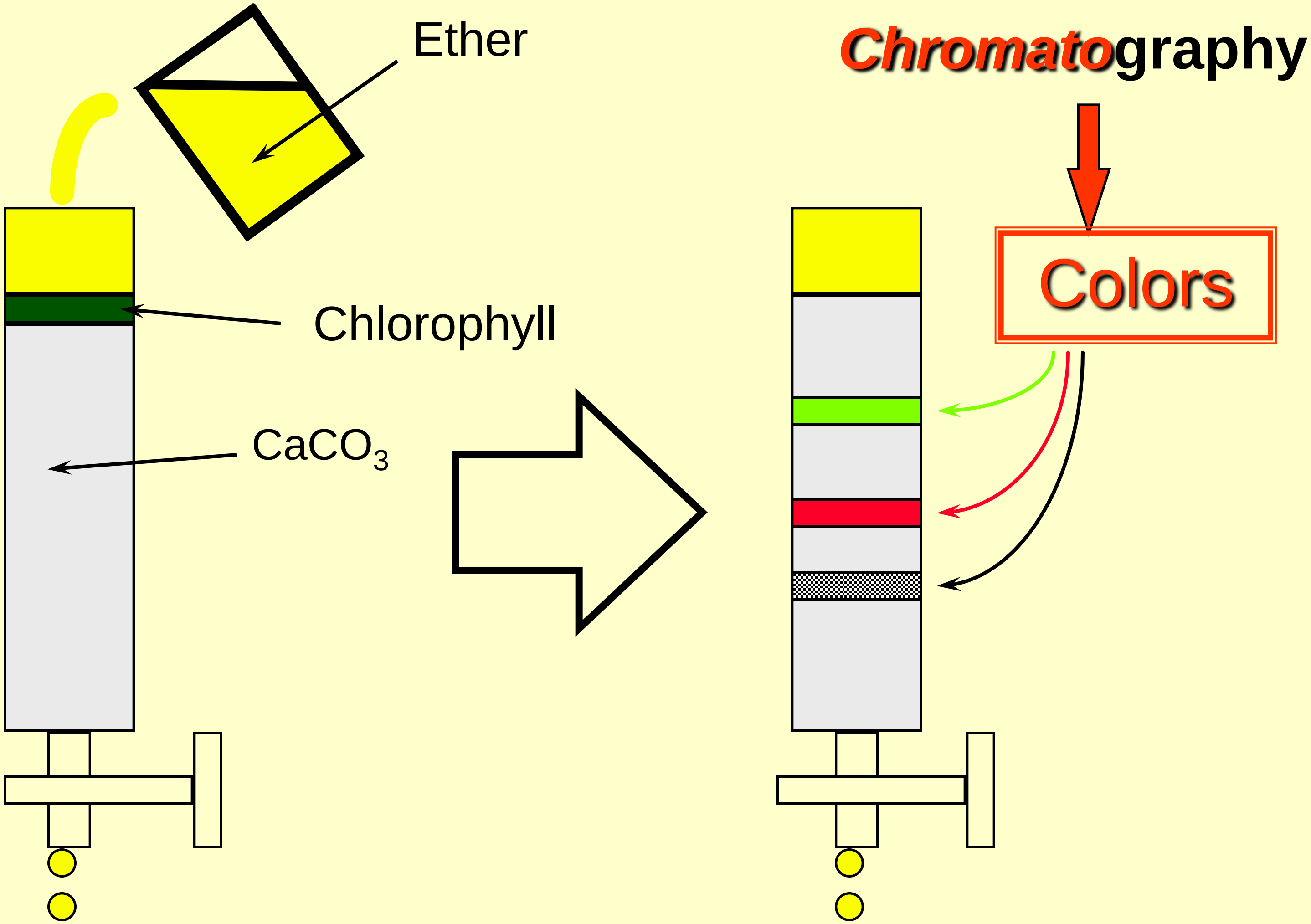
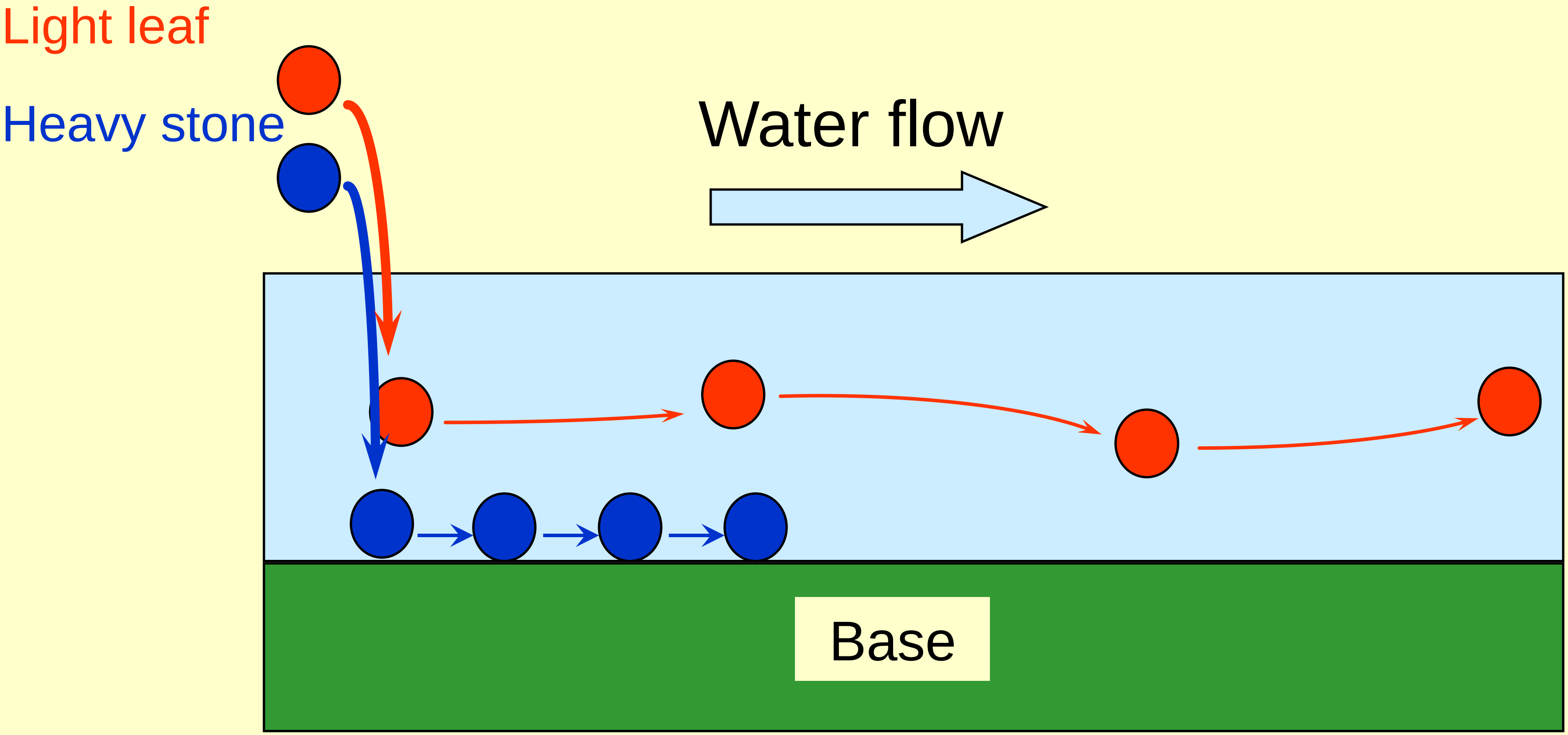
What Is HPLC?

Basic Principles

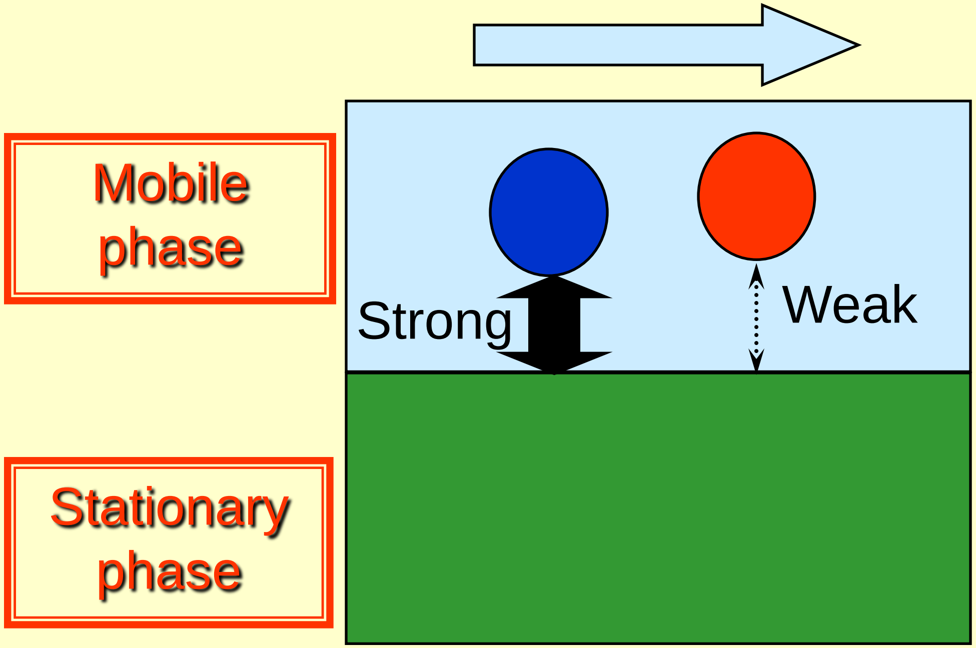
Invention of Chromatography by M. Tswett



Comparing Chromatography to the Flow of a River...



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

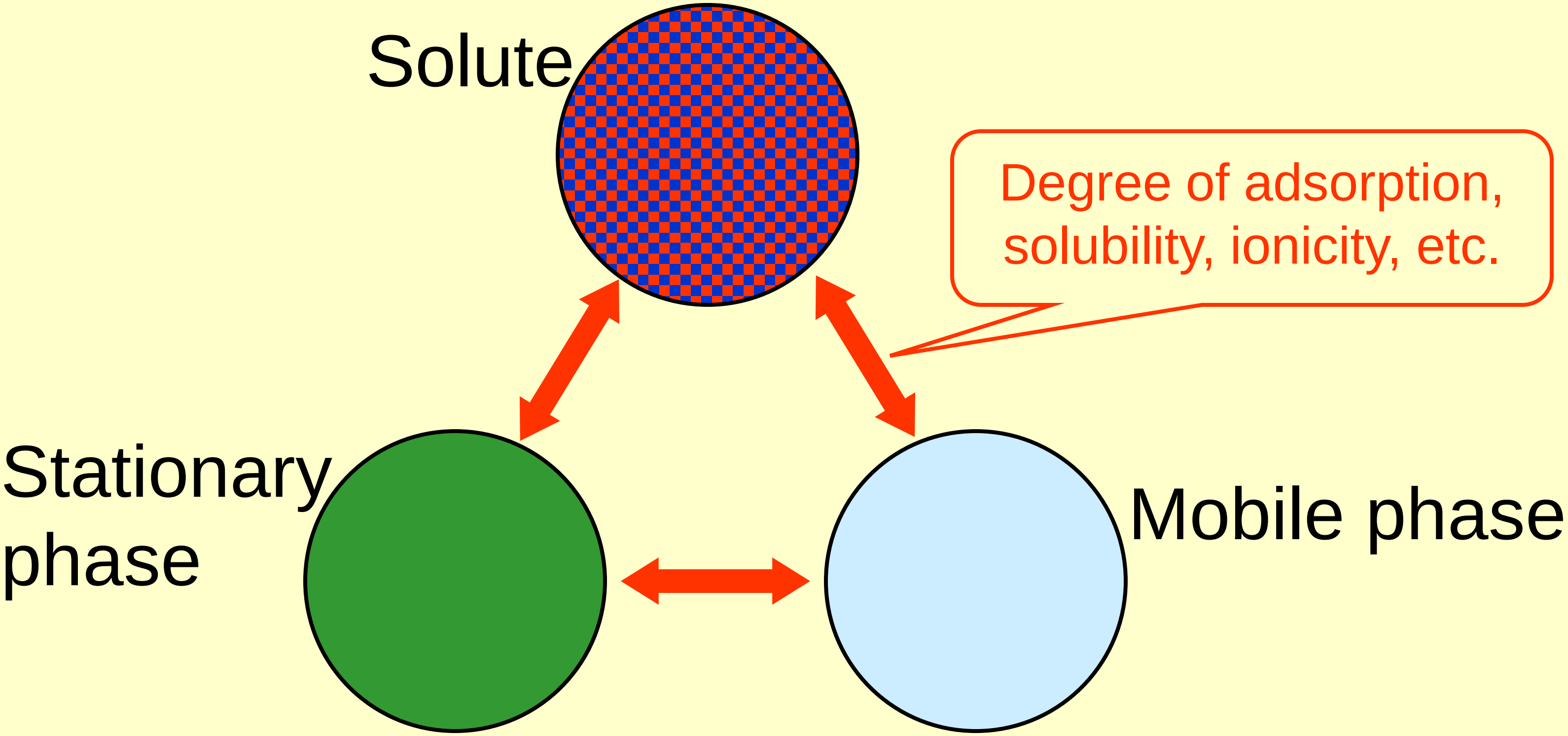
		Mobile phase		
		Gas	Liquid	Solid
Stationary phase	Gas	╳	╳	╳
	Liquid	**Gas chromatography**	**Liquid chromatography**	╳
	Solid			╳

Liquid Chromatography

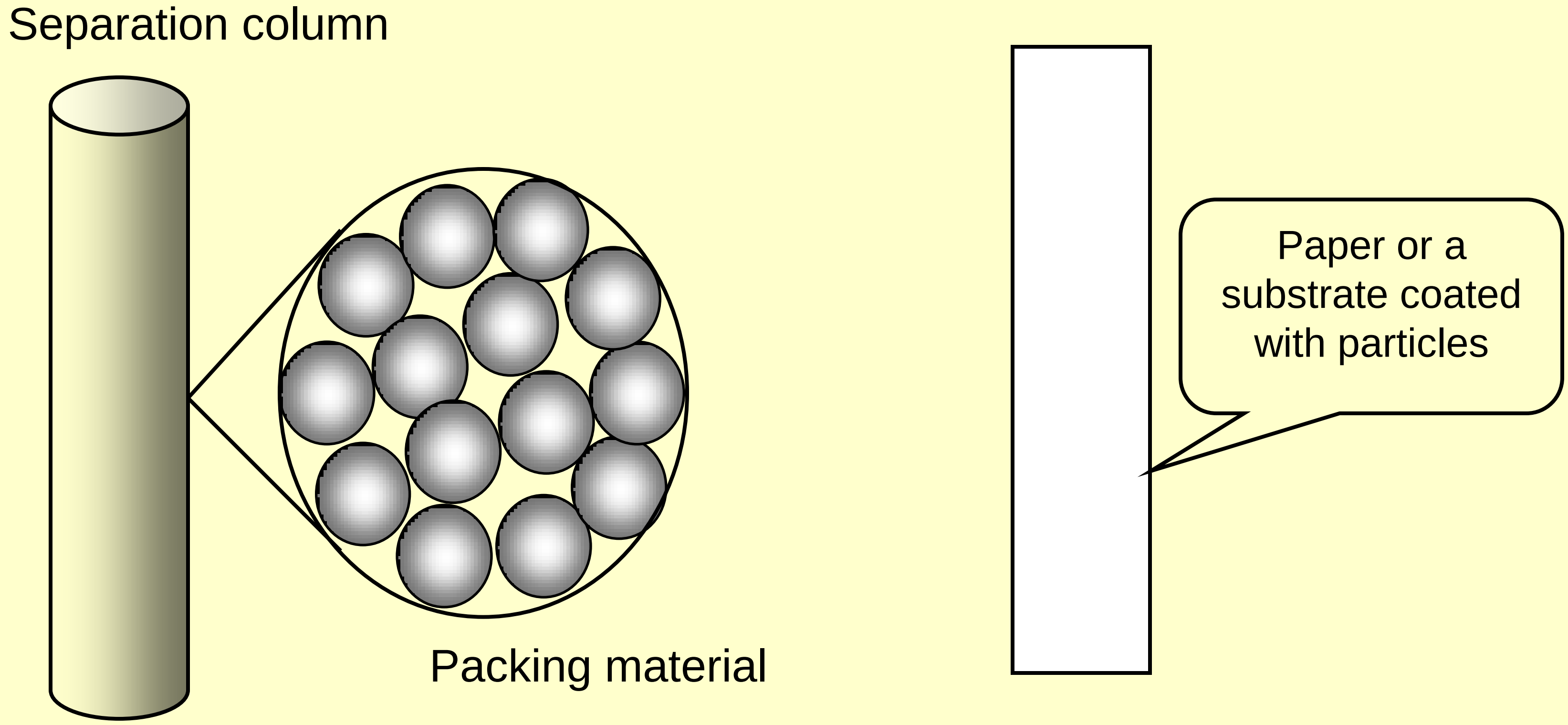
- Chromatography in which the mobile phase is a liquid.
  - The liquid used as the mobile phase is called the "eluent".
- The stationary phase is usually a solid or a liquid.
- In general, it is possible to analyze any substance that can be stably dissolved in the mobile phase.

Interaction Between Solutes, Stationary Phase, and Mobile Phase

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



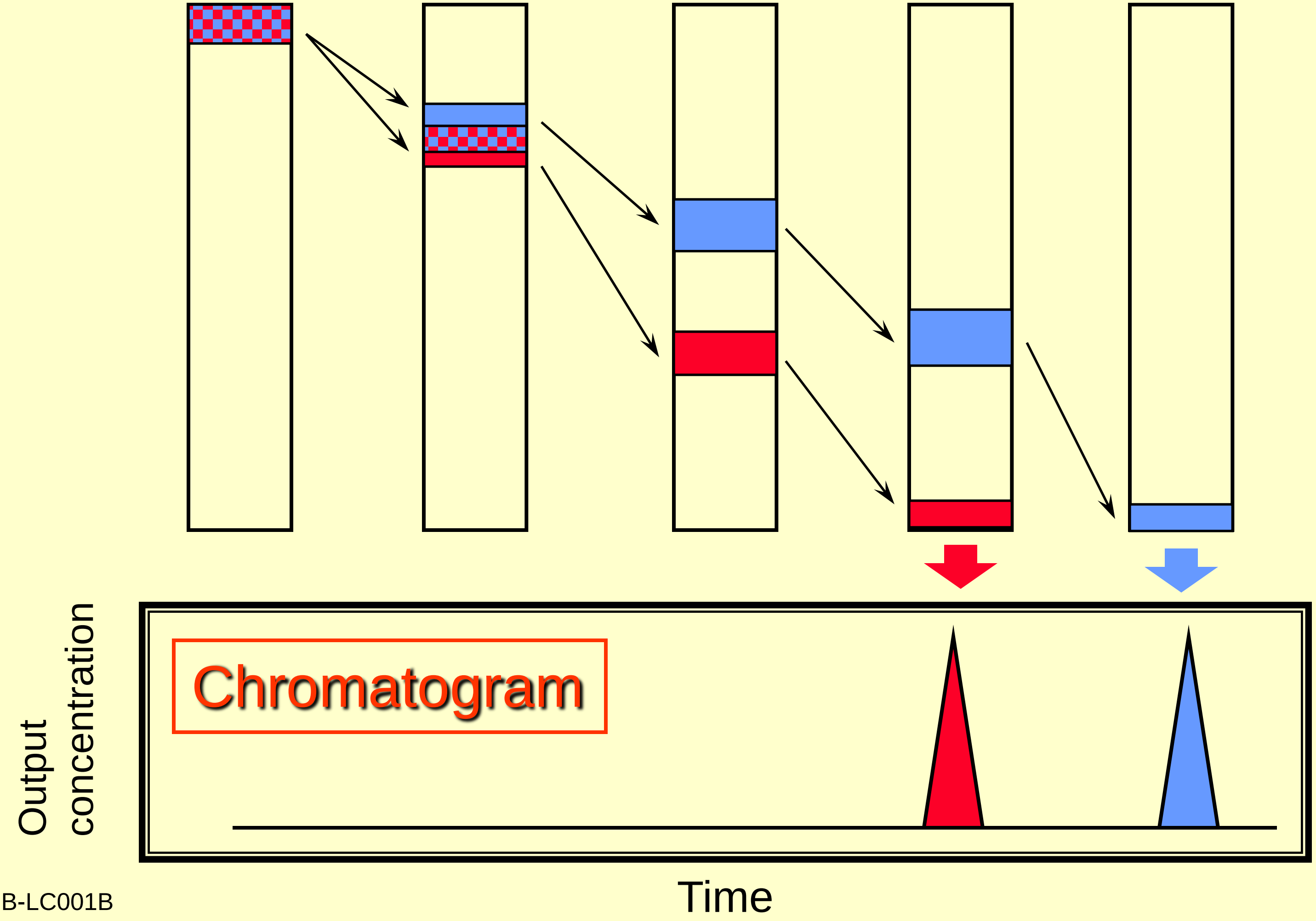
Column Chromatography and Planar Chromatography



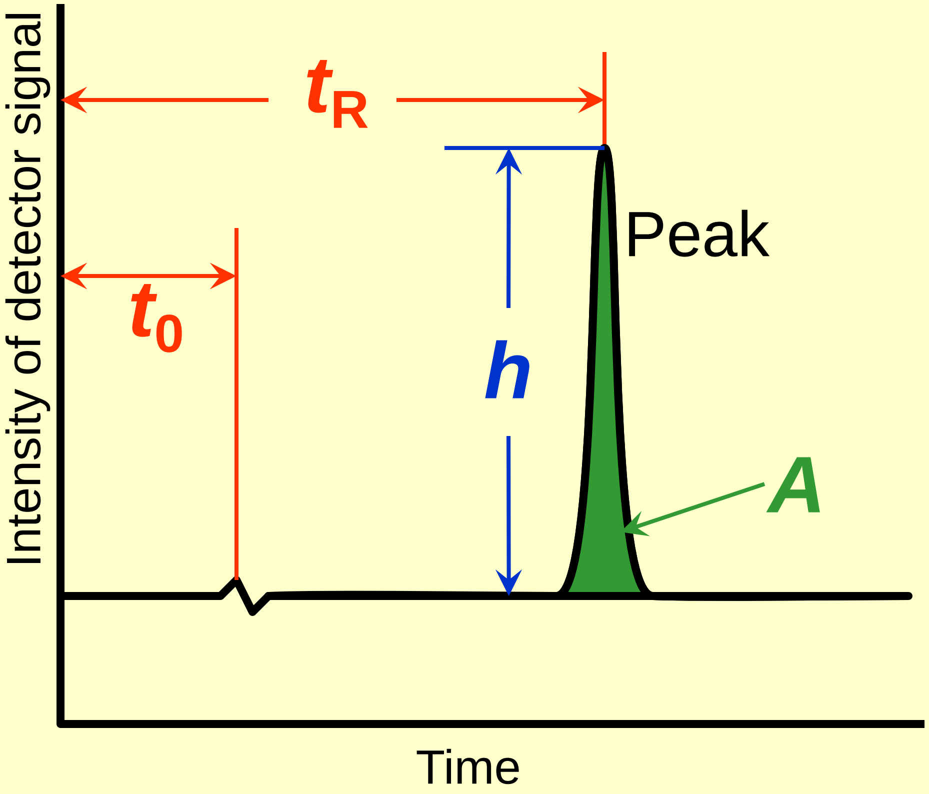
Column Chromatography

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

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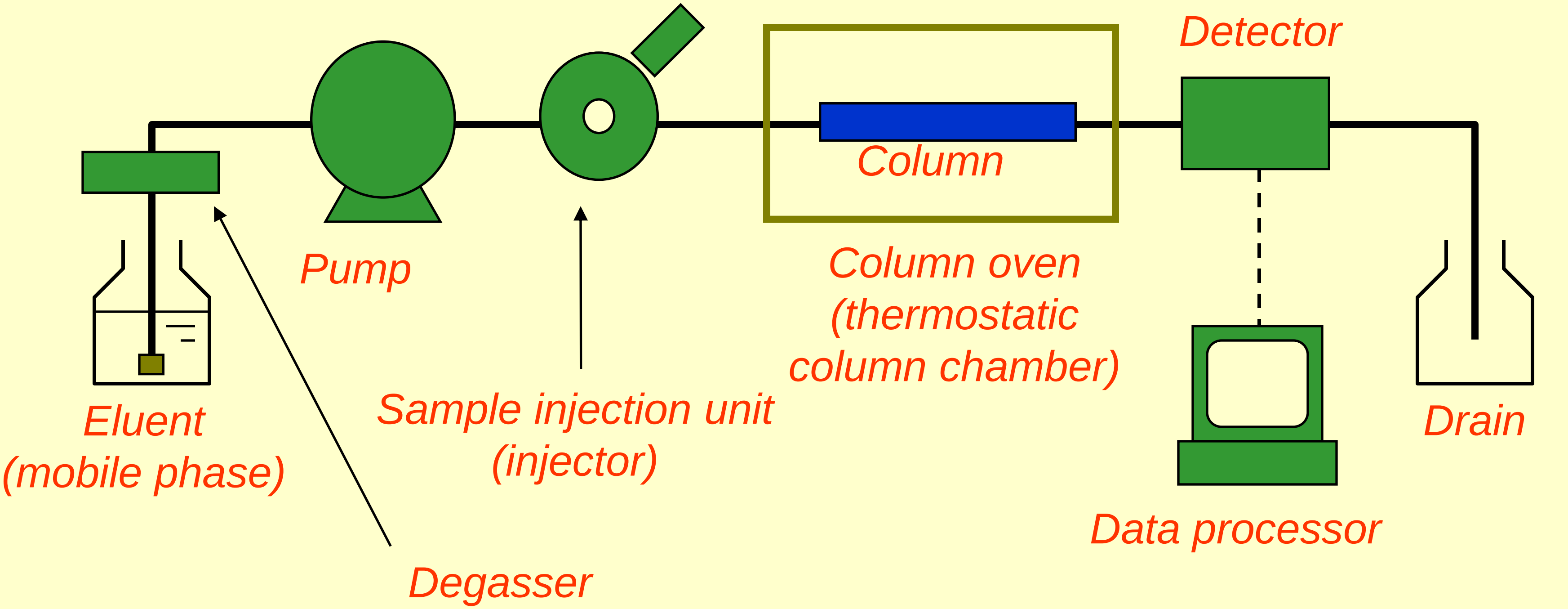
The arrival of high performance liquid chromatography!

Flow Channel Diagram for High Performance Liquid Chromatograph



Detector

Column

Pump

Column oven
(thermostatic
column chamber)

Sample injection unit
(injector)

Eluent
(mobile phase)

Drain

Data processor

Degasser

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

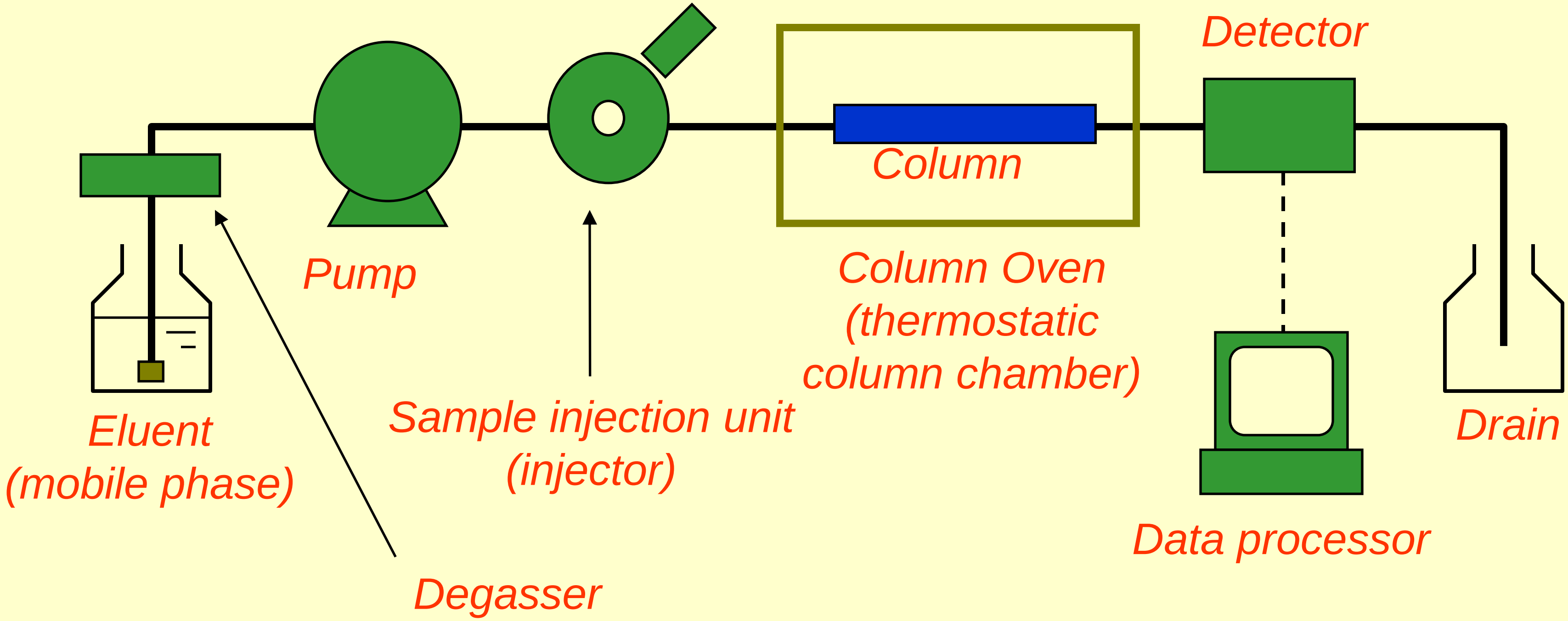
Fields in Which High Performance Liquid Chromatography Is Used

- 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



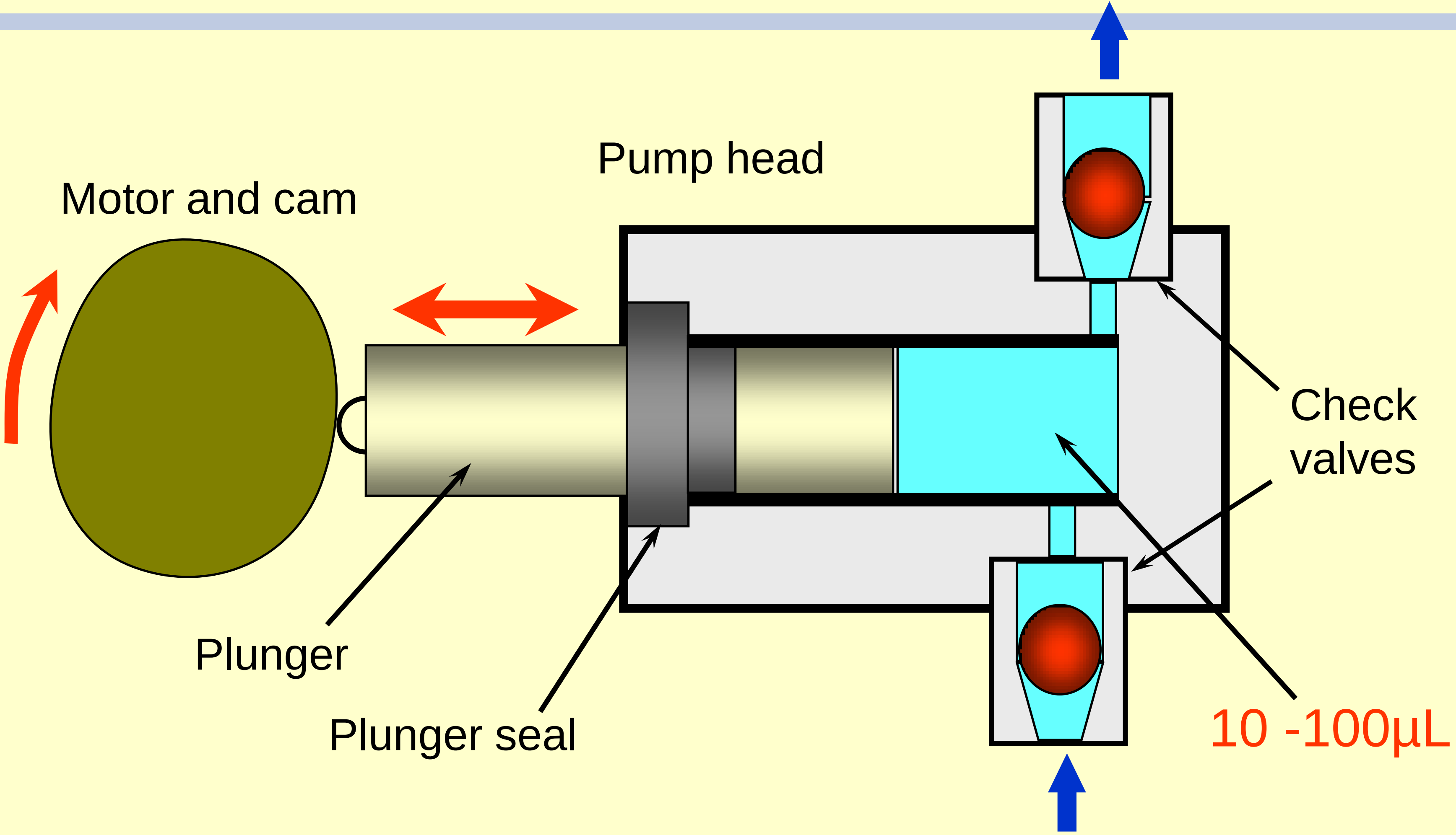
Solvent Delivery Pump

- 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.

