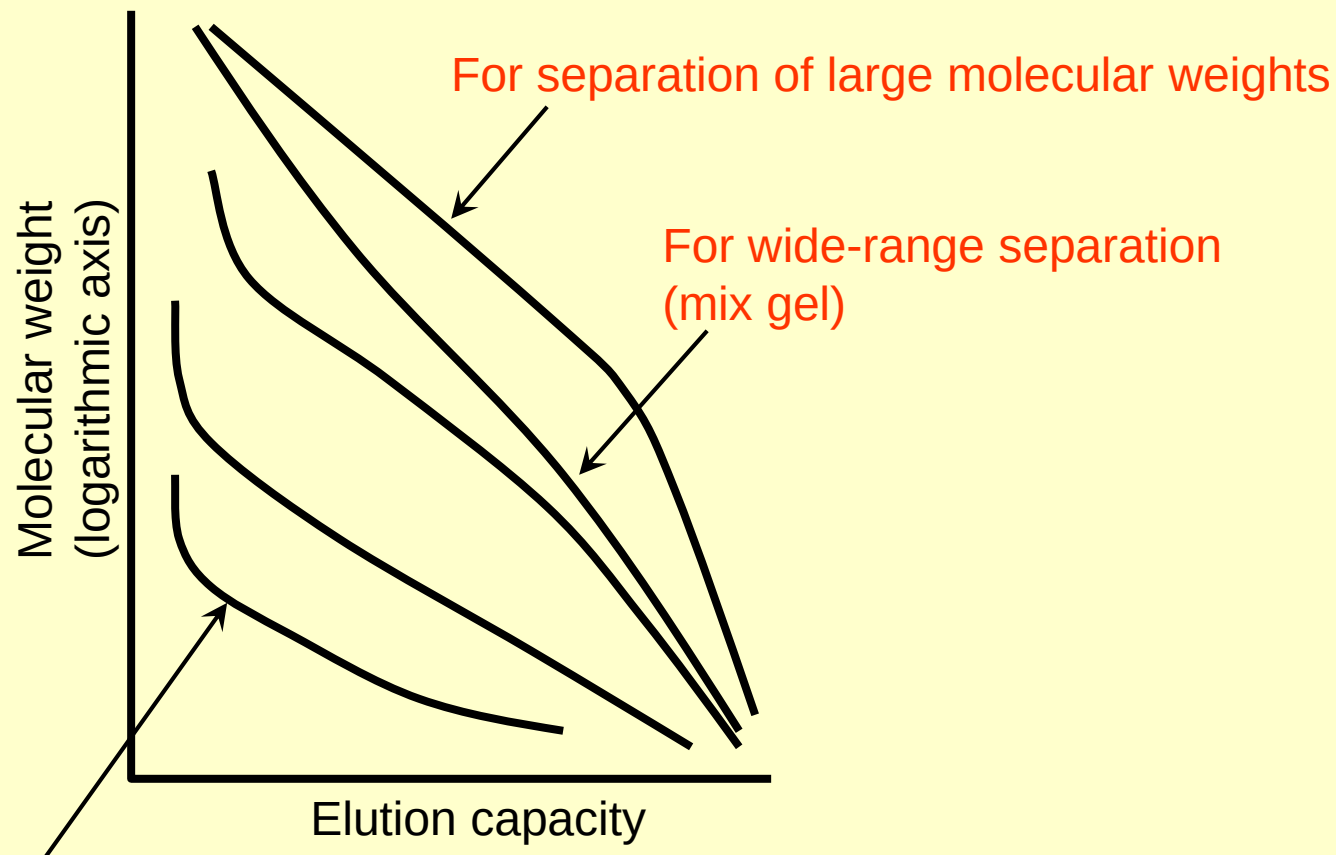


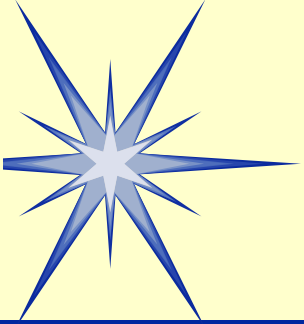
Relationship Between Molecular Weight and Retention Time in Size Exclusion Mode



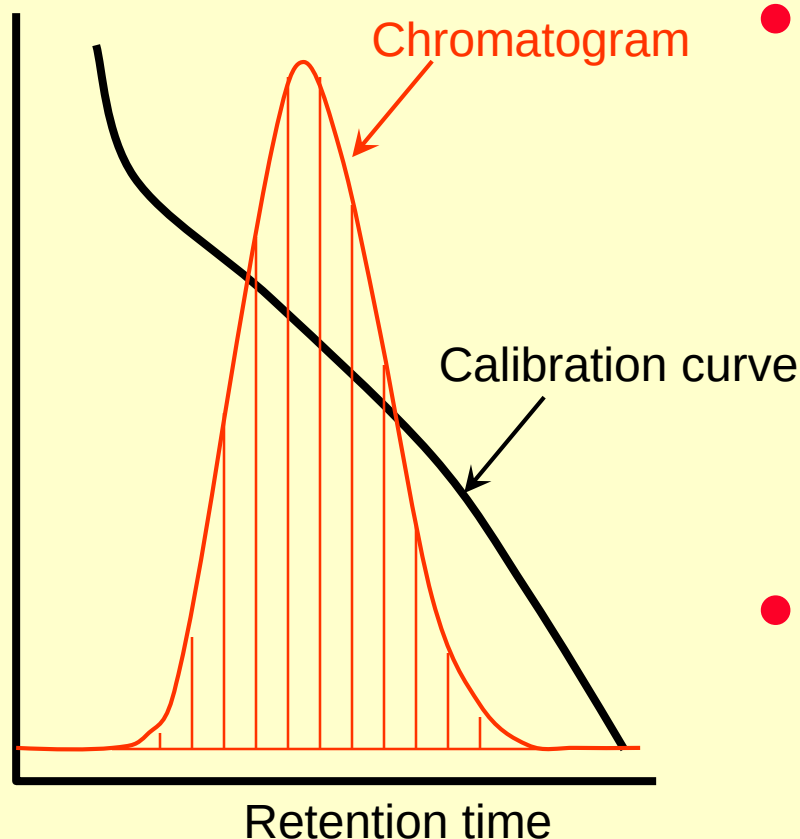


Creating a Molecular Weight Calibration Curve

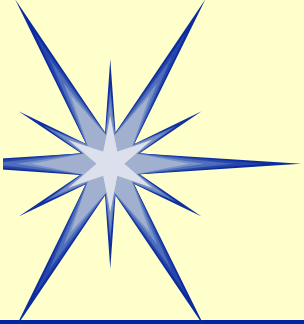




Calculating Molecular Weights



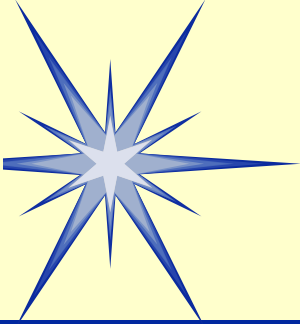
- Various Average Molecular Weights
 - ❖ M_n : Number-average molecular weight
 - ❖ M_w : Weight-average molecular weight
 - ❖ M_z : Z-average molecular weight, etc.
- Molecular weights and molecular weight distributions are calculated using special calculation software.



Guidelines for Selecting Separation Mode (1)

Required Information

- Soluble solvent
- Molecular weight
- Structural formula and chemical properties
 - ❖ Do the substances ionize?
 - ❖ Is there UV absorption or fluorescence?
 - ❖ Is derivatization possible? etc.



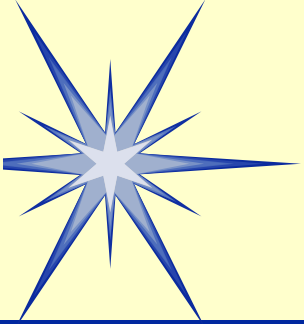
Guidelines for Selecting Separation Mode (2)

Basic Policy

- **Reversed phase mode** using an ODS column is the first choice!
- **Exceptions**
 - ❖ Large molecular weight ($> 2,000$) → **Size exclusion**
 - ❖ Optical isomers → **Chiral column**
 - ❖ Stereoisomers, positional isomers → **Normal phase / adsorption**
 - ❖ Inorganic ions → **Ion chromatography**
 - ❖ Sugars, amino acids, short-chain fatty acids → **Special column**

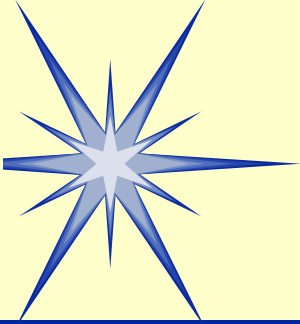
HPLC Hardware: Part 2

Detectors and Their Ranges of Application



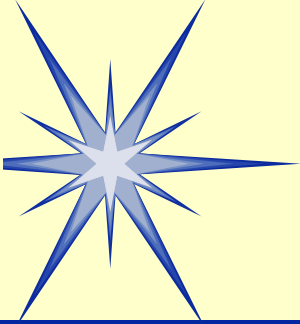
Detection Condition Requirements

- Sensitivity
 - ❖ The detector must have the appropriate level of sensitivity.
- Selectivity
 - ❖ The detector must be able to detect the target substance without, if possible, detecting other substances.
- Adaptability to separation conditions
- Operability, etc.

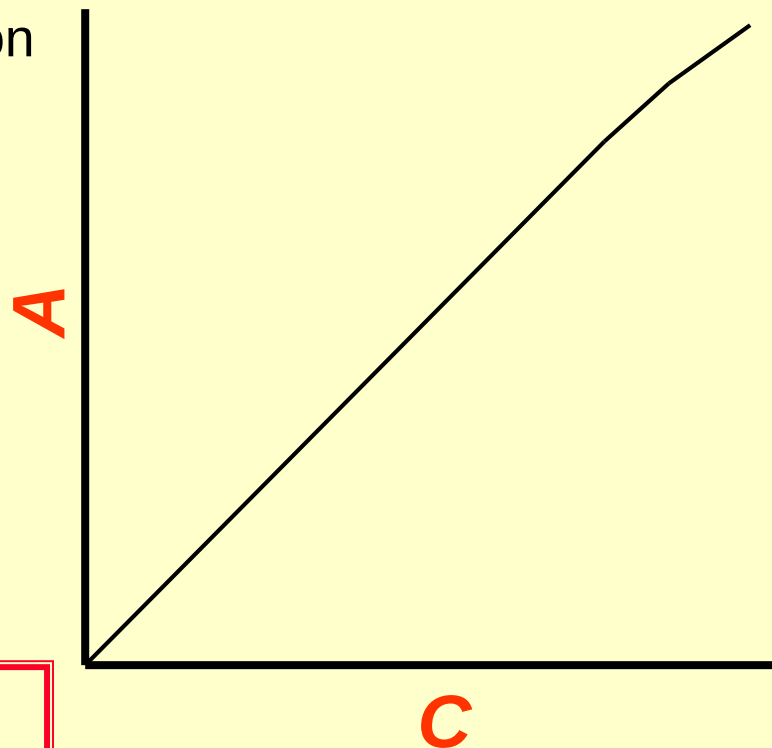
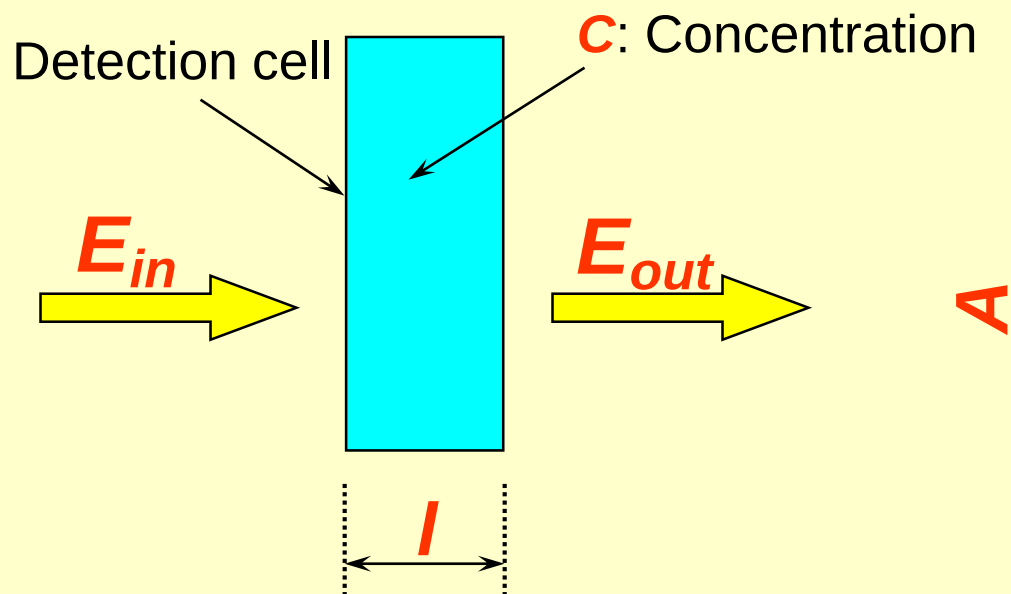


Representative HPLC Detectors

- UV-VIS absorbance detector
- Photodiode array-type UV-VIS absorbance detector
- Fluorescence detector
- Refractive index detector
- Evaporative light scattering detector
- Electrical conductivity detector
- Electrochemical detector
- Mass spectrometer

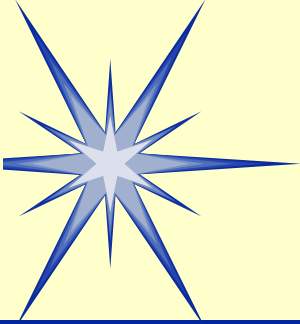


UV-VIS Absorbance Detector

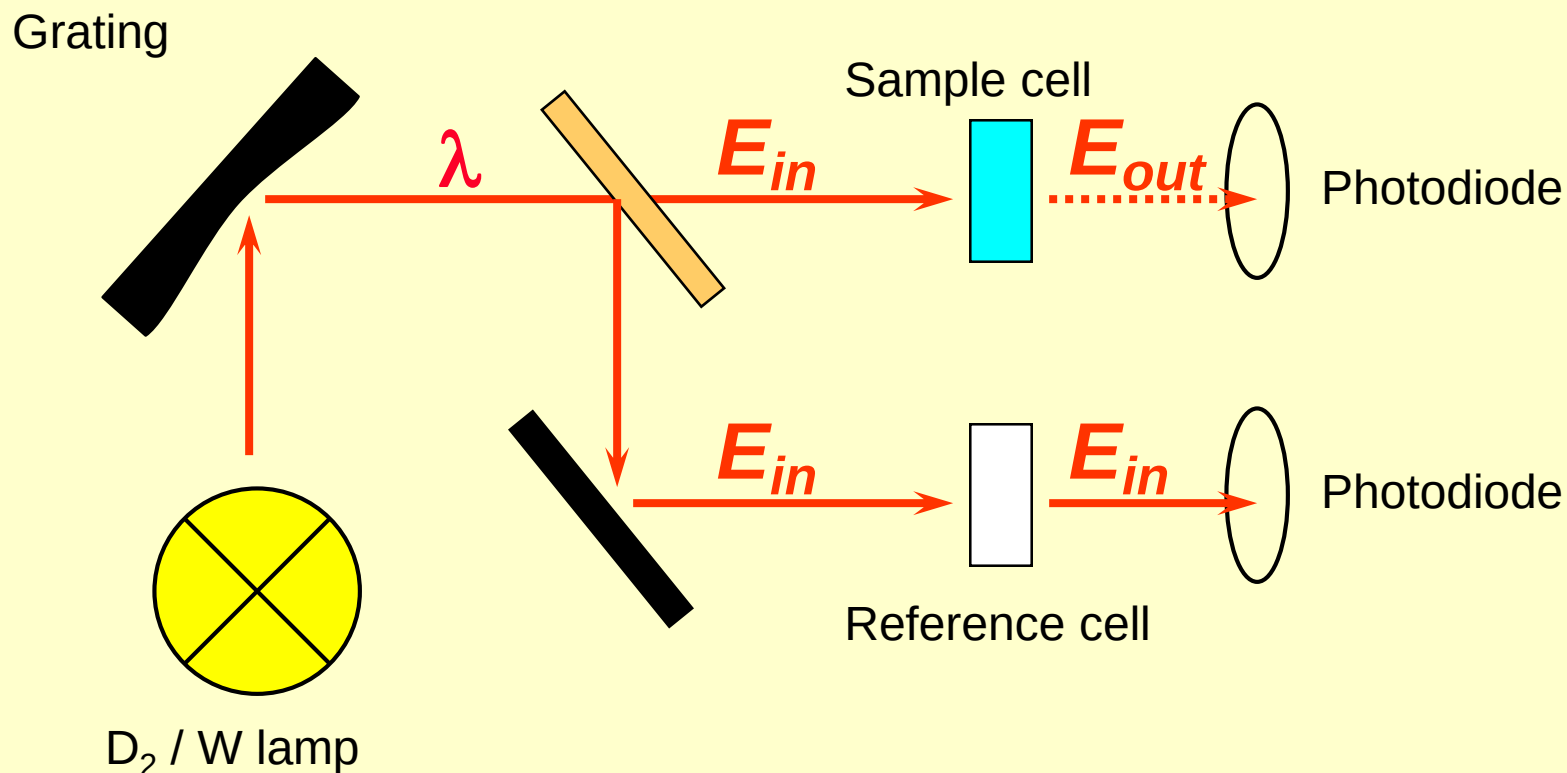


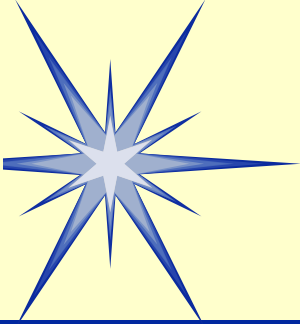
$$A = \varepsilon \cdot C \cdot I = -\log (E_{out} / E_{in})$$

