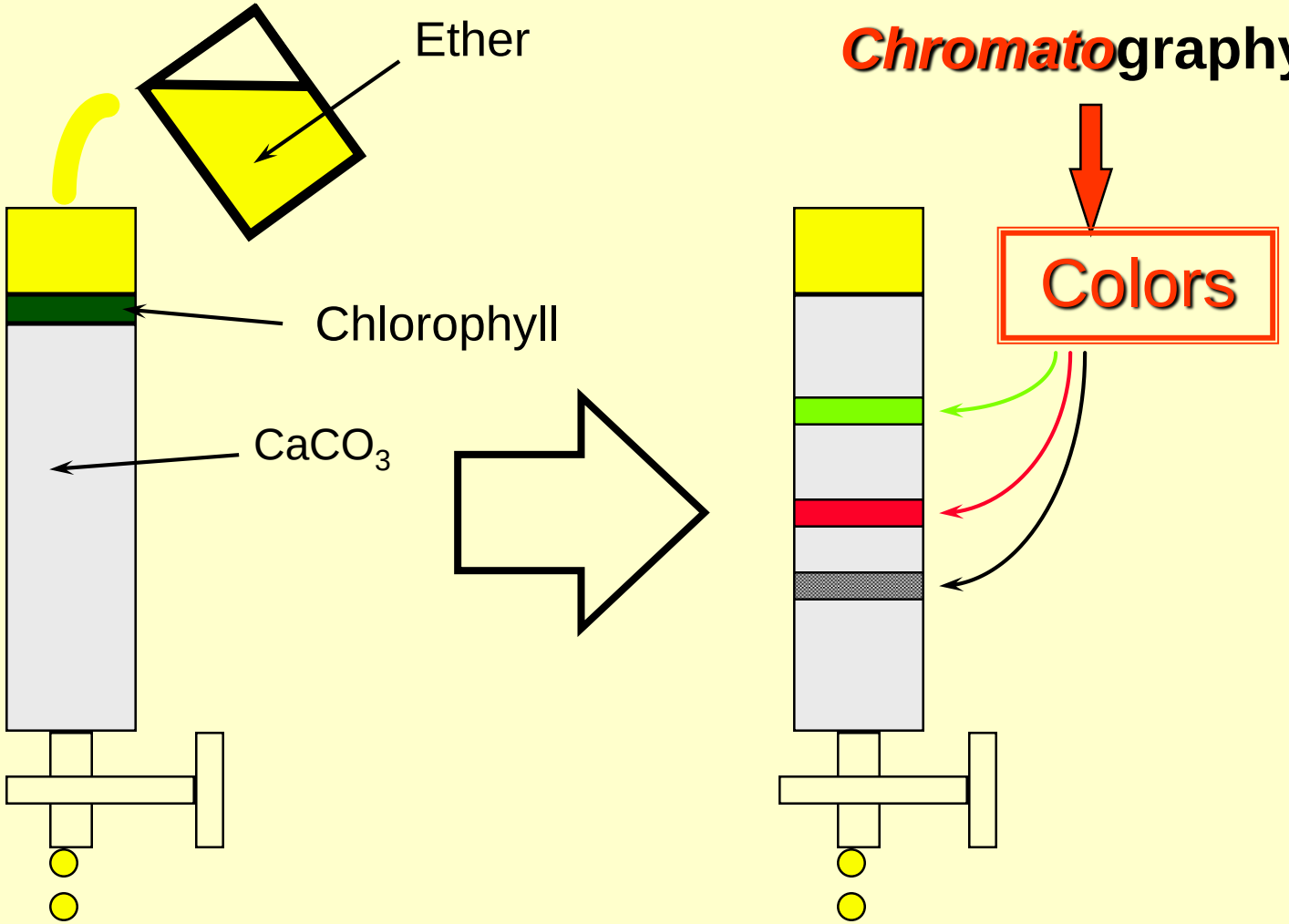
What Is HPLC?