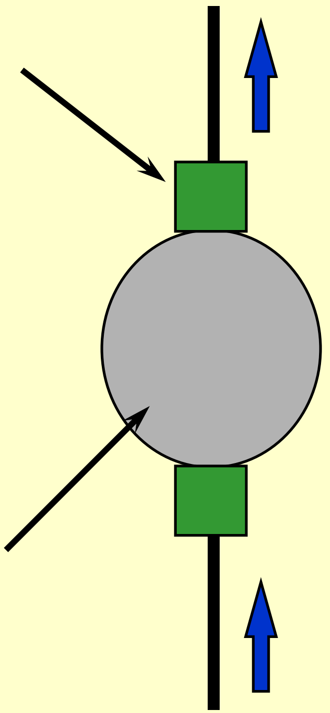
Solvent Delivery Pump: Representative Pumping Methods

- Syringe pump
- Plunger pump
- Diaphragm pump

Solvent Delivery Pump: Schematic Diagram of Plunger Pump



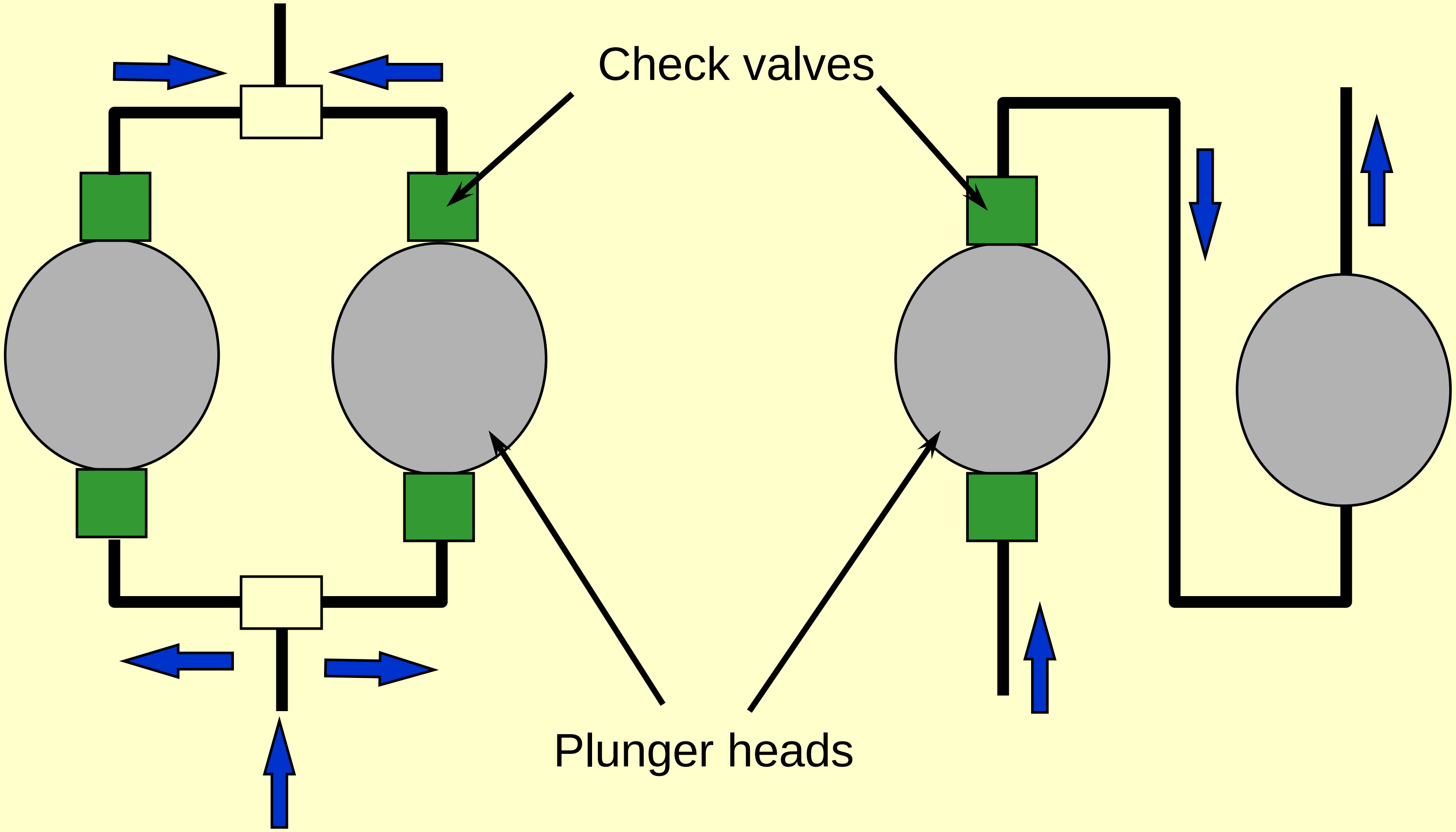
Solvent Delivery Pump: Single Plunger Type



Check valves

Plunger head

Solvent Delivery Pump: Dual Plunger Type



Check valves

Plunger heads

Type

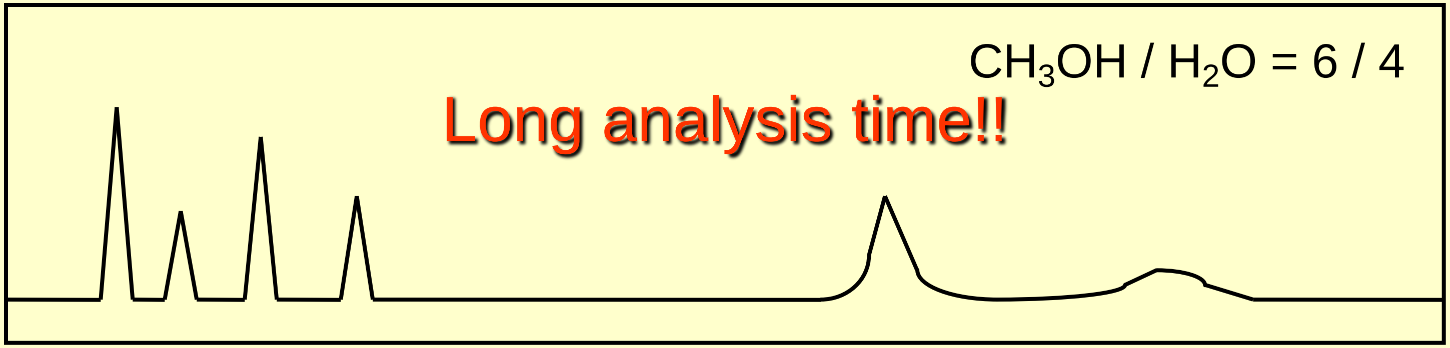
Type

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



$CH_3OH / H_2O = 6 / 4$

Long analysis time!!

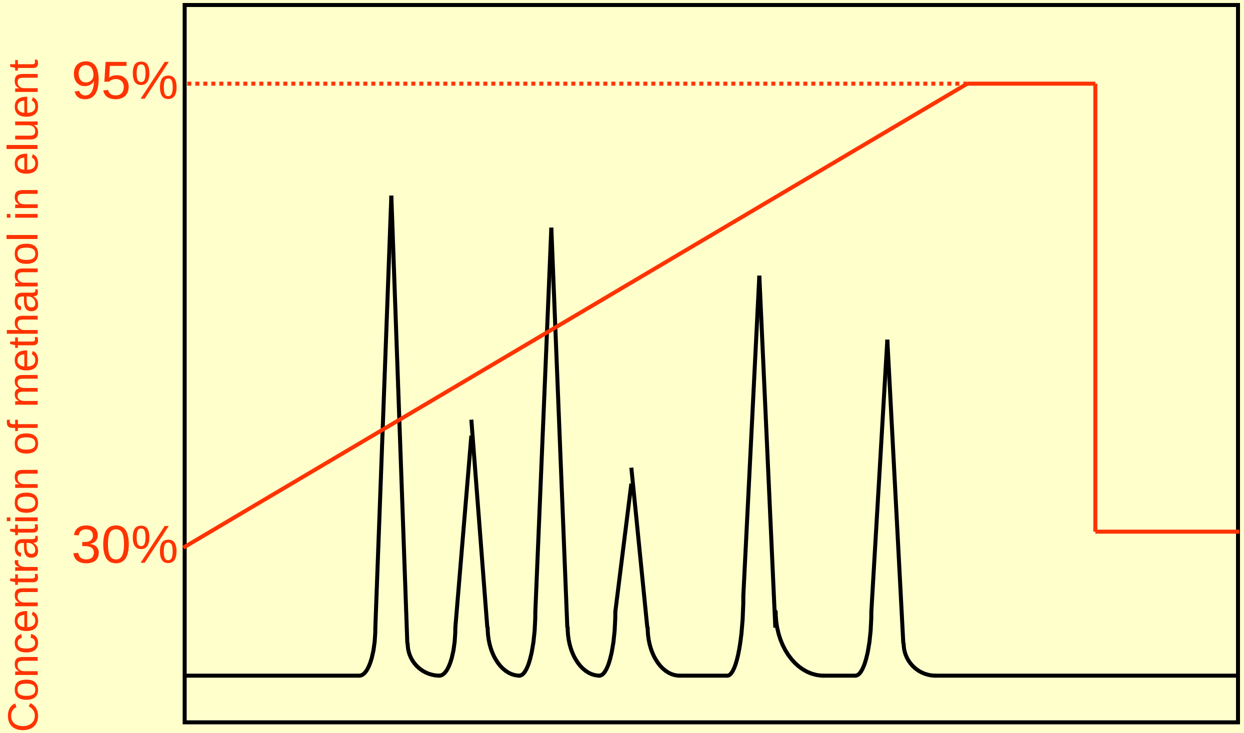
Poor separation!!

$CH_3OH / H_2O = 8 / 2$

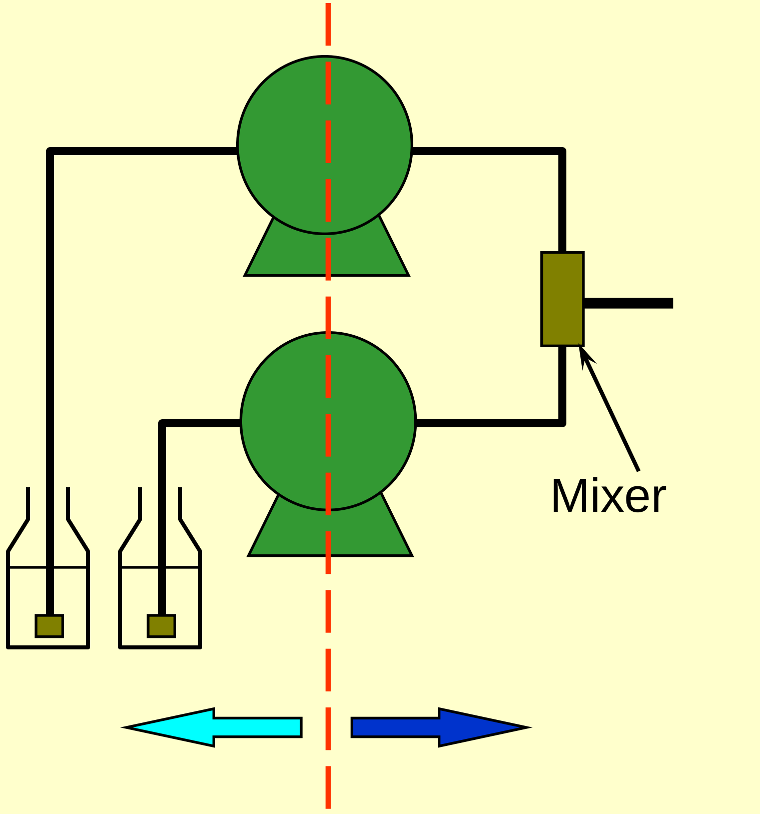
(Column: ODS type)

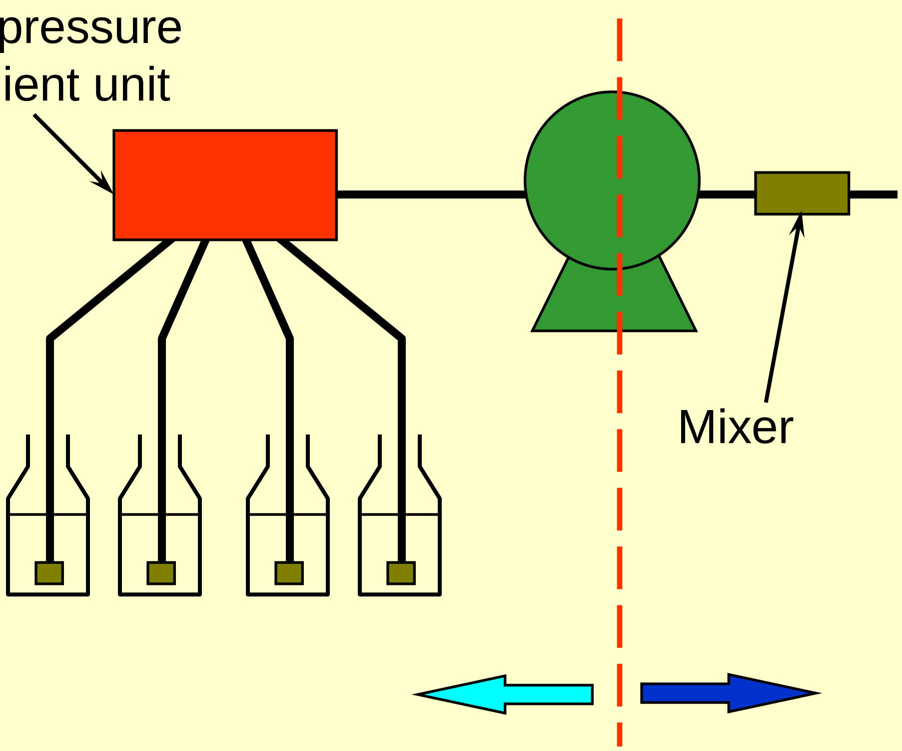
Aim of Gradient System (2)

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



High- / Low-Pressure Gradient System





High-pressure gradient

Low-pressure gradient

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

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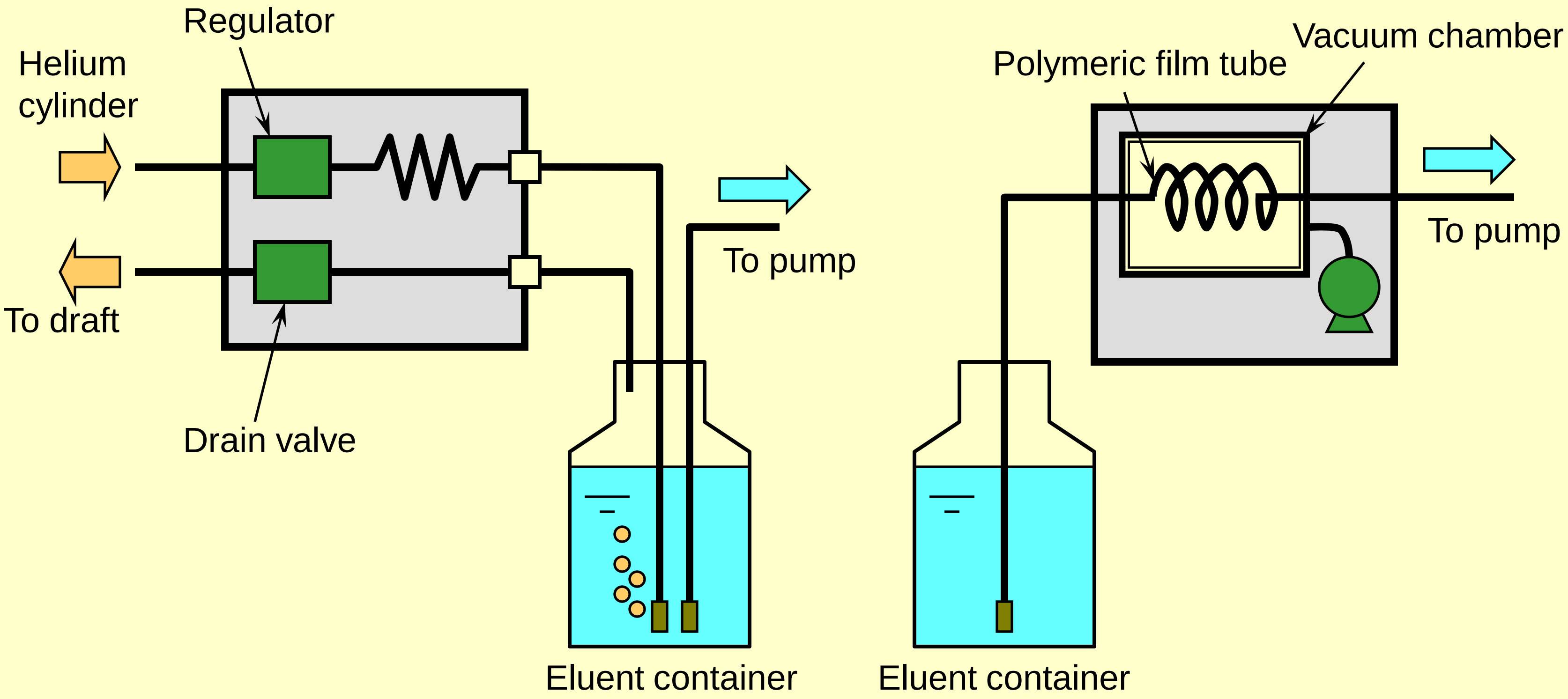
In order to avoid these problems, the eluent must be degassed.

Online Degasser



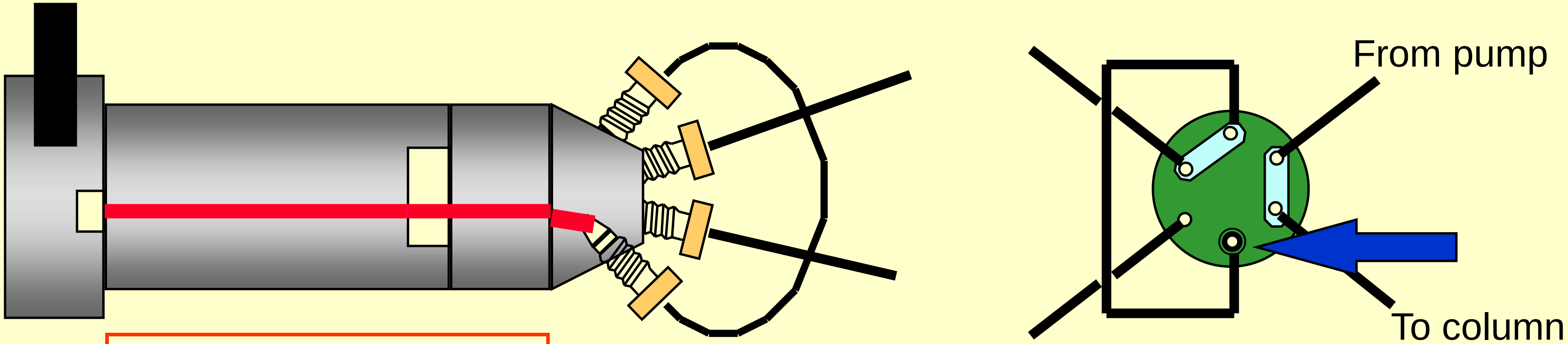
Helium purge method

Gas-liquid separation membrane method

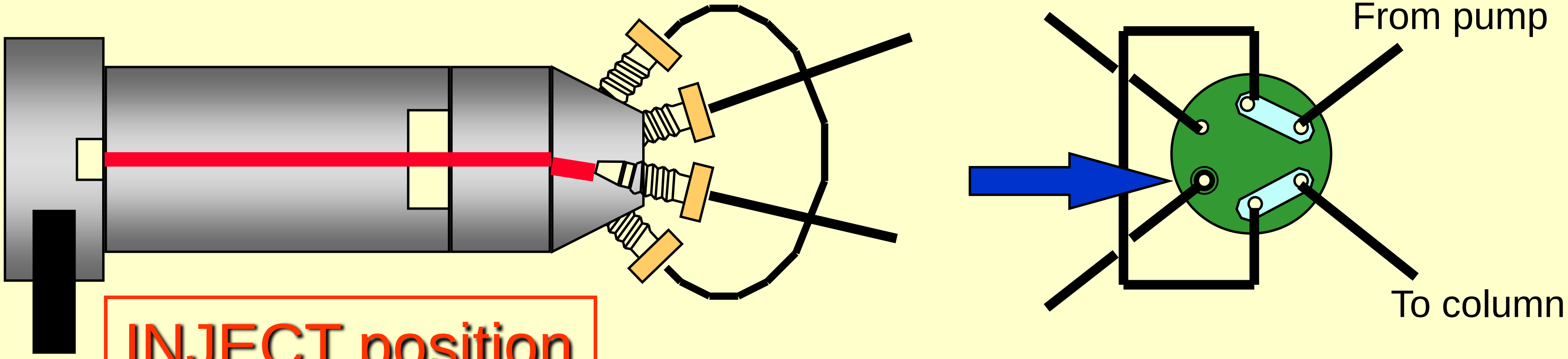
Sample Injection Unit (Injector)

- 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

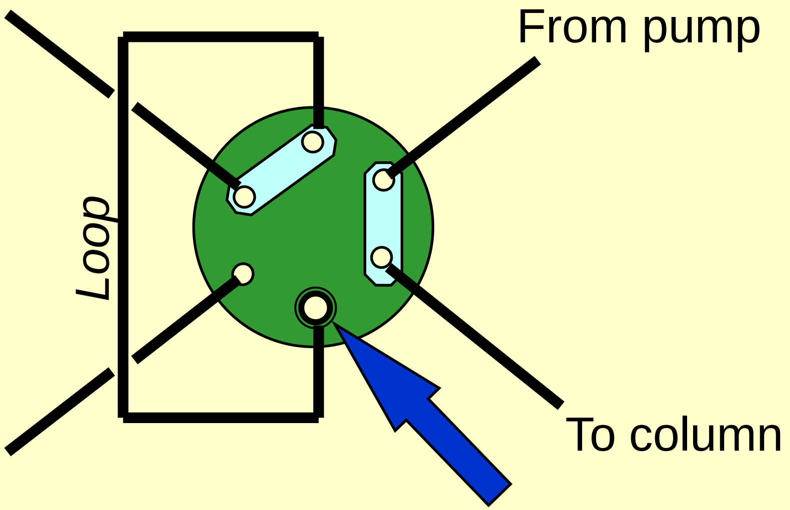


LOAD position

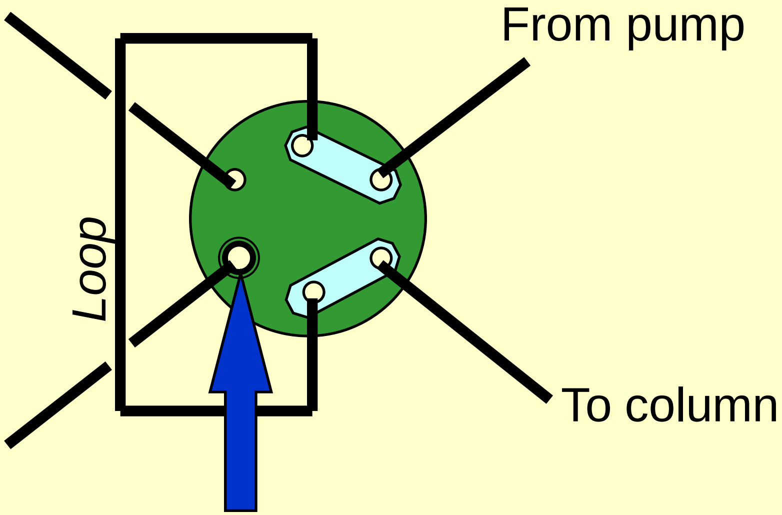


INJECT position

Manual Injector:
Operating Principle of Sample Injection



LOAD

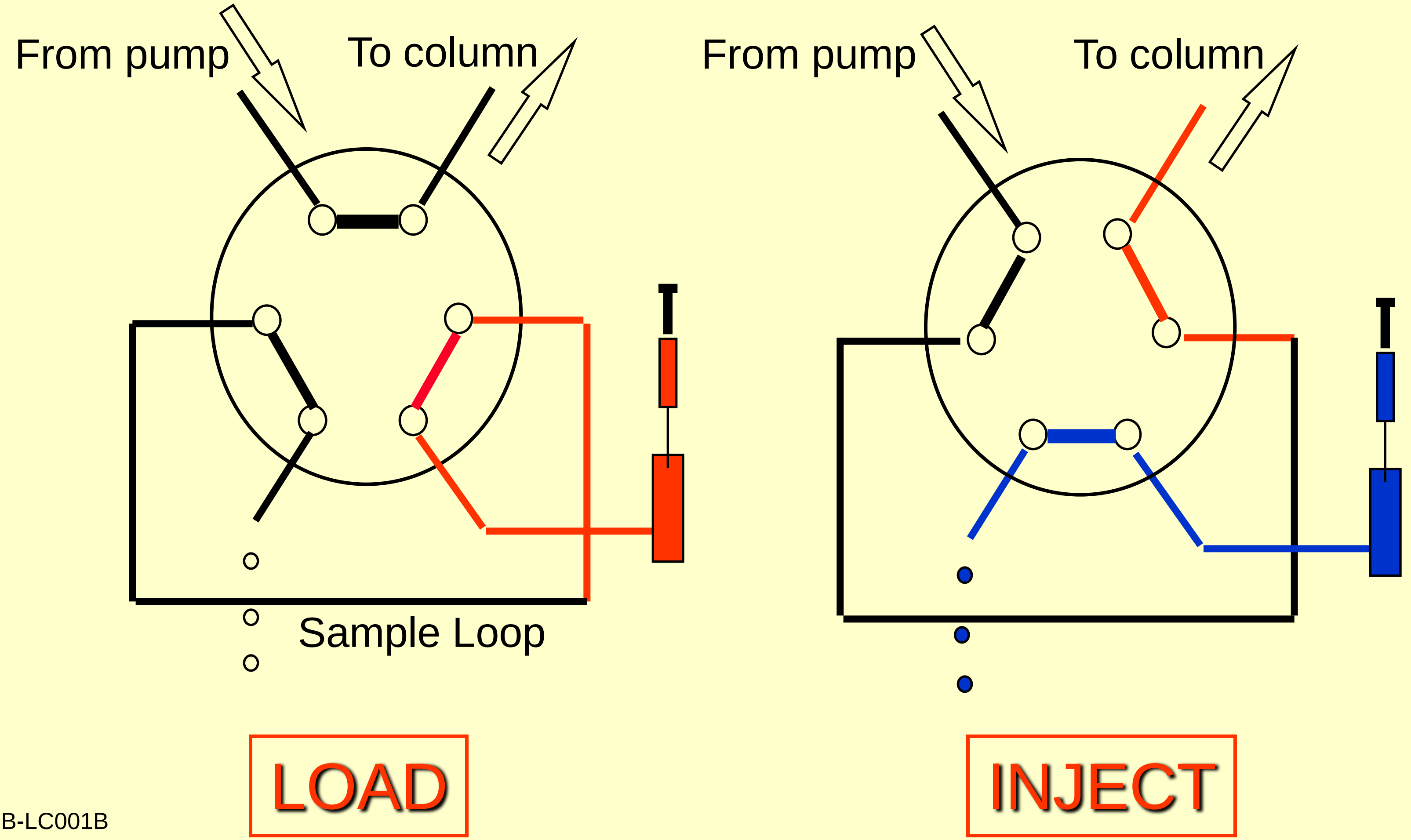


INJECT

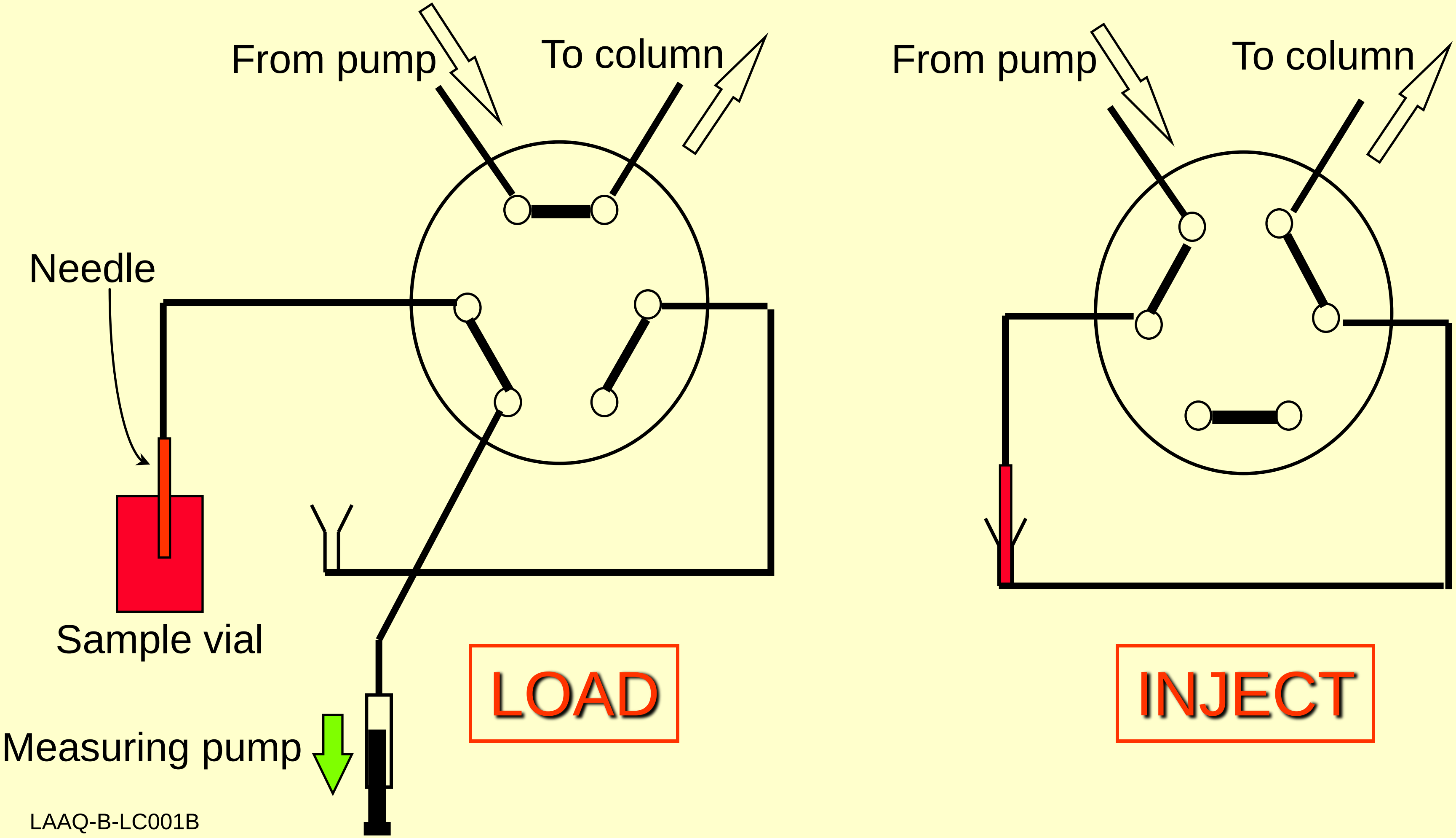
Manual Injector: Injection Method

- 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)



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

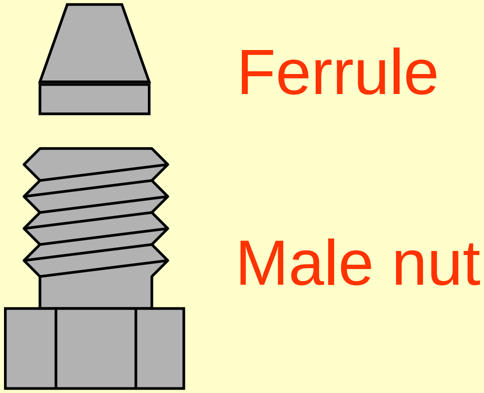
Prior to Analysis

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

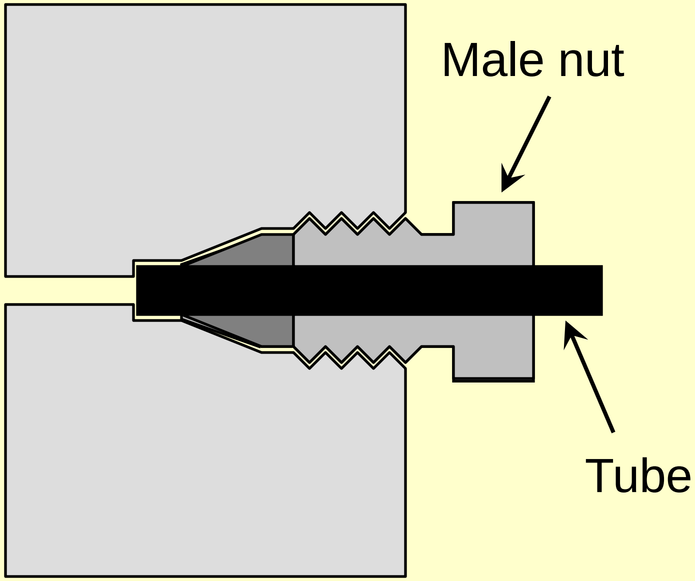
- Male nut (SUS)
  Ferrule (SUS)
  - Sealing possible up to 40 MPa



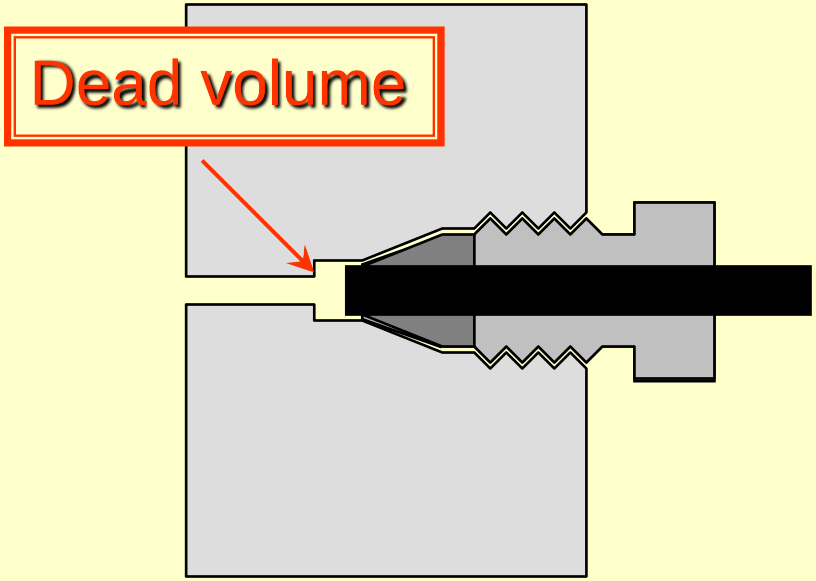
- Male nut (PEEK)
  - Can be connected without any tools
  - Resists pressures of up to approx. 25 MPa

Dead Volume (Extra-column volume)

- Dead volume can cause peaks broadening.



Excellent connection



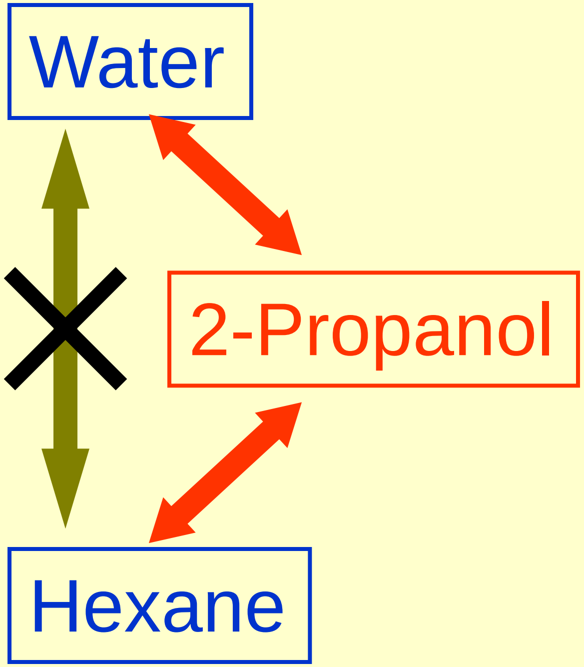
Poor connection

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.