(A : absorbance, ε : absorption coefficient)

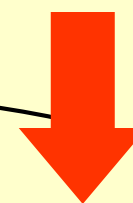
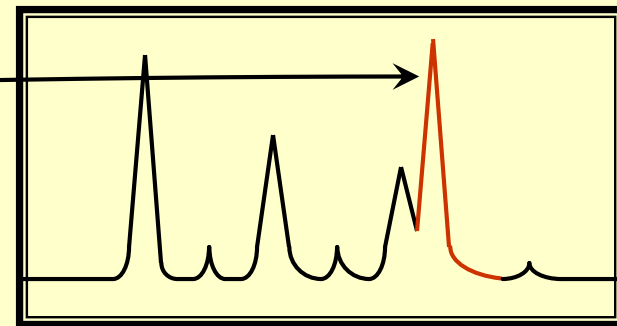
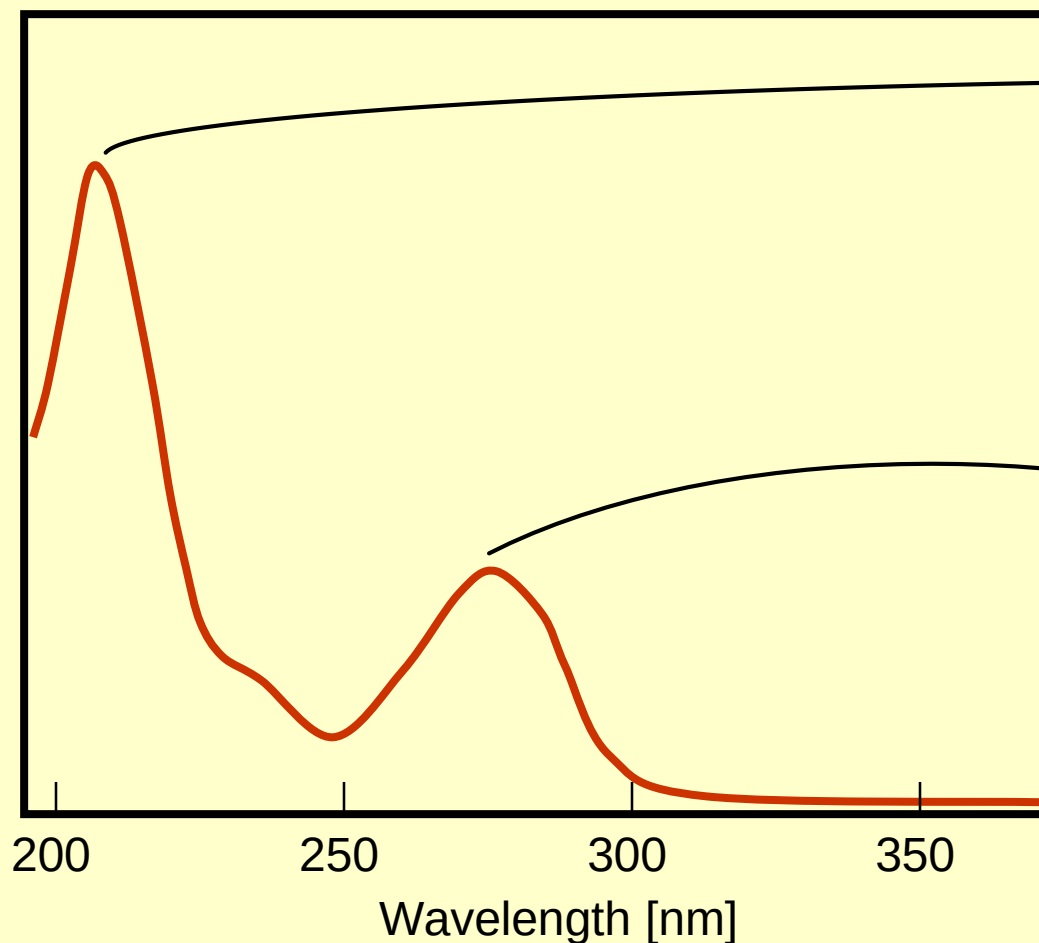


Optical System of UV-VIS Absorbance Detector

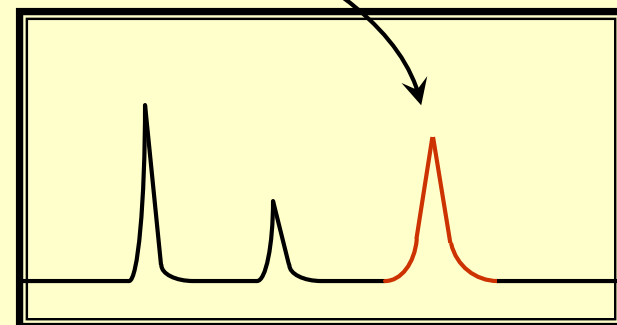


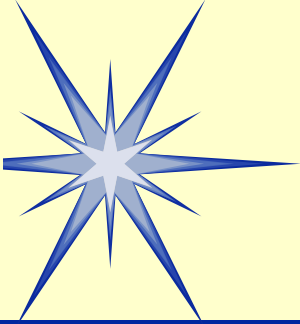


Spectrum and Selection of Detection Wavelength

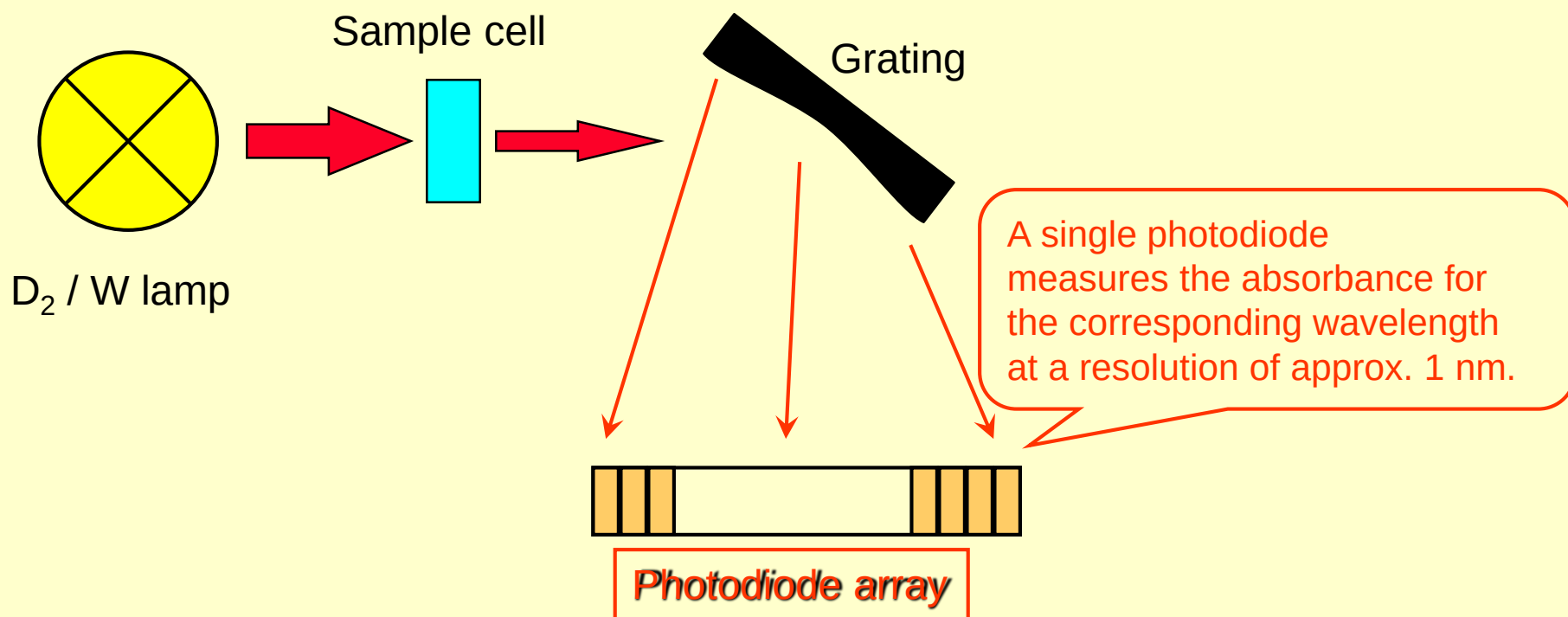


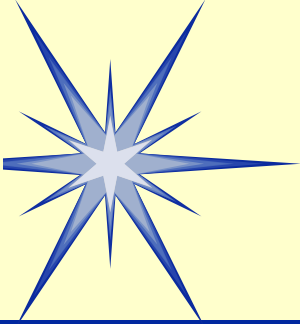
The longer wavelength is more selective.



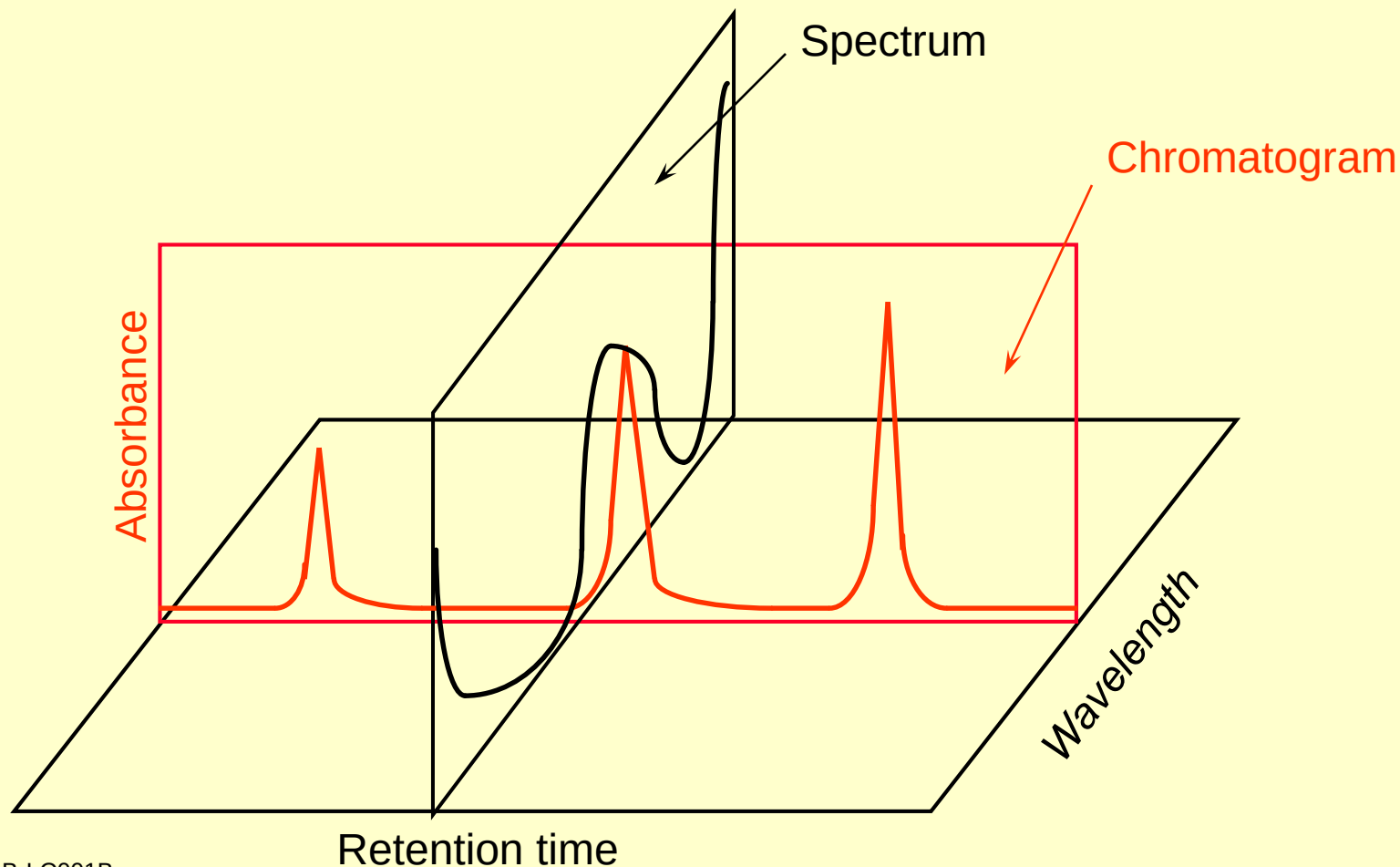


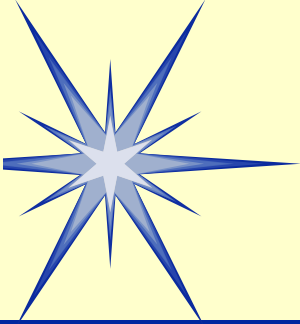
Optical System of Photodiode Array Detector





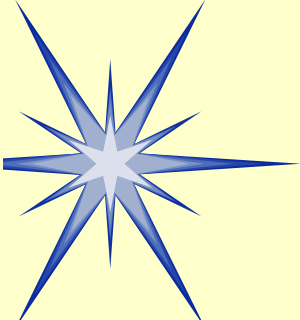
Data Obtained with a Photodiode Array Detector



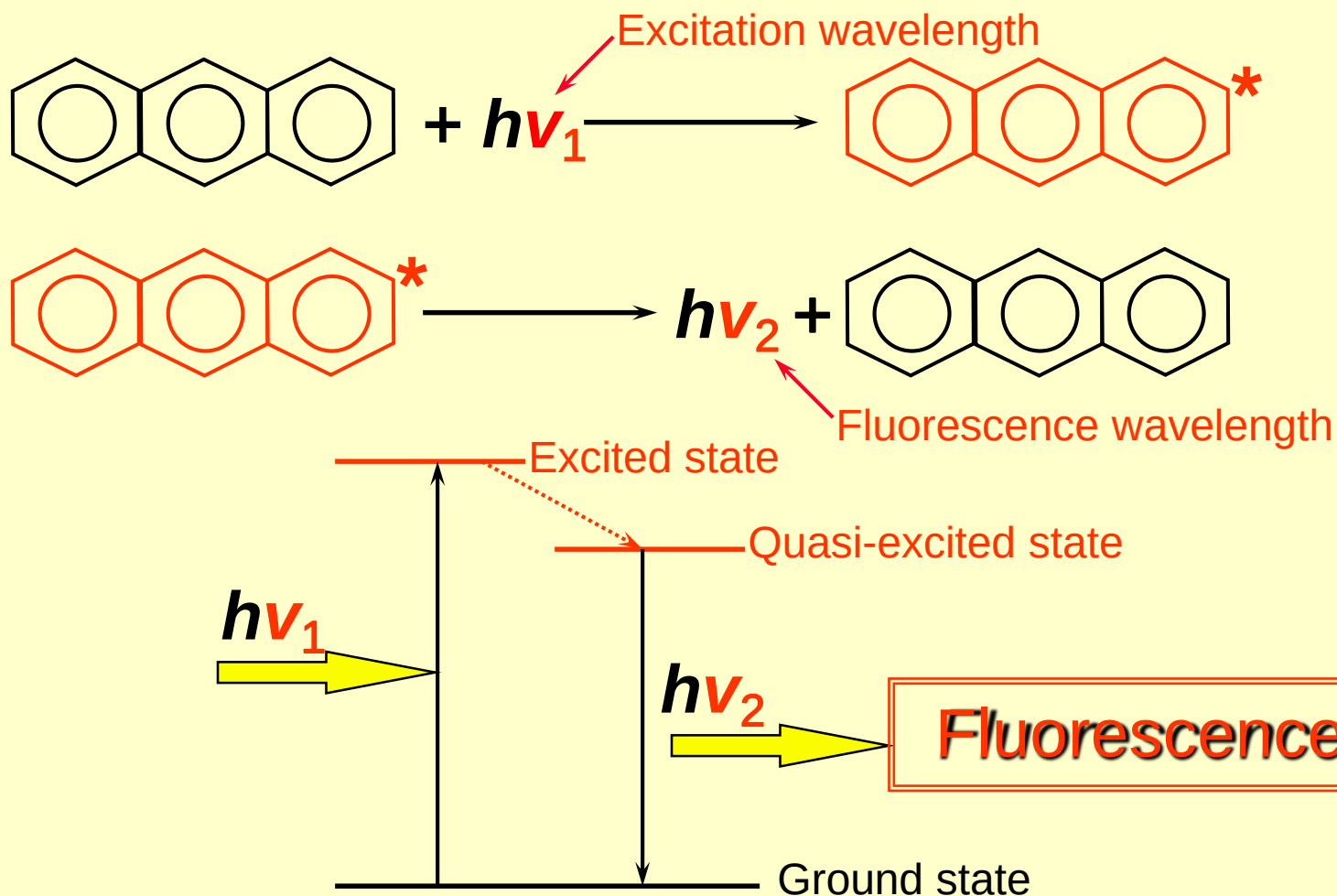


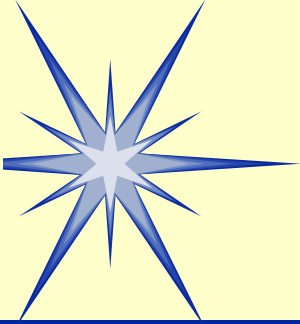
Advantages of Photodiode Array Detectors