Basic Principles

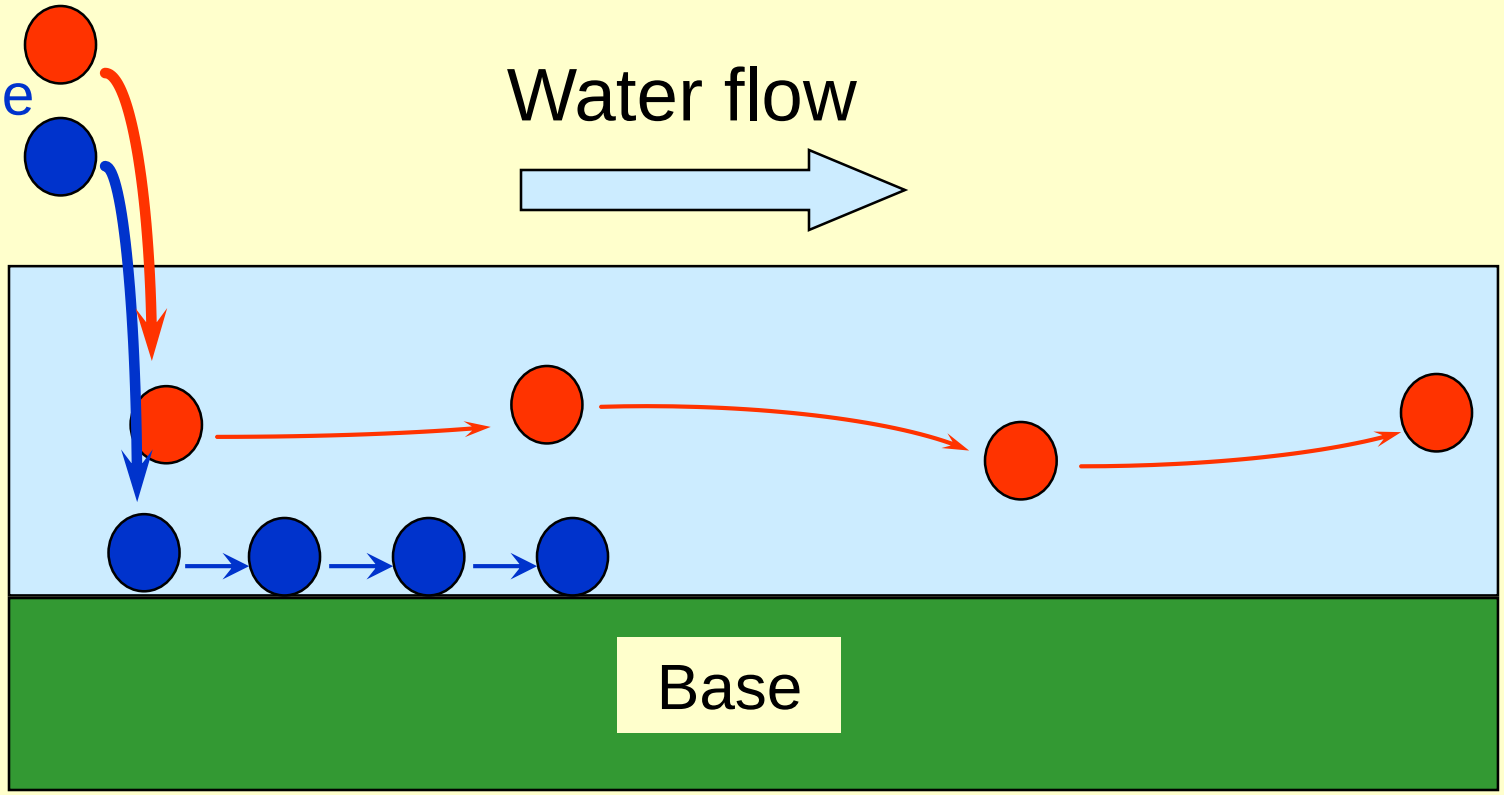
Invention of Chromatography by M. Tswett