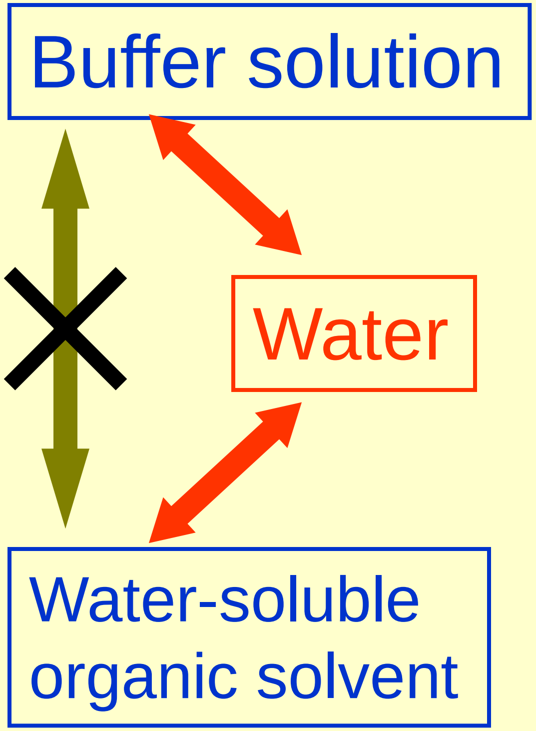


Water

2-Propanol

Hexane

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

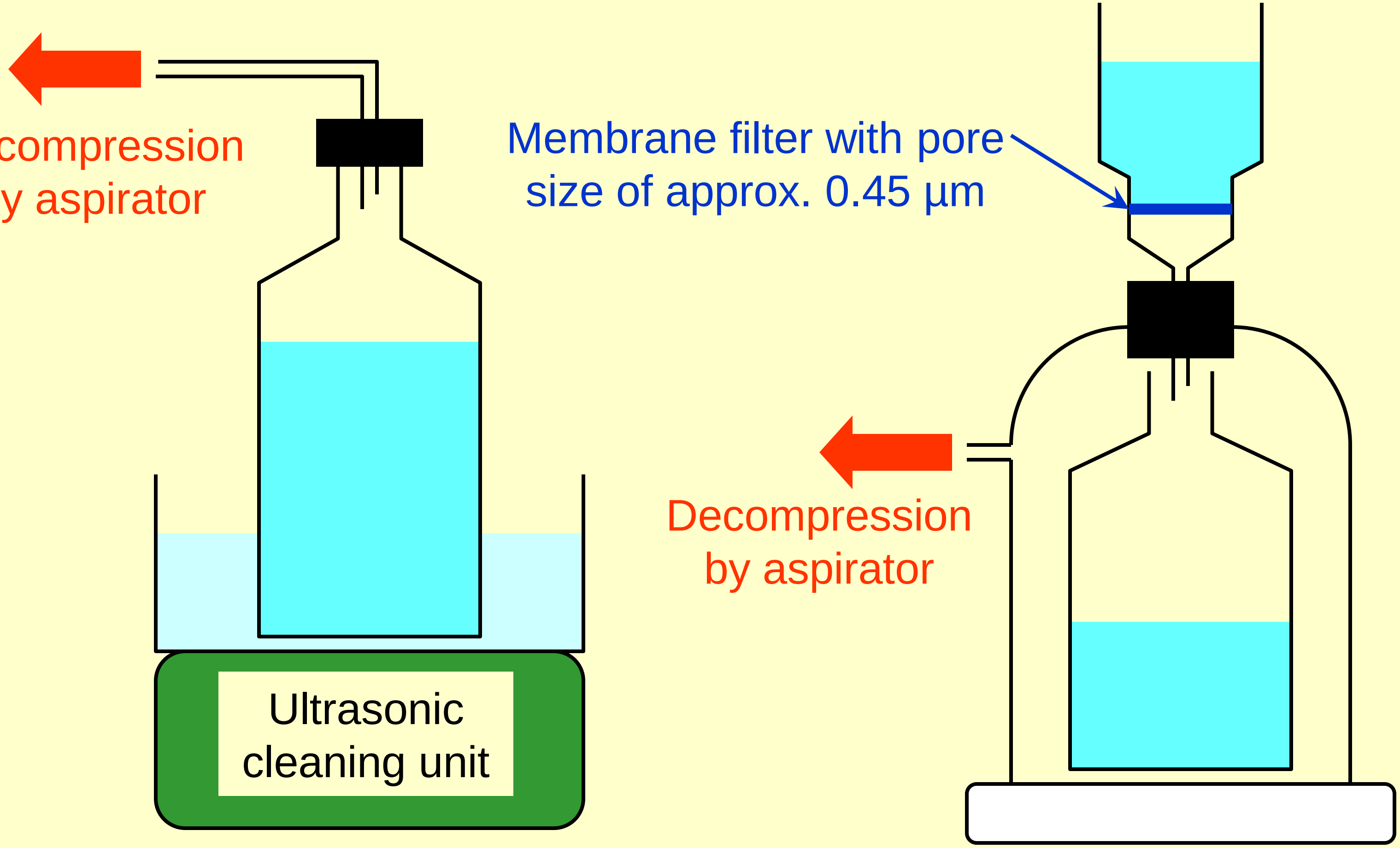


Buffer solution

Water

Water-soluble organic solvent

Mixing, Filtration, and Offline Degassing of the Eluent



Decompression by aspirator

Membrane filter with pore size of approx. 0.45 µm

Decompression by aspirator

Ultrasonic cleaning unit

Reversed Phase Chromatography
Part 1

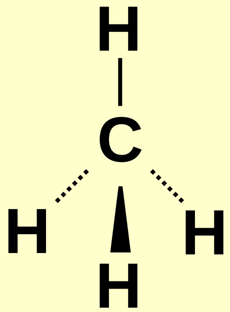
Basic Principles

Polarity of Substances

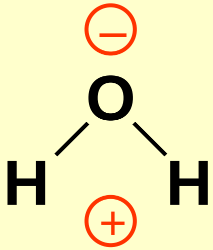
- Polarity
  - Property of a substance whereby the positions of the electrons give rise to positive and negative poles
  - Water: Polar

    Methane: Nonpolar

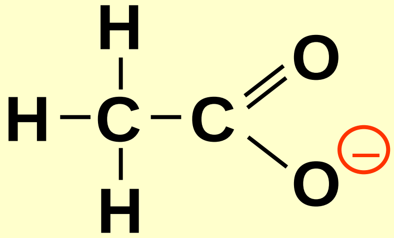
- Miscibility of solvents
  - Solvents of similar polarities can be easily dissolved together.
  - Polar and nonpolar molecules have a similar relationship to that of water and oil.



Methane



Water



Acetic acid

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

- Nonpolar Functional Groups
  - $-(CH_2)_nCH_3$
    - Alkyl groups
  - $-C_6H_5$
    - Phenyl groups
- Polar Functional Groups
  - $-COOH$
    - Carboxyl groups
  - $-NH_2$
    - Amino groups
  - $-OH$
    - 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

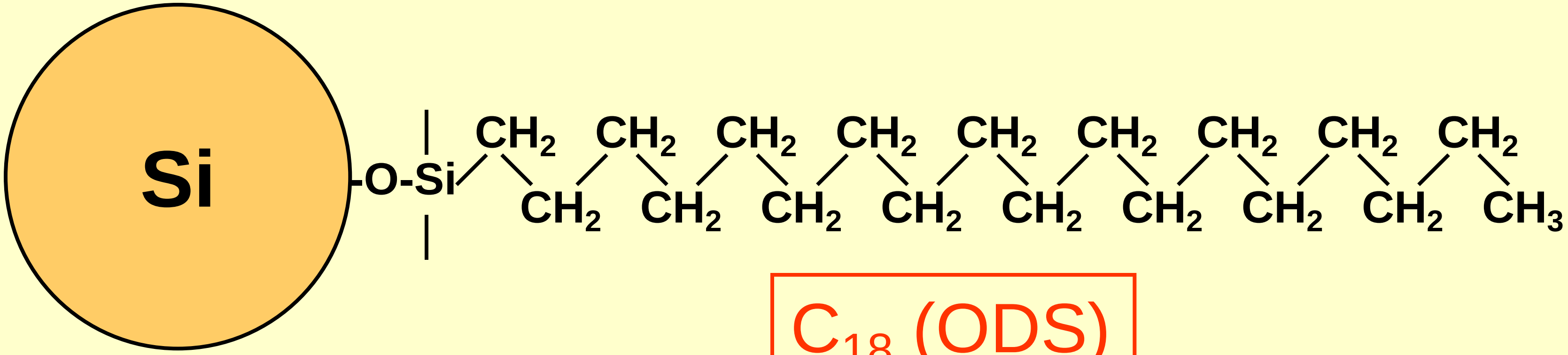
	Stationary phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)

Reversed Phase Chromatography

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

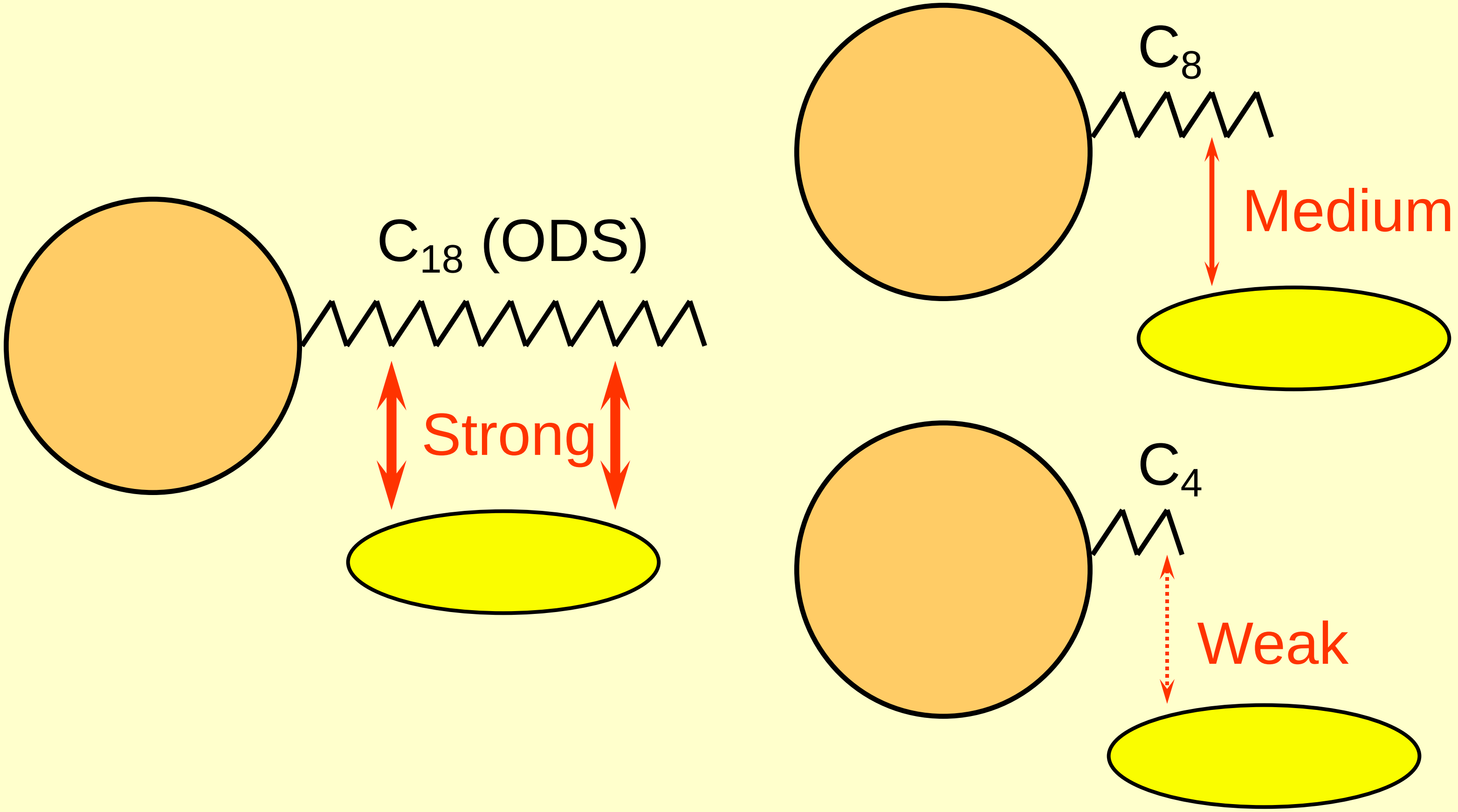
Separation Column for Reversed Phase Chromatography

- C_{18} (ODS) type
- C_8 (octyl) type
- C_4 (butyl) type
- Phenyl type
- TMS type
- Cyano type

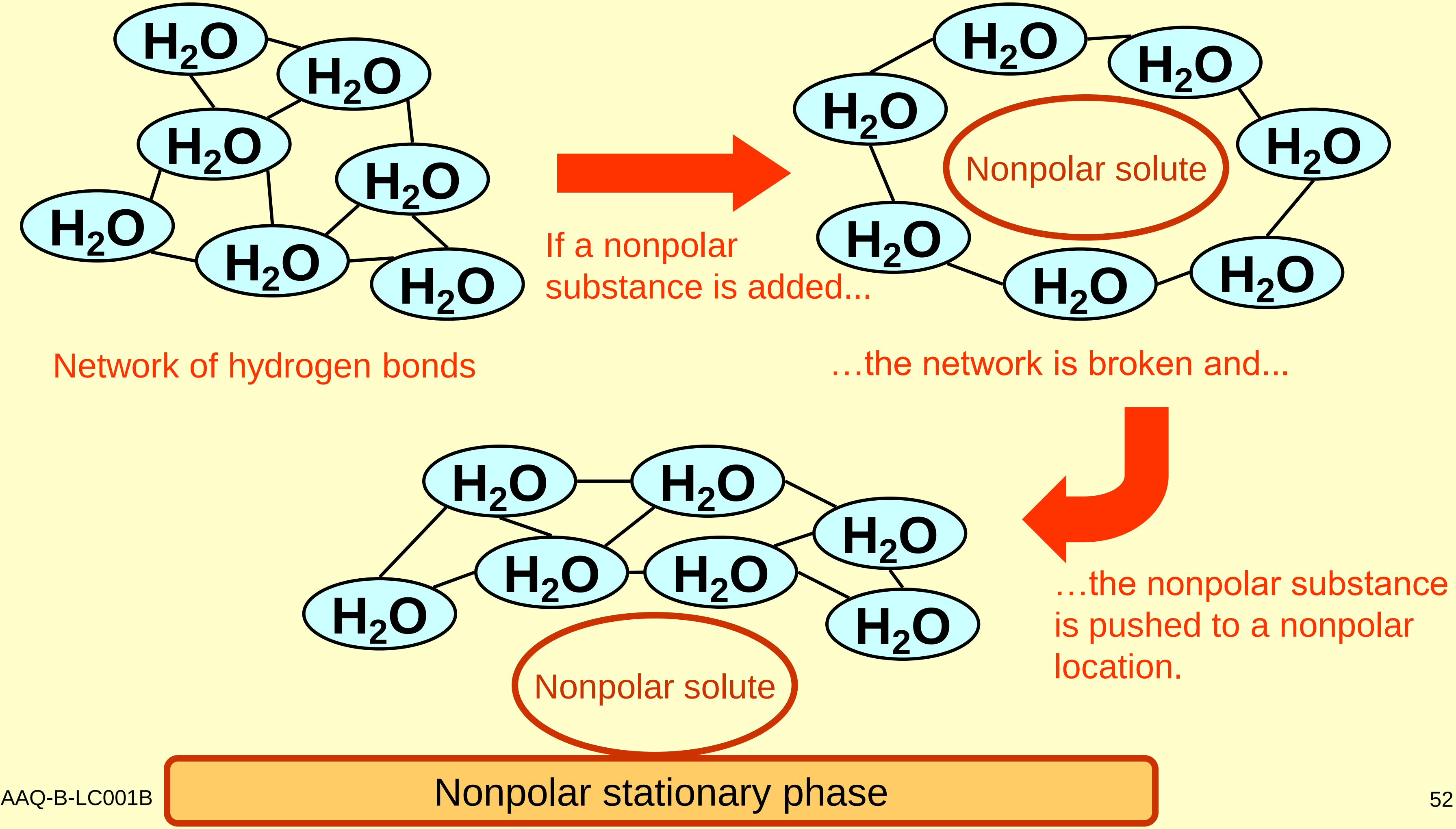


C_{18} (ODS)

Effect of Chain Length of Stationary Phase



Hydrophobic Interaction



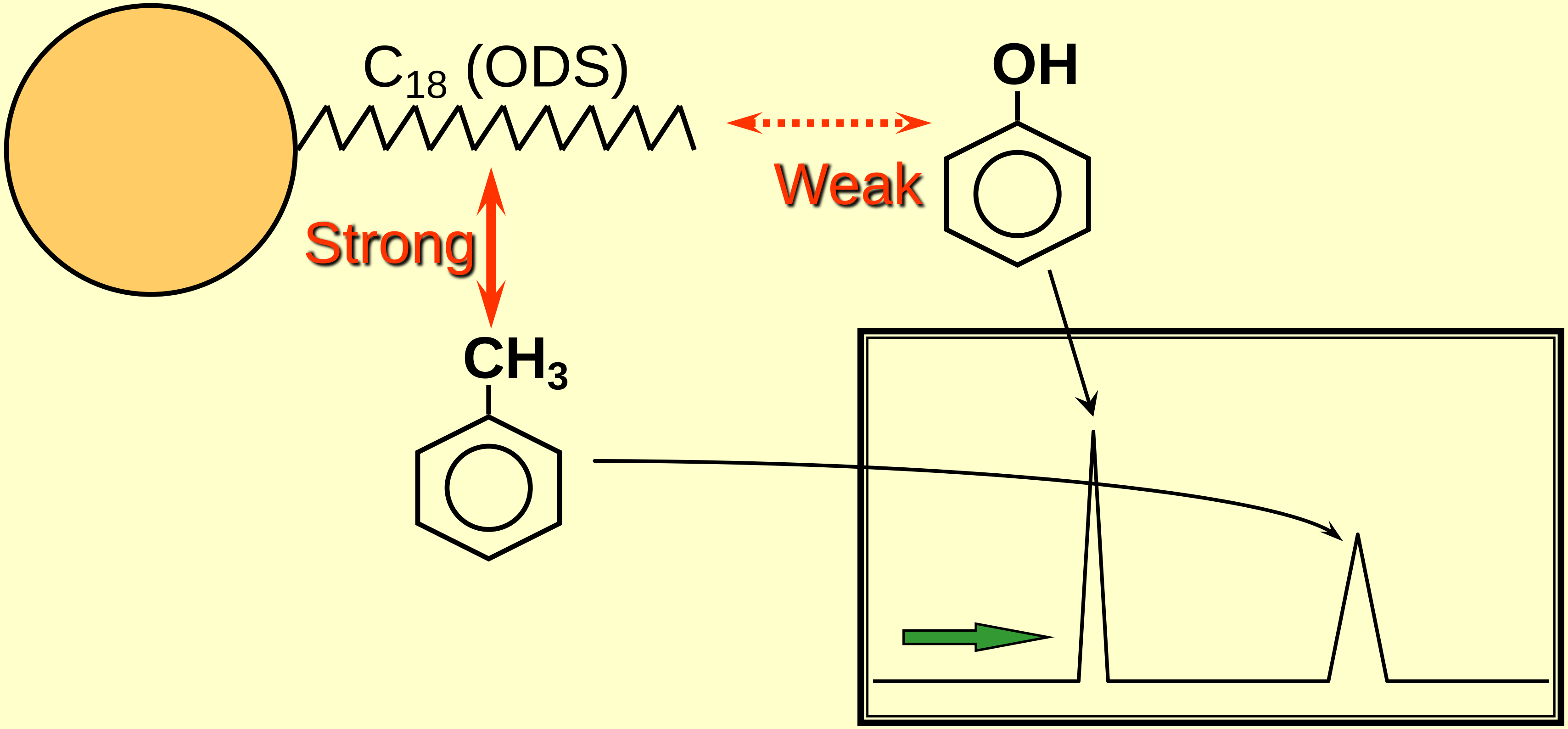
Network of hydrogen bonds

If a nonpolar substance is added...

…the network is broken and...

…the nonpolar substance is pushed to a nonpolar location.

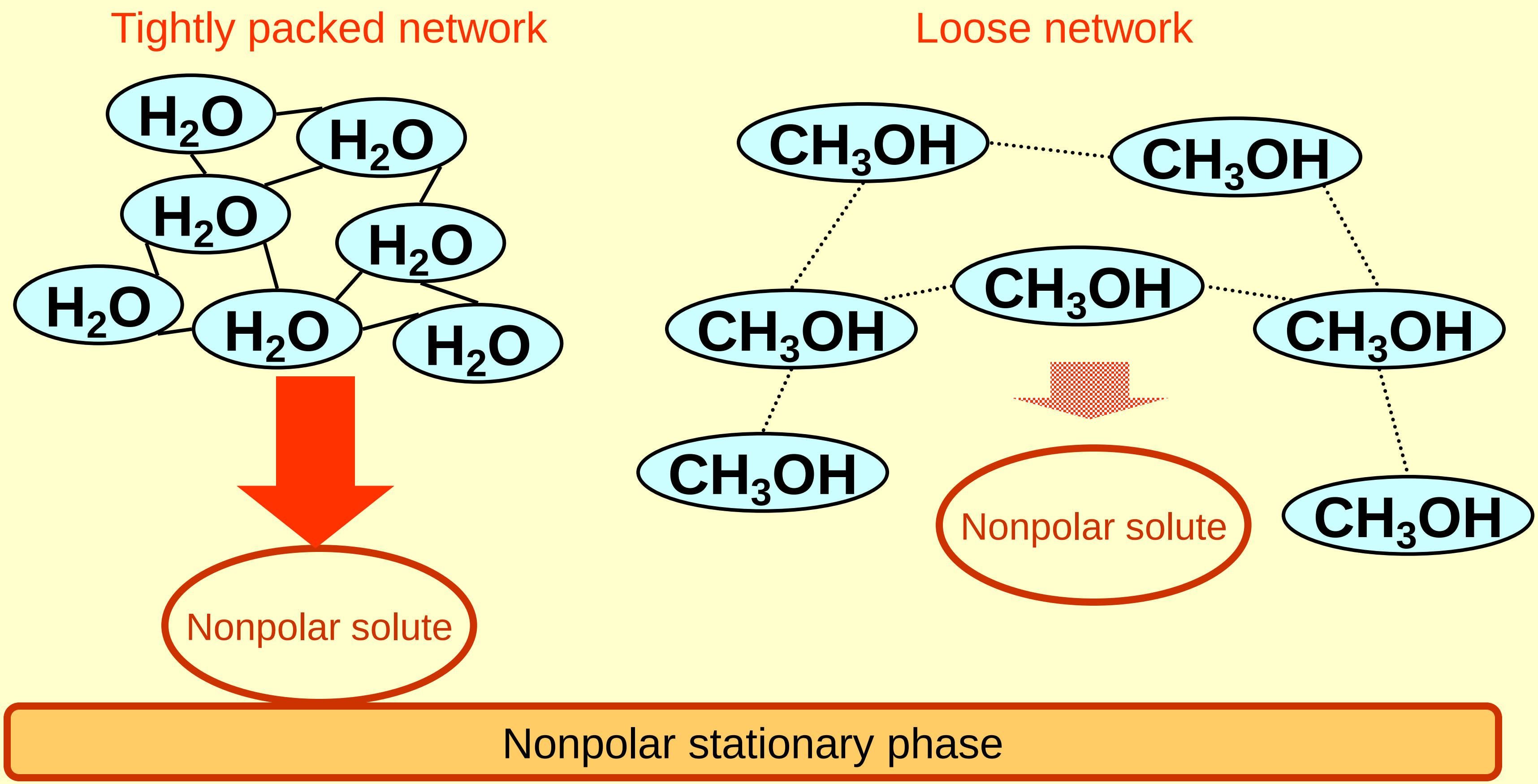
Relationship Between Retention Time and Polarity



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

H_2O

H_2O

H_2O

H_2O

H_2O

H_2O

H_2O

Nonpolar solute

Loose network

CH_3OH

CH_3OH

CH_3OH

CH_3OH

CH_3OH

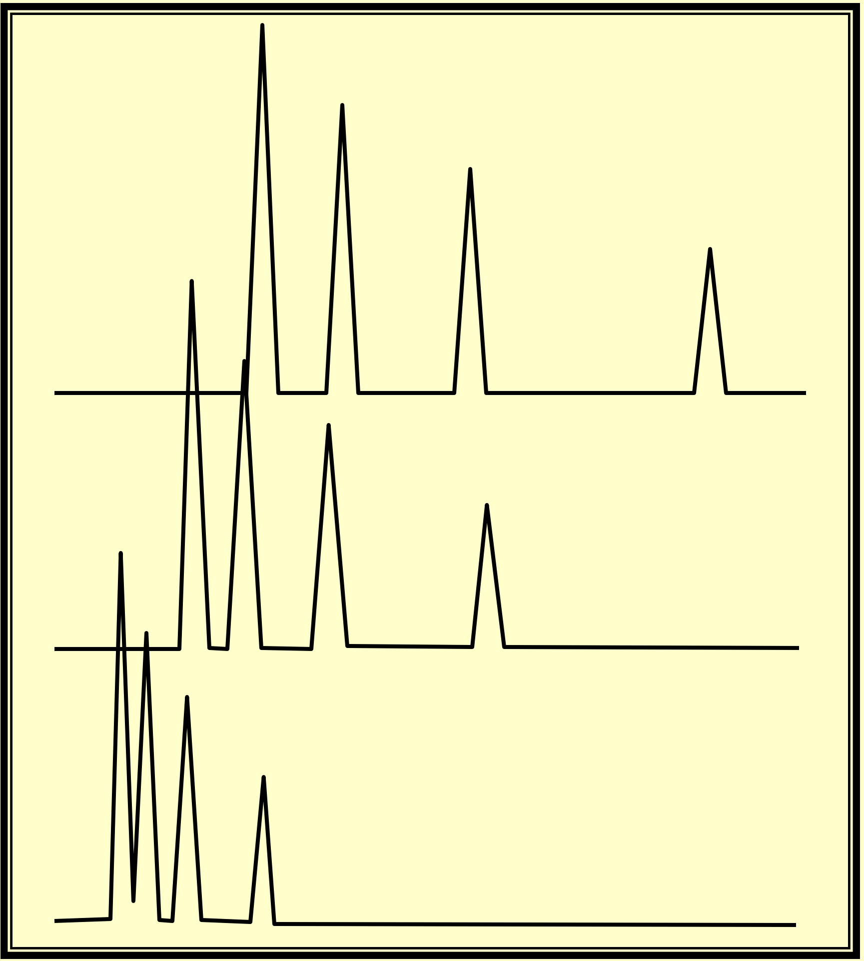
CH_3OH

CH_3OH

Nonpolar solute

Nonpolar stationary phase

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



Eluent: Methanol / Water

60/40

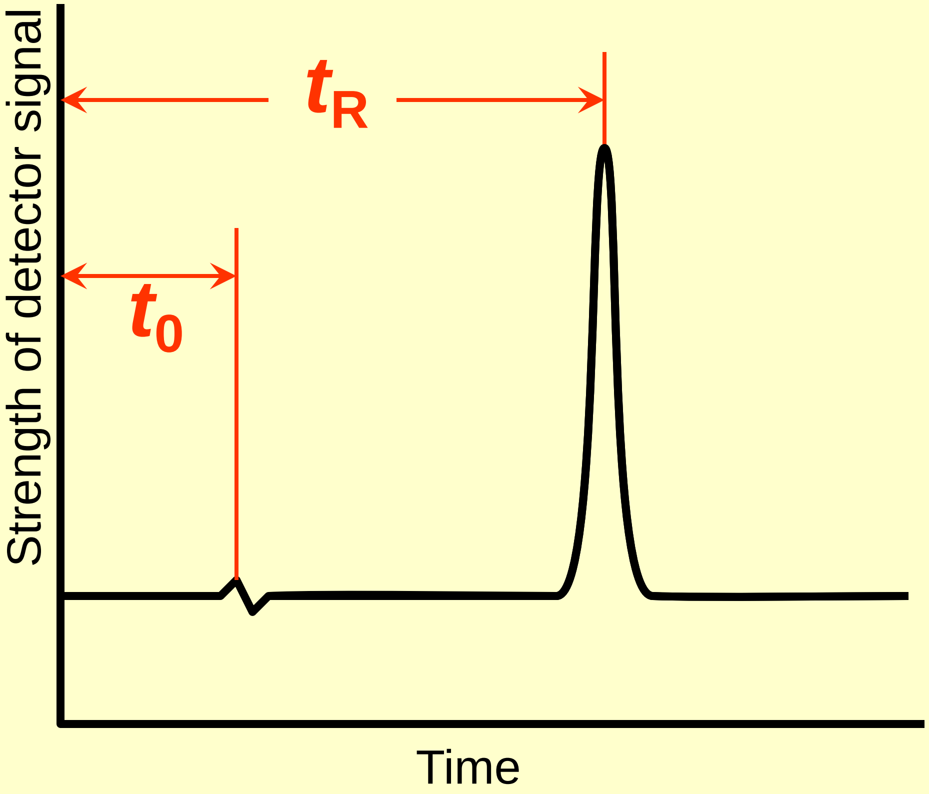
70/30

80/20

Chromatogram Parameters

Methods for Expressing Separation and Column Performance

Retention Factor, *k*

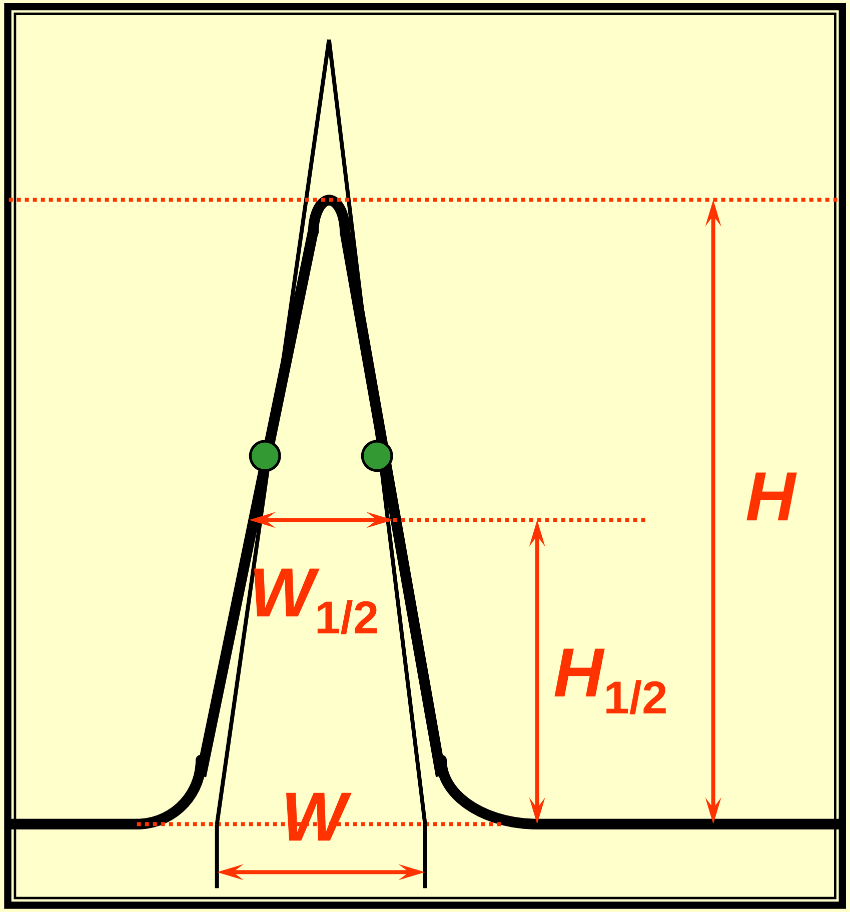


$$k = \frac{t_R - t_0}{t_0}$$

t_R: Retention time

t_0: Non-retention time

Theoretical Plate Number, *N*



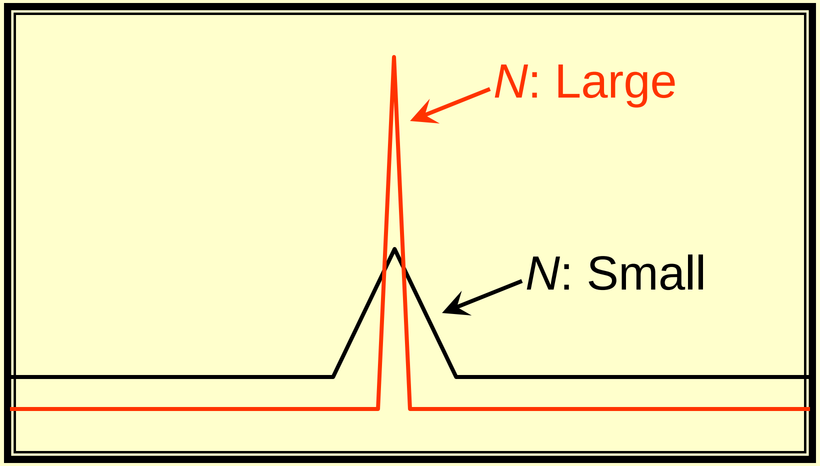
$$N = 16\left(\frac{t_R}{W}\right)^2$$

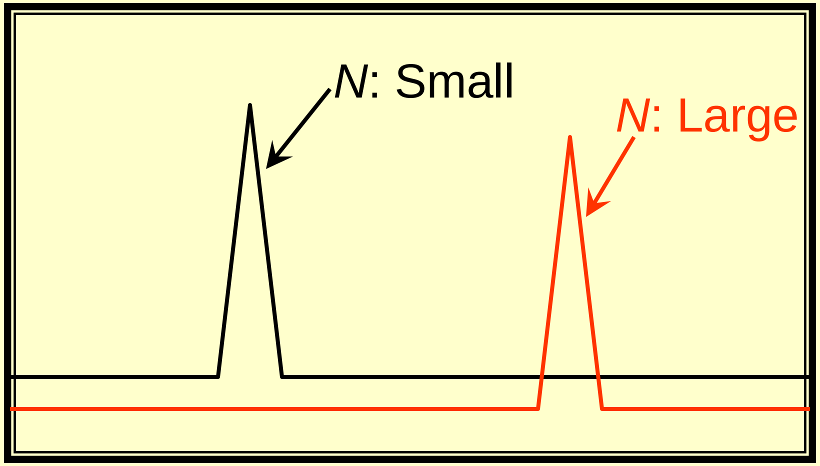
$$= 5.54\left(\frac{t_R}{W_{1/2}}\right)^2$$

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

Evaluation of Column Efficiency Based on Theoretical Plate Number

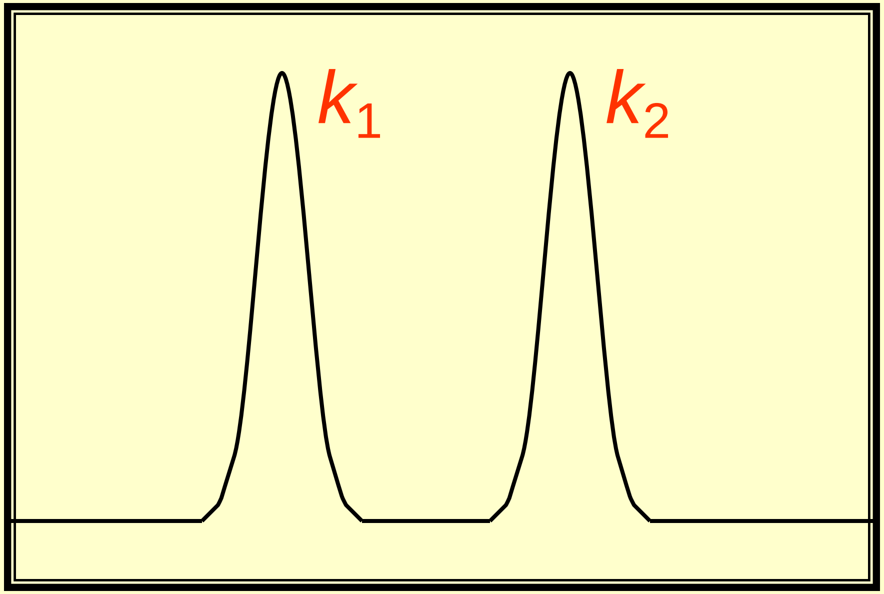
- If the retention times are the same, the peak width is smaller for the one with the larger theoretical plate number.
- If the peak width is the same, the retention time is longer for the one with the larger theoretical plate number.