- Peak Identification Using Spectra
 - ❖ Complementation of identification based on retention time
 - ❖ Library searches
- Evaluation of Peak Purity
 - ❖ Purity evaluation performed by comparison of the shape of spectra from the peak detection start point to the peak detection end point

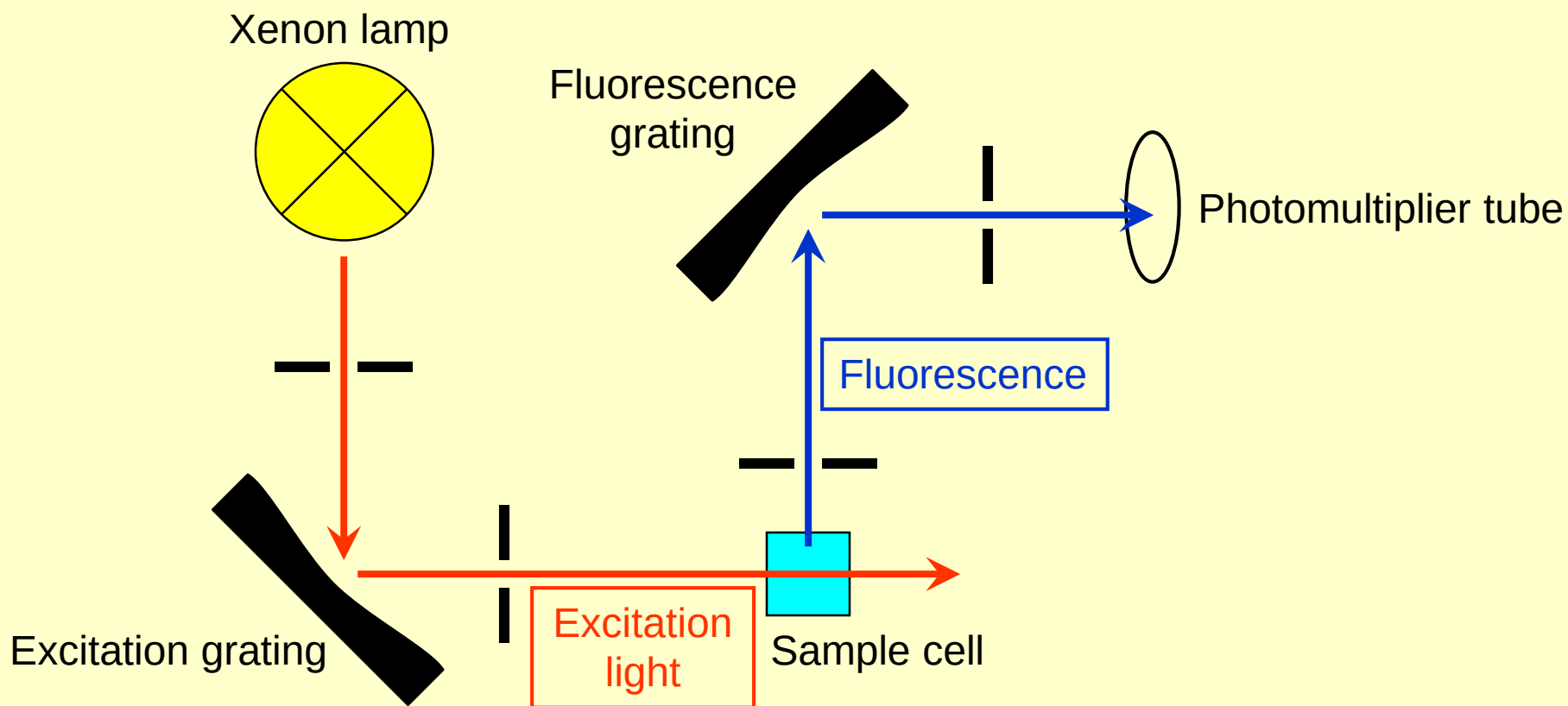


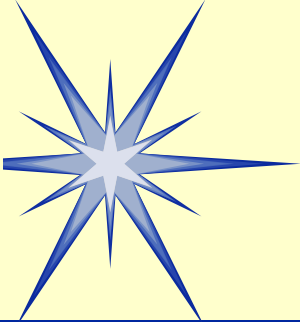
Fluorescence Detector





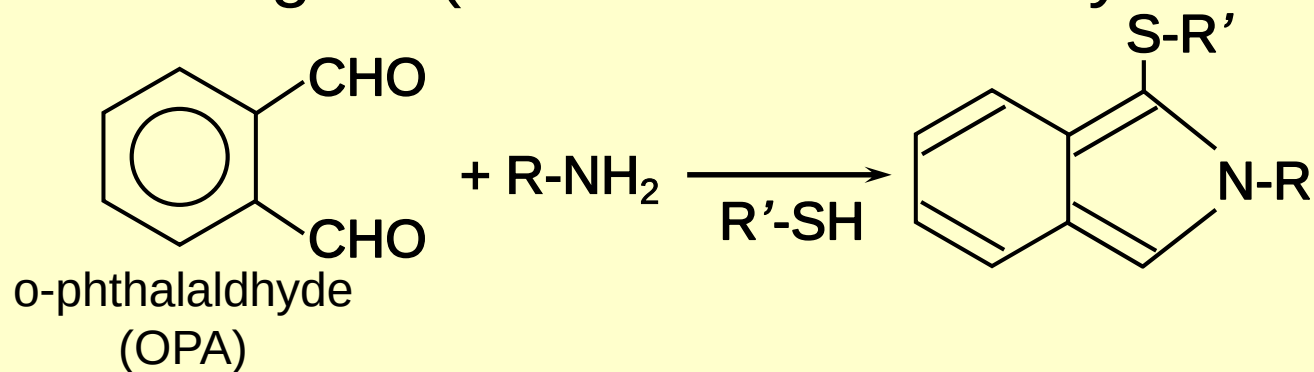
Optical System of Fluorescence Detector



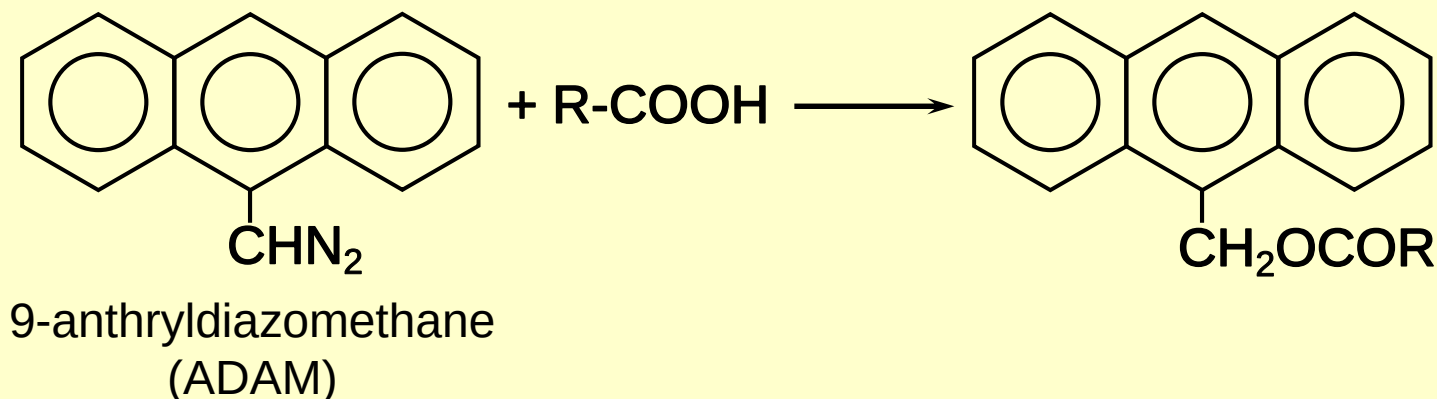


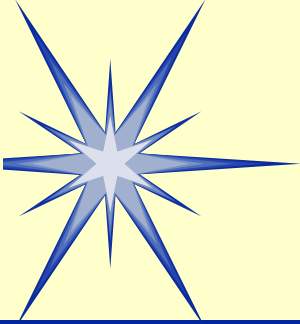
Fluorescence Derivatization Reagents

- OPA Reagent (Reacts with Primary Amines)



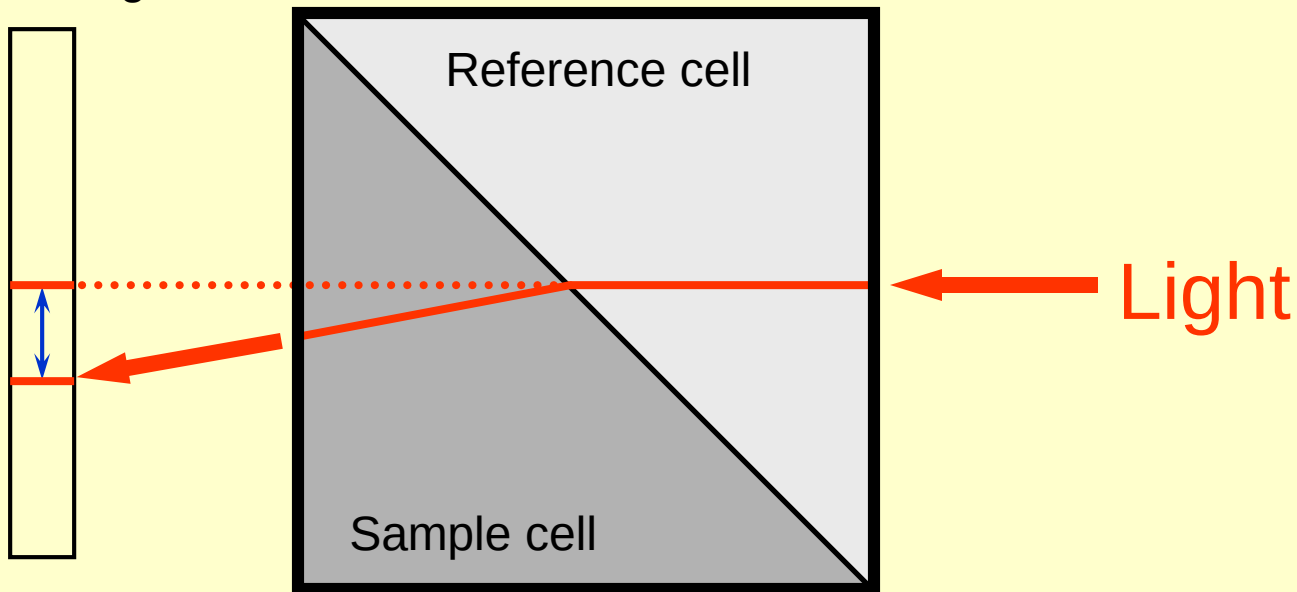
- ADAM Reagent (Reacts with Fatty Acids)

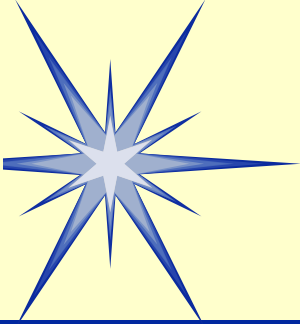




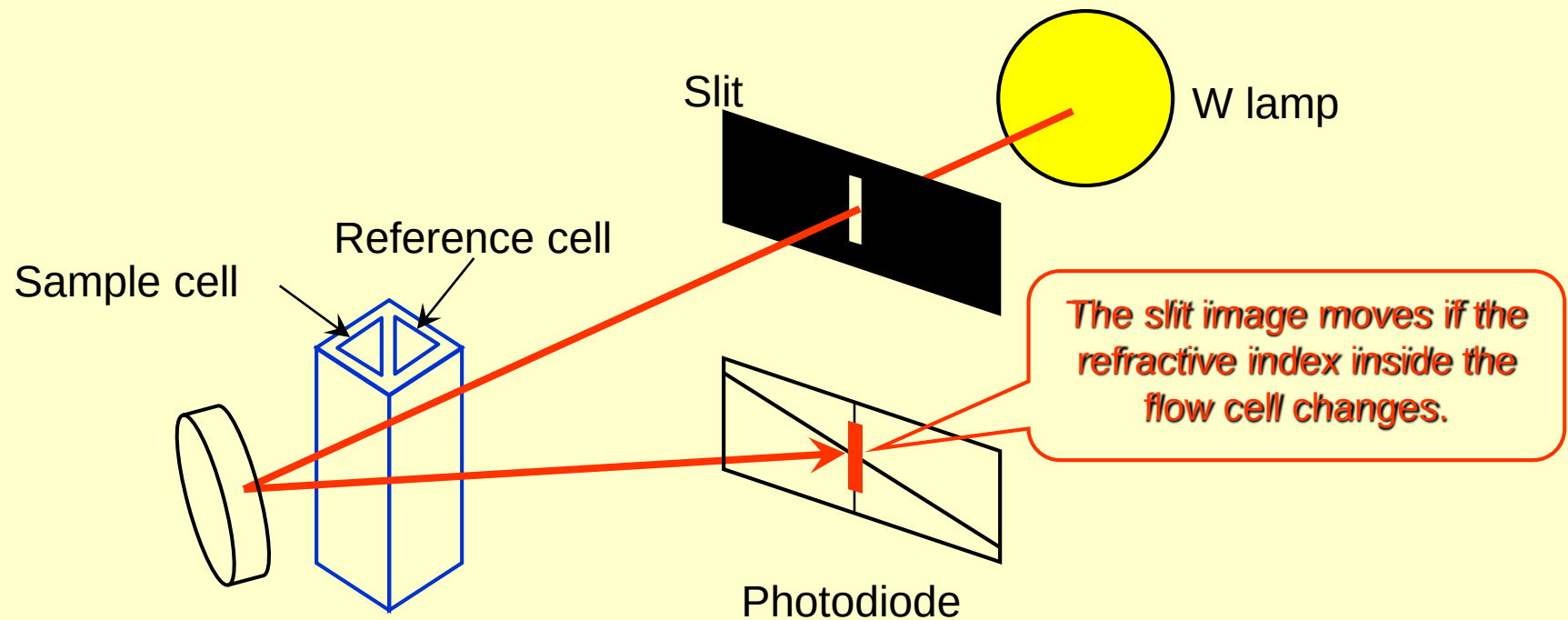
Differential Refractive Index Detector (Deflection-Type)

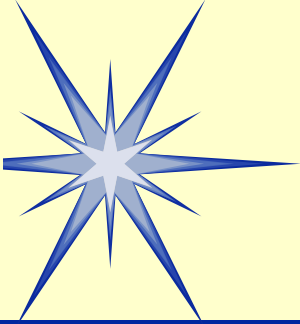
Light-receiving unit



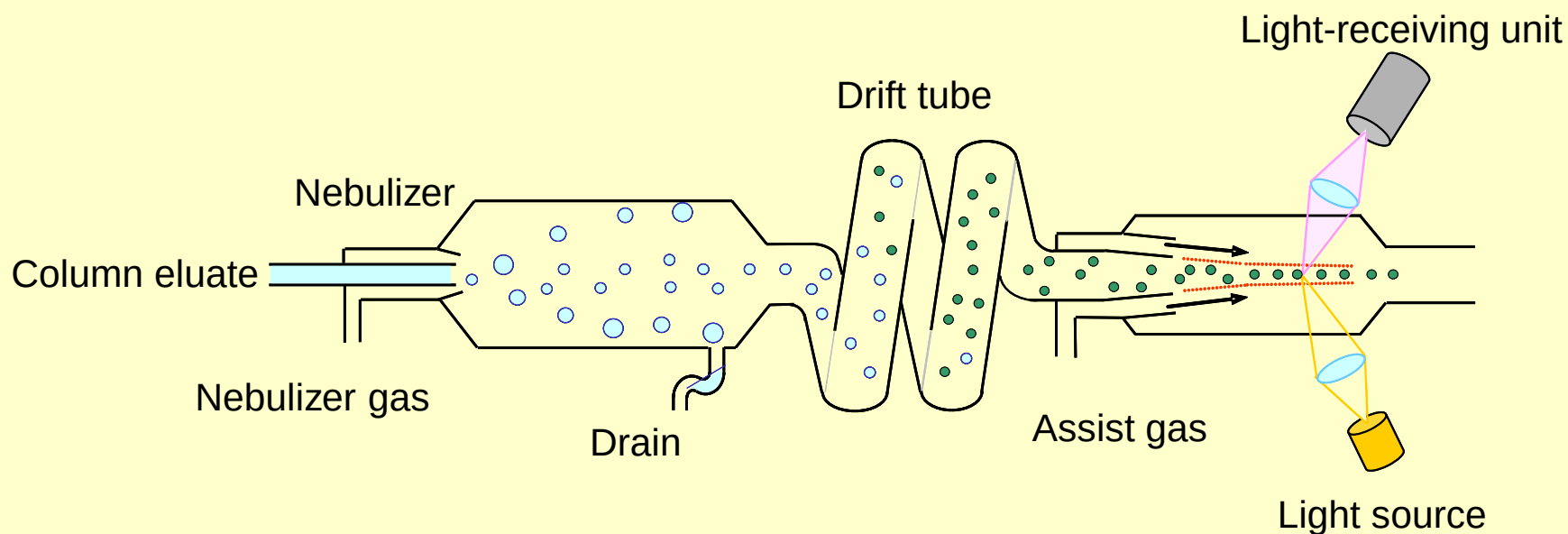


Optical System of Differential Refractive Index Detector (Deflection-Type)