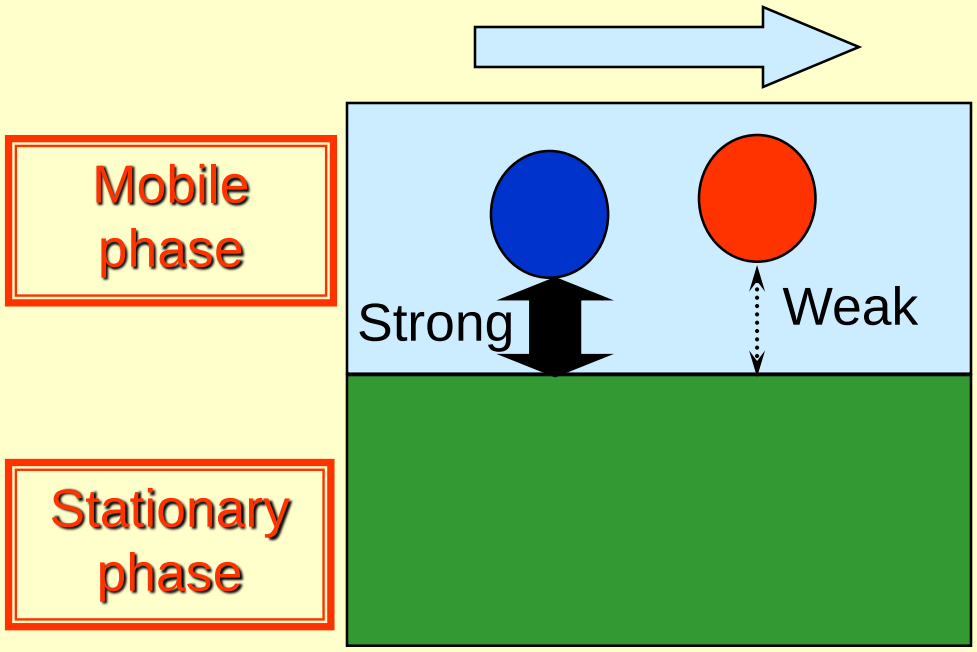
Comparing Chromatography to the Flow of a River...