Separation Factor, a

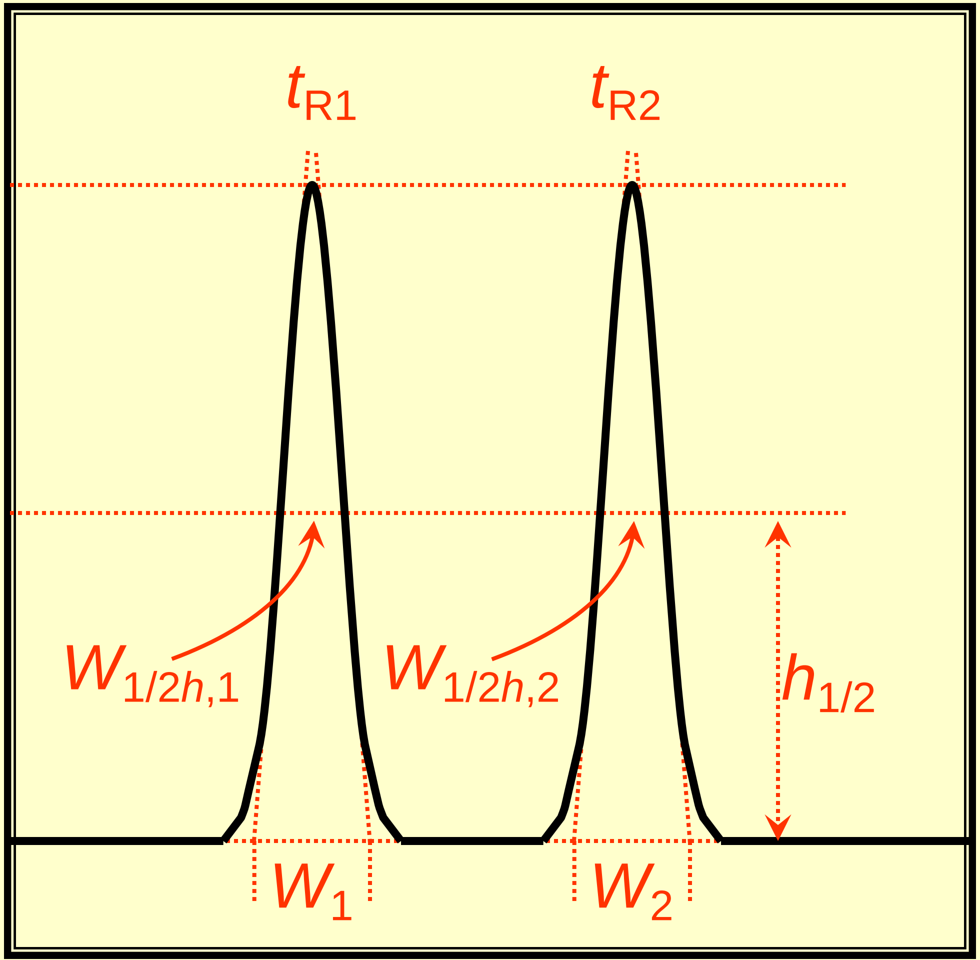
- Separation factor: Ratio of *k*'s of two peaks



$$\alpha = \frac{k_2}{k_1}$$

$$(k_2 > k_1)$$

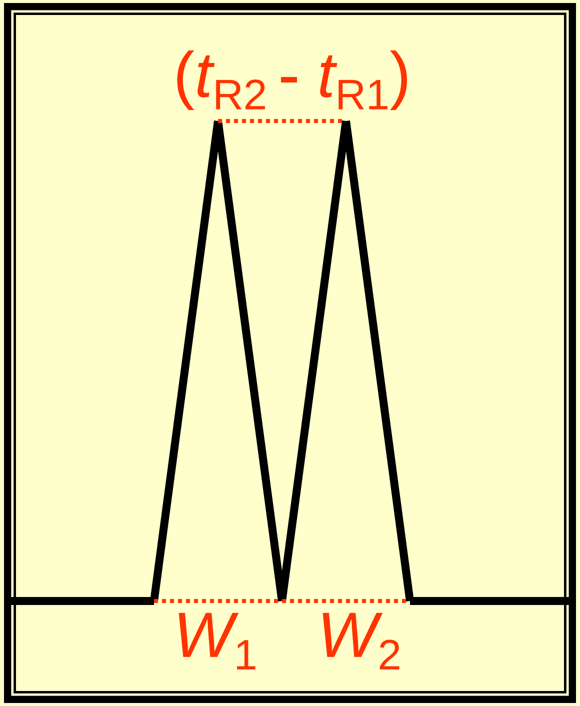
Resolution, R_S



$$R_S = \frac{t_{R_2} - t_{R_1}}{\frac{1}{2}(W_1 + W_2)}$$

$$= 1.18 \times \frac{t_{R_2} - t_{R_1}}{W_{1/2h,1} + W_{1/2h,2}}$$

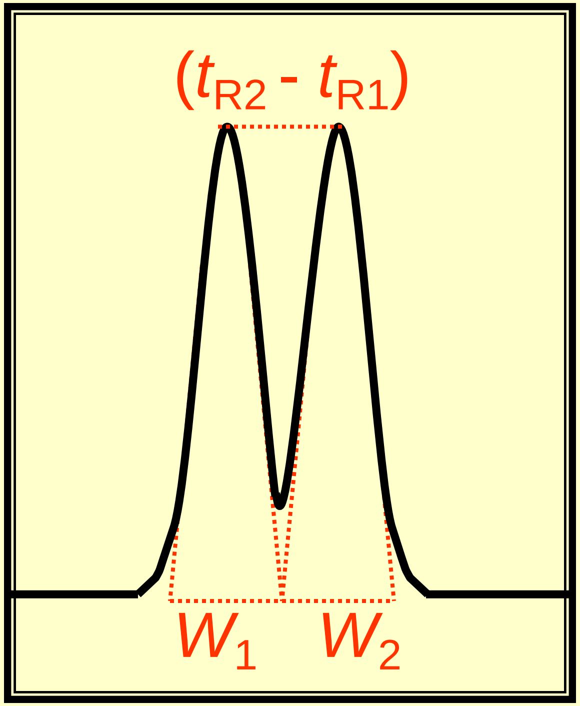
Resolution Required for Complete Separation



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

$$R_S = 1$$

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

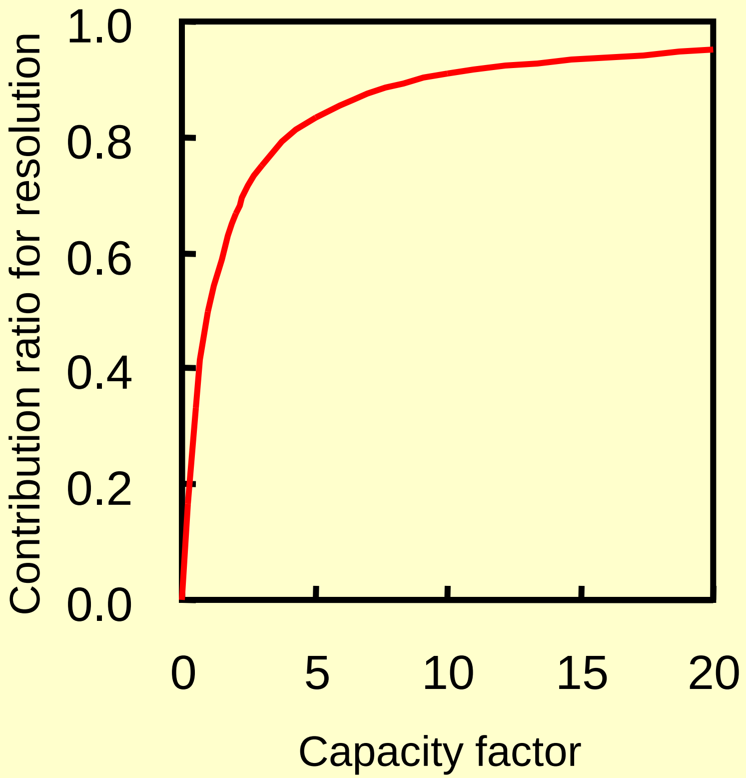
- The resolution is a function of the separation factor, the theoretical plate number, and the retention factor.
- The separation can be improved by improving these 3 parameters!

$$R_S = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)}$$

$$= \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{k_2' + 1}\right)$$

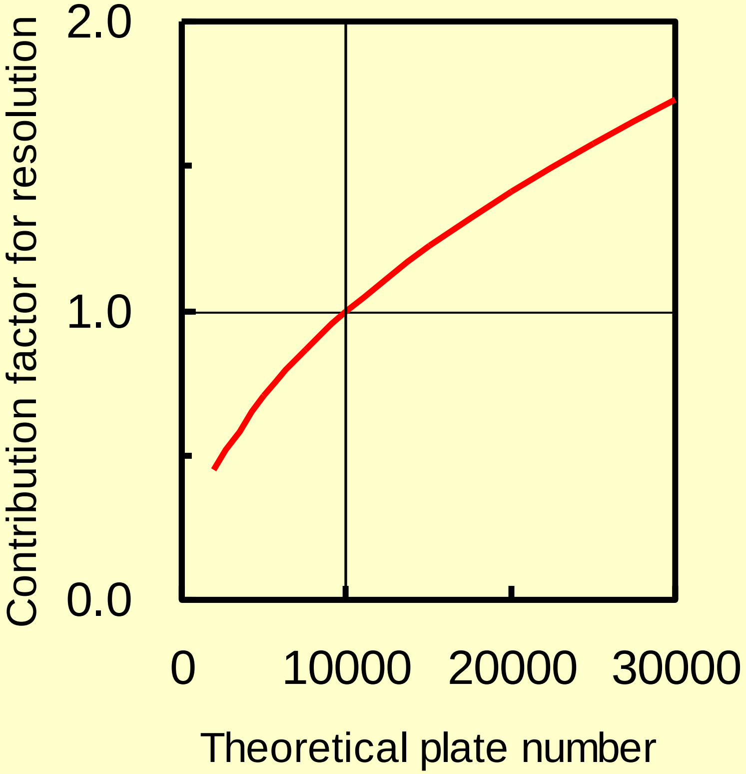
Contribution of Capacity Factor to Resolution

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

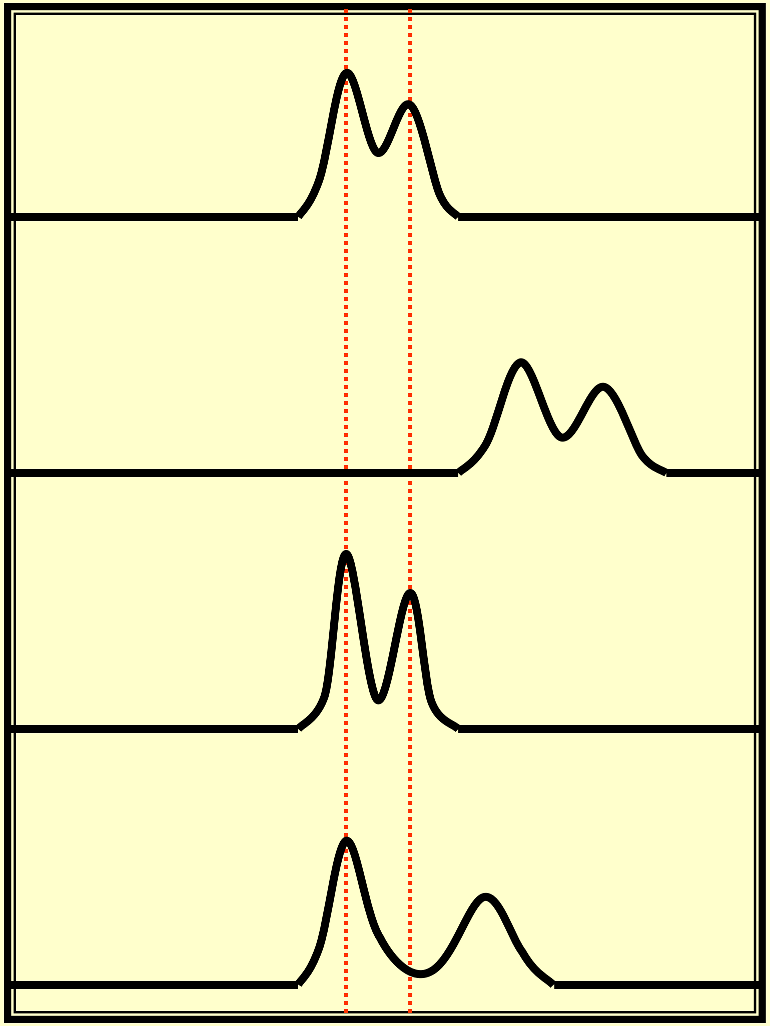


Contribution of Theoretical Plate Number to Resolution

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



To Improve Separation...



Before adjustment

k' increased

Eluent replaced with one of lower elution strength.

N increased

Column replaced with one of superior performance.
Column lengthened.

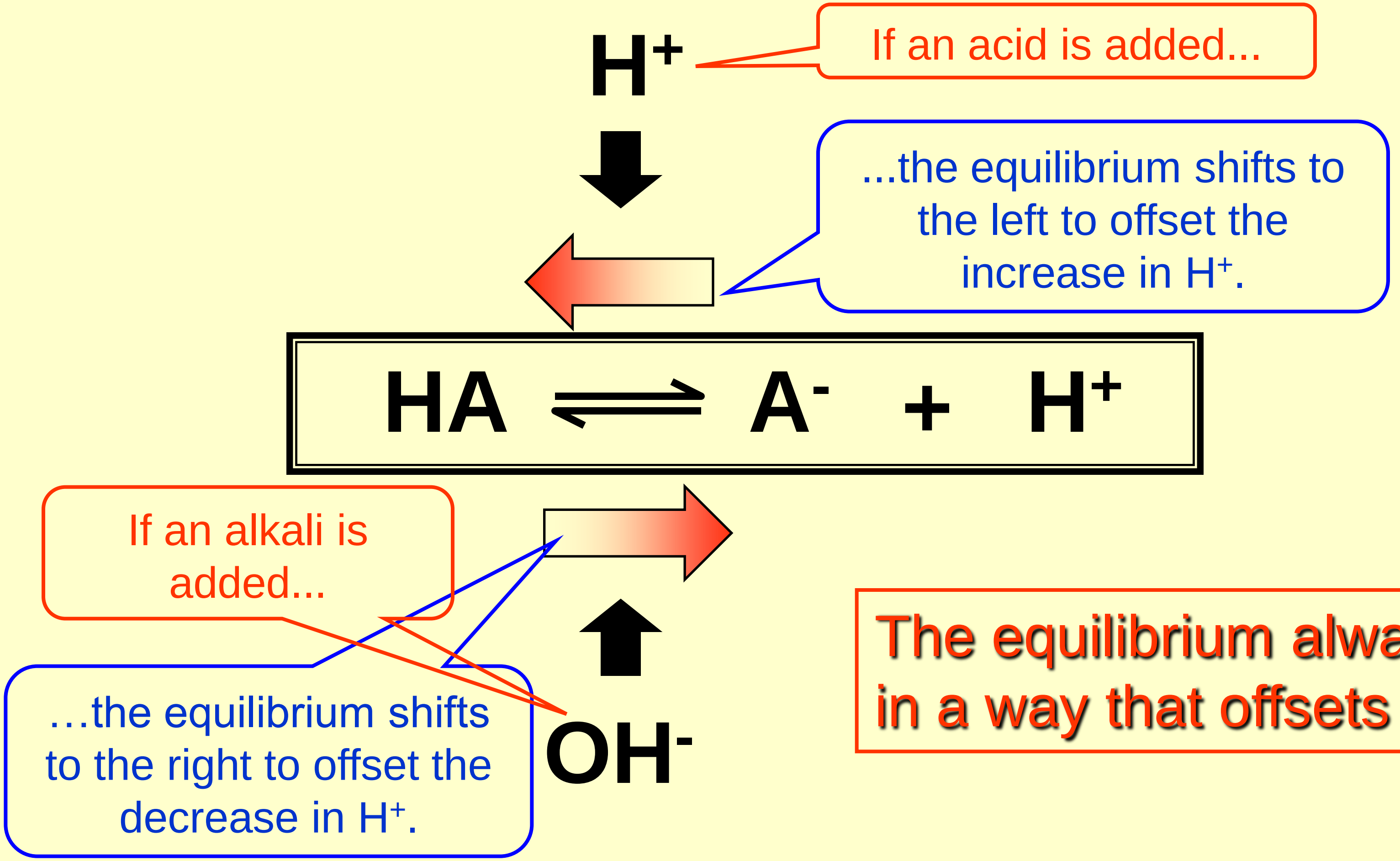
α increased

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



H^+

If an acid is added...

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

$$HA \rightleftharpoons A^- + H^+$$

If an alkali is added...

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

OH^-

The equilibrium always shifts in a way that offsets changes.

Acid Dissociation Constant and pH-Based Abundance Ratio

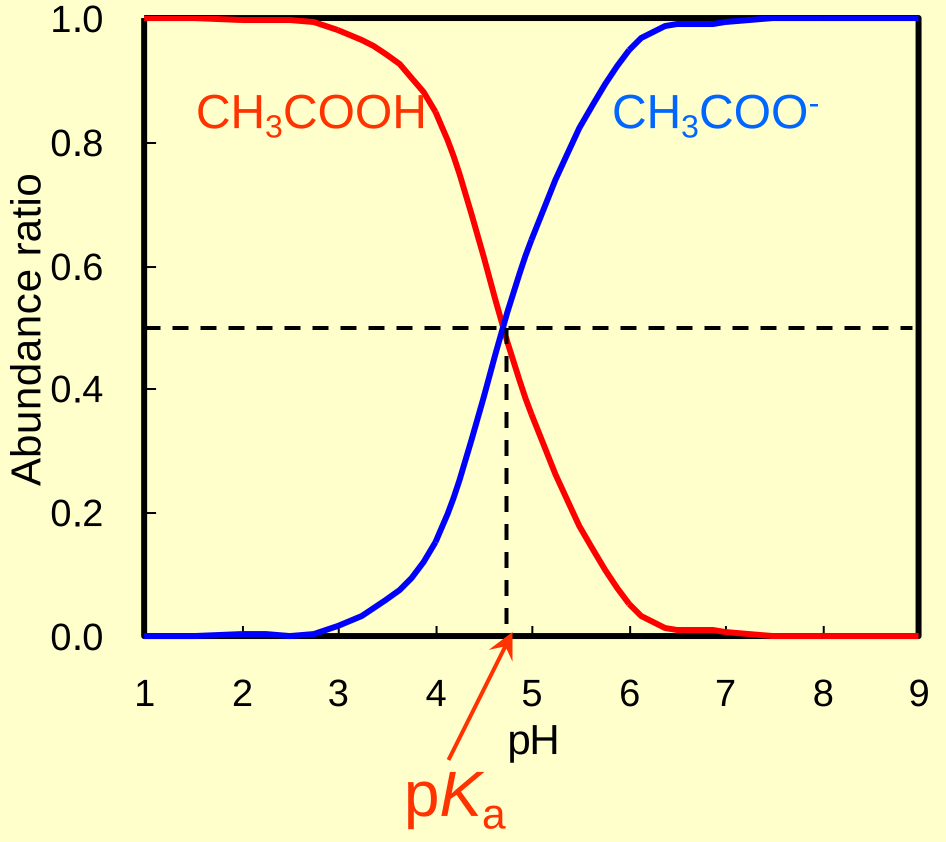
$$\boxed{\mathrm{HA} \rightleftharpoons \mathrm{A}^- + \mathrm{H}^+}$$

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

$$K_\mathrm{a} = \frac{[\mathrm{A}^-][\mathrm{H}^+]}{[\mathrm{HA}]}$$

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]}$$

$$\left(\begin{aligned} \mathrm{pH} &= -\log[\mathrm{H}^+] \\ \mathrm{p}K_\mathrm{a} &= -\log K_\mathrm{a} \end{aligned}\right)$$



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 ($\mathrm{p}K_a$ 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.

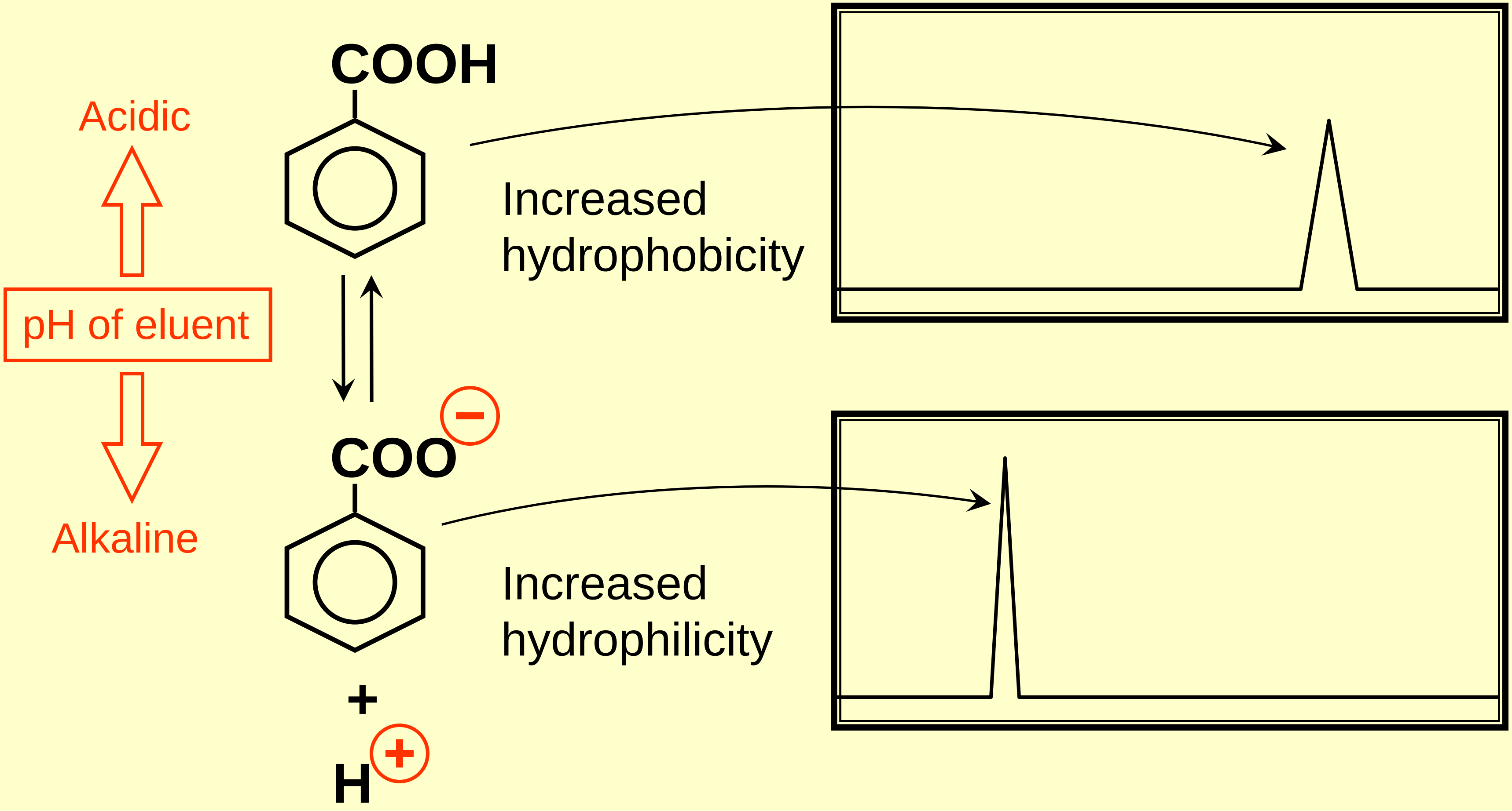
Reversed Phase Chromatography Part 2

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



Acidic

pH of eluent

Alkaline

Increased hydrophobicity

Increased hydrophilicity

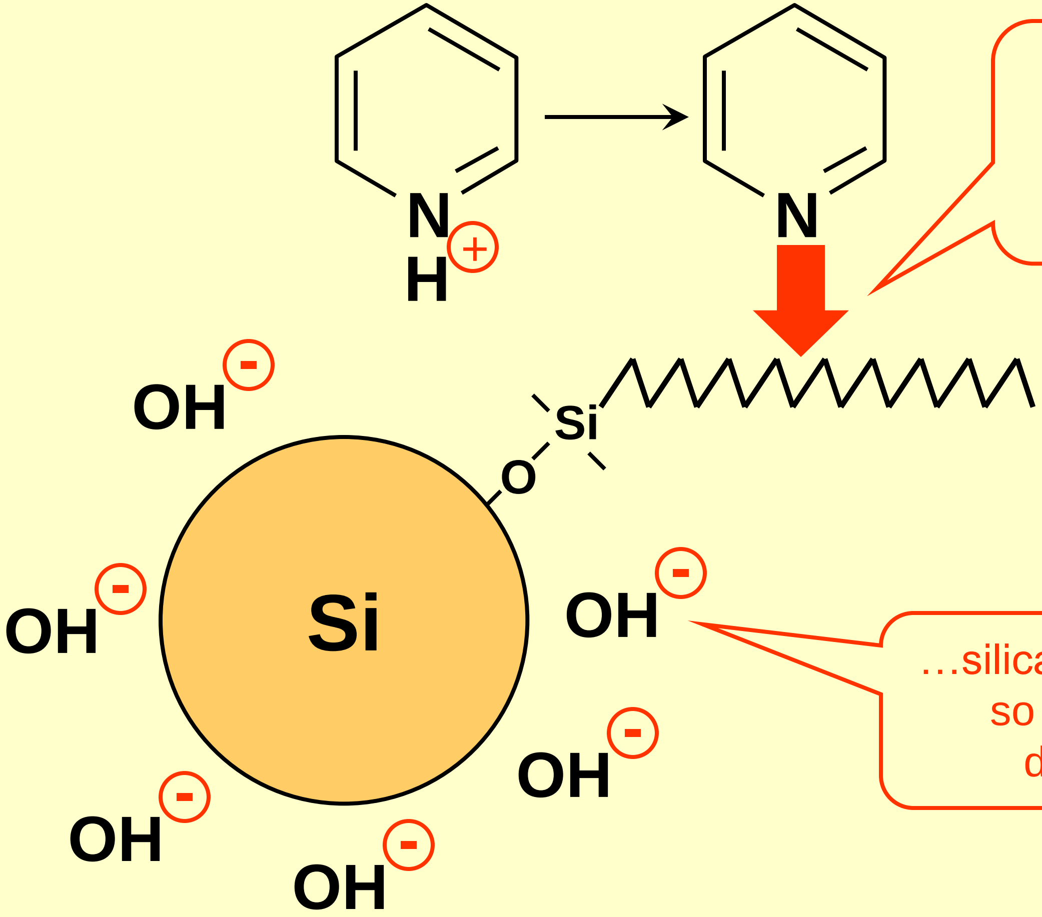
Guidelines for Setting Mobile Phase Conditions (2)

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

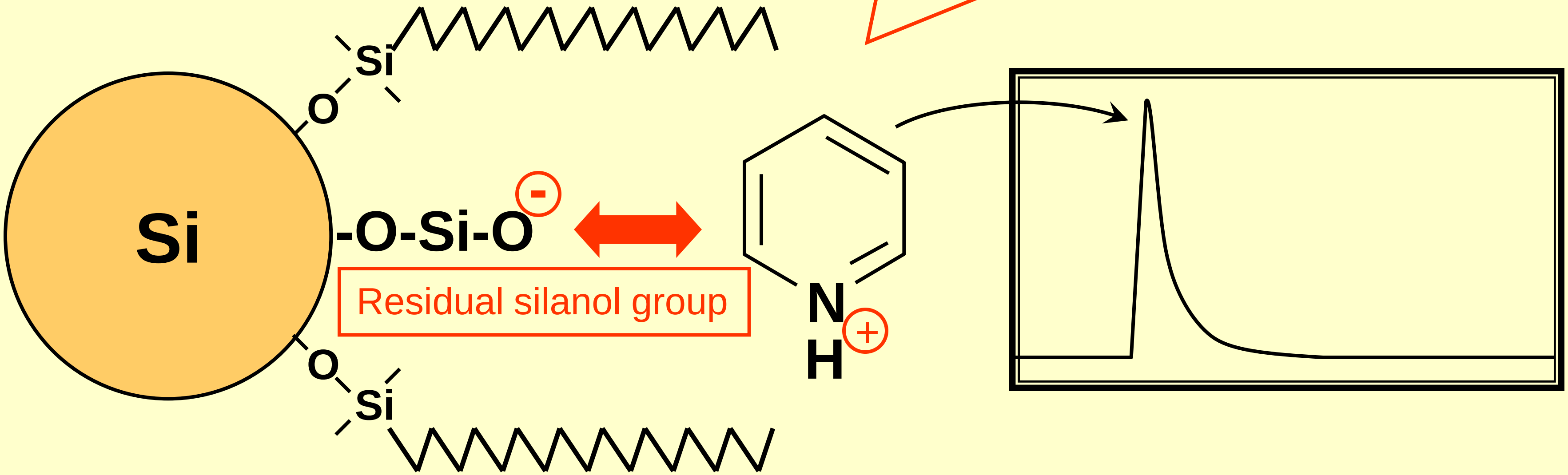


With alkaline eluents, although the ionization of basic substances is suppressed, and the retention strength increases...

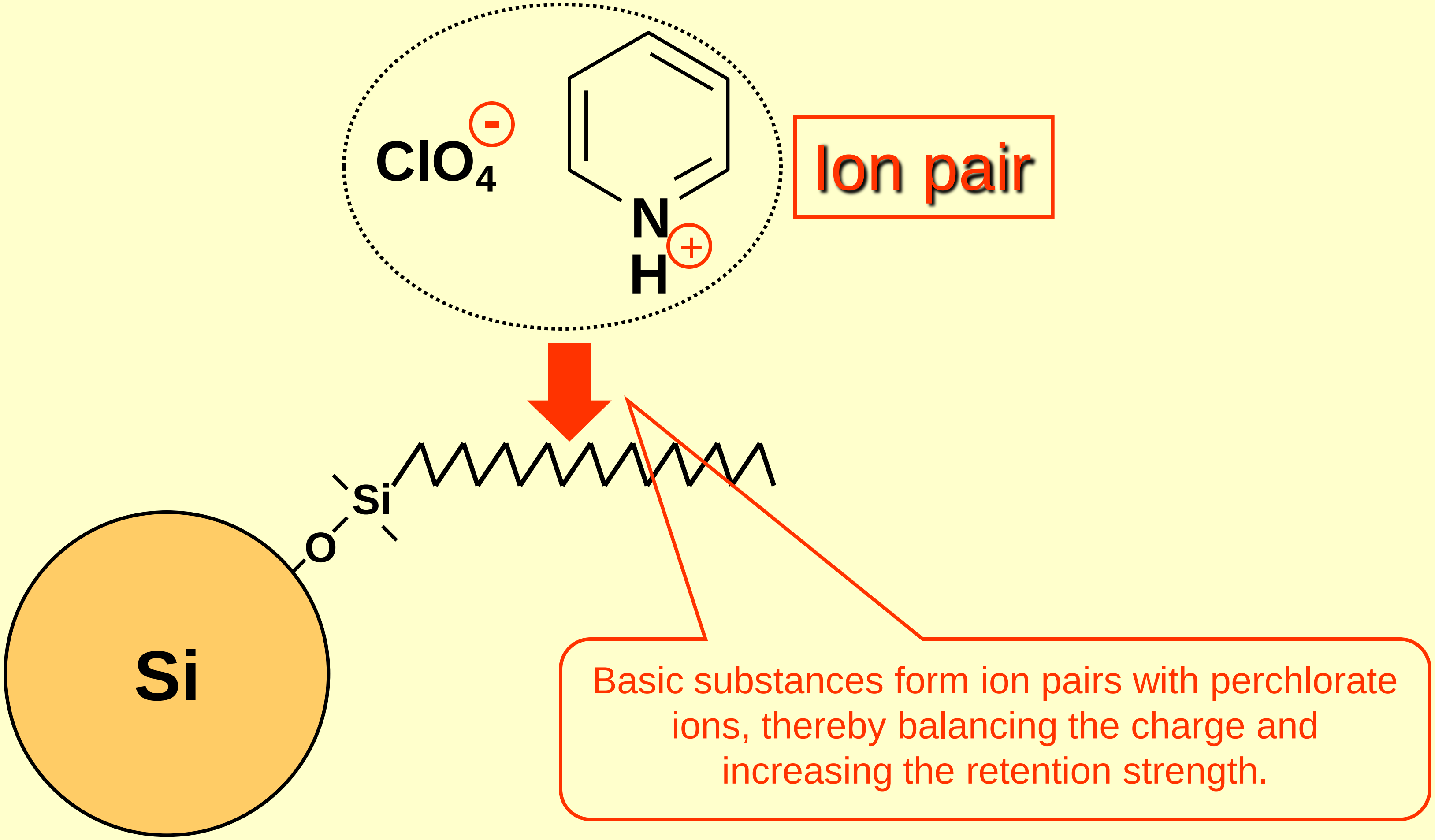
…silica gel dissolves in alkalis, so the packing material deteriorates rapidly.

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



Basic substances form ion pairs with perchlorate ions, thereby balancing the charge and increasing the retention strength.