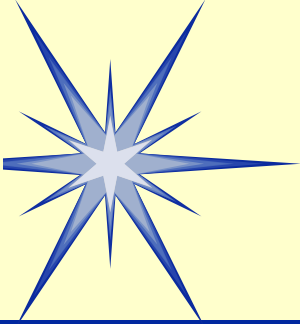




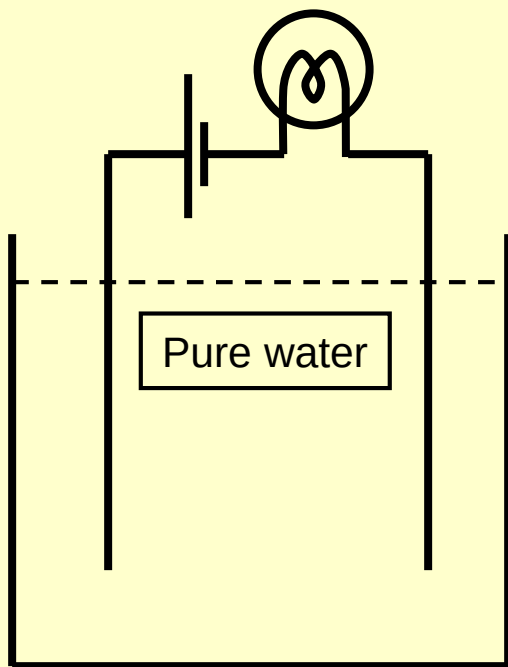
Evaporative Light Scattering Detector



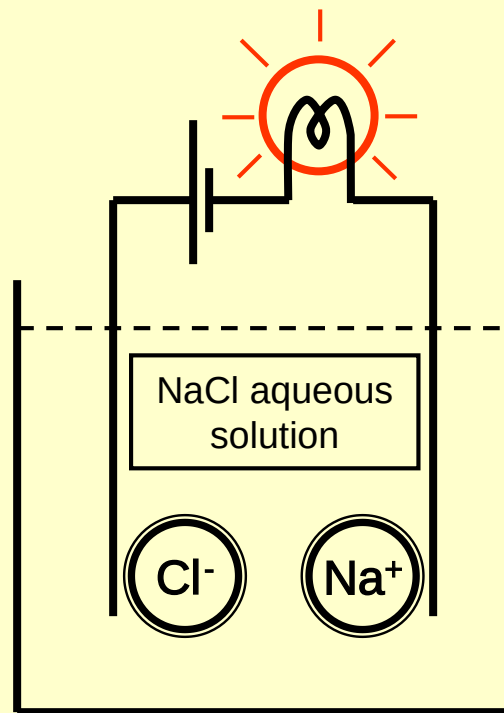
The column eluate is evaporated and the light scattered by the particles of nonvolatile substances is detected.



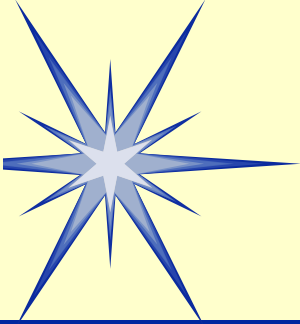
Electrical Conductivity Detector



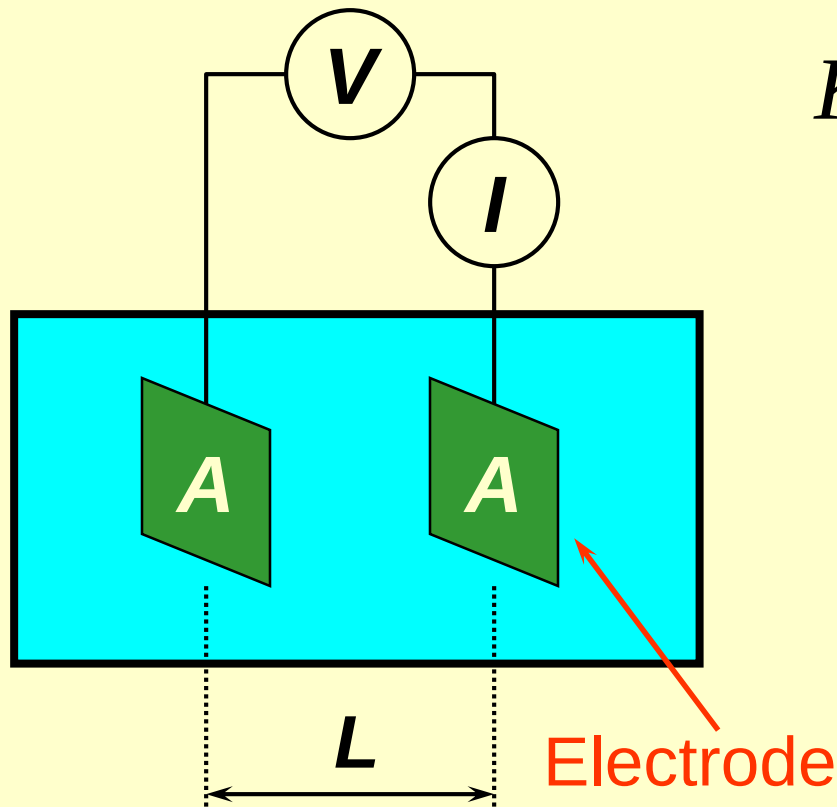
The bulb does not light with water.



The bulb lights if there are ions.



Principle of Electrical Conductivity Detector



$$K = \frac{I}{E} = \frac{A}{L} \bullet k$$

$$k = \frac{L}{A} \bullet K$$

K : Electrical conductivity [S]

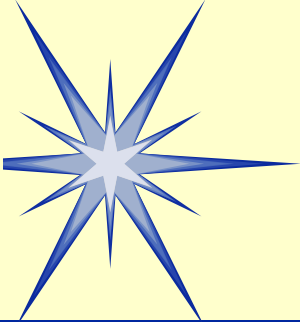
I : Electric current [A]

E : Voltage [V]

A : Electrode surface area [cm²]

L : Distance between electrodes [cm]

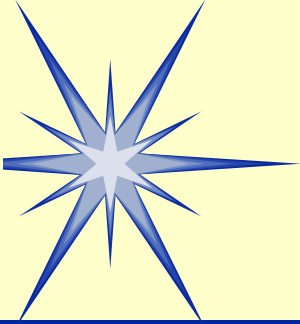
k : Specific electrical conductivity [S•cm⁻¹]



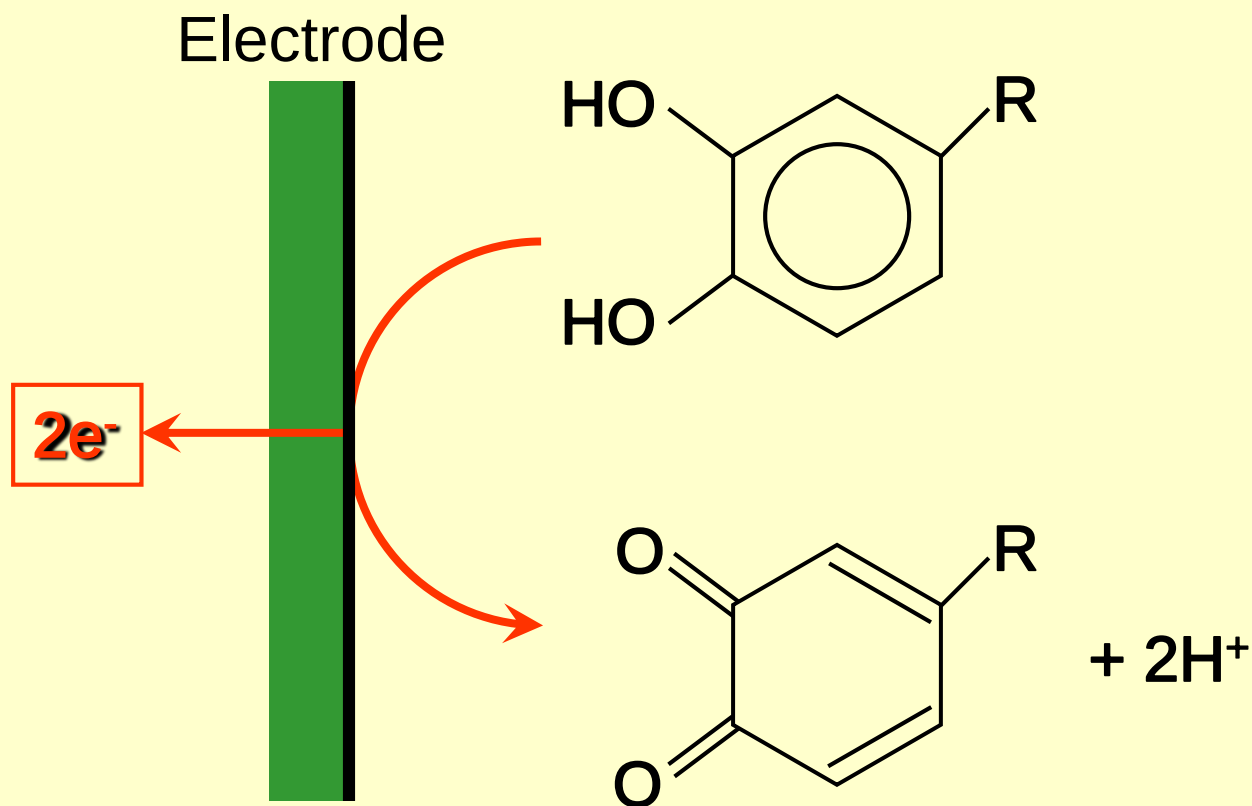
Limiting Equivalent Ion Conductance, λ [S•cm²/mol], in Aqueous Solution (25°C)

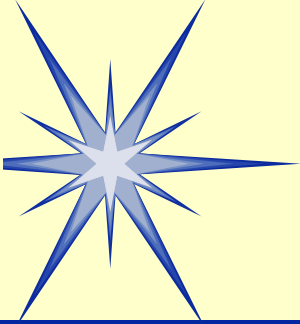
Cation	λ
H ⁺	349.8
Li ⁺	38.6
Na ⁺	50.1
K ⁺	73.5
NH ₄ ⁺	73.5
(CH ₃) ₃ NH ⁺	47.2
Mg ²⁺	53.0
Ca ²⁺	59.5

Anion	λ
OH ⁻	198.3
F ⁻	55.4
Cl ⁻	76.3
Br ⁻	78.1
NO ₃ ⁻	71.4
CH ₃ COO ⁻	40.9
C ₆ H ₅ COO ⁻	32.3
SO ₄ ²⁻	80.0