Light leaf

Heavy stone



Mobile Phase / Stationary Phase

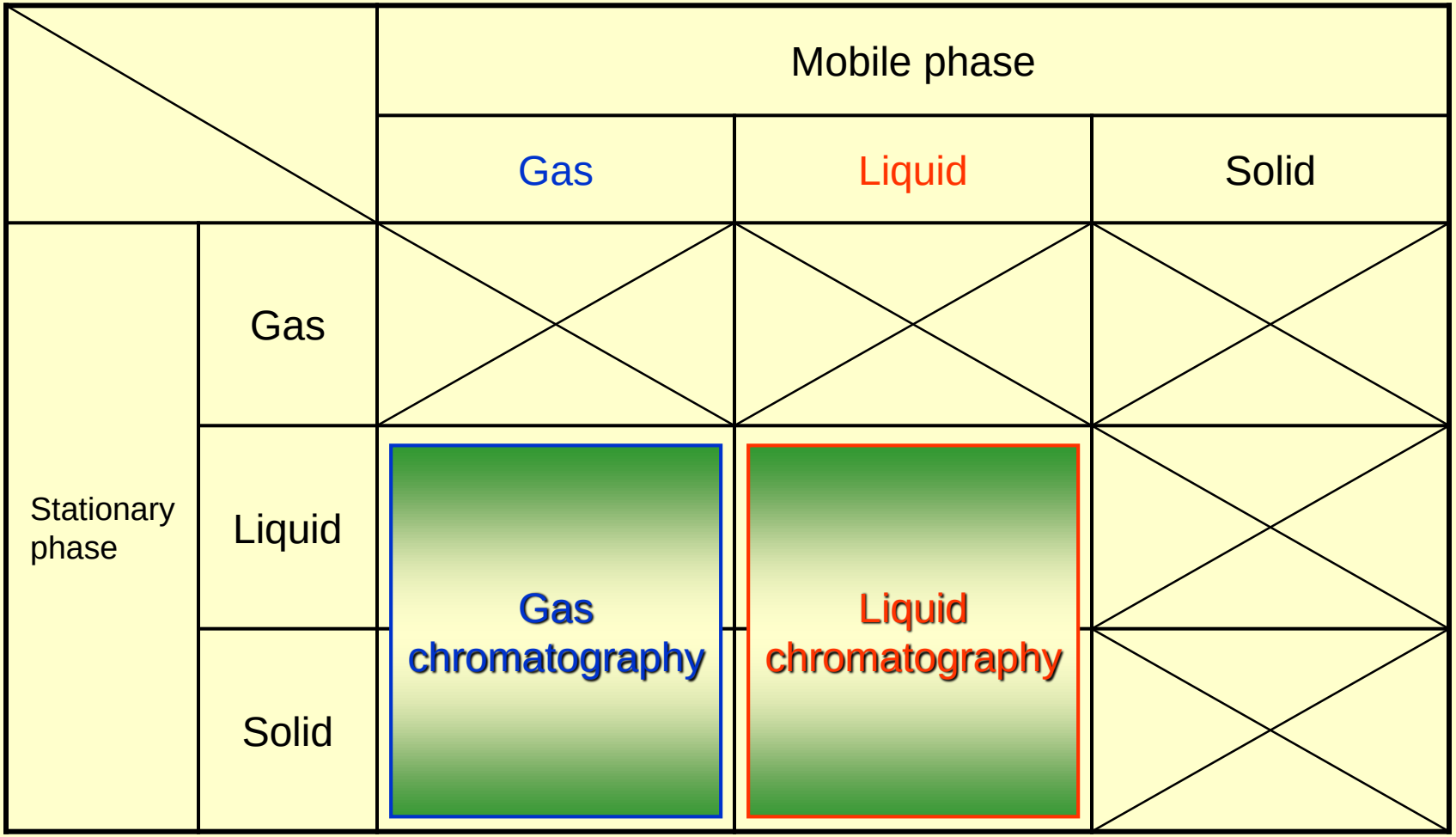


- A site in which a moving phase (mobile phase) and a non-moving phase (stationary phase) make contact via an interface that is set up.
- The affinity with the mobile phase and stationary phase varies with the solute. → Separation occurs due to differences in the speed of motion.