Guidelines for Setting Mobile Phase Conditions (3)
Basic Substances (Cationic Substances)

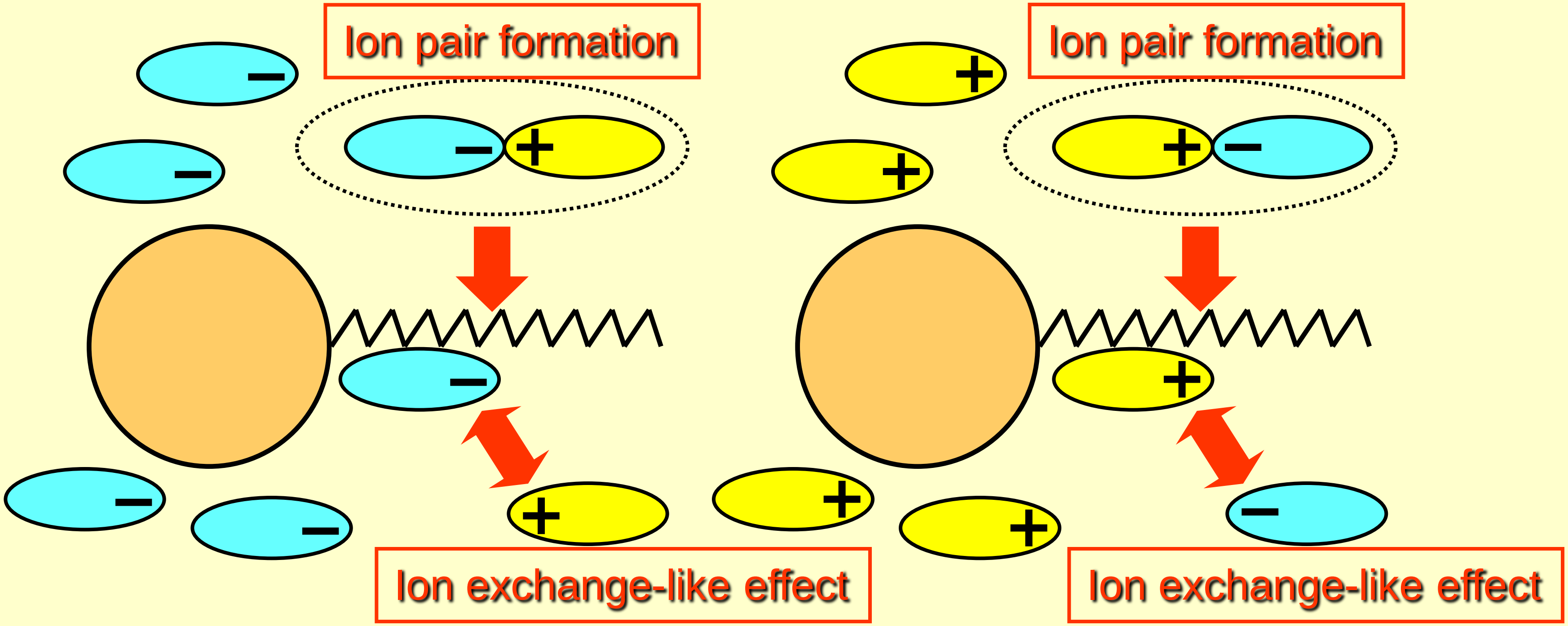
- Eluent Composition
  - Acidic buffer solution containing anions with a low charge density (e.g., perchlorate ions) / acetonitrile
  - As above / methanol

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.



Ion pair formation

Ion exchange-like effect

Basic Substance

Ion pair formation

Ion exchange-like effect

Acidic Substance

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

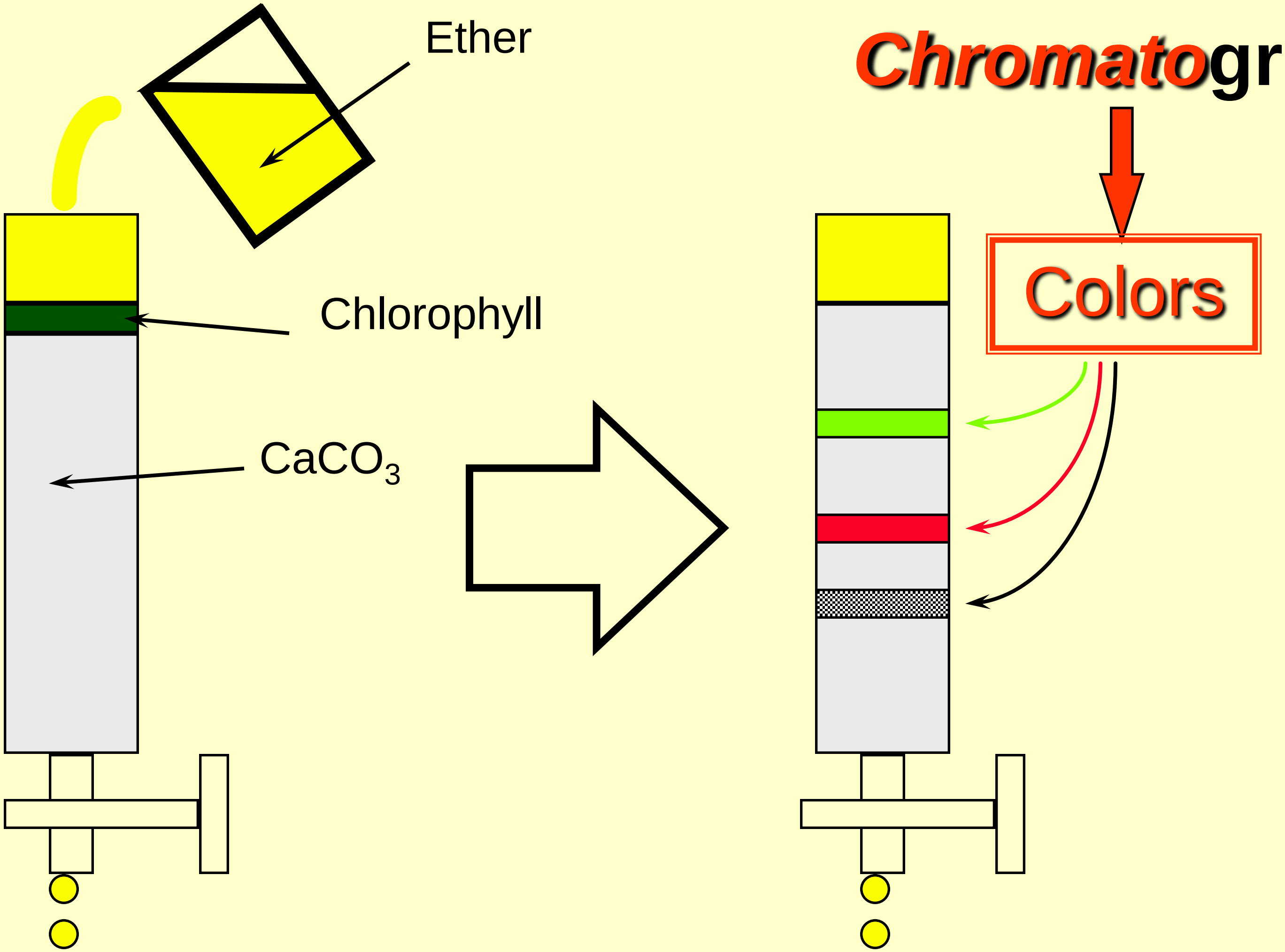
Normal Phase and Reversed Phase

	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

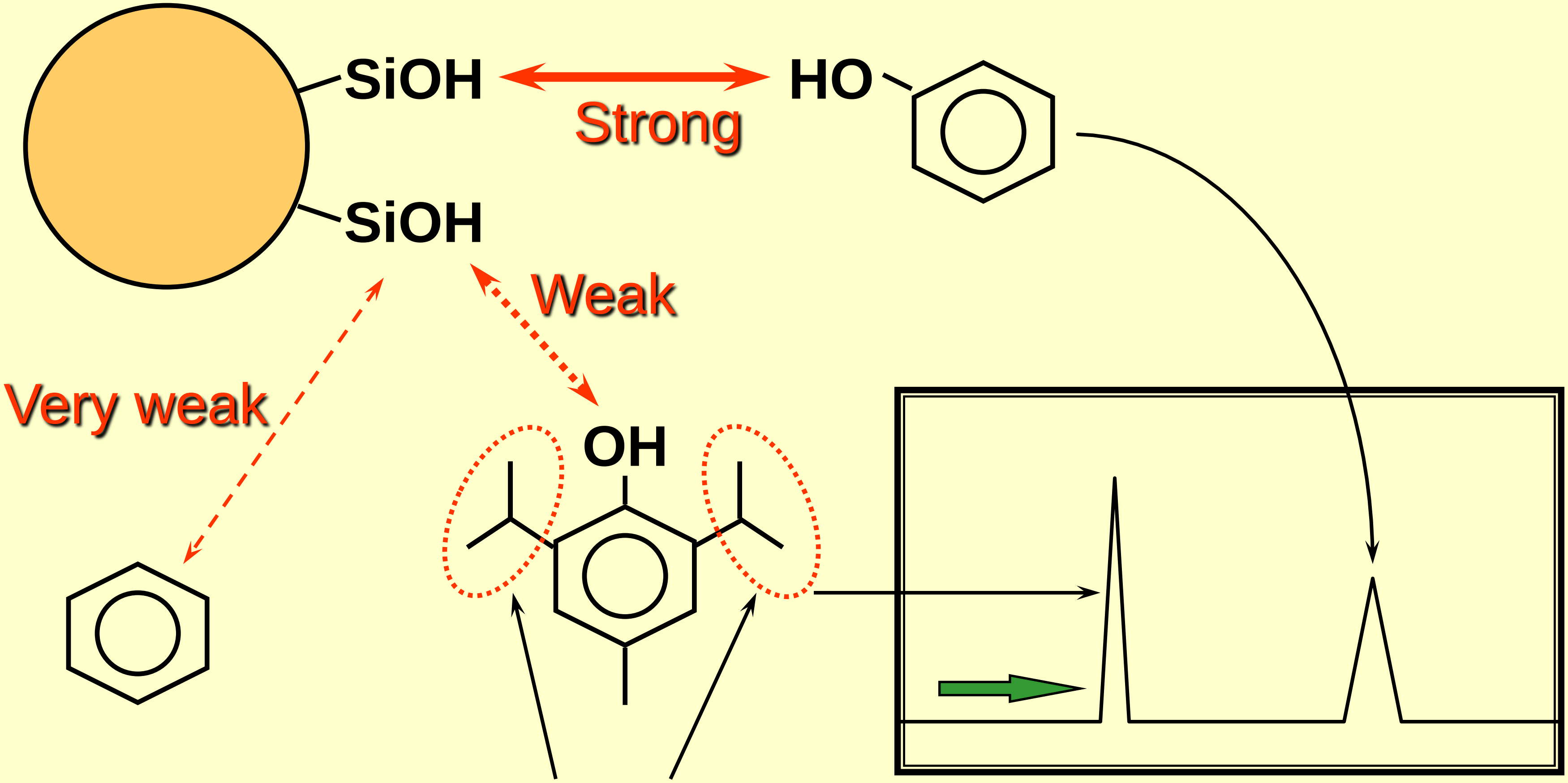
Invention of Chromatography by M. Tswett



Stationary Phase and Mobile Phase Used in Normal Phase Mode

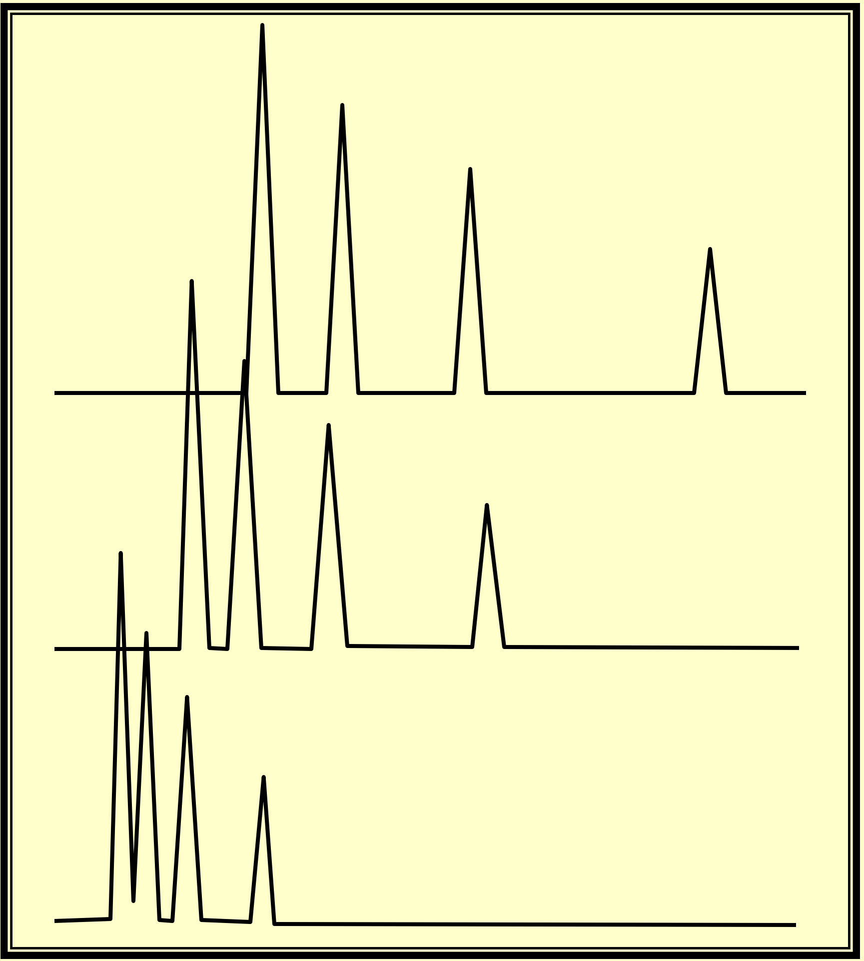
- Stationary Phase
  - Silica gel: -Si-OH
  - Cyano type: $-Si-CH_2CH_2CH_2CN$
  - Amino type: $-Si-CH_2CH_2CH_2NH_2$
  - Diol type: $-Si-CH_2CH_2CH_2OCH(OH)-CH_2OH$
- Mobile Phase
  - Basic solvents: Aliphatic hydrocarbons, aromatic hydrocarbons, etc.
  - Additional solvents: Alcohols, ethers, etc.

Relationship between Hydrogen Bonding and Retention Time in Normal Phase Mode



Steric hindrance

Relationship Between Eluent Polarity and Retention Time in Normal Phase Mode



Eluent: Hexane/methanol

100/0

98/2

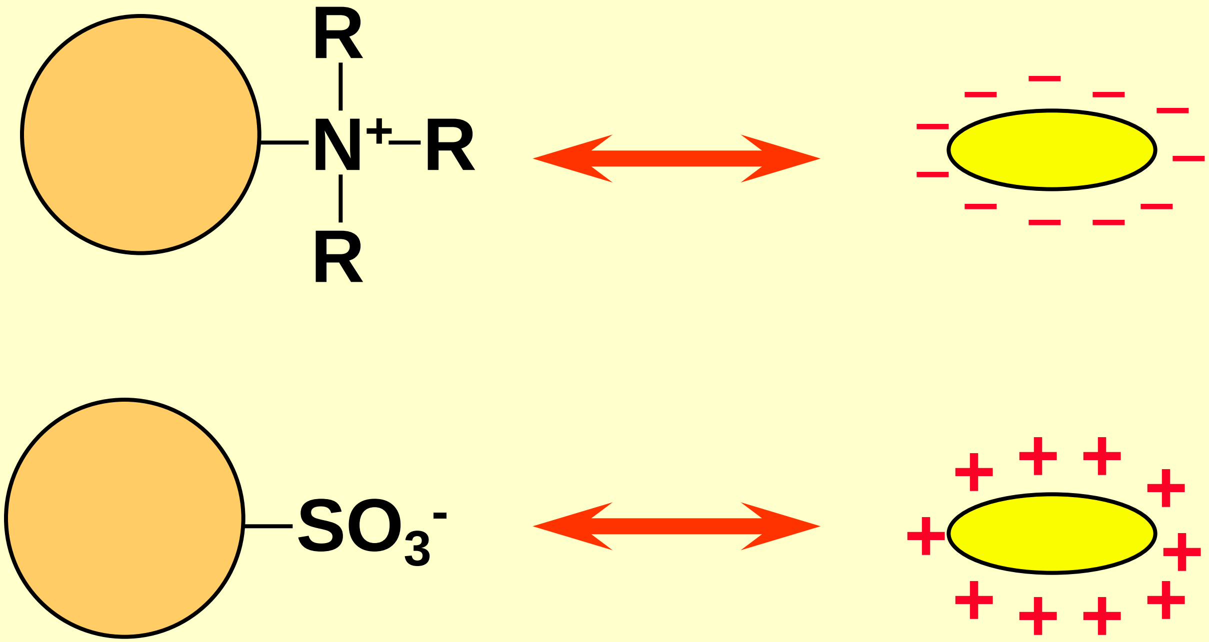
95/5

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



Anion exchange

Cation exchange

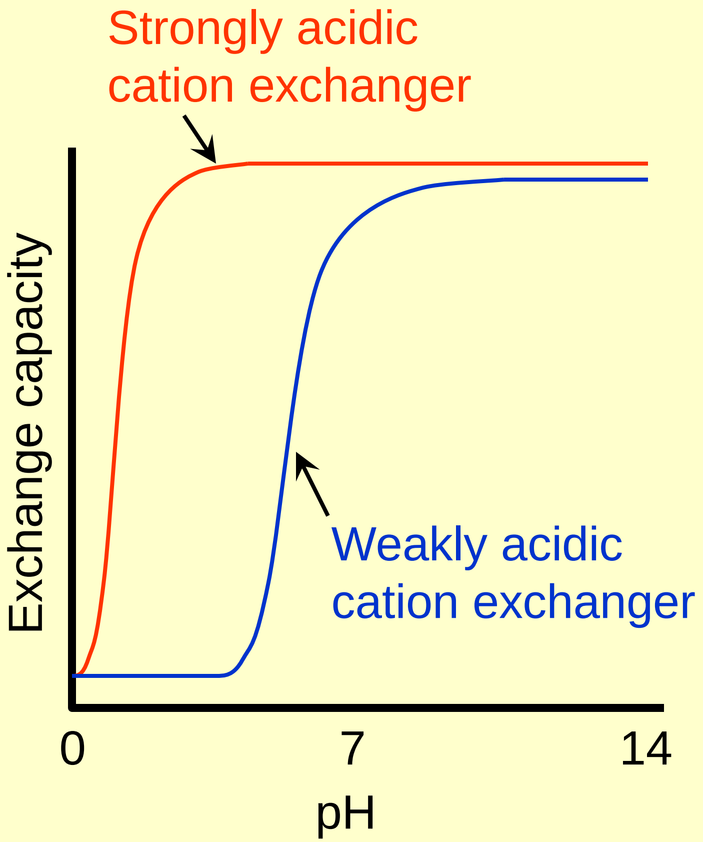
Electrostatic interaction
(Coulomb force)

Stationary Phase Used in Ion Exchange Mode

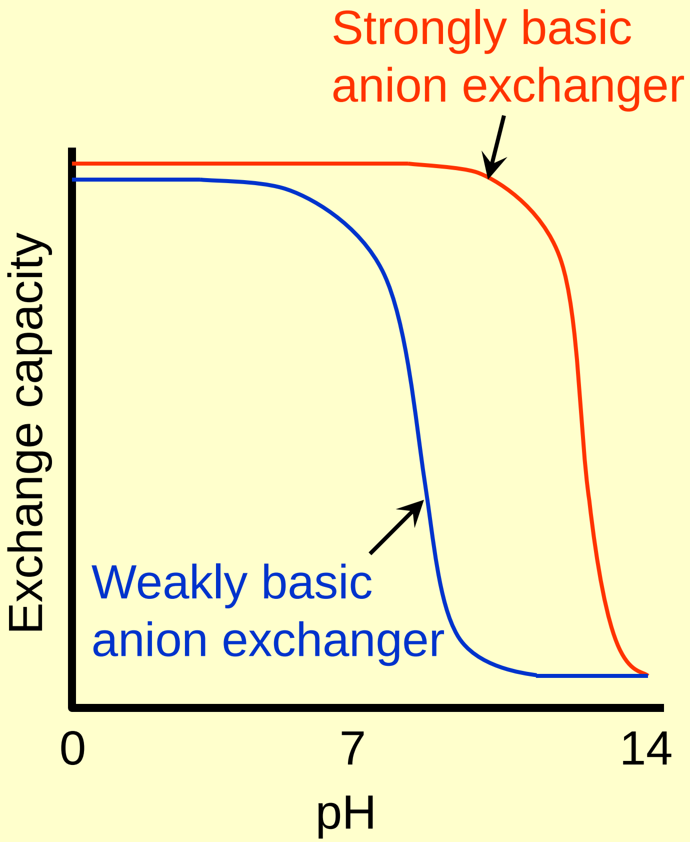
- Base Material
  - Resin is often used.
  - Silica gel is also used.

- Cation Exchange Column
  - Strong cation exchange (SCX) **$-SO_3^-$**
  - Week cation exchange (WCX) **$-COO^-$**
- Anion Exchange Column
  - Strong anion exchange (SAX) **$-NR_3^+$**
  - Week anion exchange (WAX) **$-NHR_2^+$**

Dependence of Exchange Capacity of Ion Exchanger on pH of Eluent

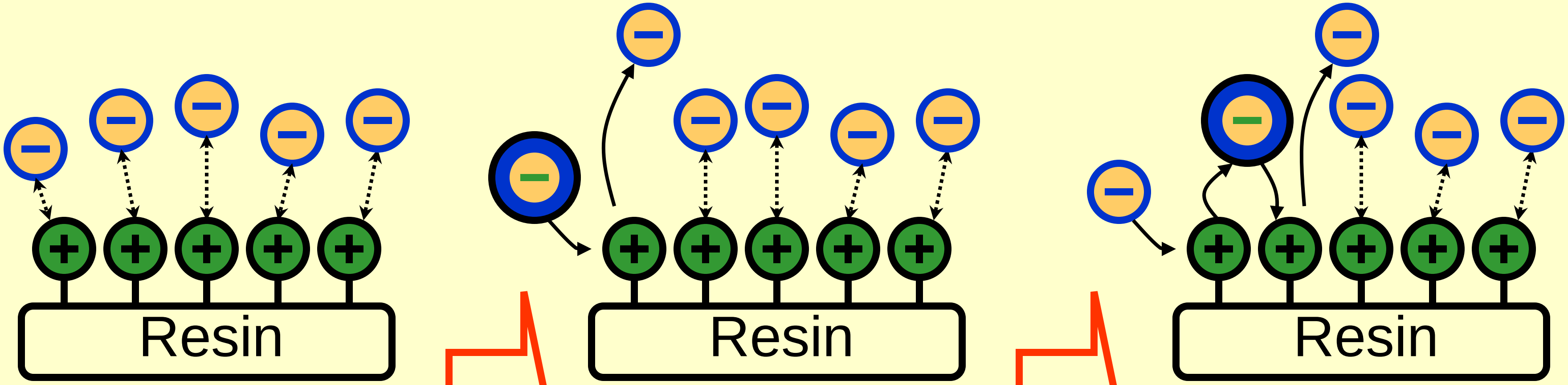


Cation exchange mode



Anion exchange mode

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



The exchange groups are in equilibrium with anions in the eluent.

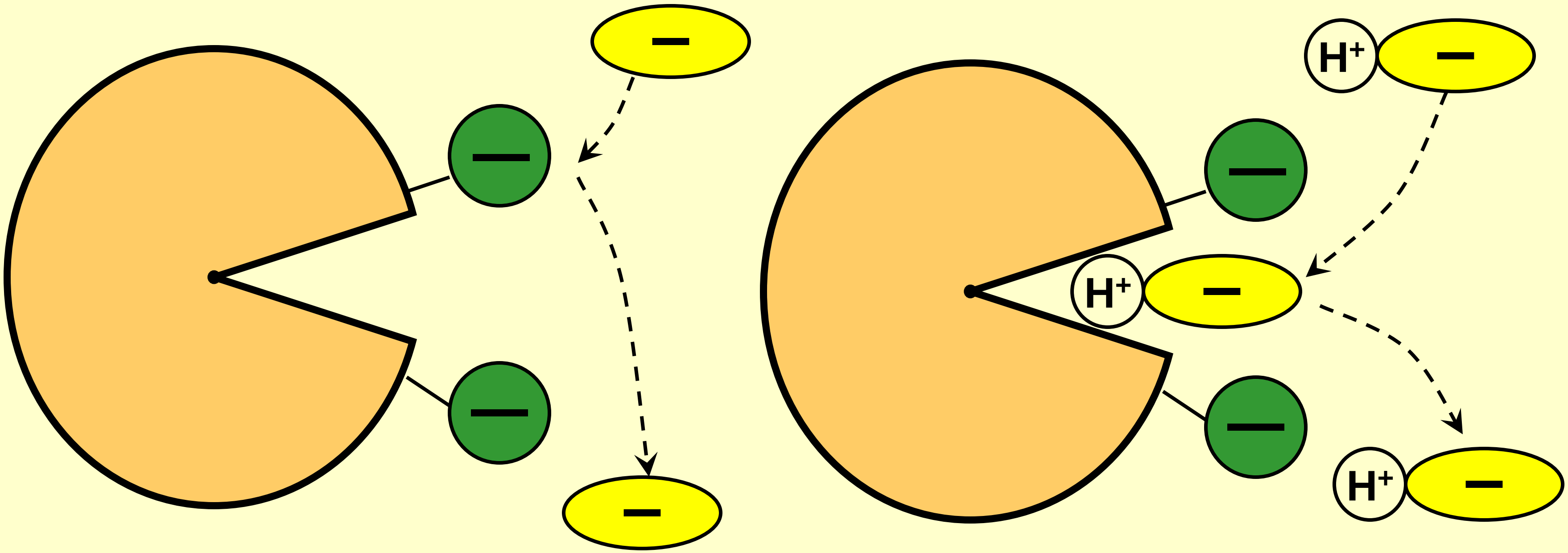
An eluent ion is driven away and a solute ion is adsorbed.

The solute ion is driven away by an eluent ion and is adsorbed by the next exchange group.

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.

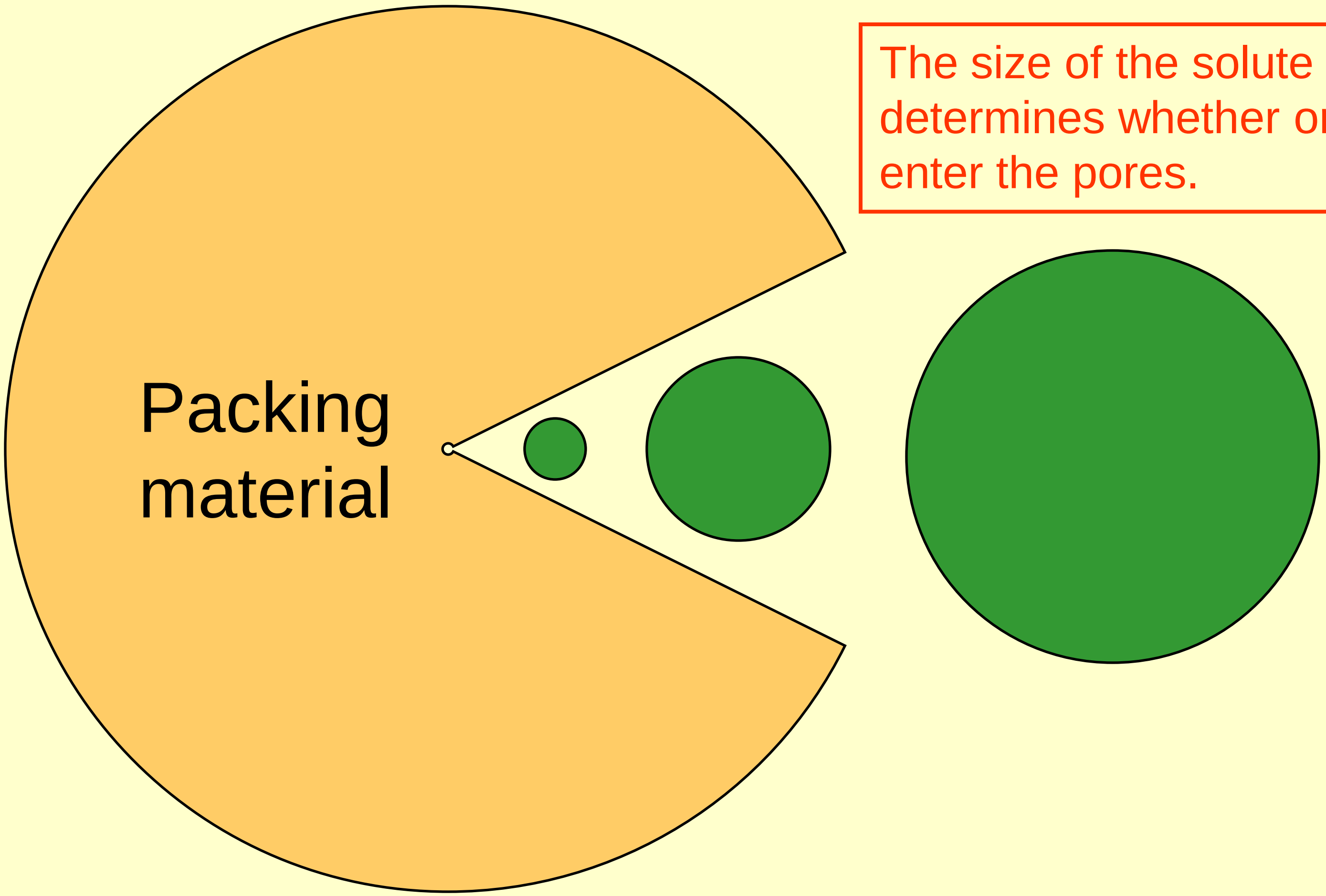
Depending on the level of dissociation, some weak acid ions can enter the pore.

Size Exclusion Chromatography

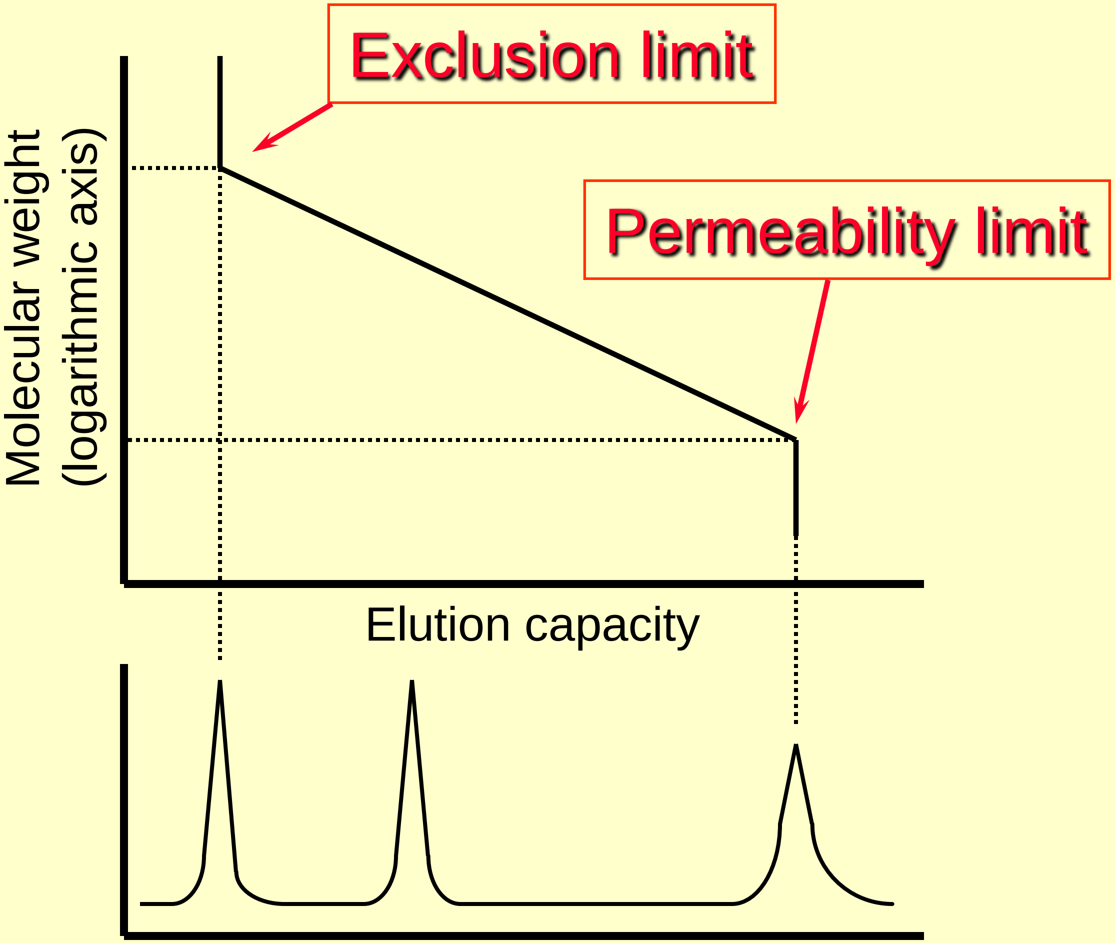
- Separation is based on the size (bulkiness) of molecules.
- The name varies with the application field!
  - Size Exclusion Chromatography (SEC)
  - Gel Permeation Chromatography (GPC)
    - Chemical industry fields, synthetic polymers, nonaqueous systems
  - Gel Filtration Chromatography (GFC)
   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

Principle of Size Exclusion Mode

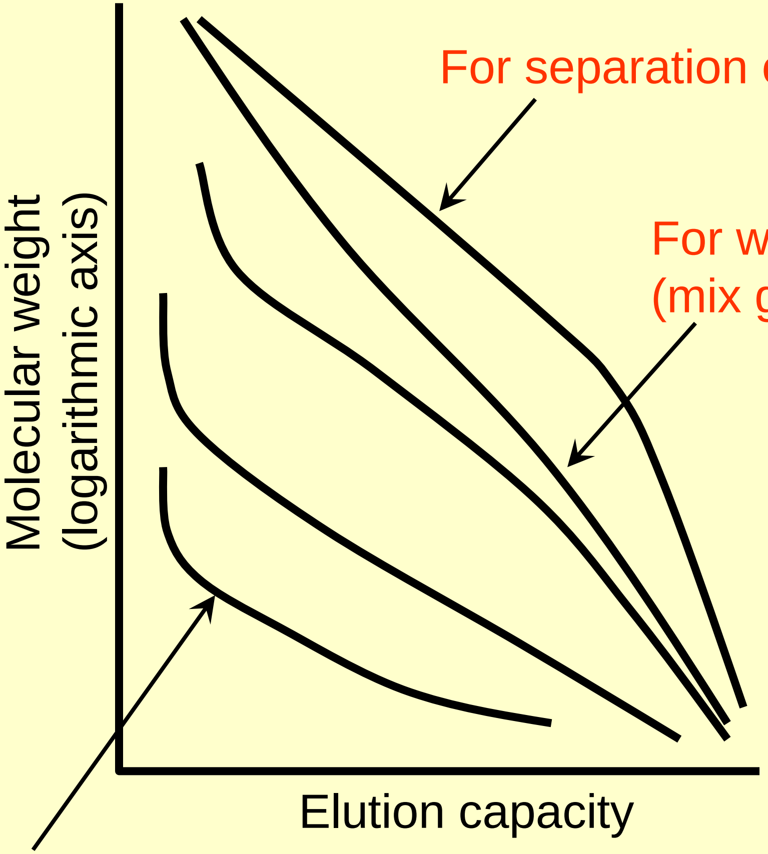
The size of the solute molecules determines whether or not they can enter the pores.