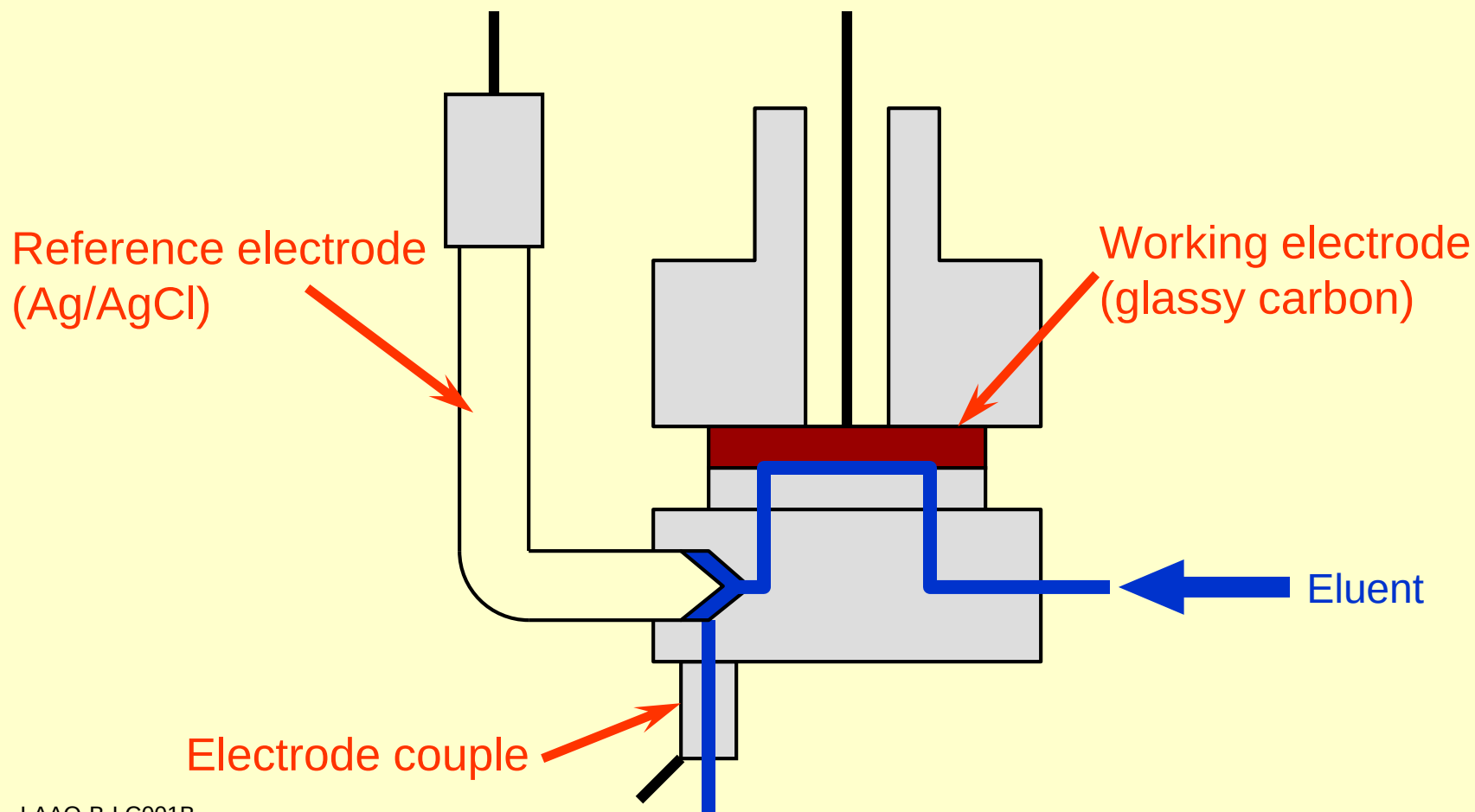


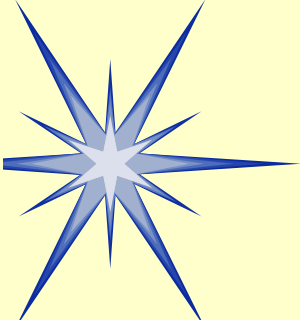
Electrochemical Detector



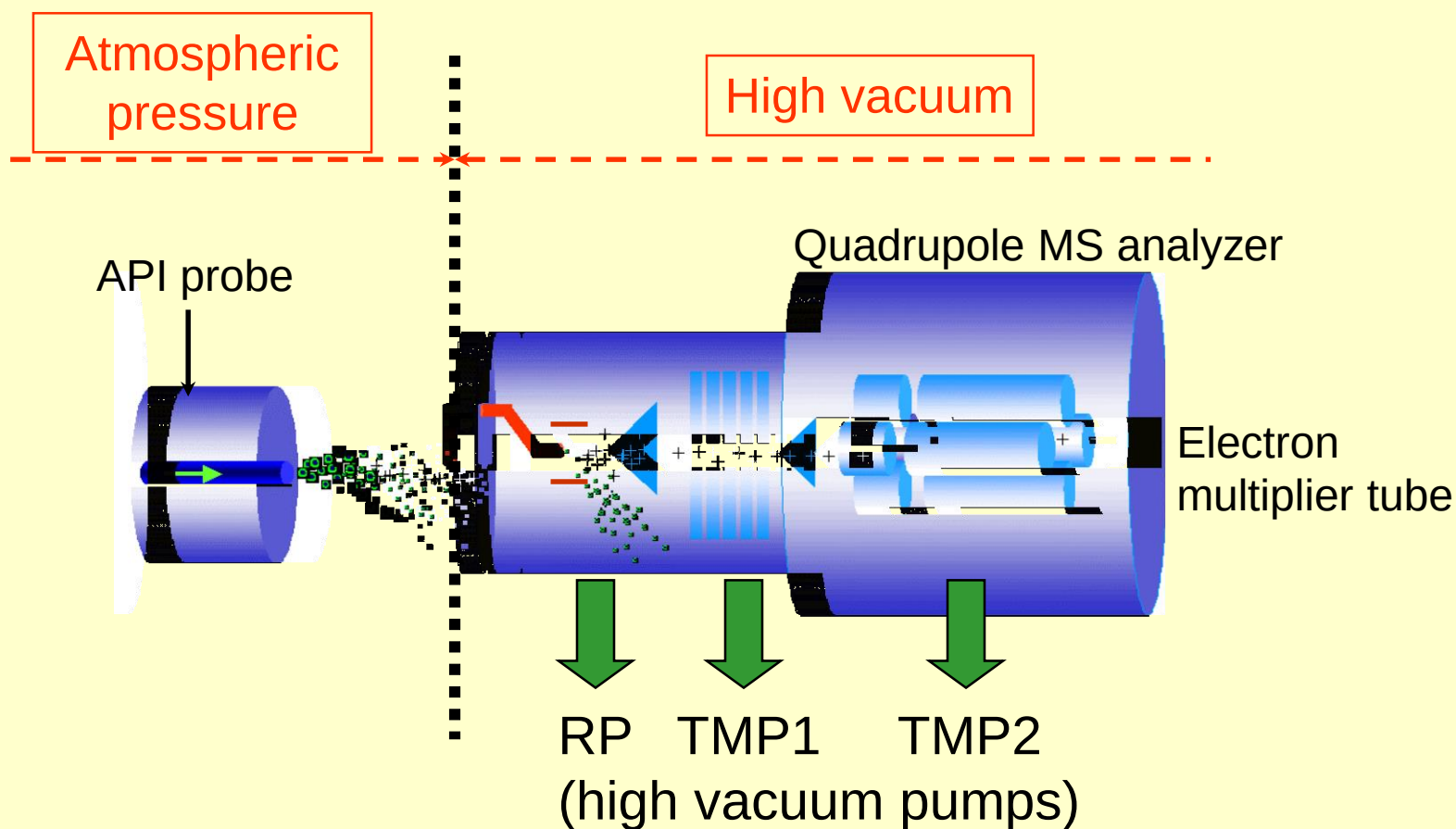


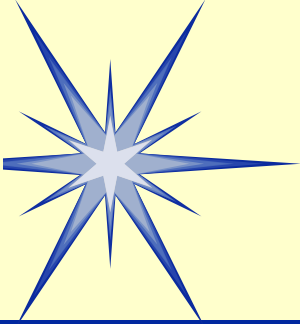
Cell Structure of Electrochemical Detector (Amperometric Type)





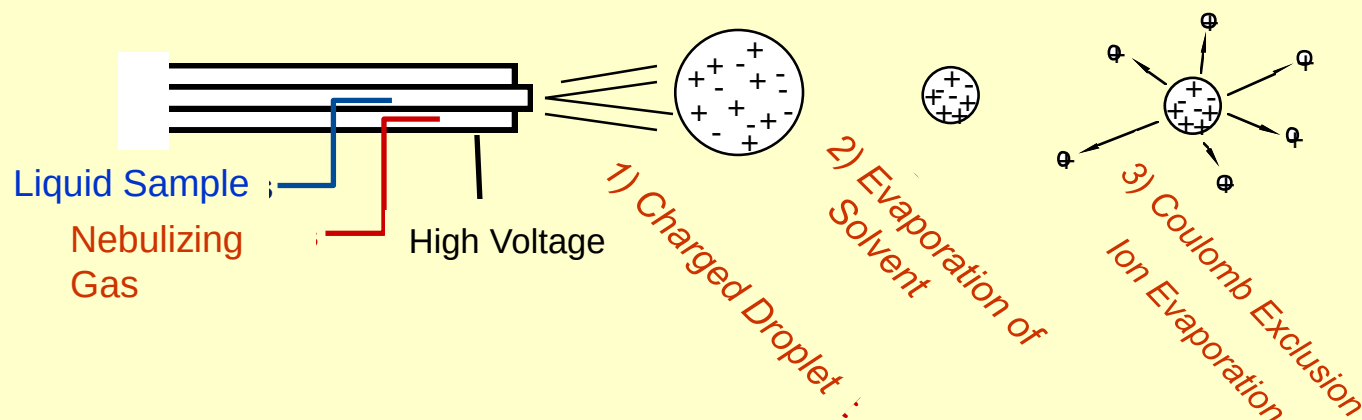
Mass Spectrometer (LCMS)



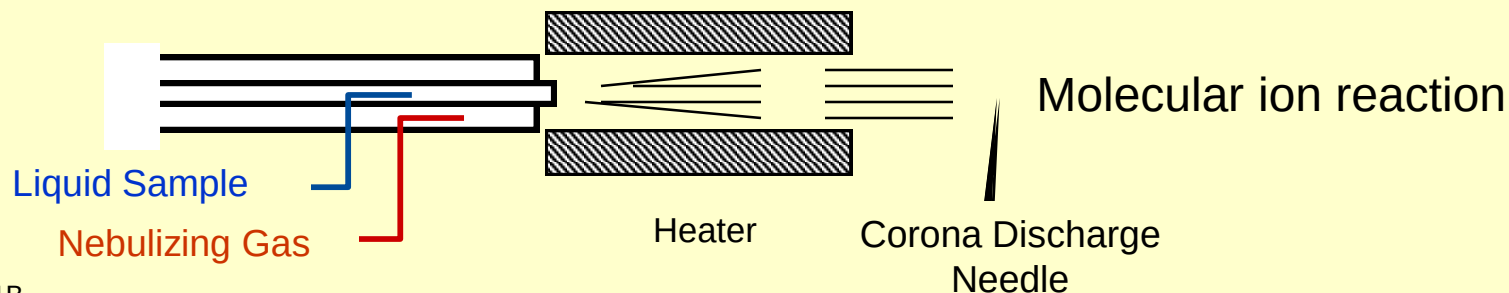


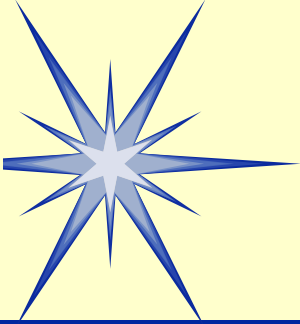
Atmospheric Pressure Ionization

Electrospray Ionization (ESI)



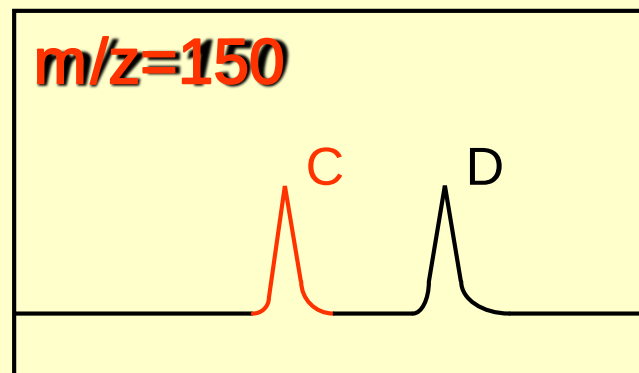
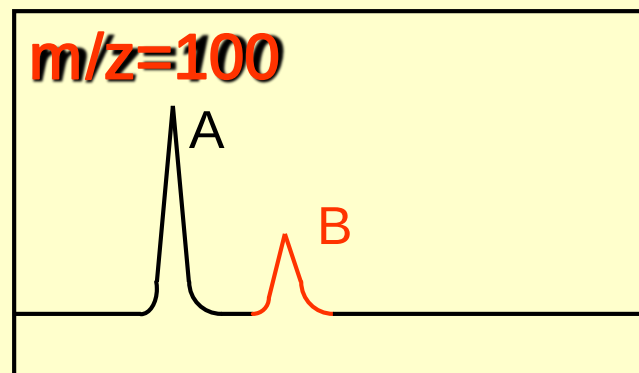
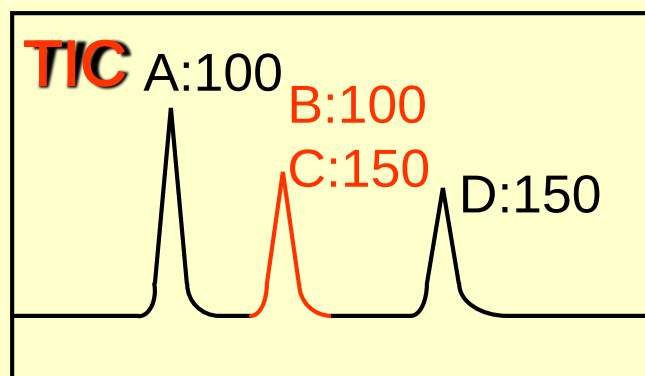
Atmospheric Pressure Chemical Ionization (APCI)

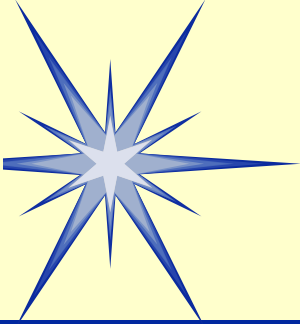




Advantages of LCMS (1)

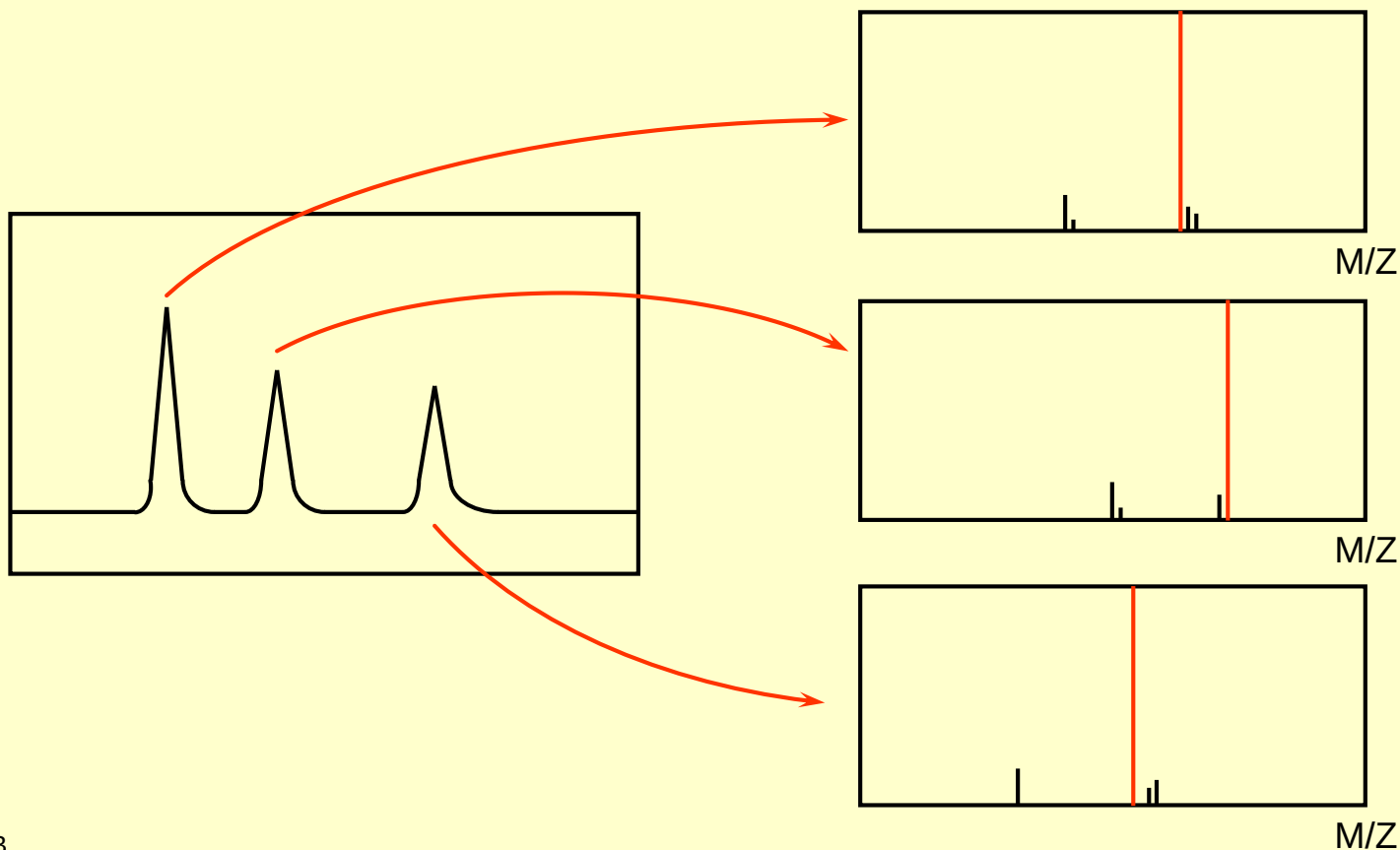
- Quantitative analysis with excellent selectivity

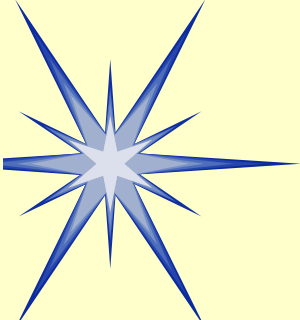




Advantages of LCMS (2)

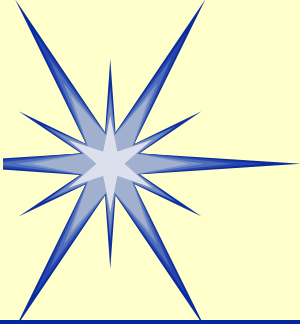
- Peaks can be identified with MS spectra.



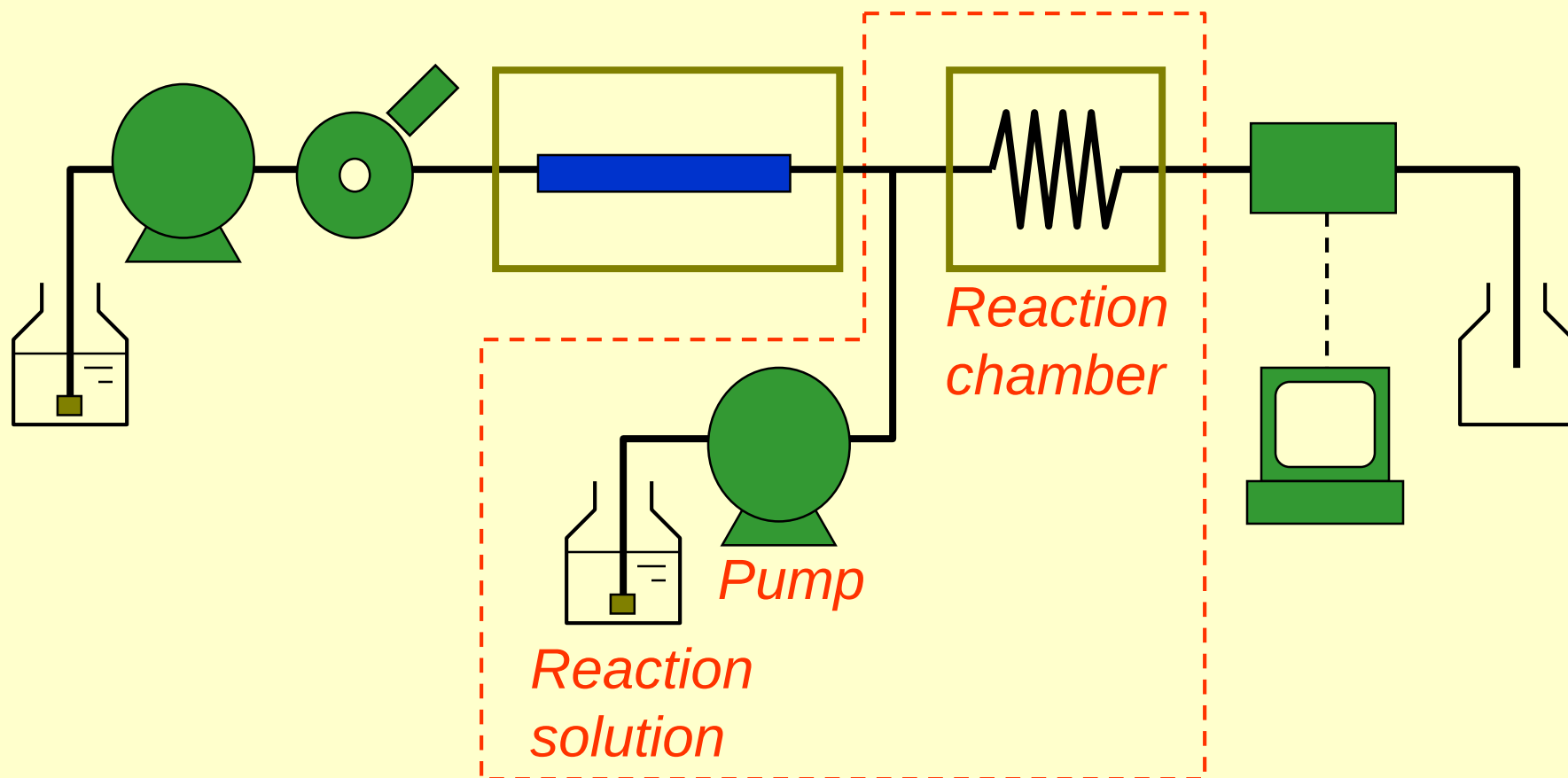


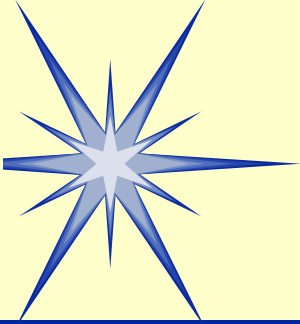
Comparison of Detectors

	Selectivity	Sensitivity	Possibility of Gradient System
Absorbance	Light-absorbing substances	ng	Possible
Fluorescence	Fluorescent substances	pg	Possible
Differential refractive index	None	μg	Impossible
Evaporative light scattering	Nonvolatile substances	μg	Possible
Electrical conductivity	Ionic substances	ng	Partially possible
Electrochemical	Oxidizing / reducing substances	pg	Partially possible