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

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

Three States of Matter and Chromatography Types

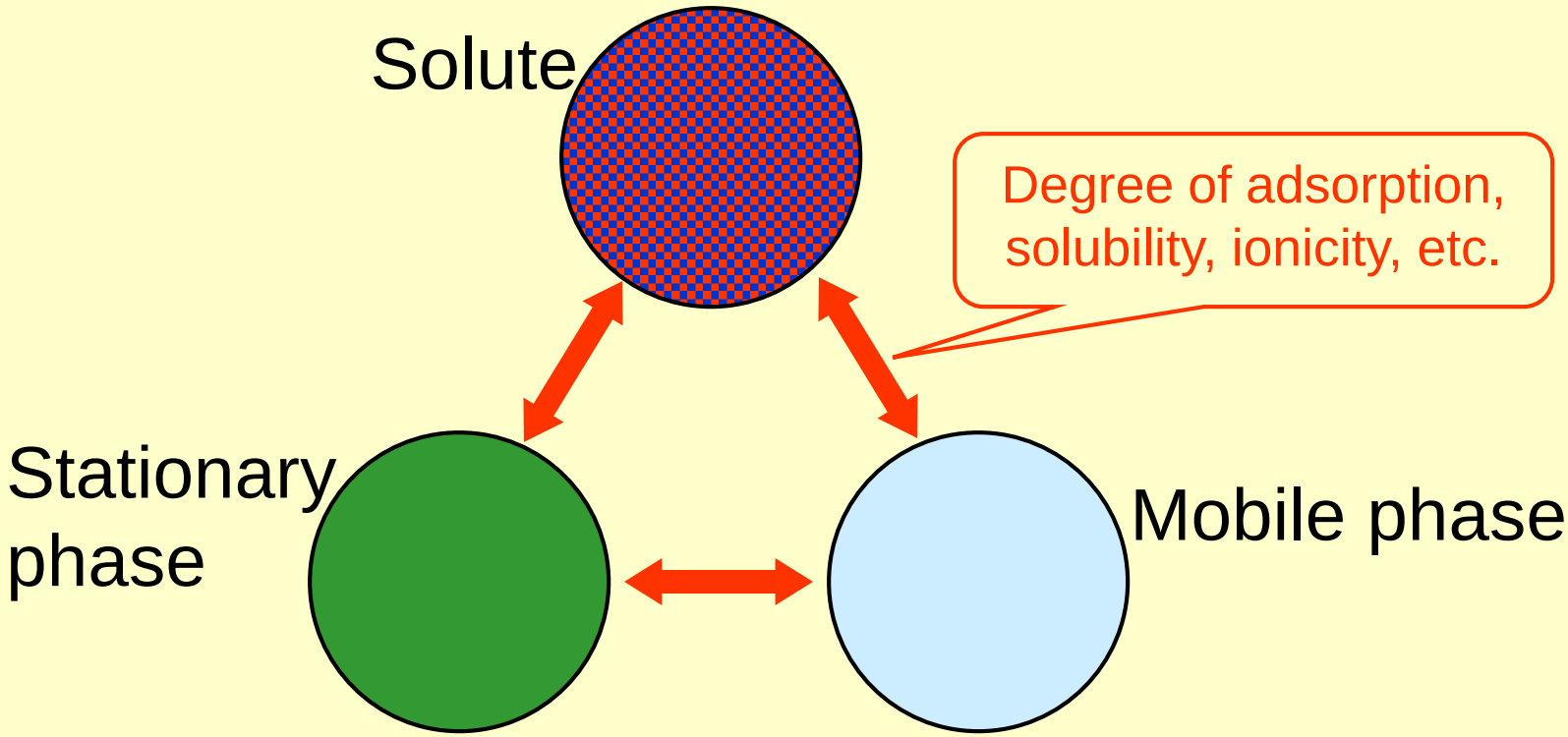


Liquid Chromatography

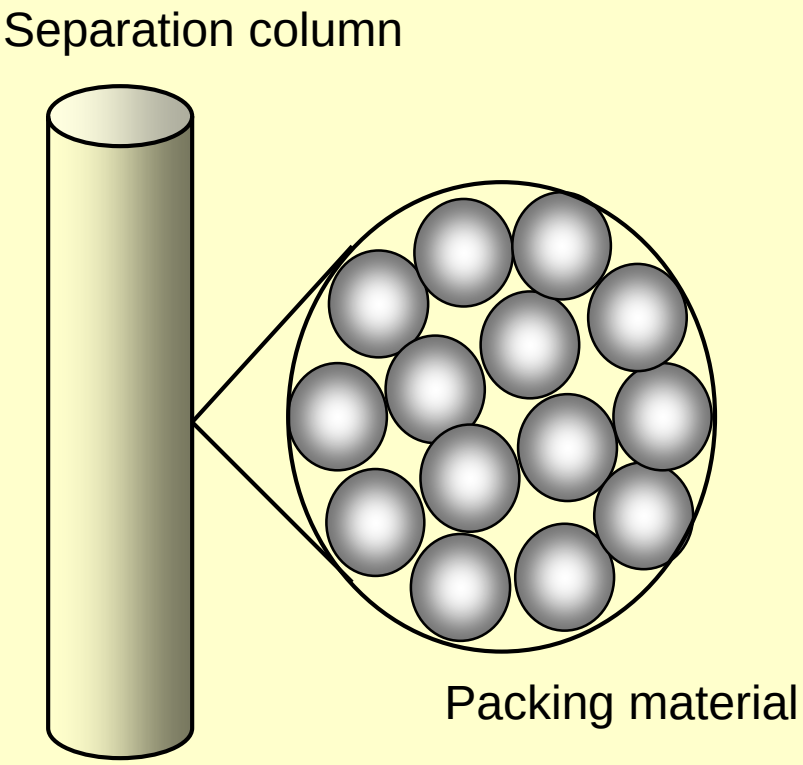
- Chromatography in which the mobile phase is a liquid.
  - The liquid used as the mobile phase is called the "eluent".
- The stationary phase is usually a solid or a liquid.
- In general, it is possible to analyze any substance that can be stably dissolved in the mobile phase.