Relationship Between Molecular Weight and Retention Time in Size Exclusion Mode



Creating a Molecular Weight Calibration Curve

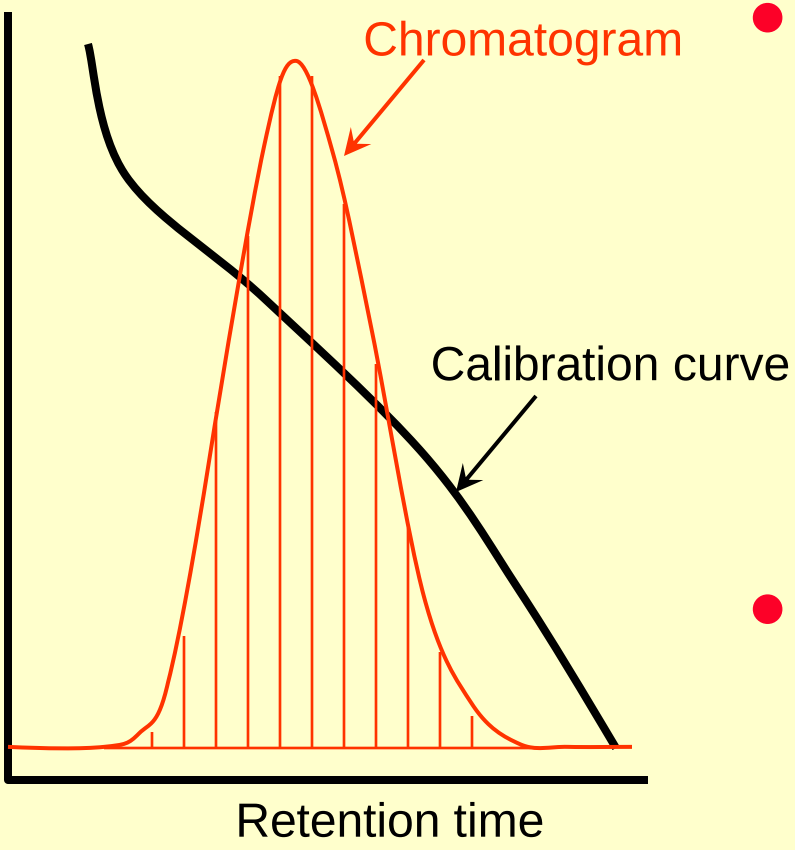


For separation of large molecular weights

For wide-range separation
(mix gel)

For separation of small molecular weights

Calculating Molecular Weights



- Various Average Molecular Weights
  - Mn: Number-average molecular weight
  - Mw: Weight-average molecular weight
  - Mz: 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

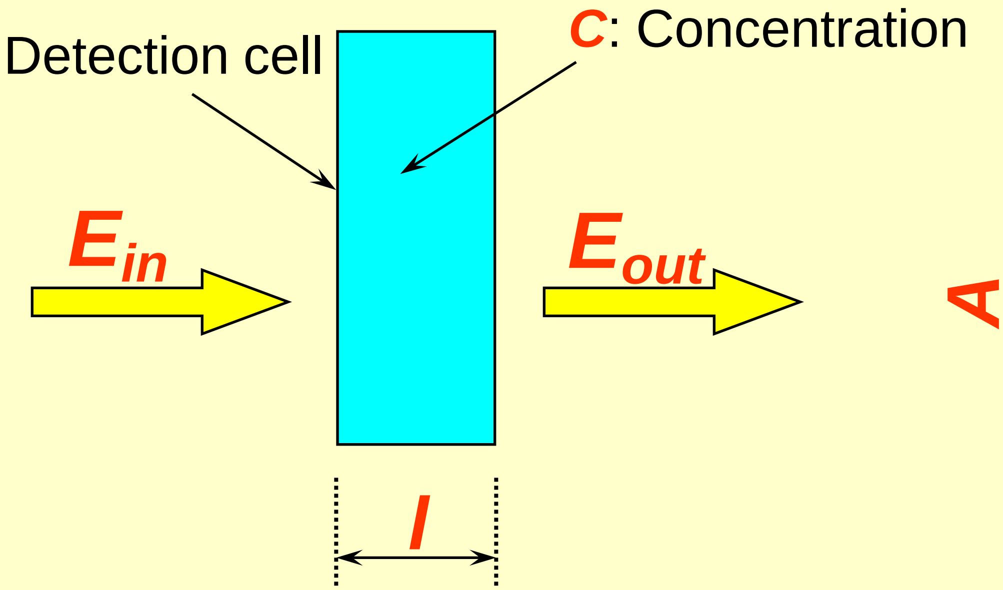
Detection Condition Requirements

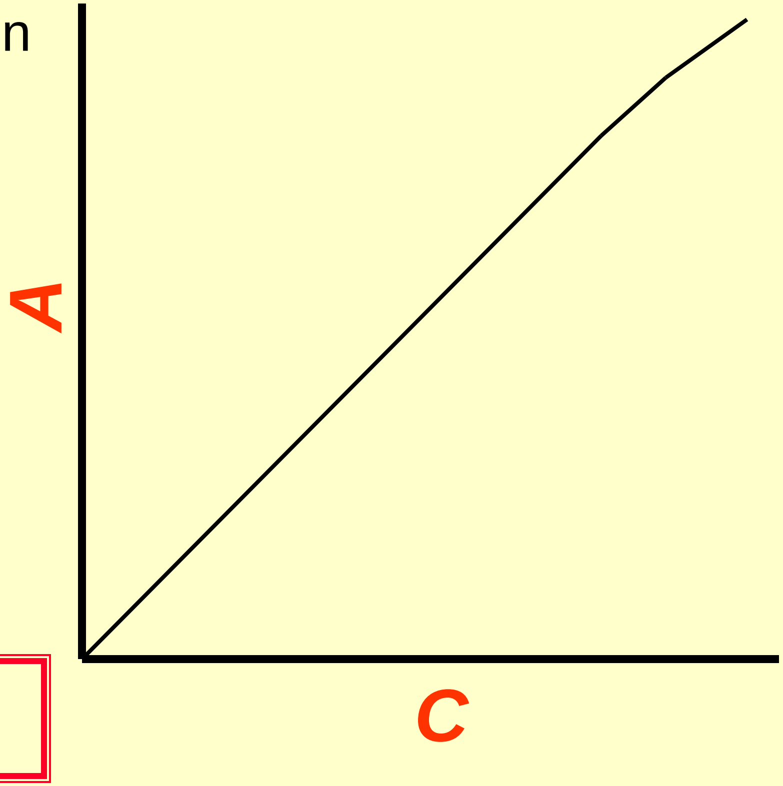
- Sensitivity
  - The detector must have the appropriate level of sensitivity.
- Selectivity
  - The detector must be able to detect the target substance without, if possible, detecting other substances.
- Adaptability to separation conditions
- Operability, etc.

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

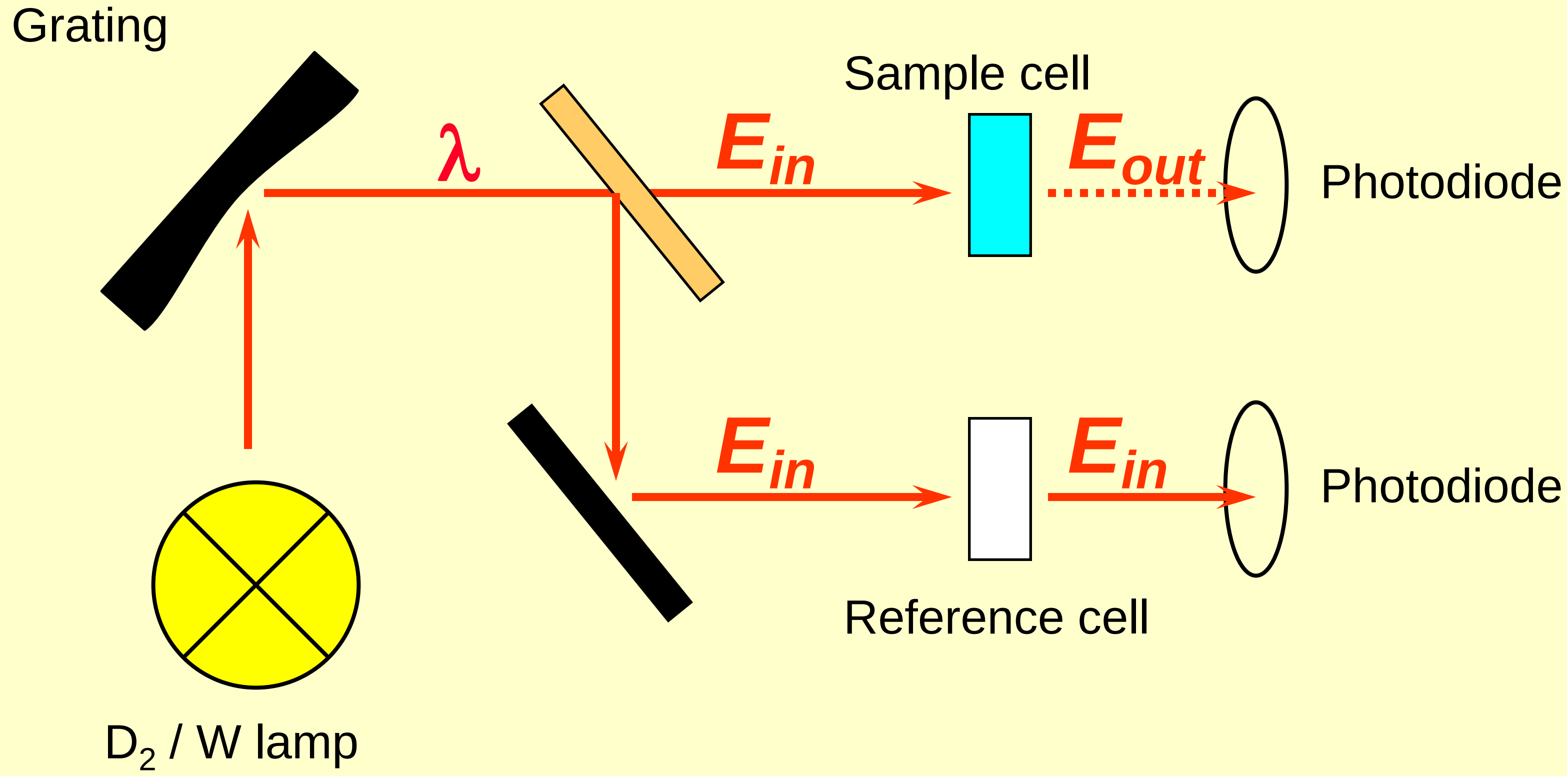




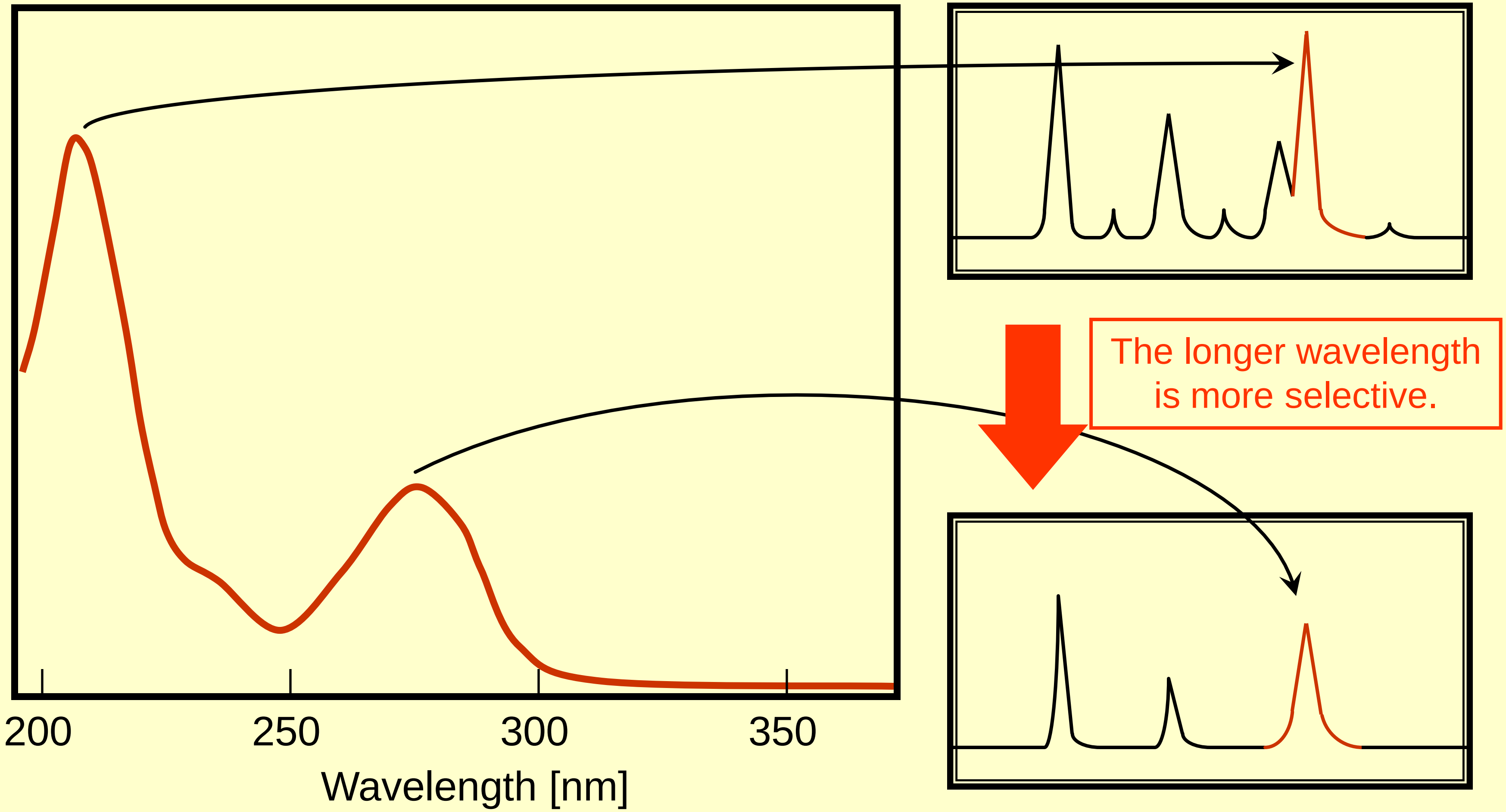
$$\boxed{A = \varepsilon \cdot C \cdot l = -\log\left(E_{out} / E_{in}\right)}$$

(***A***: absorbance, ***E***: absorption coefficient)

Optical System of UV-VIS Absorbance Detector

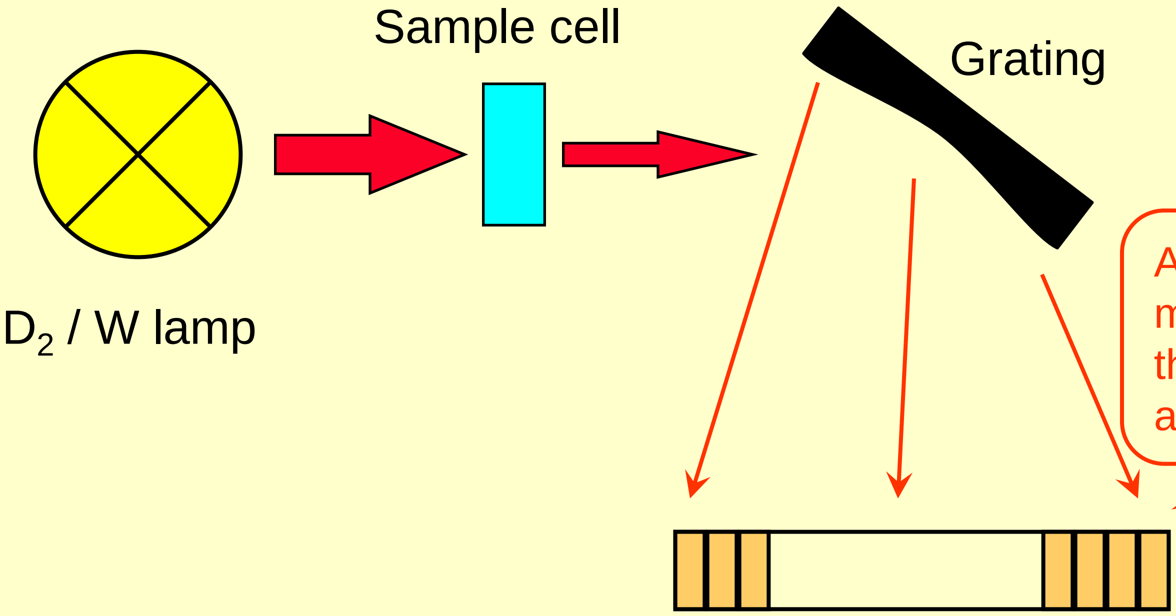


Spectrum and Selection of Detection Wavelength



The longer wavelength is more selective.

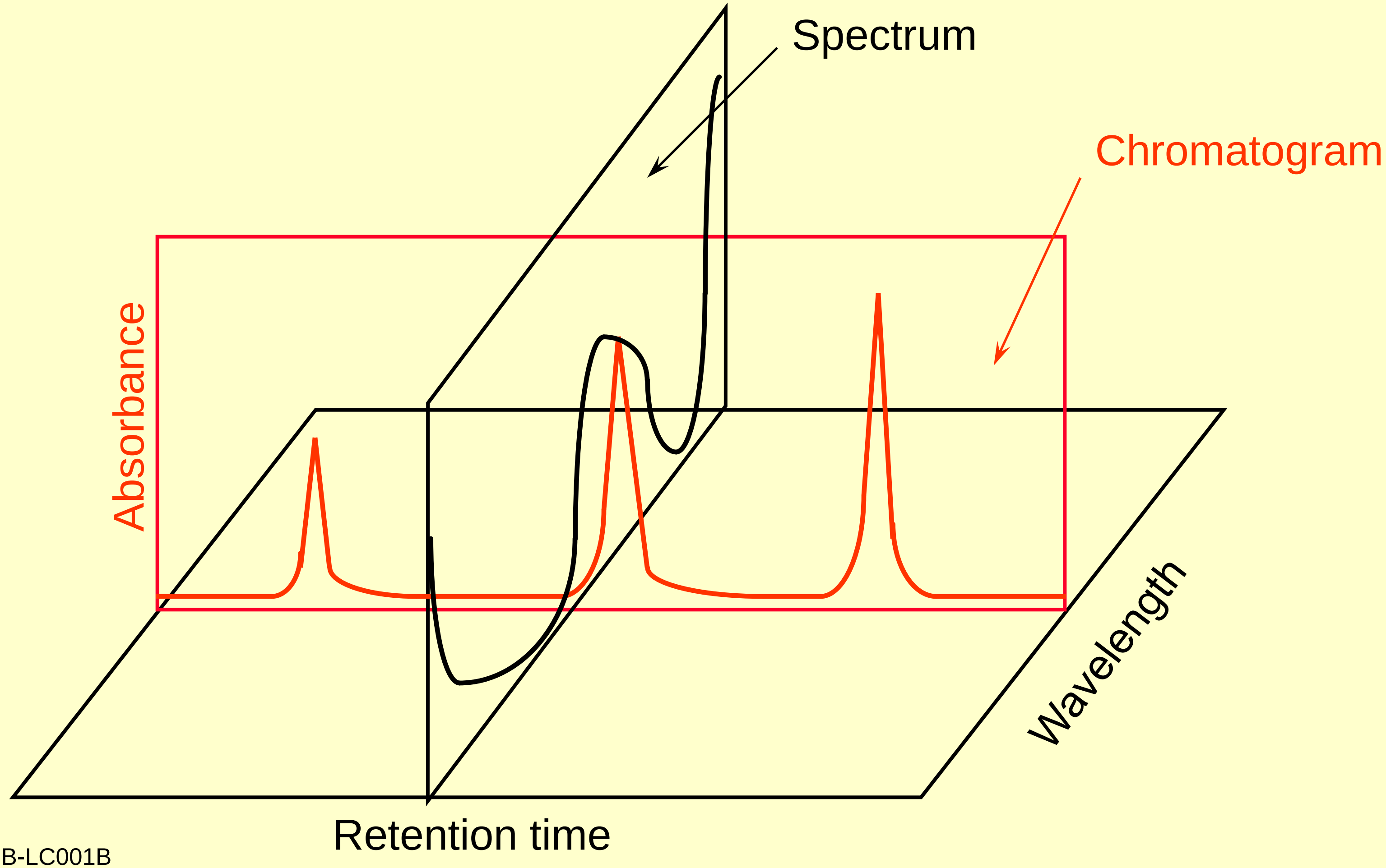
Optical System of Photodiode Array Detector



A single photodiode measures the absorbance for the corresponding wavelength at a resolution of approx. 1 nm.

Photodiode array

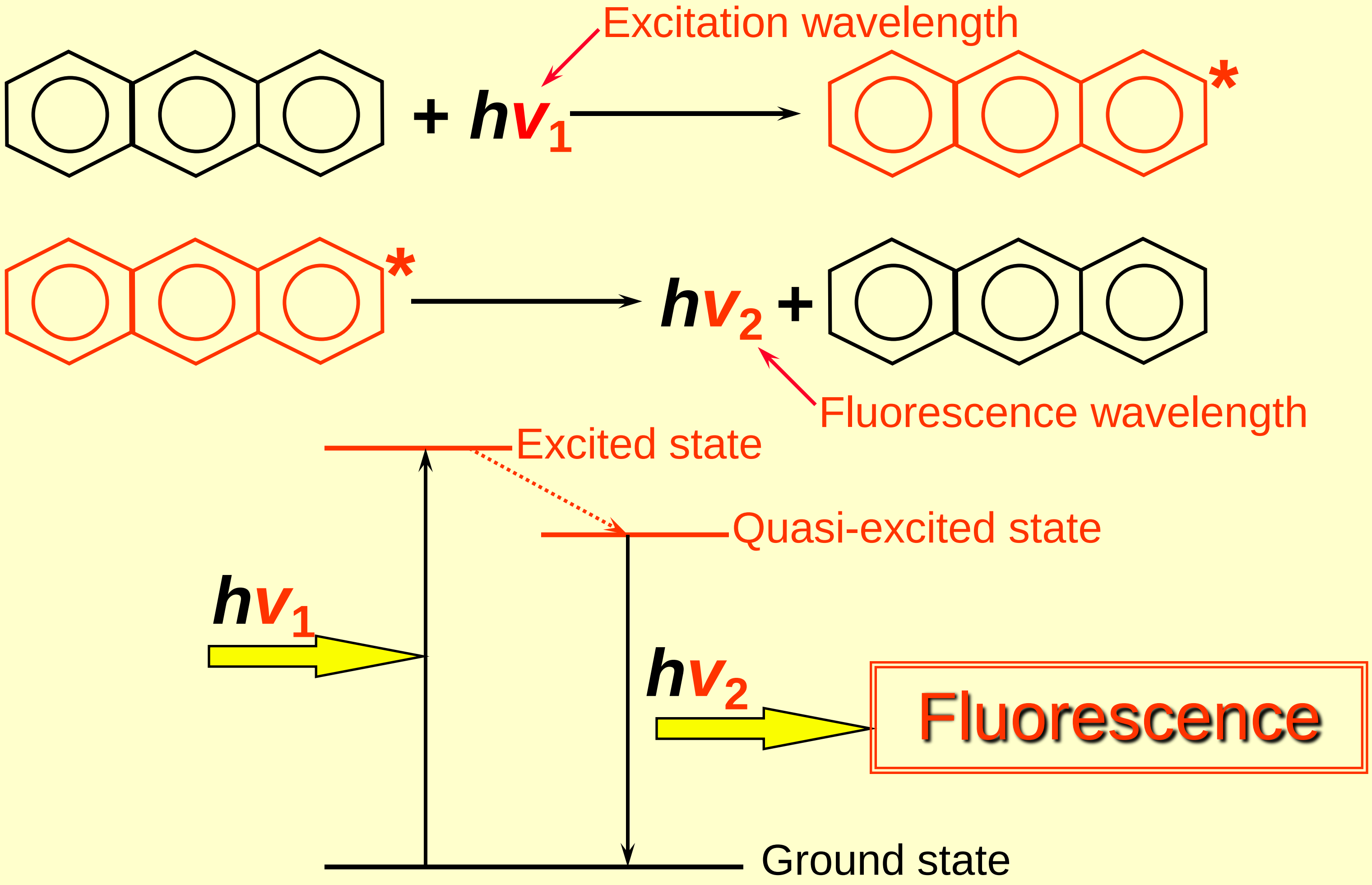
Data Obtained with a Photodiode Array Detector



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



Excitation wavelength

+ $h\nu_1$ → *

* → $h\nu_2$ +

Fluorescence wavelength

Excited state

Quasi-excited state

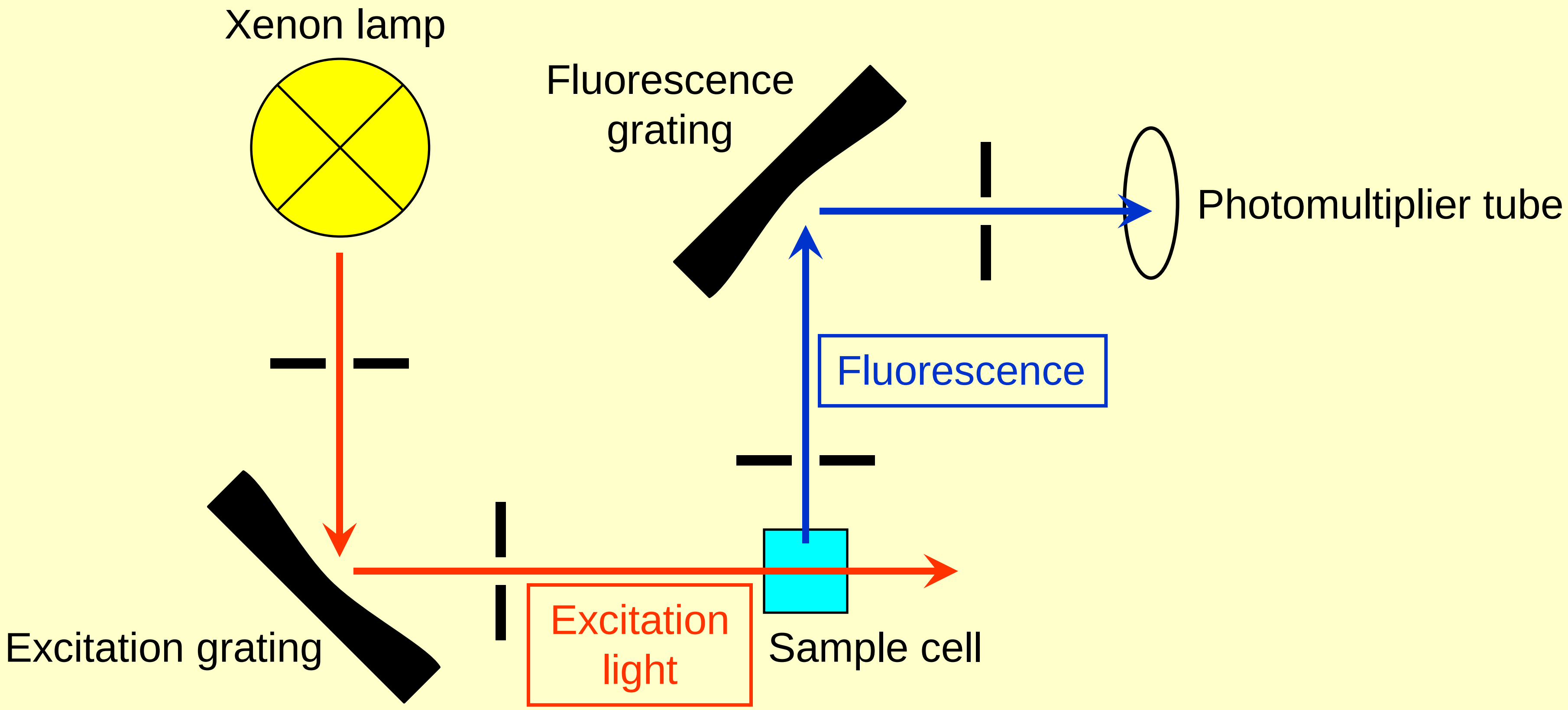
$h\nu_1$

$h\nu_2$

Fluorescence

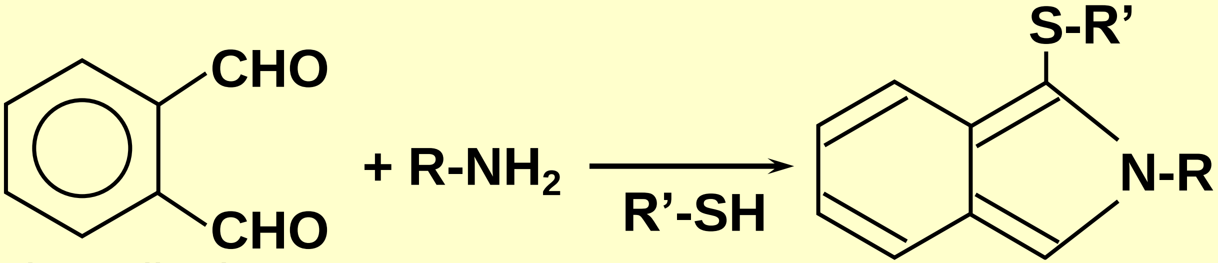
Ground state

Optical System of Fluorescence Detector



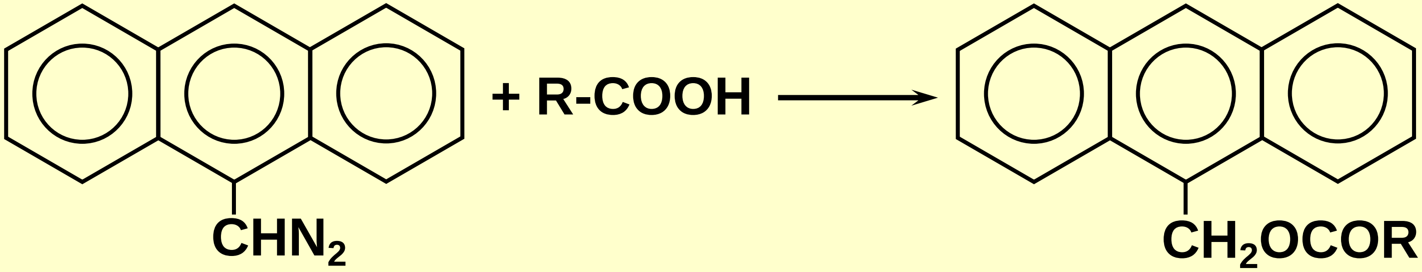
Fluorescence Derivatization Reagents

- OPA Reagent (Reacts with Primary Amines)



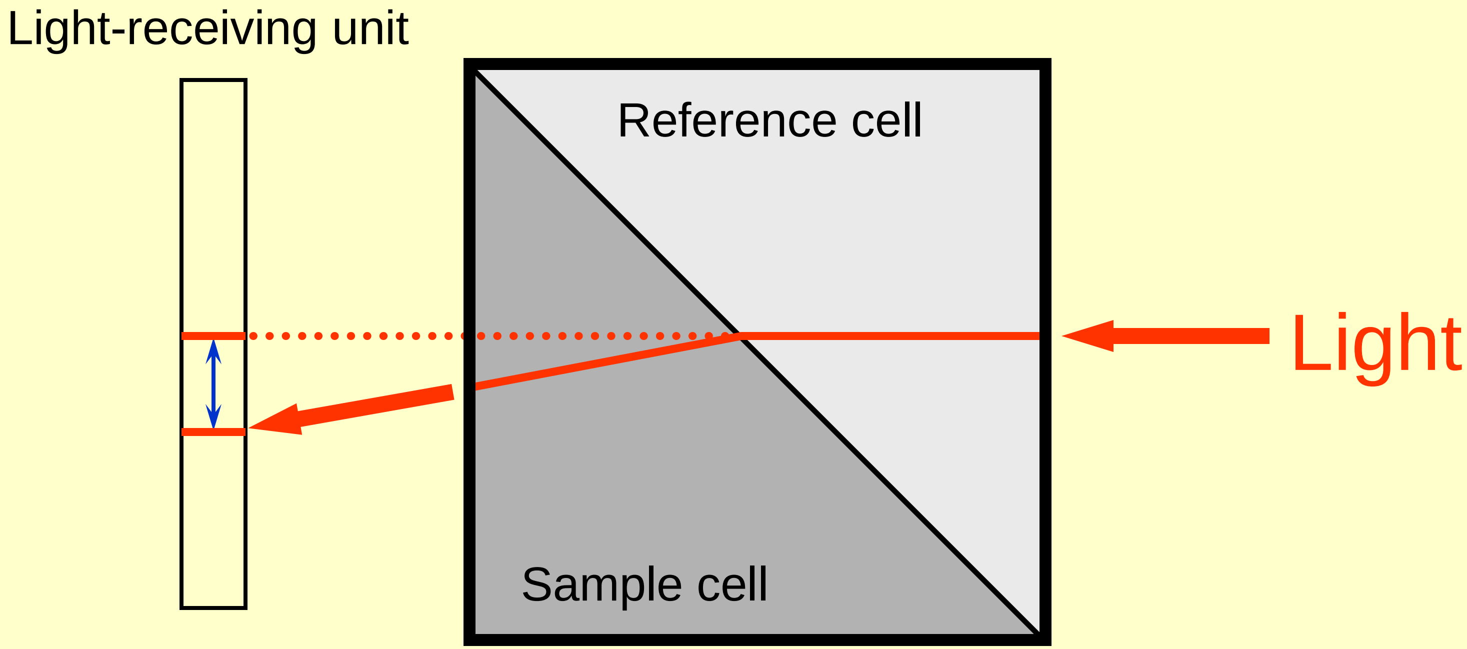
o-phthalaldhyde
(OPA)

- ADAM Reagent (Reacts with Fatty Acids)