Post-Column Derivatization





Application Examples of Post-Column Methods

- Amino Acids

- ❖ Orthophthalic acid, OPA (fluorescence)
- ❖ Ninhydrin (visible absorption)

- Reducing Sugars

- ❖ Arginine (fluorescence)

- Carbamate Pesticides

- ❖ Alkaline hydrolysis - OPA (fluorescence)

- Bromate Ions

- ❖ Tribromide ionization (ultraviolet absorption)
- ❖ o-Dianisidine (visible absorption)

- Cyanide Ions

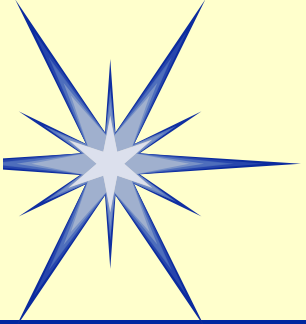
- ❖ Chlorination - pyrazolone (visible absorption)

- Transition Metal Ions

- ❖ 4-(2-Pyridylazo) resorcinol, PAR (visible absorption)

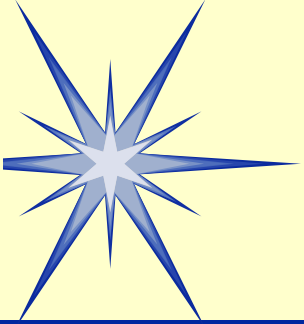
Quantitative Analysis

Absolute Calibration Curve Method
and Internal Standard Method



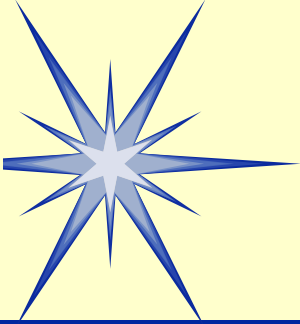
Qualitative Analysis

- Identification based on retention time
- Acquisition of spectra with detector
 - ❖ UV spectra
 - ❖ MS spectra
- Transfer to other analytical instruments after preparative separation

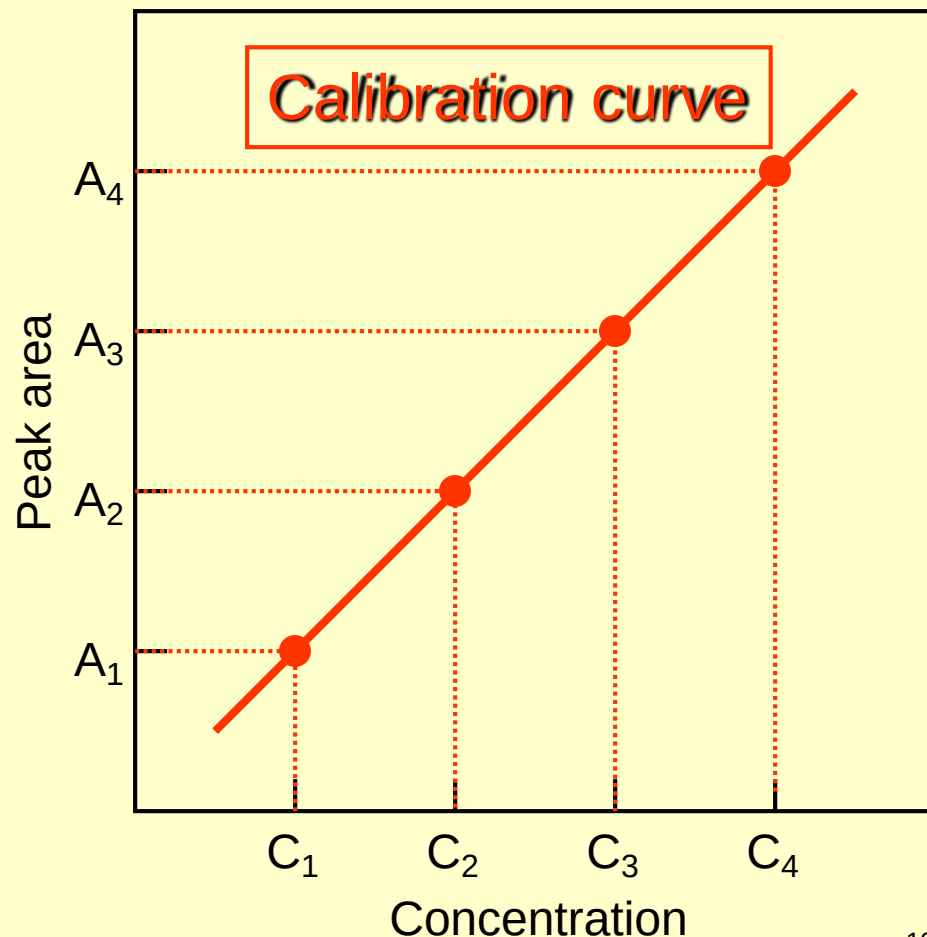
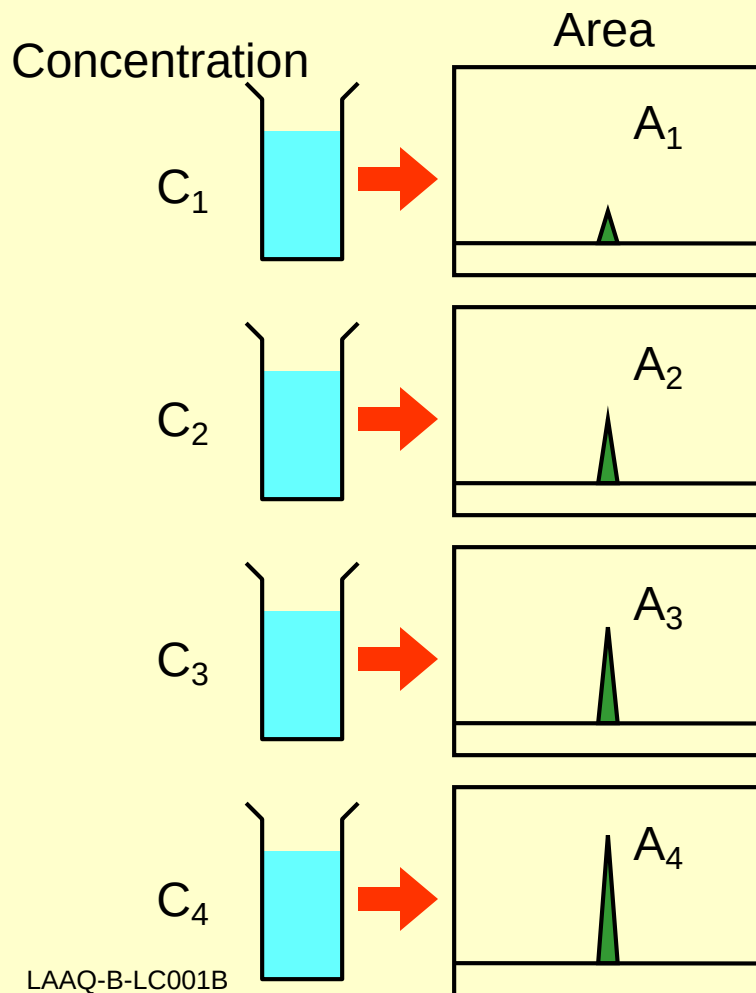


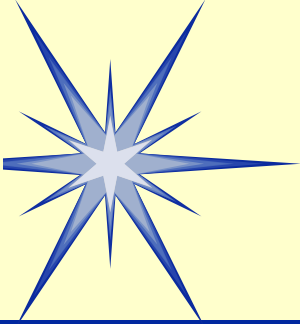
Quantitative Analysis

- Quantitation performed with peak area or height.
- Calibration curve created beforehand using a standard.
 - ❖ Absolute calibration curve method
 - ❖ Internal standard method
 - ❖ Standard addition method

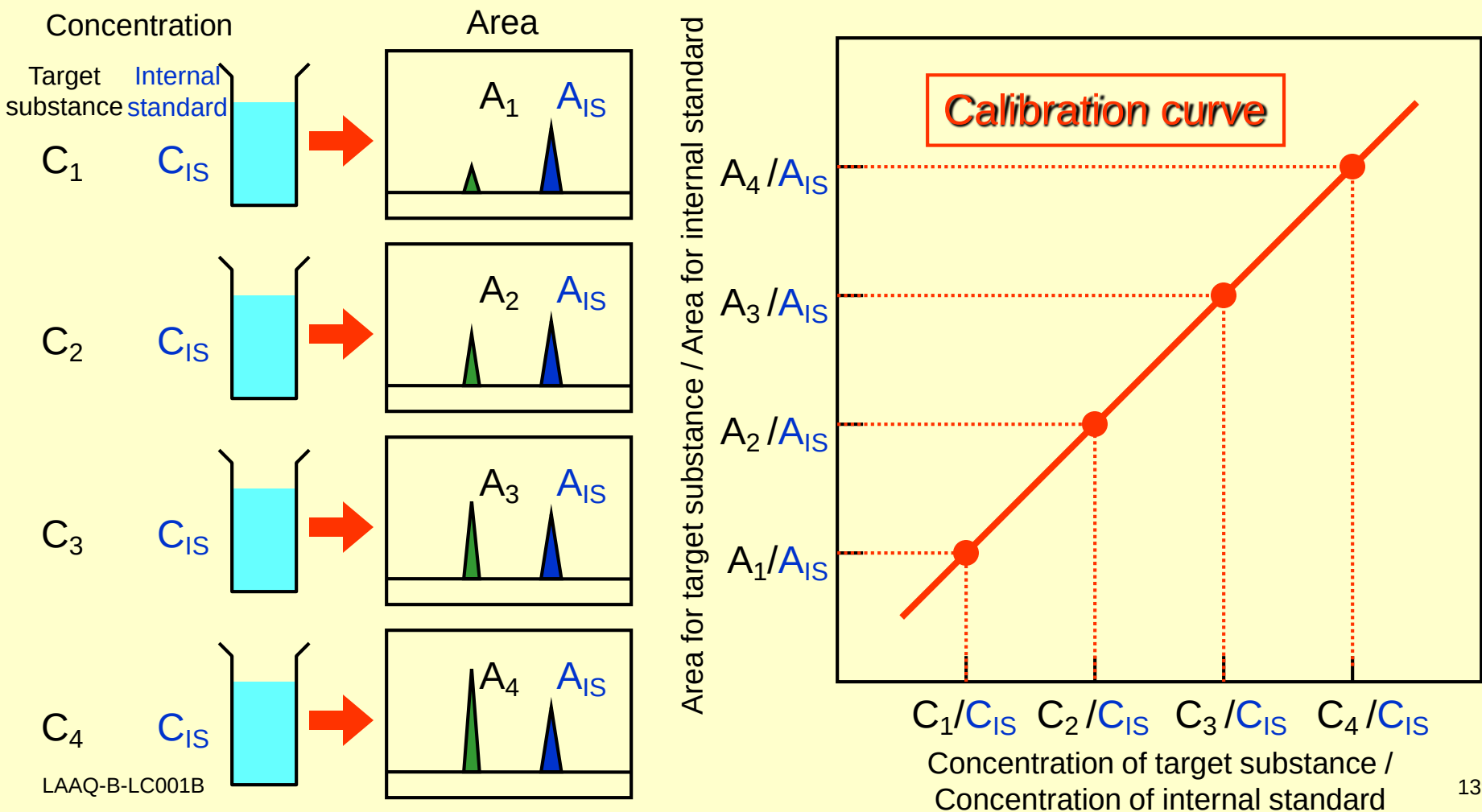


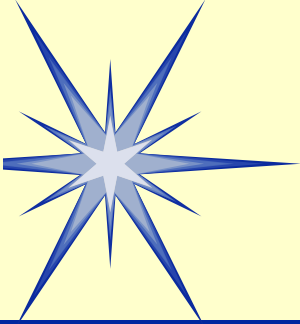
Calibration Curve for Absolute Calibration Curve Method





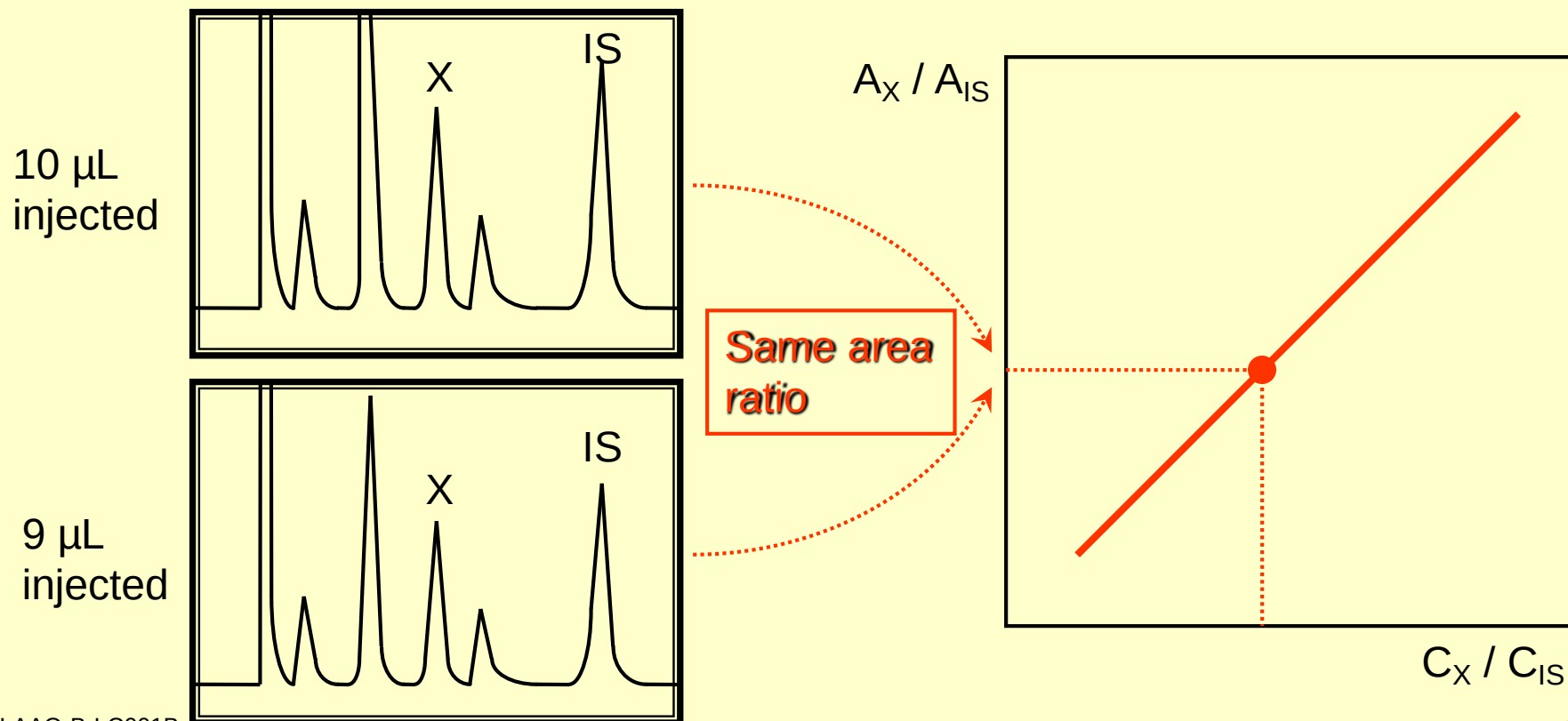
Calibration Curve for Internal Standard Method

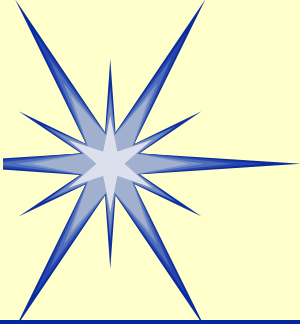




Advantages of Internal Standard Method (1)