Interaction Between Solutes, Stationary Phase, and Mobile Phase

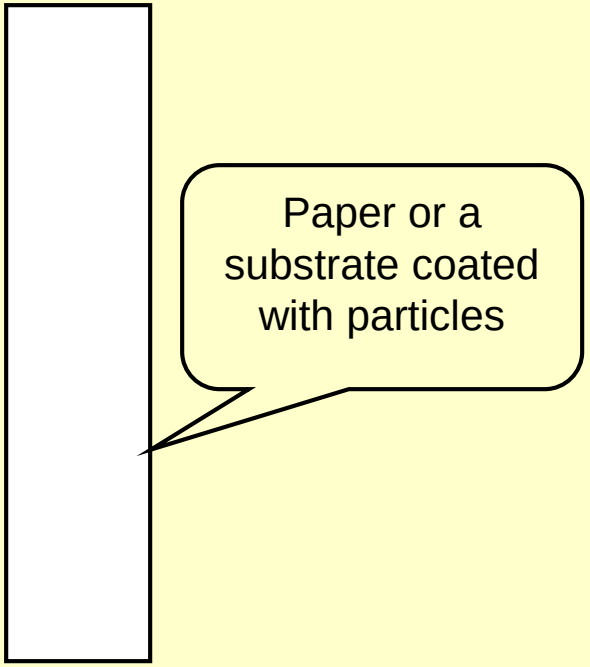
- Differences in the interactions between the solutes and stationary and mobile phases enable separation.