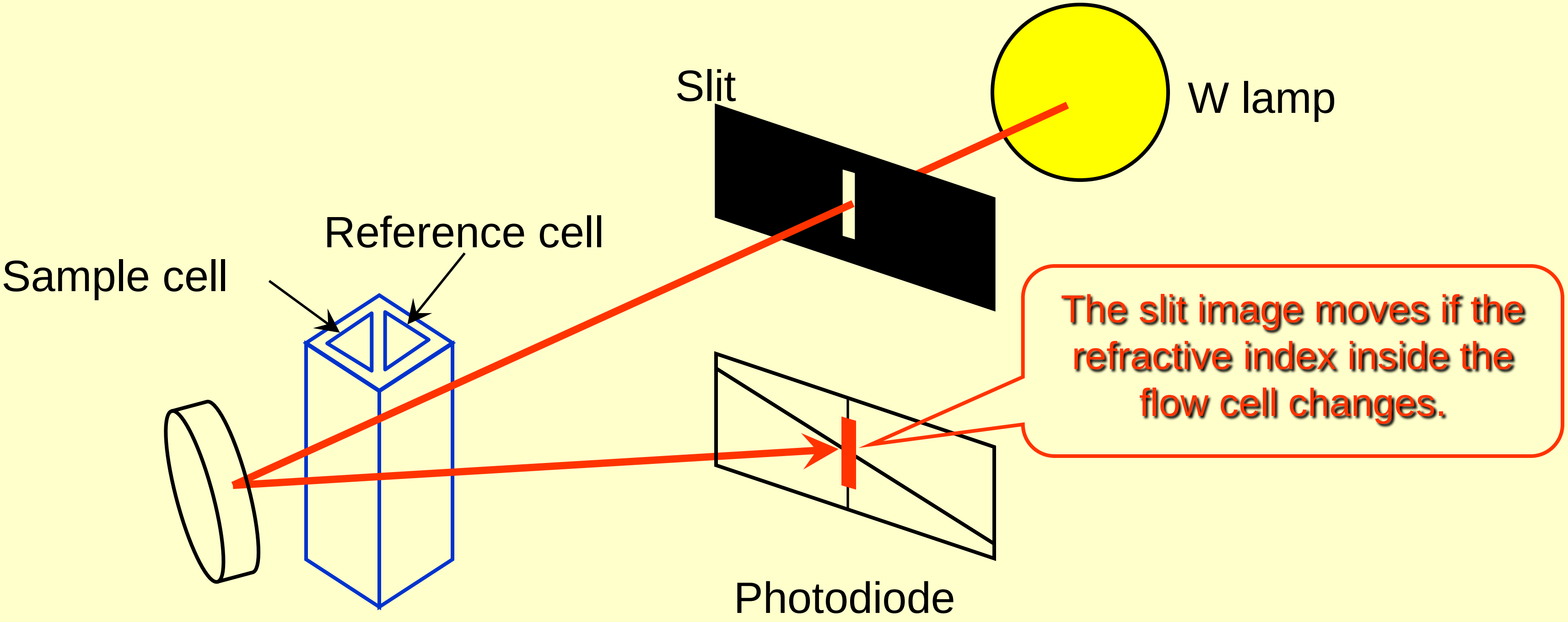


9-anthryldiazomethane
(ADAM)

Differential Refractive Index Detector (Deflection-Type)

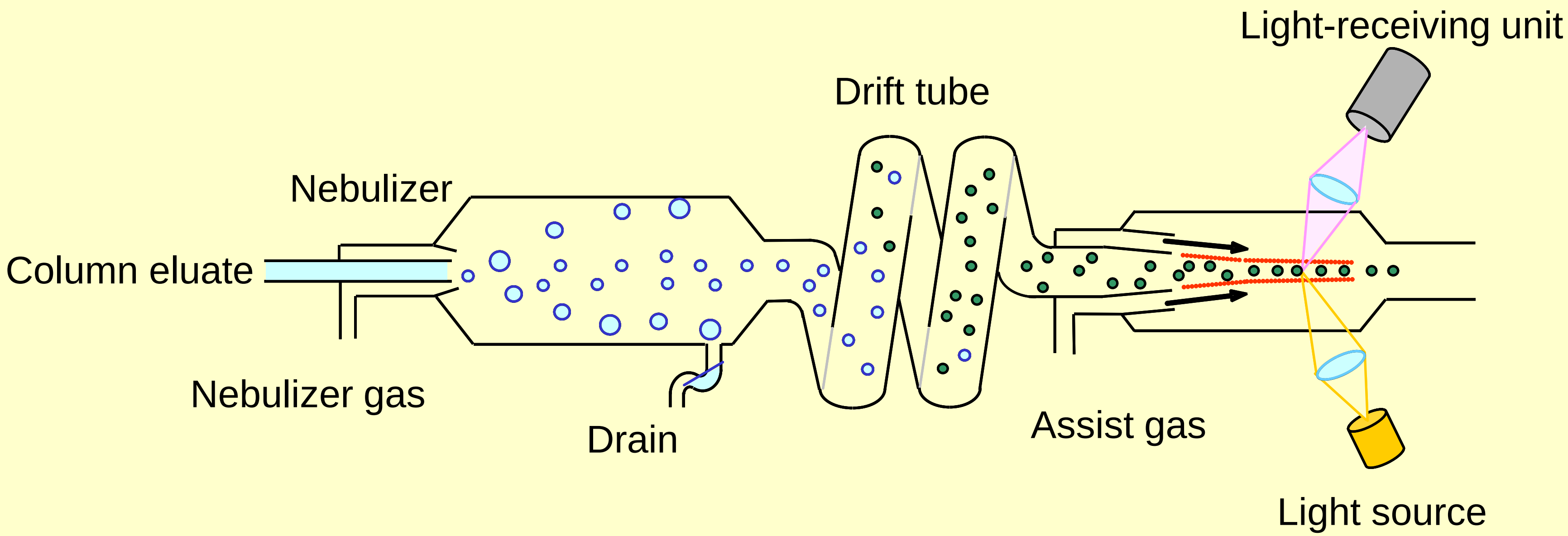


Optical System of Differential Refractive Index Detector (Deflection-Type)



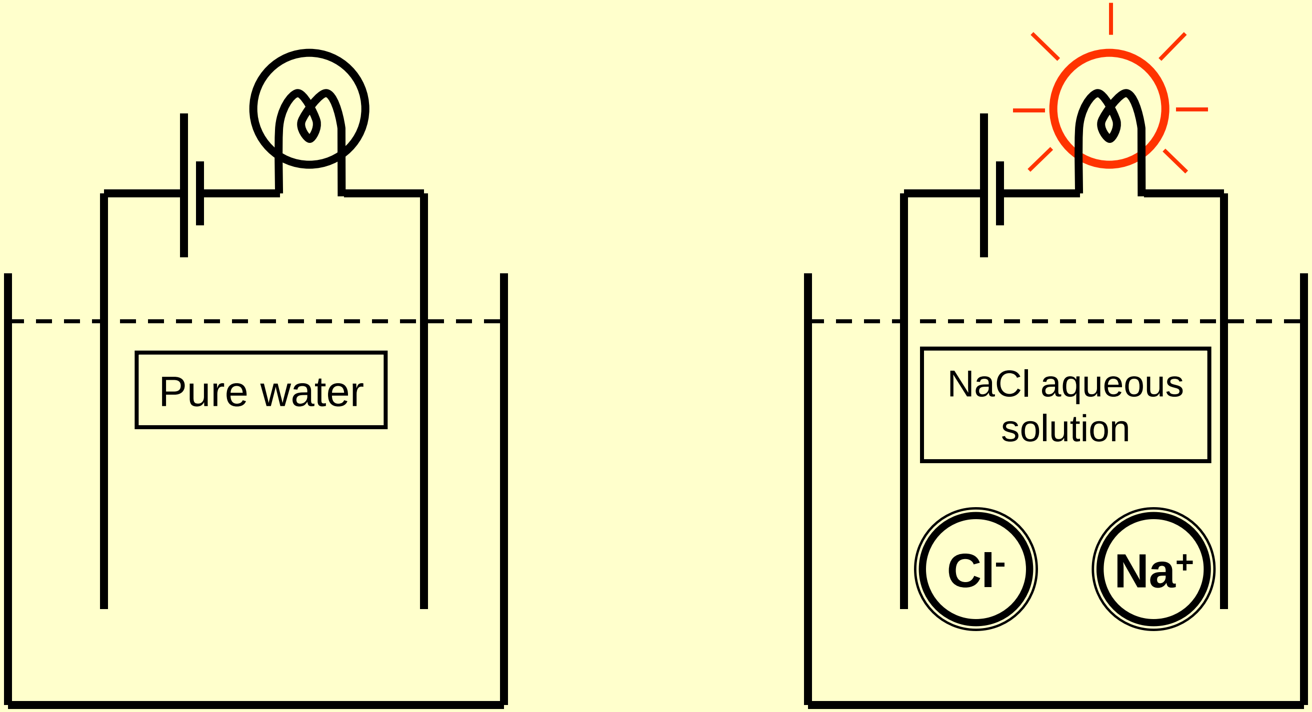
The slit image moves if the refractive index inside the flow cell changes.

Evaporative Light Scattering Detector



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

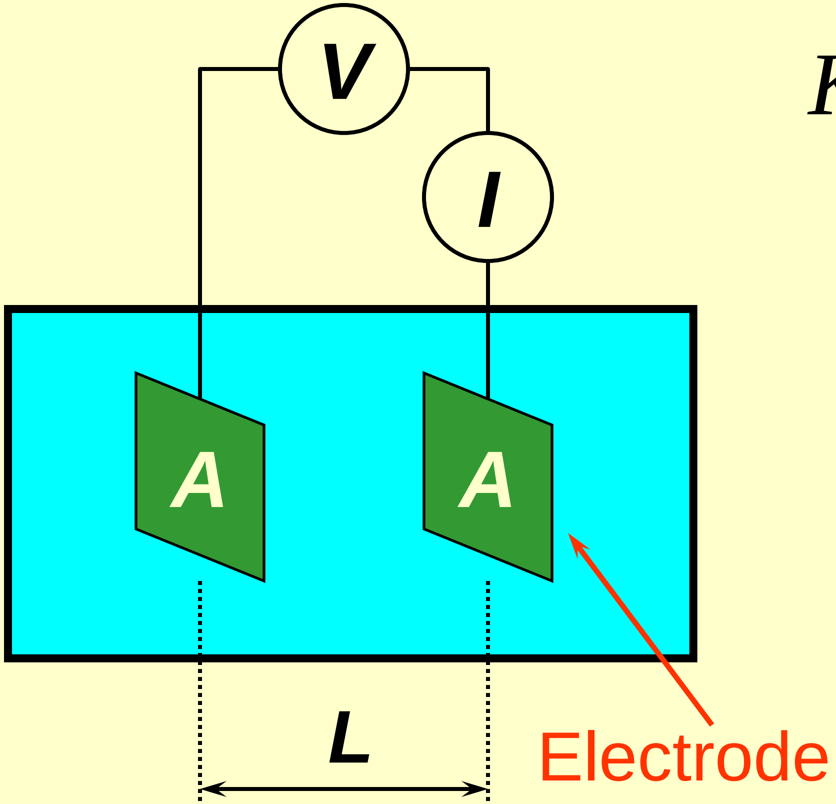
Electrical Conductivity Detector



The bulb does not light with water.

The bulb lights if there are ions.

Principle of Electrical Conductivity Detector



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

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

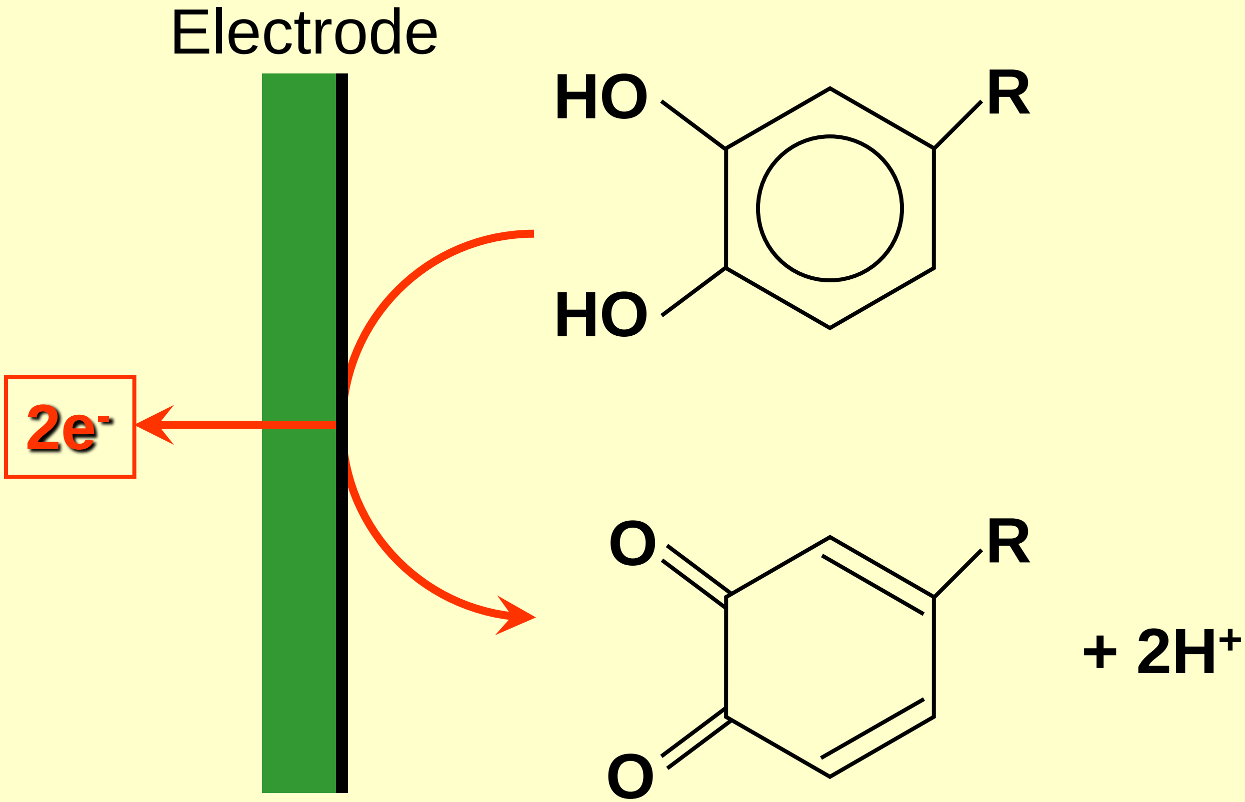
K: Electrical conductivity [S]
I: Electric current [A]
E: Voltage [V]
A: Electrode surface area [cm^2]
L: Distance between electrodes [cm]
k: Specific electrical conductivity [$\mathrm{S \bullet cm^{-1}}$]

Limiting Equivalent Ion Conductance, λ [$S \cdot cm^2/mol$], in Aqueous Solution (25ºC)

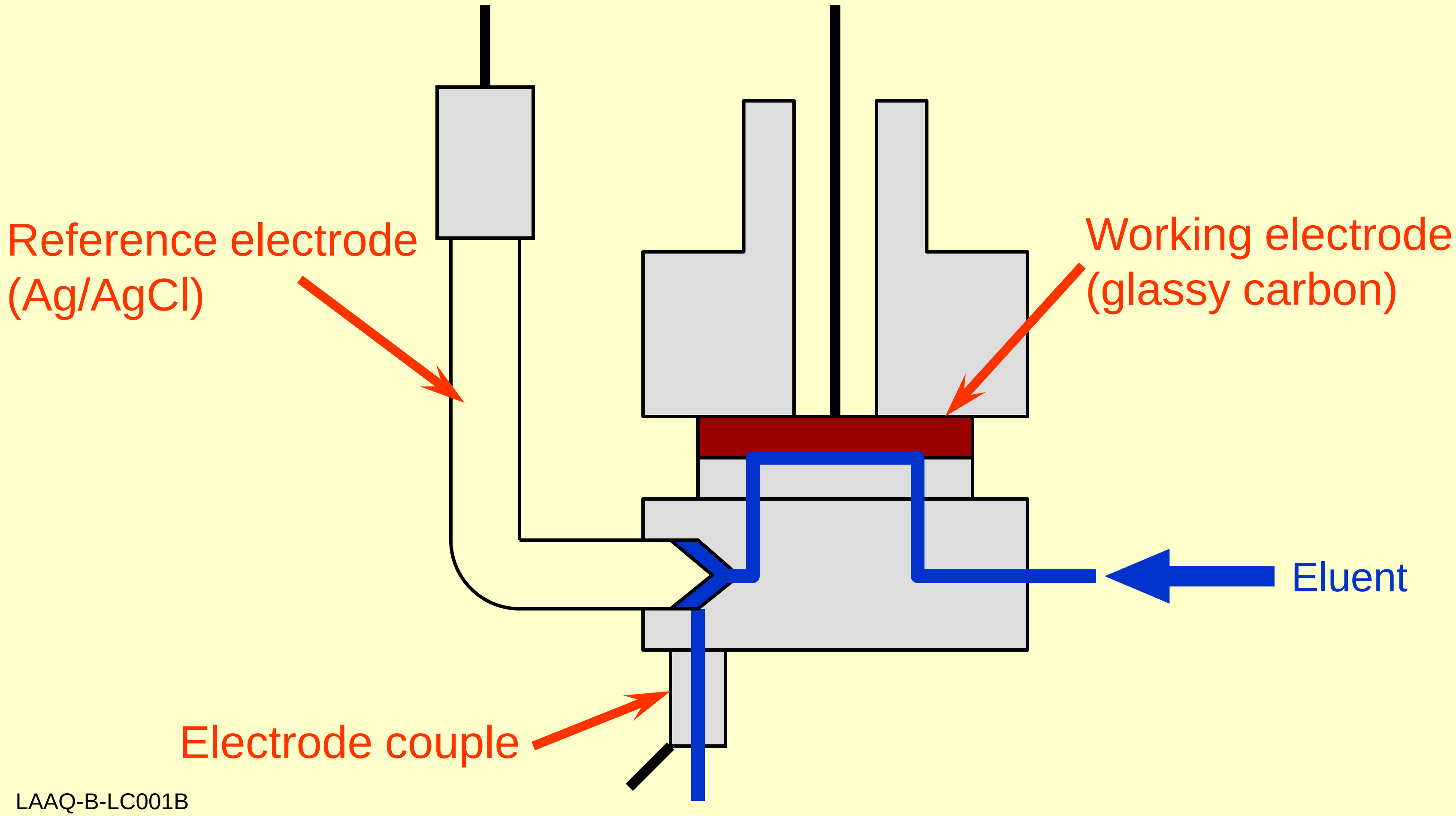
Cation	λ
H^+	349.8
Li^+	38.6
Na^+	50.1
K^+	73.5
NH_4^+	73.5
$(CH_3)_3NH^+$	47.2
Mg^{2+}	53.0
Ca^{2+}	59.5

Anion	λ
OH^-	198.3
F^-	55.4
Cl^-	76.3
Br^-	78.1
NO_3^-	71.4
CH_3COO^-	40.9
$C_6H_5COO^-$	32.3
SO_4^{2-}	80.0

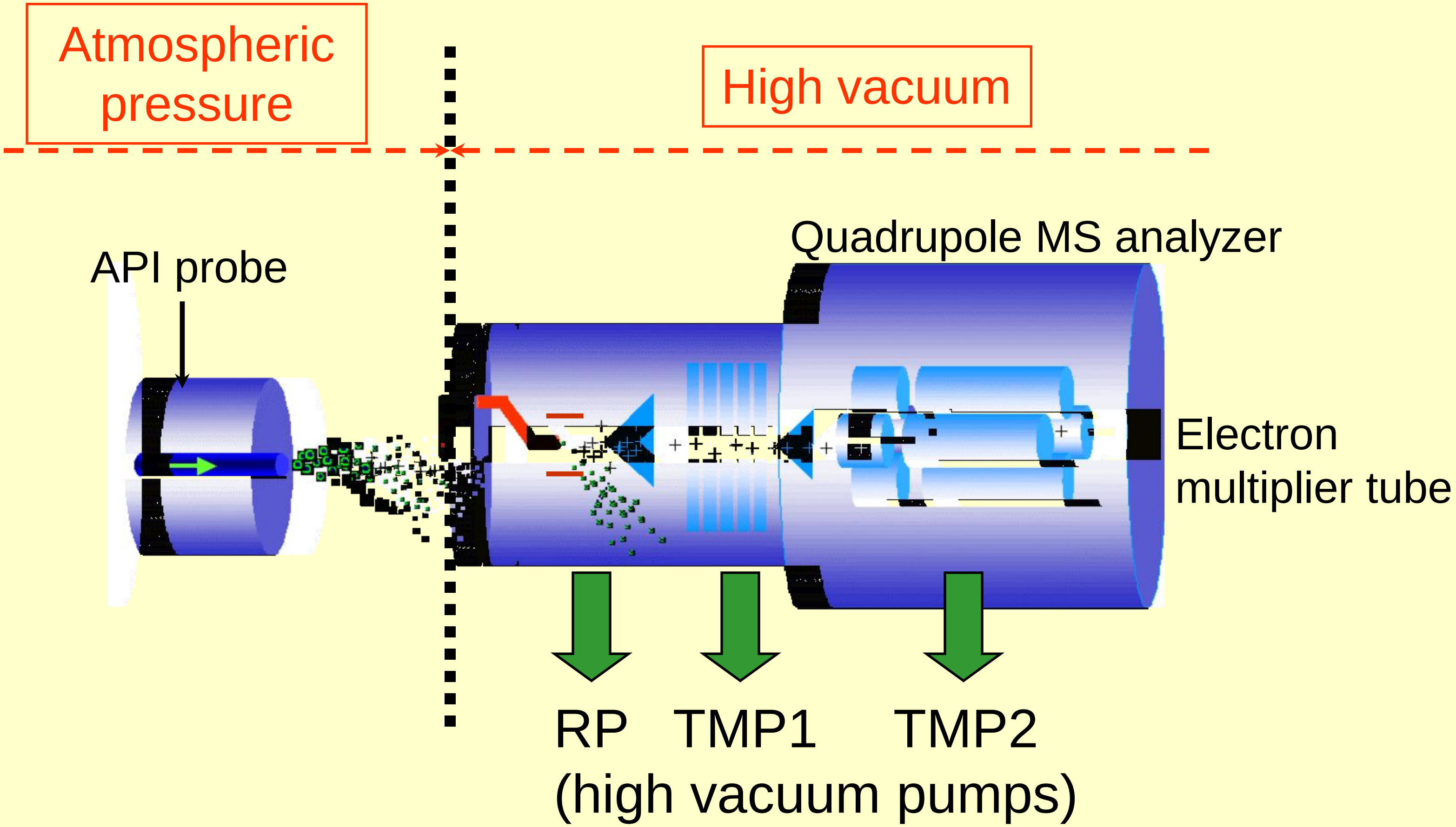
Electrochemical Detector



Cell Structure of Electrochemical Detector (Amperometric Type)



Mass Spectrometer (LCMS)



Atmospheric pressure

High vacuum

API probe

Quadrupole MS analyzer

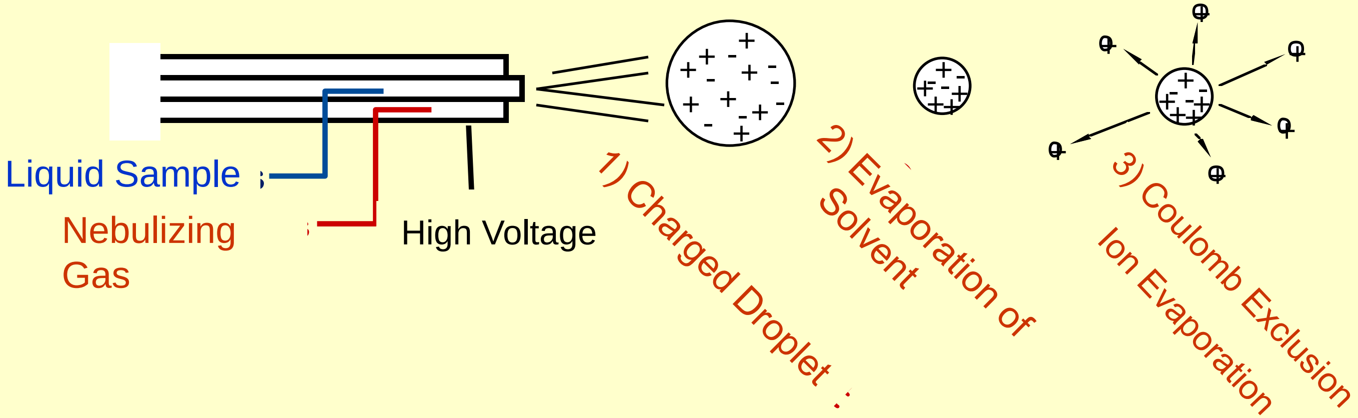
Electron multiplier tube

RP TMP1 TMP2

(high vacuum pumps)

Atmospheric Pressure Ionization

Electrospray Ionization (ESI)



Liquid Sample

Nebulizing Gas

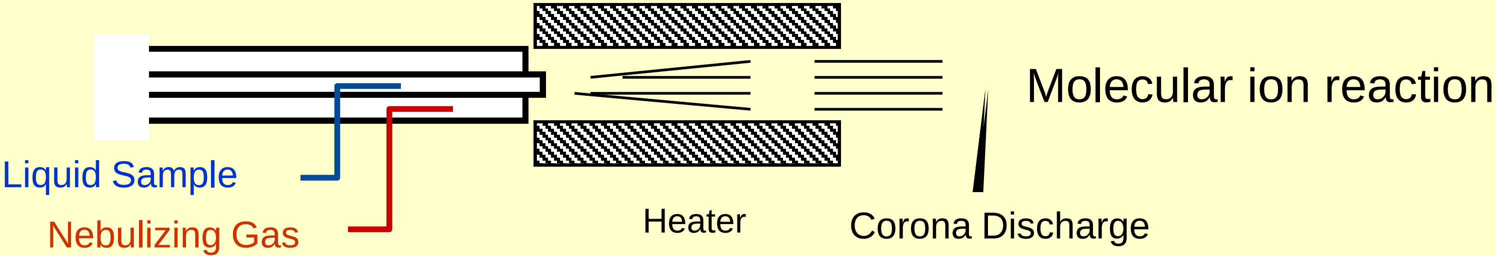
High Voltage

1) Charged Droplet

2) Evaporation of Solvent

3) Coulomb Exclusion Ion Evaporation

Atmospheric Pressure Chemical Ionization (APCI)



Liquid Sample

Nebulizing Gas

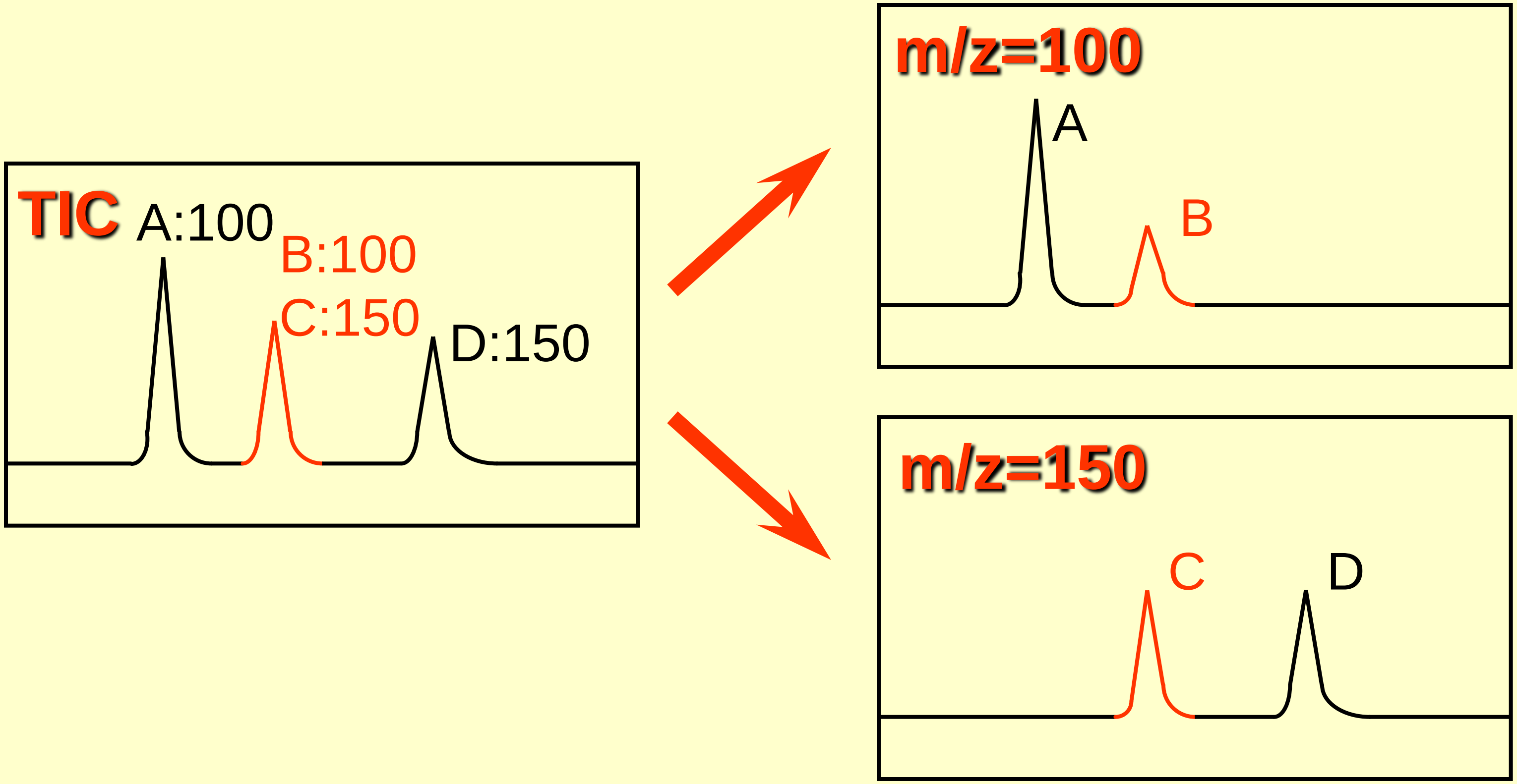
Heater

Corona Discharge Needle

Molecular ion reaction

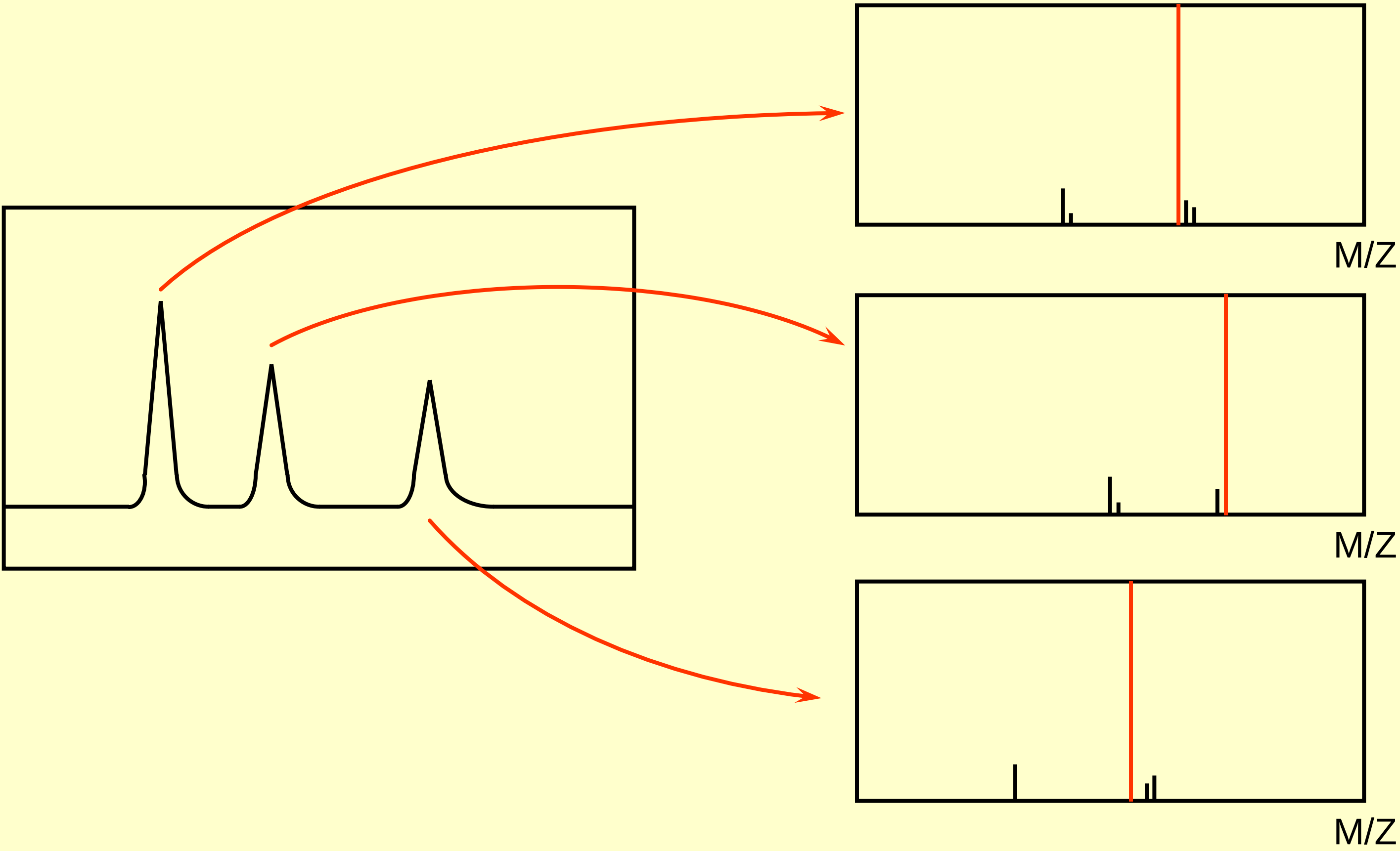
Advantages of LCMS (1)

- Quantitative analysis with excellent selectivity



Advantages of LCMS (2)

- Peaks can be identified with MS spectra.