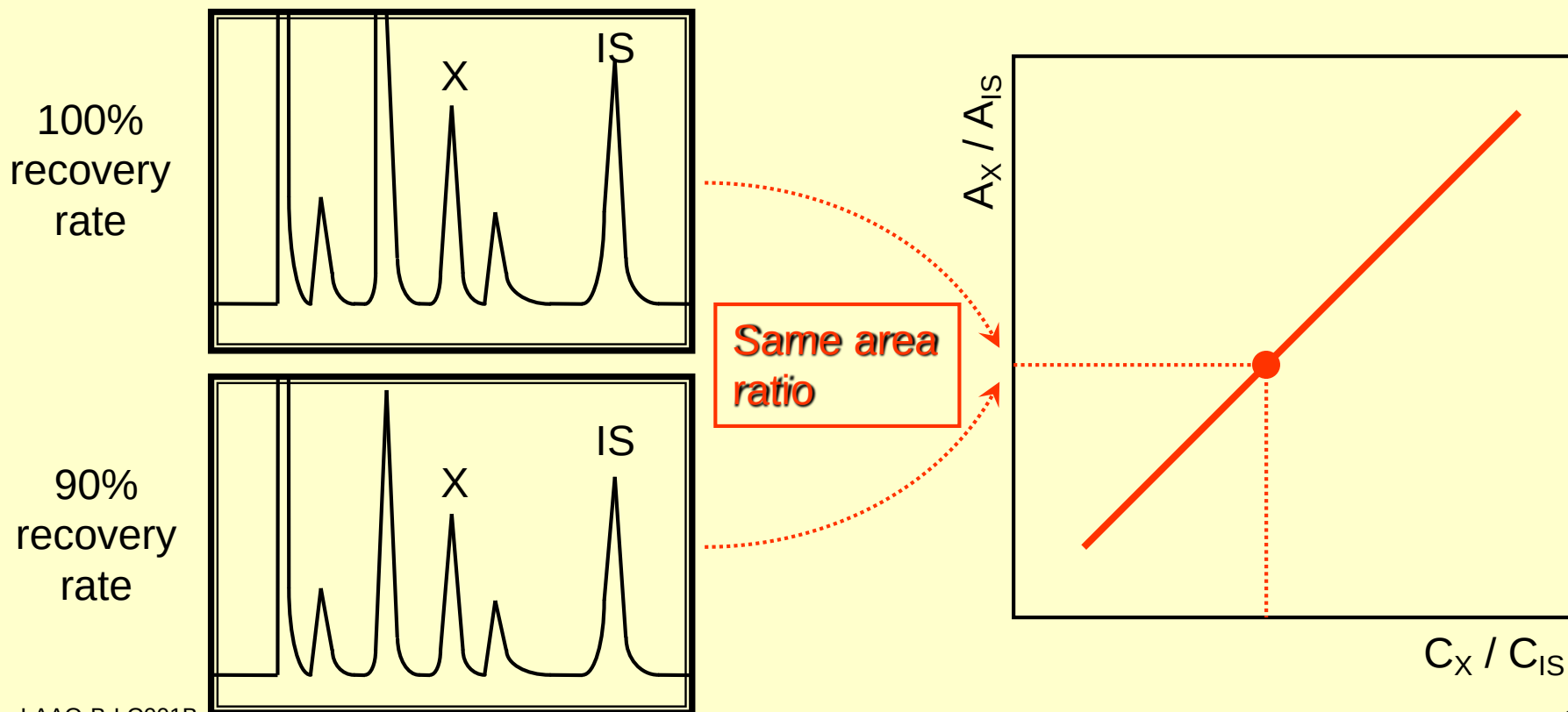
- Not affected by inconsistencies in injection volume.

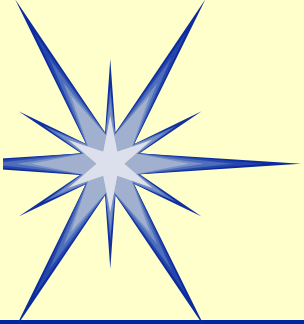




Advantages of Internal Standard Method (2)

- Not affected by the pretreatment recovery rate.



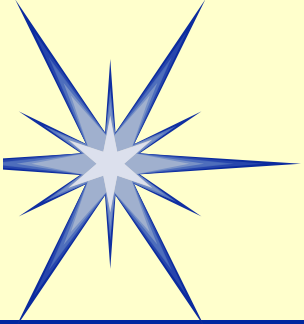


Selection Criteria for Internal Standard

- It must have similar chemical properties to the target substance.
- Its peak must appear relatively near that of the target substance.
- It must not already be contained in the actual samples.
- Its peak must be completely separated from those of other sample components.
- It must be chemically stable.

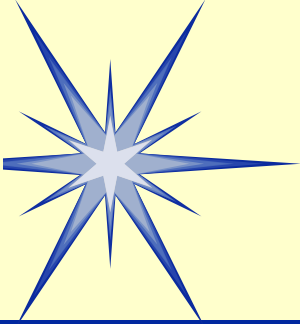
Sample Pretreatment

Tasks Performed Before Injection



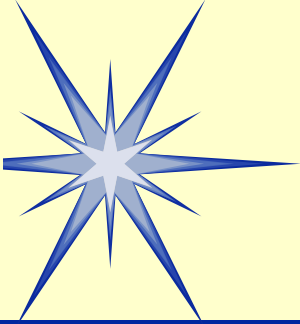
Objectives of Pretreatment

- To improve the accuracy of quantitative values
- To improve sensitivity and selectivity
- To protect and prevent the deterioration of columns and analytical instruments
- To simplify measurement operations and procedures
- To stabilize target substances



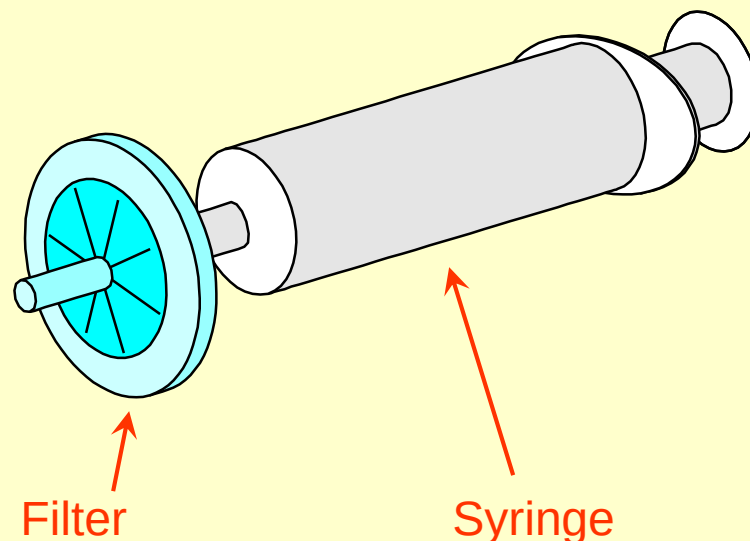
Substances That Must Not Be Injected into the Column

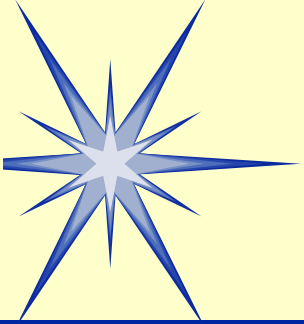
- Insoluble substances (e.g., microscopic particles and precipitation)
- Substances that are precipitated in the eluent
- Substances that irreversibly adsorb to the packing material
- Substances that dissolve, or chemically react, with the packing material



Filtration and Centrifugal Separation

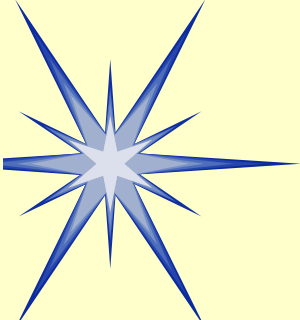
- In general, filter every sample before injection!
- It is convenient to use a disposable filter with a pore diameter of approx. $0.45\ \mu\text{m}$.
- Centrifugal separation is applicable for samples that are difficult to filter.





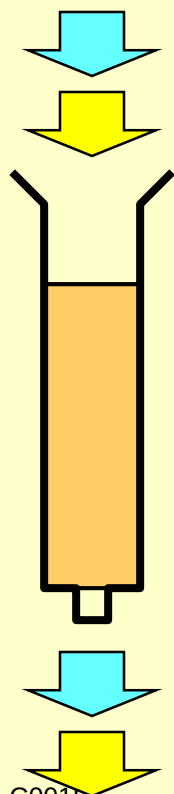
Deproteinization

- Precipitation
 - ❖ Addition of organic solvent (e.g., acetonitrile)
 - ❖ Addition of acid (e.g., trichloroacetic acid, perchloric acid)
 - ❖ Addition of heavy metal or neutral salt
- Ultrafiltration

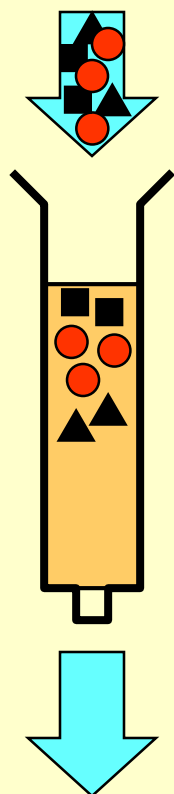


Solid Phase Extraction

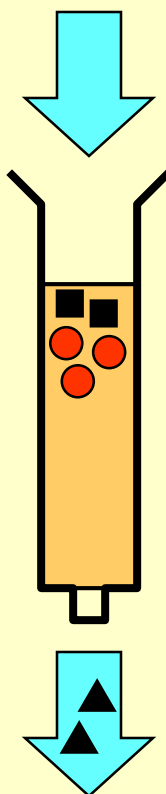
(1)
Conditioning



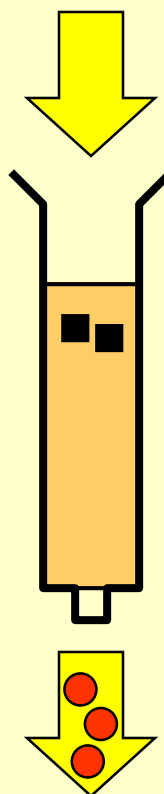
(2)
Sample addition

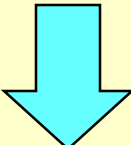


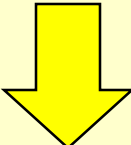
(3)
Rinsing



(4)
Elution

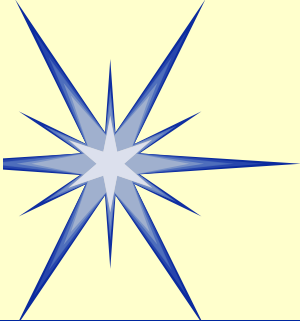


 Solvent with low elution strength

 Solvent with high elution strength

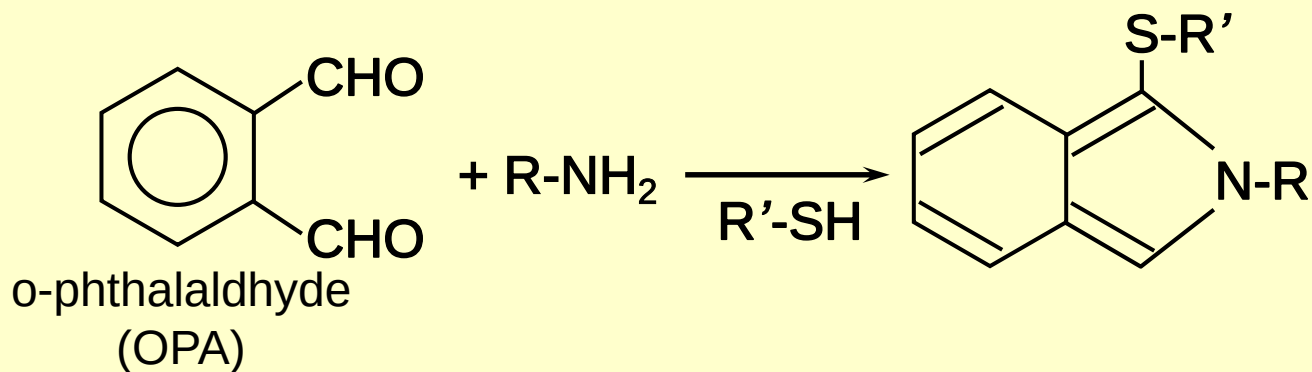
 Target component

 Unwanted components

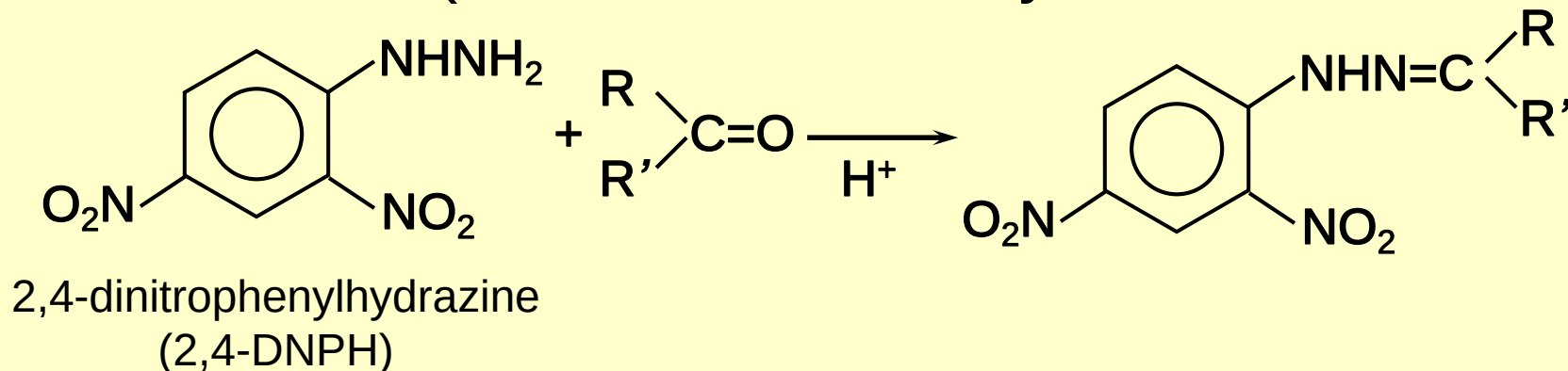



Pre-Column Derivatization

- OPA Reagent (Reacts with Primary Amines)

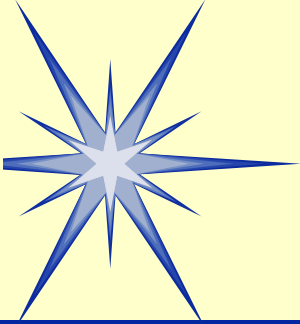


- 2,4-DNPH (Reacts with Aldehydes and Ketones)



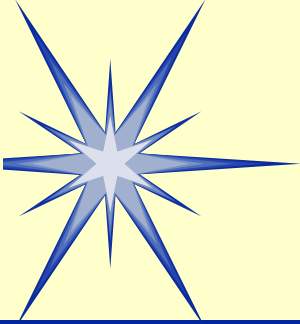
Evaluation of the Reliability of Analysis

Validation of Analytical Methods



What Is “Validation of Analytical Methods”?

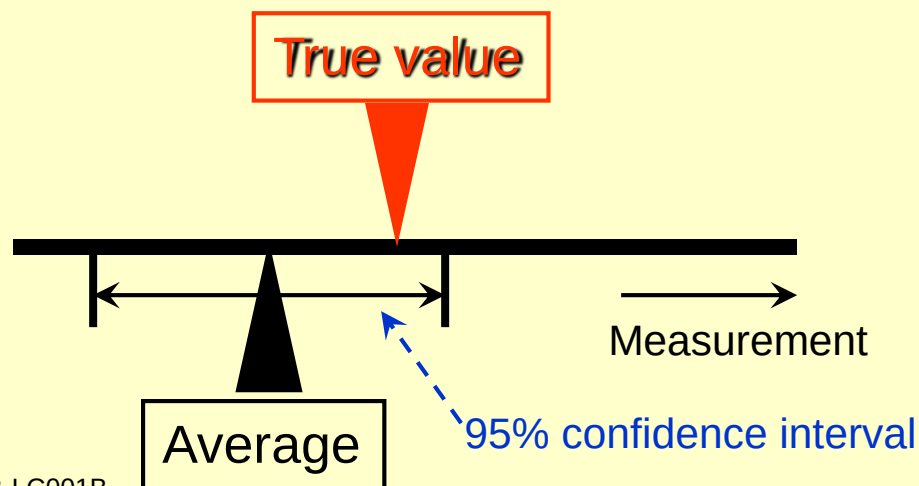
- Scientifically demonstrating that the analytical methods concur with the intended purpose (i.e., that errors are within a permissible range)
- Evaluating required items from the validation characteristics
- Validation characteristics
 - ❖ Accuracy / trueness
 - ❖ Precision
 - ❖ Specificity
 - ❖ Detection limit
 - ❖ Quantitation limit
 - ❖ Linearity
 - ❖ Range
 - ❖ (Robustness)



Accuracy / Trueness

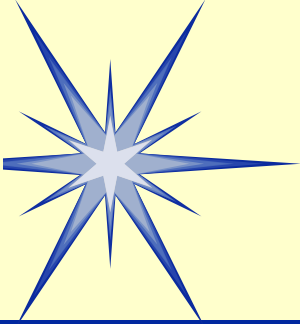
- Definition

- ❖ Degree of bias in measurements obtained with analytical procedures
- ❖ Difference between true value and grand mean of measurements



- Evaluation Method

- ❖ Comparison with theoretical values (or authenticated values)
- ❖ Comparison with results obtained using other analytical procedures for which the accuracy (trueness) is known
- ❖ Recovery test



Precision

- Definition

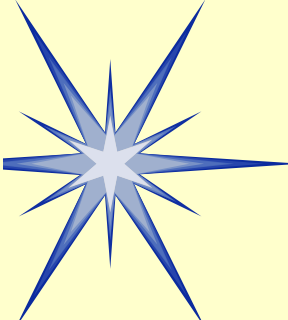
- ❖ Degree of coincidence of series of measurements obtained by repeatedly analyzing multiple samples taken from a homogenous test substance
- ❖ Variance, standard deviation, or relative standard deviation of measurements

- Repeatability / Intra-Assay Precision

- ❖ Precision of measurements taken over a short time period under the same conditions

- Intermediate Precision

- Reproducibility



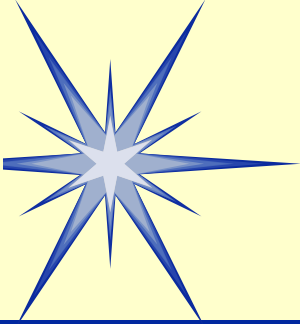
Specificity

● Definition

- ❖ The ability to accurately analyze the target substance in the presence of other expected substances
- ❖ The discrimination capability of the analytical methods
- ❖ Multiple analytical procedures may be combined in order to attain the required level of discrimination

● Evaluation Method

- ❖ Confirmation that the target substance can be discriminated (separated) from co-existing components, related substances, decomposition products, etc.
- ❖ If reference standards for impurities cannot be obtained, the measurement results for samples thought to contain the impurities are compared.