Column Chromatography and Planar Chromatography

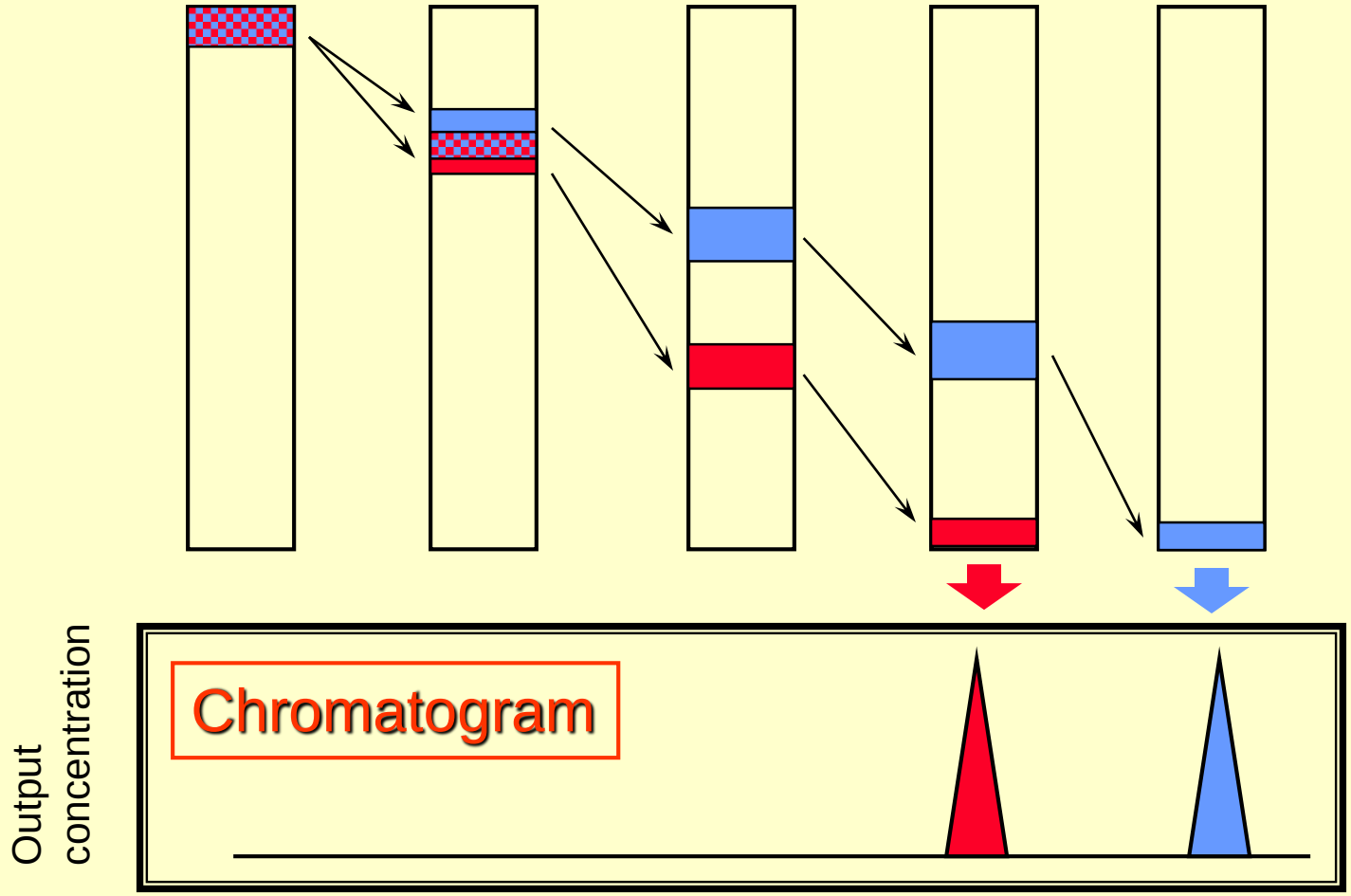


Column Chromatography

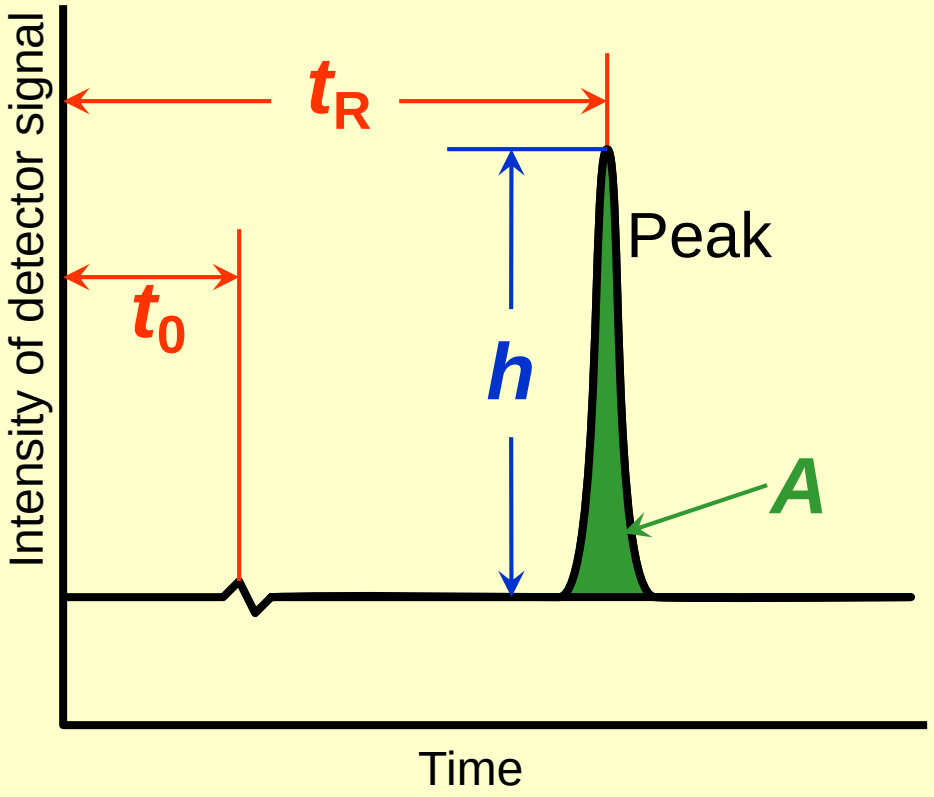


Paper Chromatography
Thin Layer Chromatography (TLC)