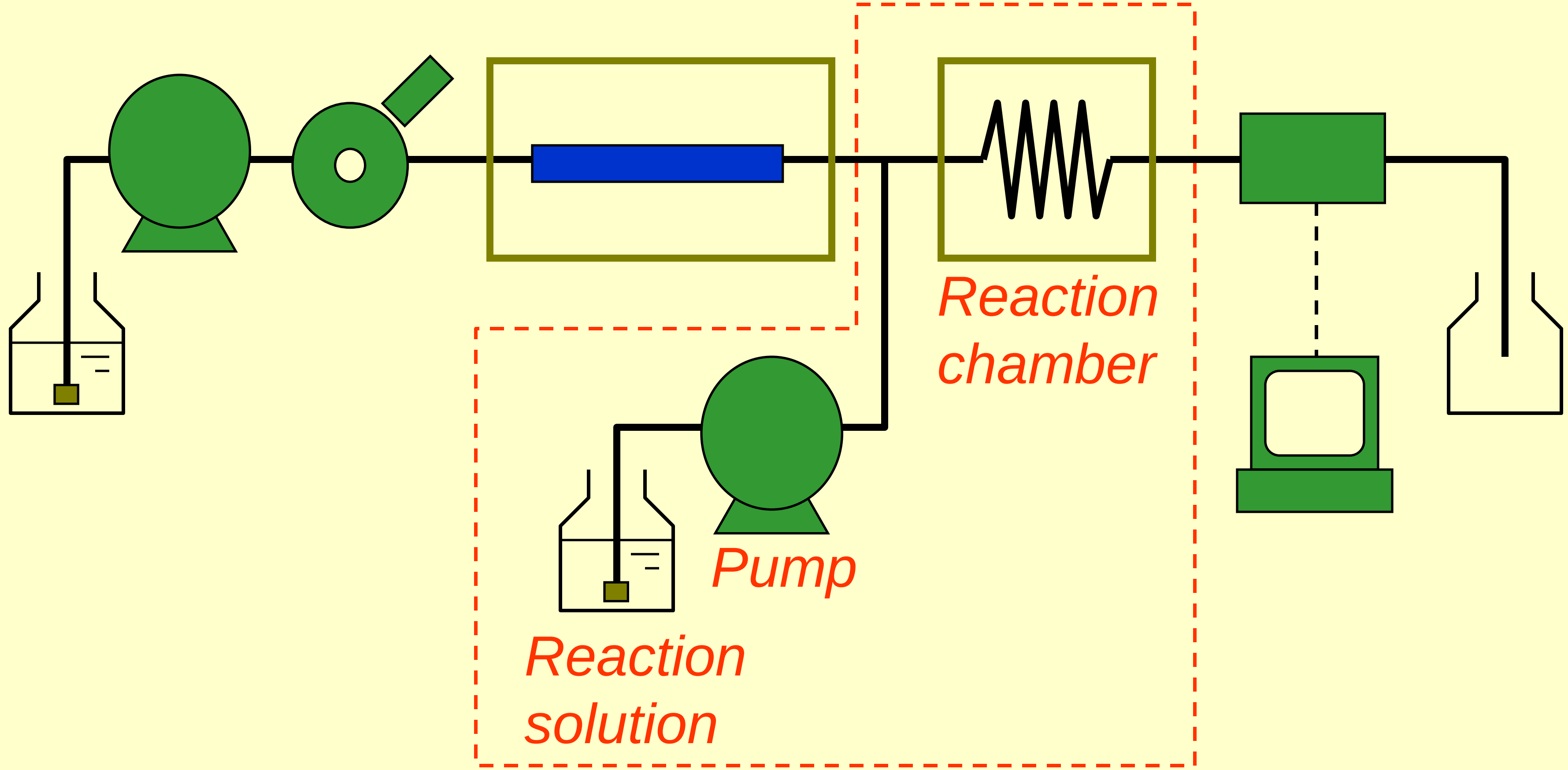


Comparison of Detectors

	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

Note: The above table indicates general characteristics. There are exceptions.

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

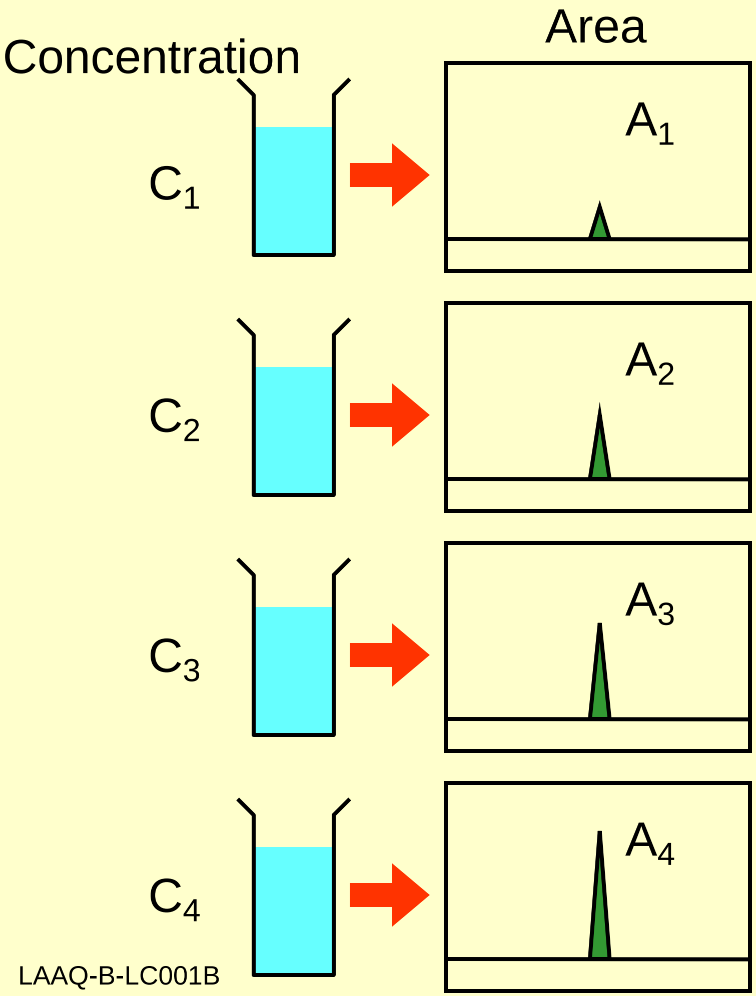
Qualitative Analysis

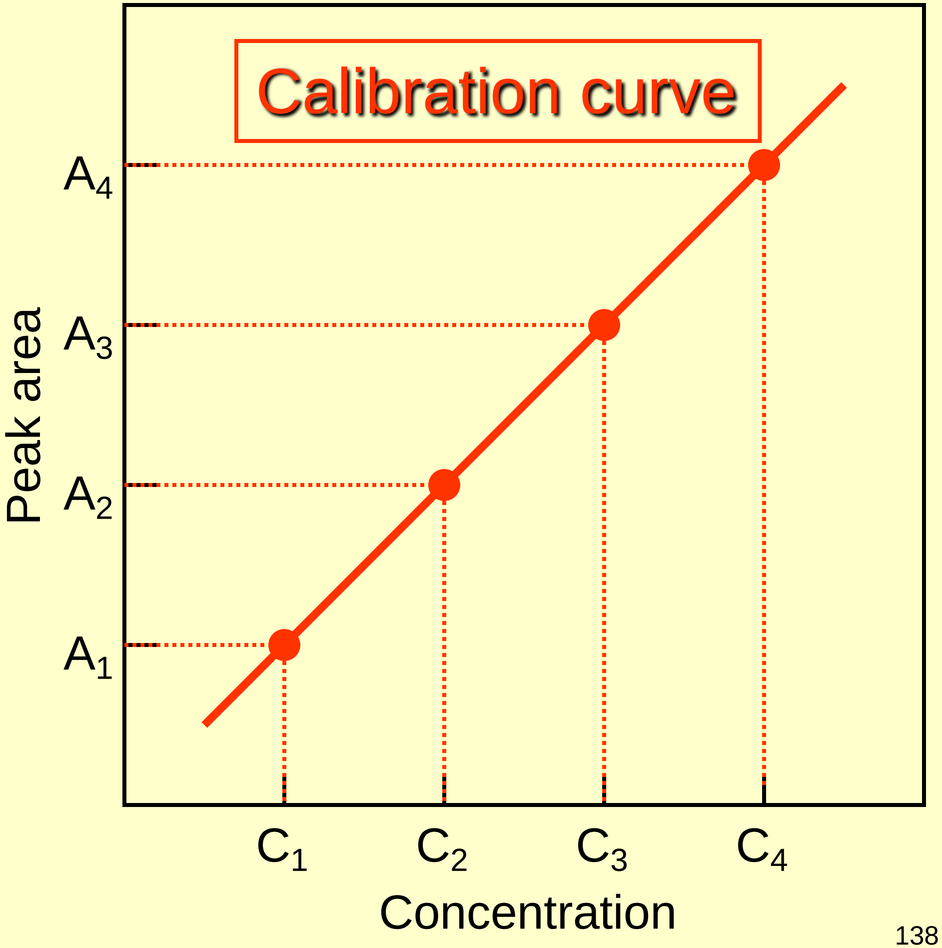
- Identification based on retention time
- Acquisition of spectra with detector
    - UV spectra
    - MS spectra
- Transfer to other analytical instruments after preparative separation

Quantitative Analysis

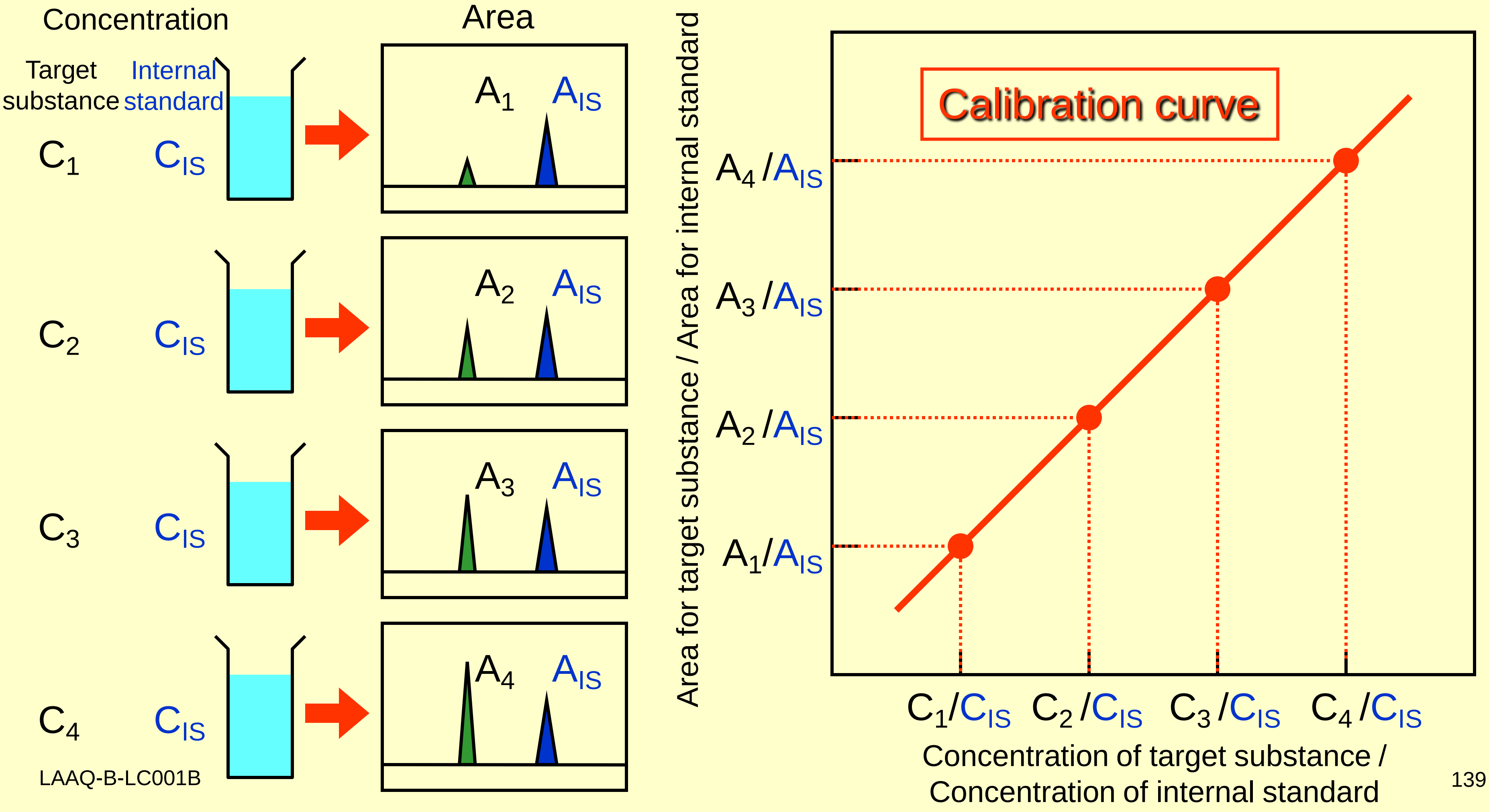
- Quantitation performed with peak area or height.
- Calibration curve created beforehand using a standard.
    - Absolute calibration curve method
    - Internal standard method
    - Standard addition method

Calibration Curve for Absolute Calibration Curve Method



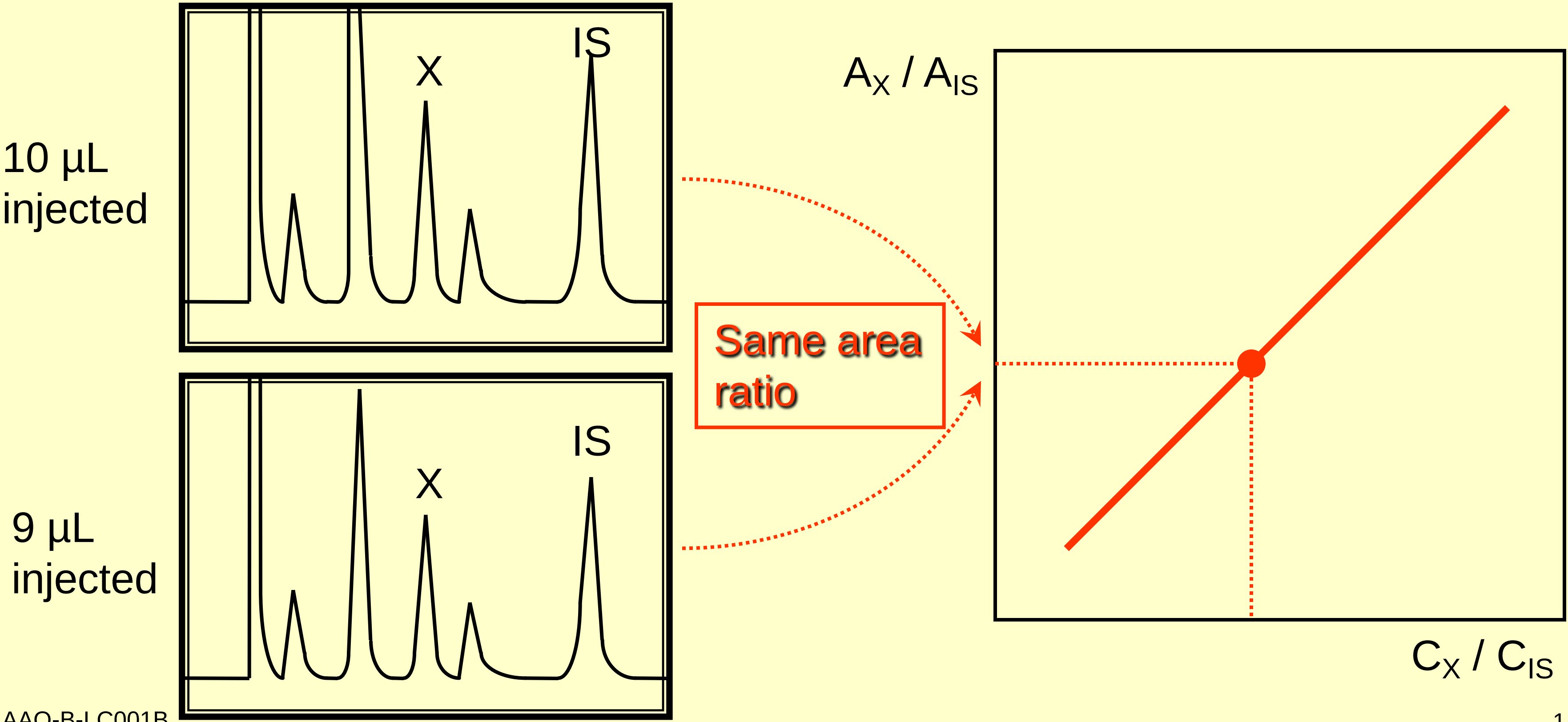


Calibration Curve for Internal Standard Method



Advantages of Internal Standard Method (1)

- Not affected by inconsistencies in injection volume.



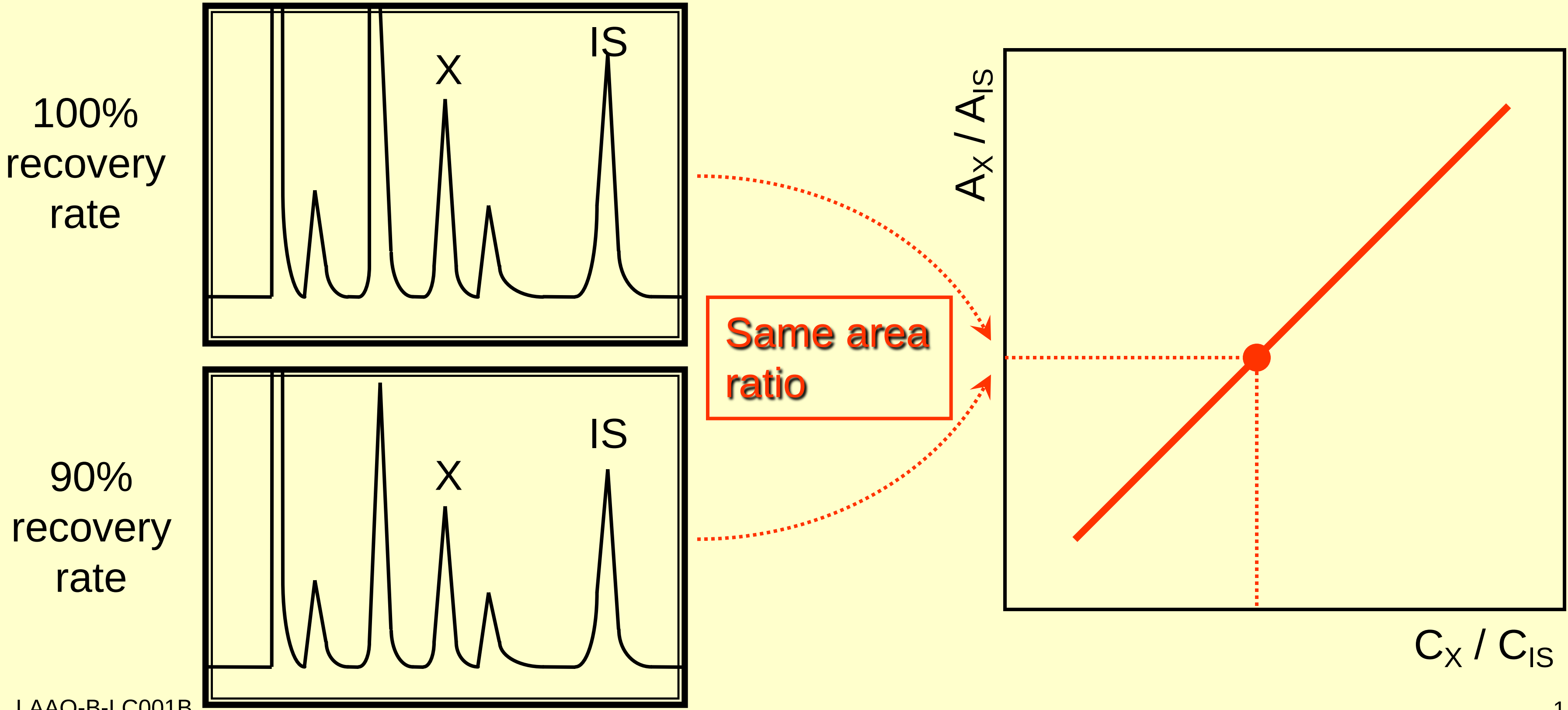
10 µL injected

9 µL injected

Same area ratio

Advantages of Internal Standard Method (2)

- Not affected by the pretreatment recovery rate.



100% recovery rate

90% recovery rate

Same area ratio

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

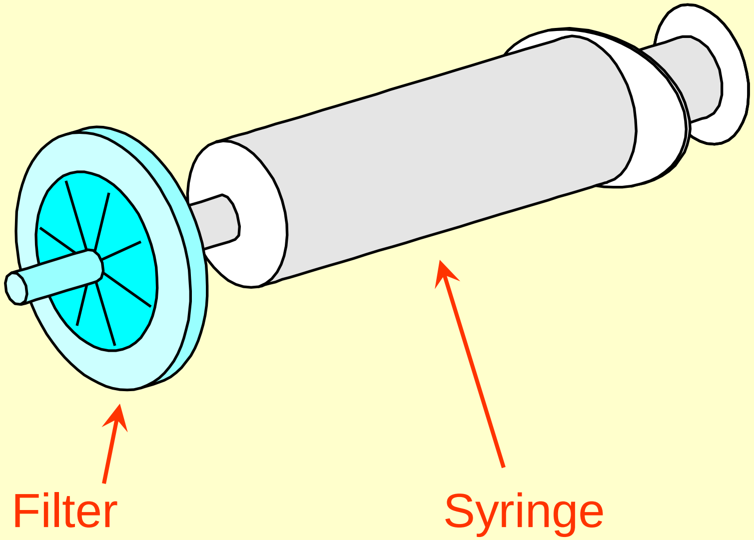
- To improve the accuracy of quantitative values
- To improve sensitivity and selectivity
- To protect and prevent the deterioration of columns and analytical instruments
- To simplify measurement operations and procedures
- To stabilize target substances

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

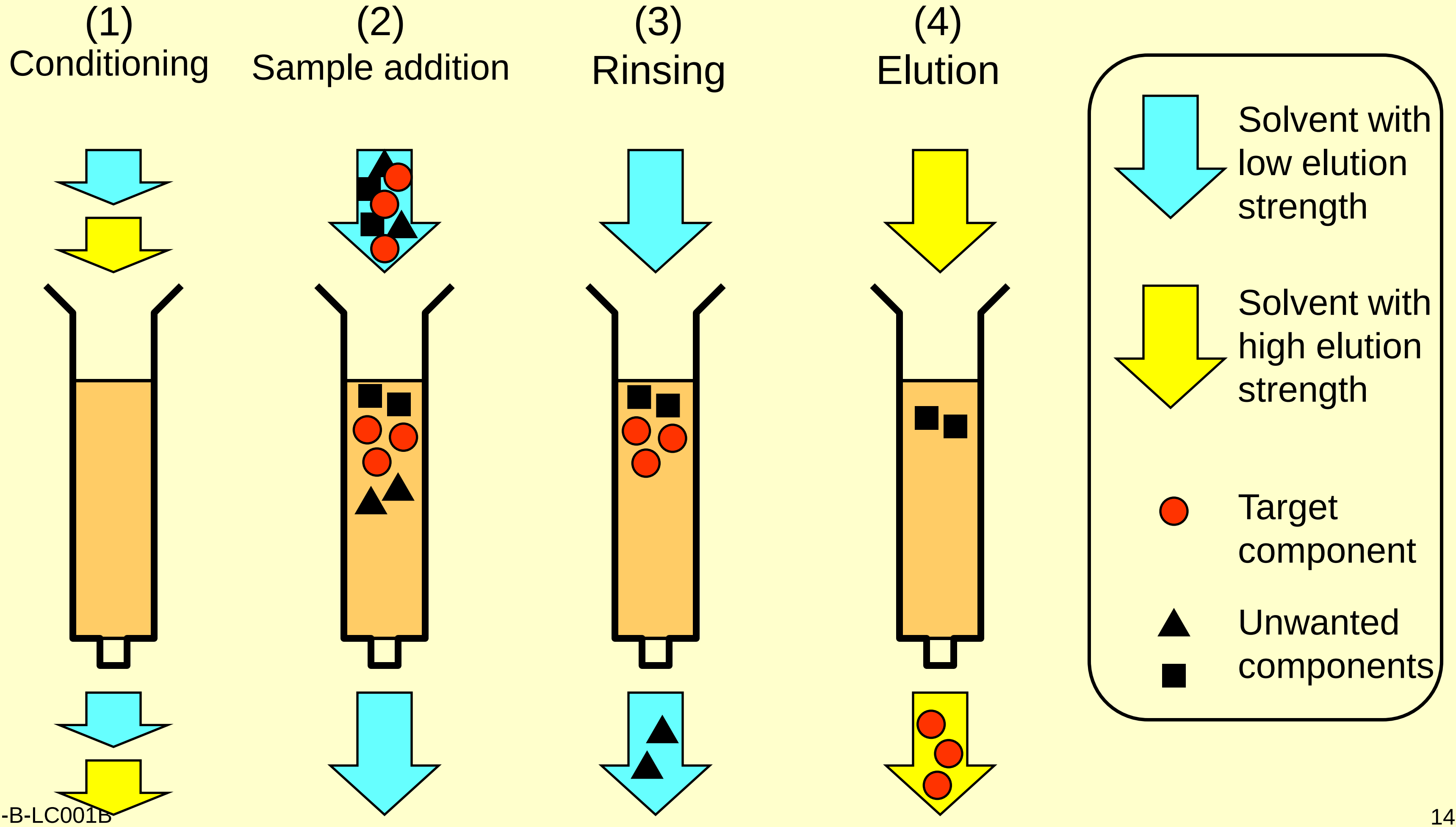
- In general, filter every sample before injection!
- It is convenient to use a disposable filter with a pore diameter of approx. 0.45 µm.
- Centrifugal separation is applicable for samples that are difficult to filter.



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

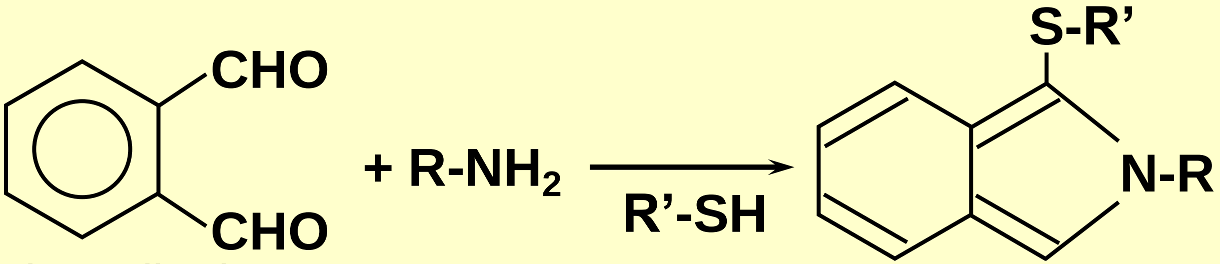
Solvent with high elution strength

● Target component

▲ ■ Unwanted components

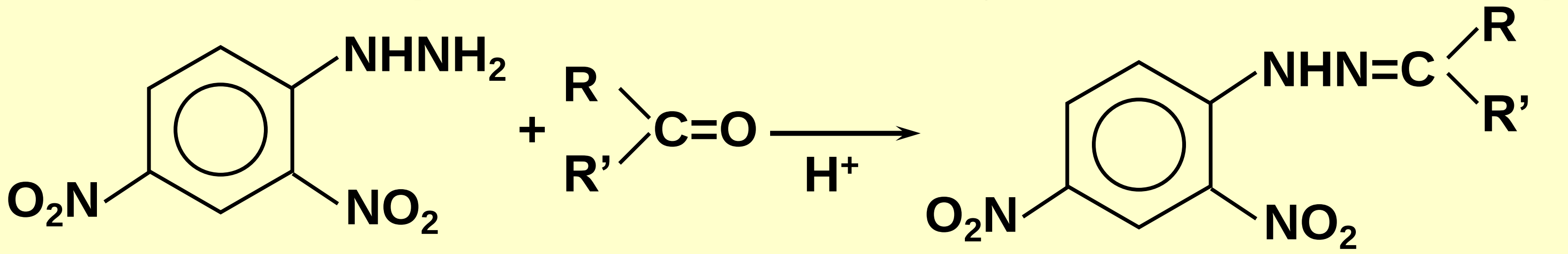
Pre-Column Derivatization

- OPA Reagent (Reacts with Primary Amines)



o-phthalaldhyde
(OPA)

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



2,4-dinitrophenylhydrazine
(2,4-DNPH)

Evaluation of the Reliability of Analysis

Validation of Analytical Methods

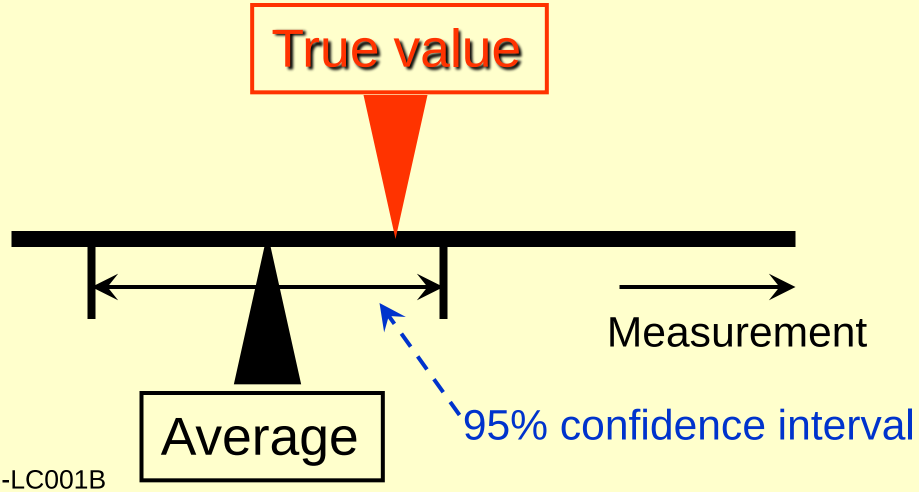
What Is “Validation of Analytical Methods”?

- 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 σ/*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.
    - $QL = 10\ \sigma/slope$
      (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.

Range

- 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	60ºC 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

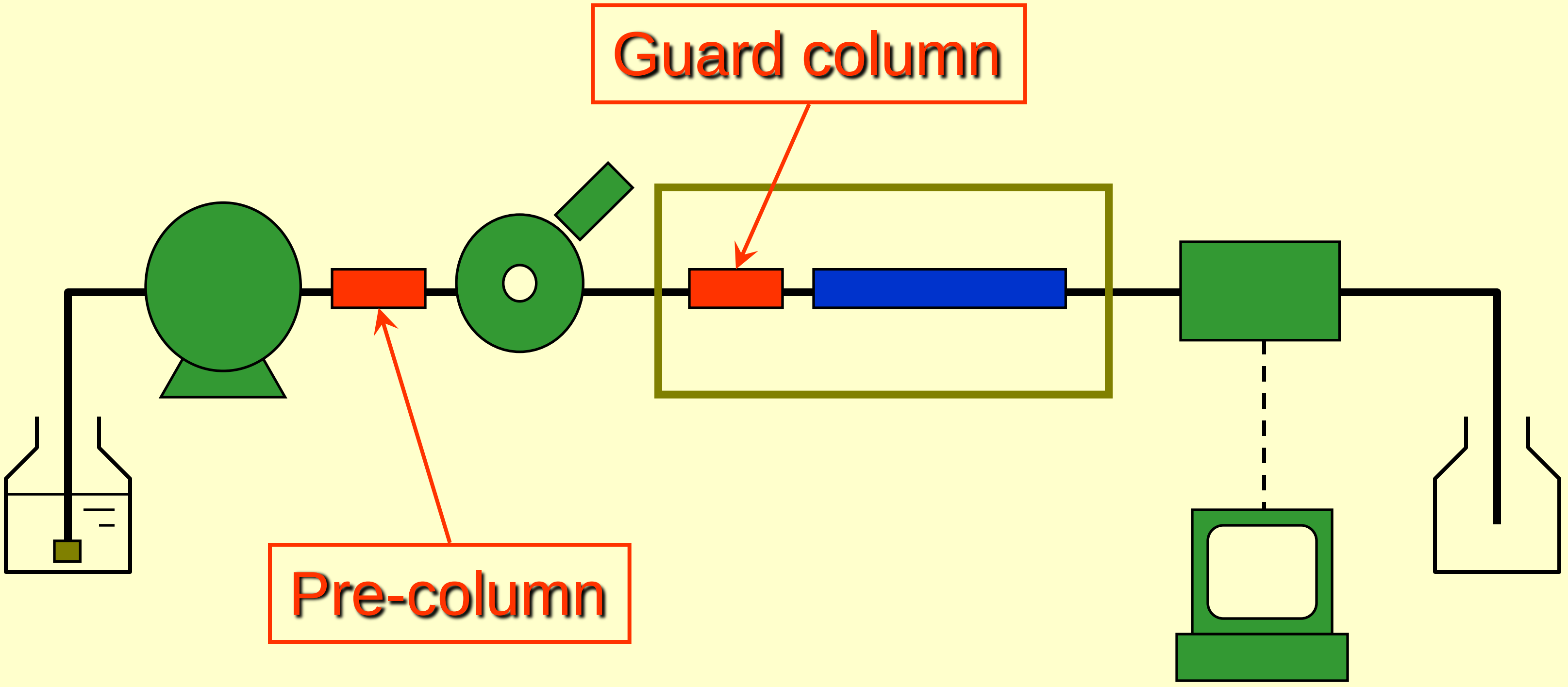
- If the column is identified as the cause...
  - ❖ Rinsing
  - ❖ Replacement

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



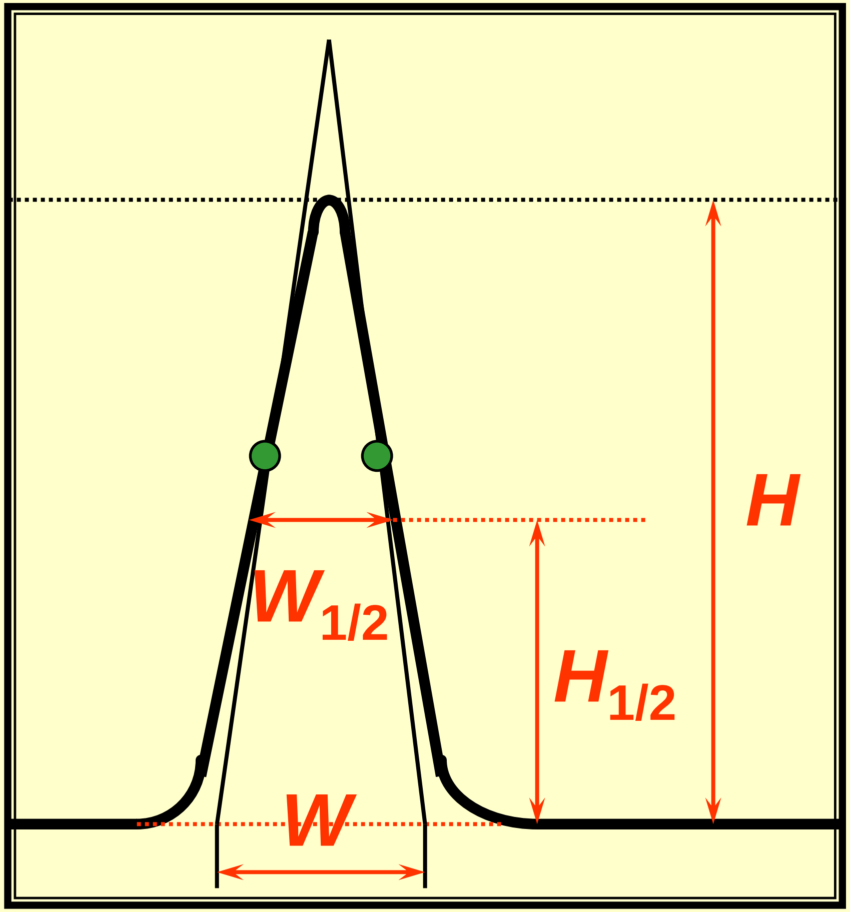
Guard column

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.