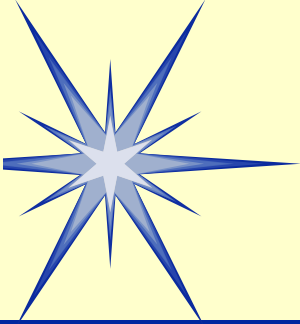
Detection Limit

● Definition

- ❖ The minimum quantity of a target substance that can be detected.
- ❖ Quantitation is not absolutely necessary.

● Evaluation Method

- ❖ Calculated from the standard deviation of measurements and the slope of the calibration curve.
 - ★ $DL = 3.3 \sigma / \text{slope}$
(σ : Standard deviation of measurements)
(Slope: Slope of calibration curve)
- ❖ Calculated from the signal-to-noise ratio.
 - ★ Concentration for which $S/N = 3$ or 2



Quantitation Limit

● Definition

- ❖ The minimum quantity of a target substance that can be quantified
- ❖ Quantitation with an appropriate level of accuracy and precision must be possible. (In general, the relative standard deviation must not exceed 10%.)

● Evaluation Method

- ❖ Calculated from the standard deviation of measurements and the slope of the calibration curve.
 - ★ $QL = 10 \sigma / \text{slope}$
(σ : Standard deviation of measurements)
(Slope: Slope of calibration curve)
- ❖ Calculated from the signal-to-noise ratio.
 - ★ Concentration for which $S/N = 10$



Linearity

● Definition

- ❖ The ability of the analytical method to produce measurements for the quantity of a target substance that satisfy a linear relationship.
- ❖ Values produced by converting quantities or measurements of the target substance using a precisely defined formula may be used.

● Evaluation Method

- ❖ Samples containing different quantities of the target substance (usually 5 concentrations) are analyzed repeatedly, and regression equations and correlation coefficients are obtained.
- ❖ Residuals obtained from the regression equations of the measurements are plotted, and it is confirmed that there is no specific slope.

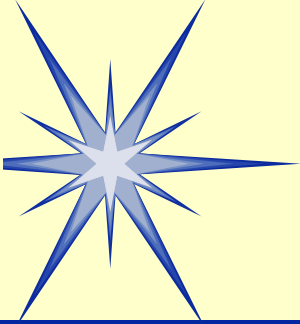


- Definition

- ❖ The region between the lower and upper limits of the quantity of a target substance that gives appropriate levels of accuracy and precision

- Evaluation Method

- ❖ The accuracy, precision, and linearity are investigated for samples containing quantities of a target substance that correspond to the lower limit, upper limit, and approximate center of the range.



Robustness

- Definition

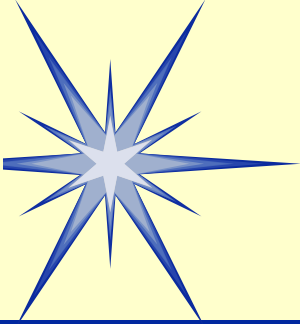
- ❖ The ability of an analytical procedure to remain unaffected by small changes in analytical conditions.

- Evaluation Method

- ❖ Some or all of the variable factors (i.e., the analytical conditions) are changed and the effects are evaluated.

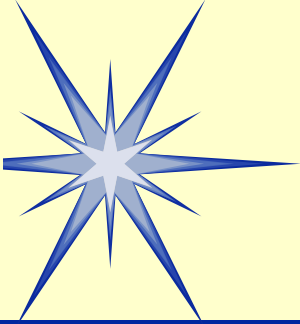
Maintenance of Separation Column

Extending the Column's Service Life



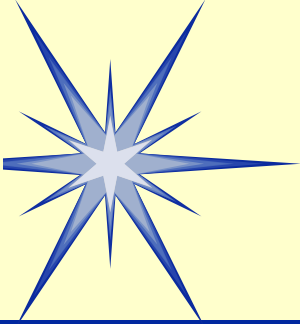
Silica-Based Packing Materials and Resin-Based Packing Materials

	Silica-Based	Resin-Based
pH range	2 - 7.5	Generally a wide range
Organic solvent	No restrictions	Significant restrictions
Pressure resistance	25 MPa max.	Low pressure resistance
Temperature	60°C max.	Depends on packing material



General Handling of Columns

- Observe restrictions related to solvents and the pH range.
- Never allow the packing material to dry.
- Do not allow solids or microscopic particles to enter the column.
 - ❖ Filter samples.
- Use as low a load pressure as possible.
 - ❖ Do not exceed the upper pressure limit.
 - ❖ Do not subject the column to sudden pressure changes.
- Do not subject the column to intense shocks.



Typical Problems (1)

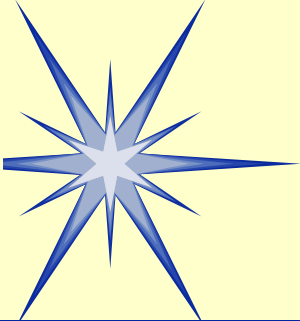
Column Clogging

● Preventive Measures

- ❖ Filter samples.
- ❖ Check that samples dissolve in the eluent.
- ❖ Get in the habit of observing pressure values.

● Corrective Action

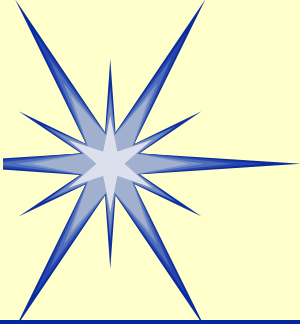
- ❖ Check for clogging in parts other than the column.
- ❖ Rinse with an appropriate solvent.
- ❖ Connect the column in reverse and flush out the insoluble substances at a low flow rate.
- ❖ Open the column end and perform ultrasonic cleaning of the filter.



Typical Problems (2)

Peak Deformation

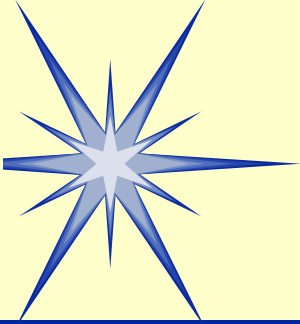
Cause	Corrective Action
Sample overload	Reduce the sample injection volume or concentration.
Inappropriate sample solvent	Replace the sample solvent with one of a low elution capacity.
Dirt	Rinse the column.
Gap in column inlet	Repair the column by supplementing it with packing material.
Influence of secondary retention effects	Rinse the column. Replace the column with one that is only minimally influenced.



Typical Problems (3)

Decrease in Retention Time

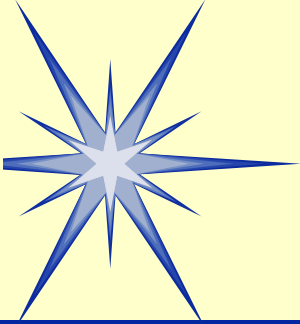
- Check whether the cause of the problem is not the column.
 - ❖ Eluent composition
 - ❖ Eluent flow rate
 - ❖ Column temperature
- If the column is identified as the cause...
 - ❖ Rinsing
 - ❖ Replacement



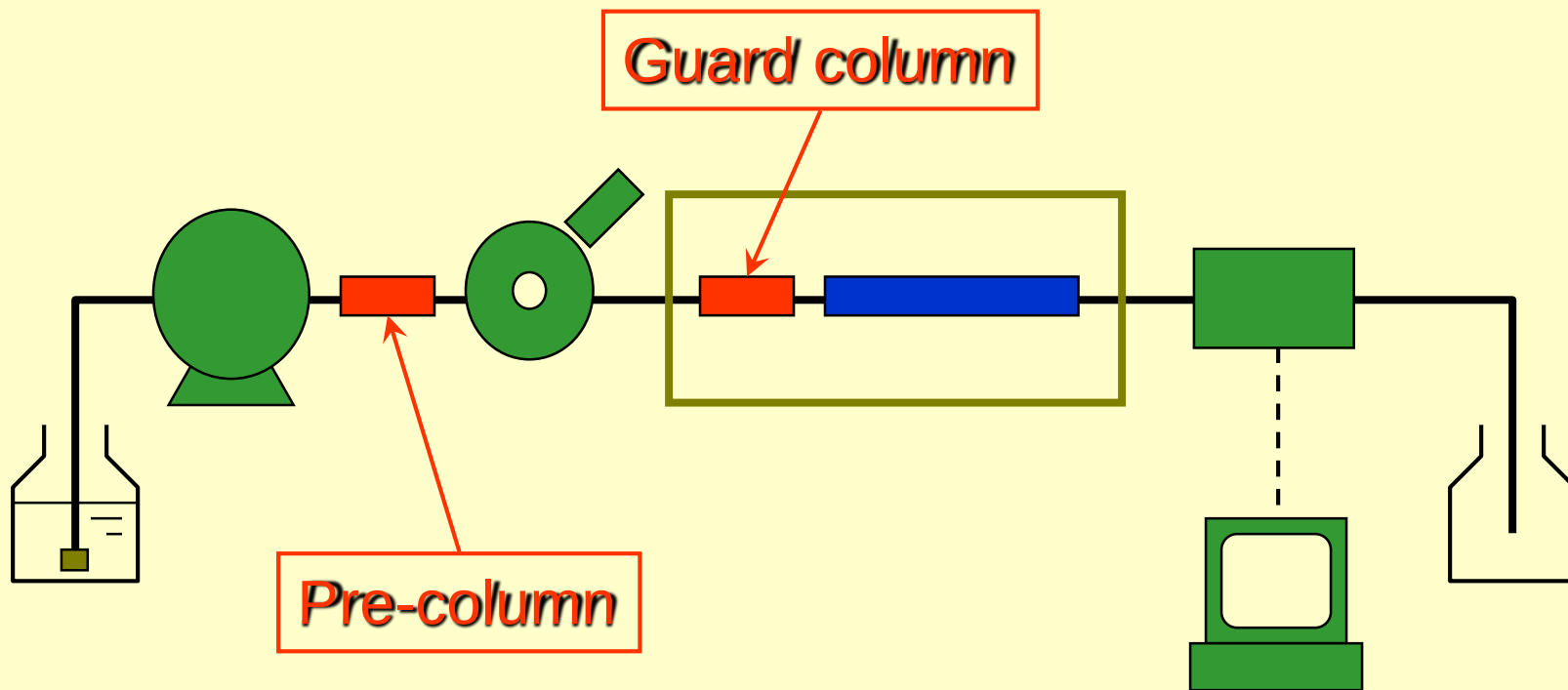
Typical Problems (4)

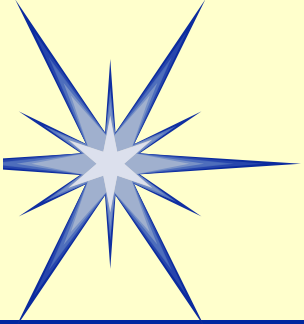
Baseline Drift

- Check whether the cause of the problem is not the column.
 - ❖ If the problem persists when the column is removed, it is caused by the eluent, the solvent delivery system (pump or degasser), or the detector.
- If the column is identified as the cause...
 - ❖ Rinsing
 - ❖ Review of temperature control
 - ❖ Replacement



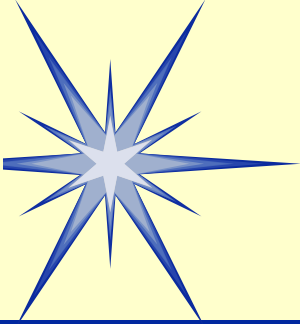
Guard Column and Pre-column



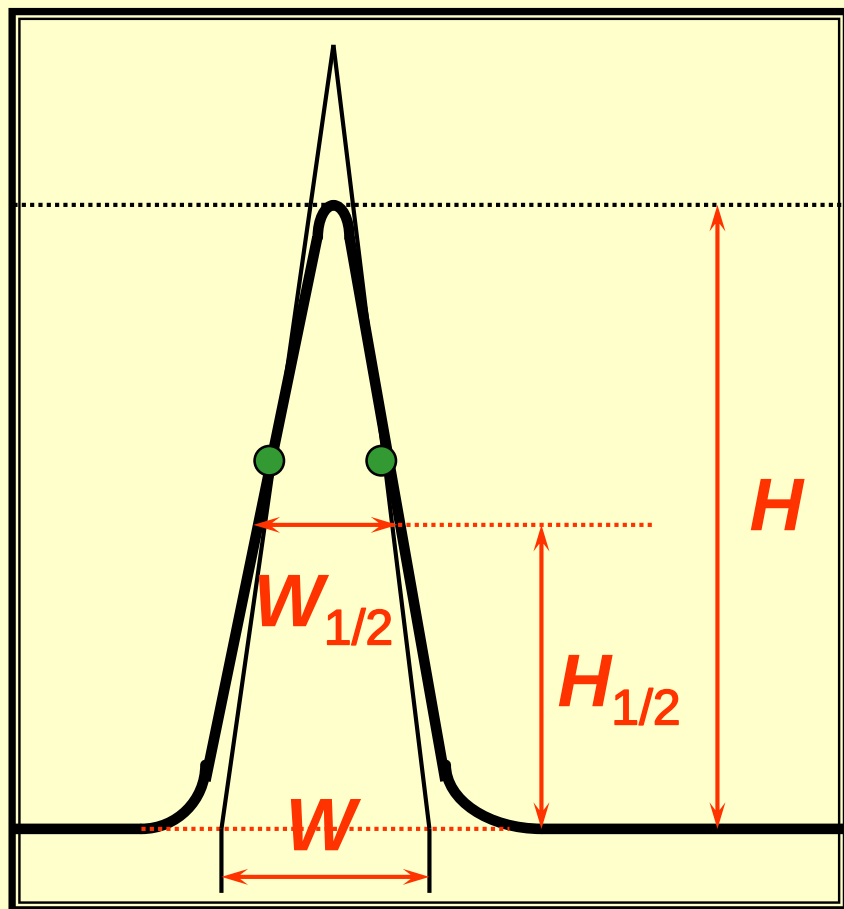


Column Rinsing

- Use an eluent with a high elution capacity
 - ❖ Reversed phase mode: Solution with a high proportion of organic solvent
 - ❖ Ion exchange mode: Solution with a high salt concentration
- Consider secondary retention effects
 - ❖ To remove basic substances from a reversed phase column → Use an acidic solution and add an ion pair reagent.
 - ❖ To remove hydrophobic substances from an ion exchange column → Add an organic solvent.



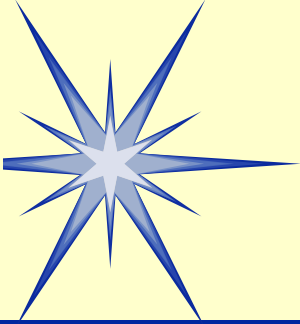
Checking Column Performance



$$N = 16 \left(\frac{t_R}{W} \right)^2$$

$$= 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2$$

$$= 2\pi \left(\frac{t_R \bullet H}{Area} \right)^2$$



Column Storage

● Storage Solution

- ❖ It is generally safe to use the same storage solution as used at shipment.
- ❖ In order to prevent putrefaction, alcohol or some other preservative substance may be added.

● Storage Conditions

- ❖ Insert an airtight stopper in the column end. **Never allow the packing material to dry.**
- ❖ Make a record of the storage solution and final usage conditions and store it together with the column.
- ❖ Store the column in a location not subject to shocks or sudden temperature changes.