Separation Process and Chromatogram
for Column Chromatography



Chromatogram



t_R : Retention time

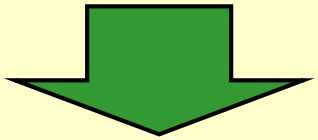
t_0 : Non-retention time

A : Peak area

h : Peak height

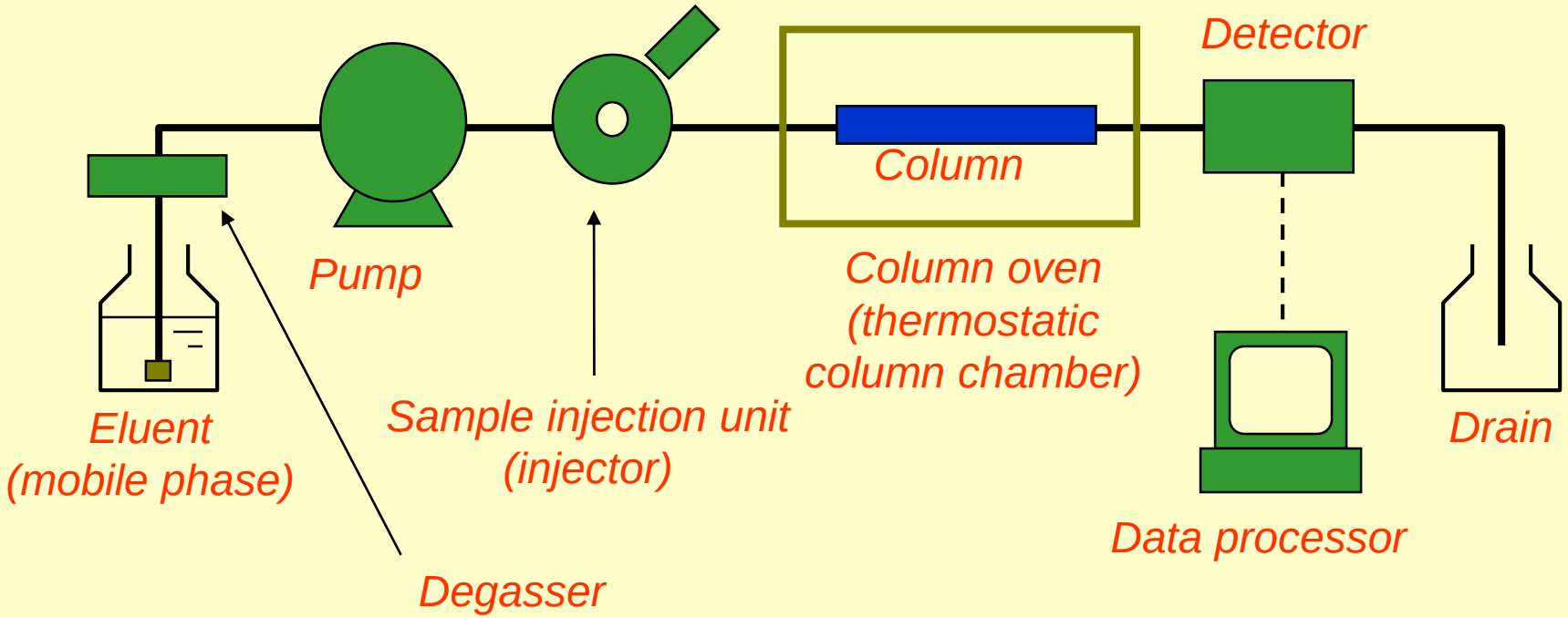
From Liquid Chromatography to High Performance Liquid Chromatography

- 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



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

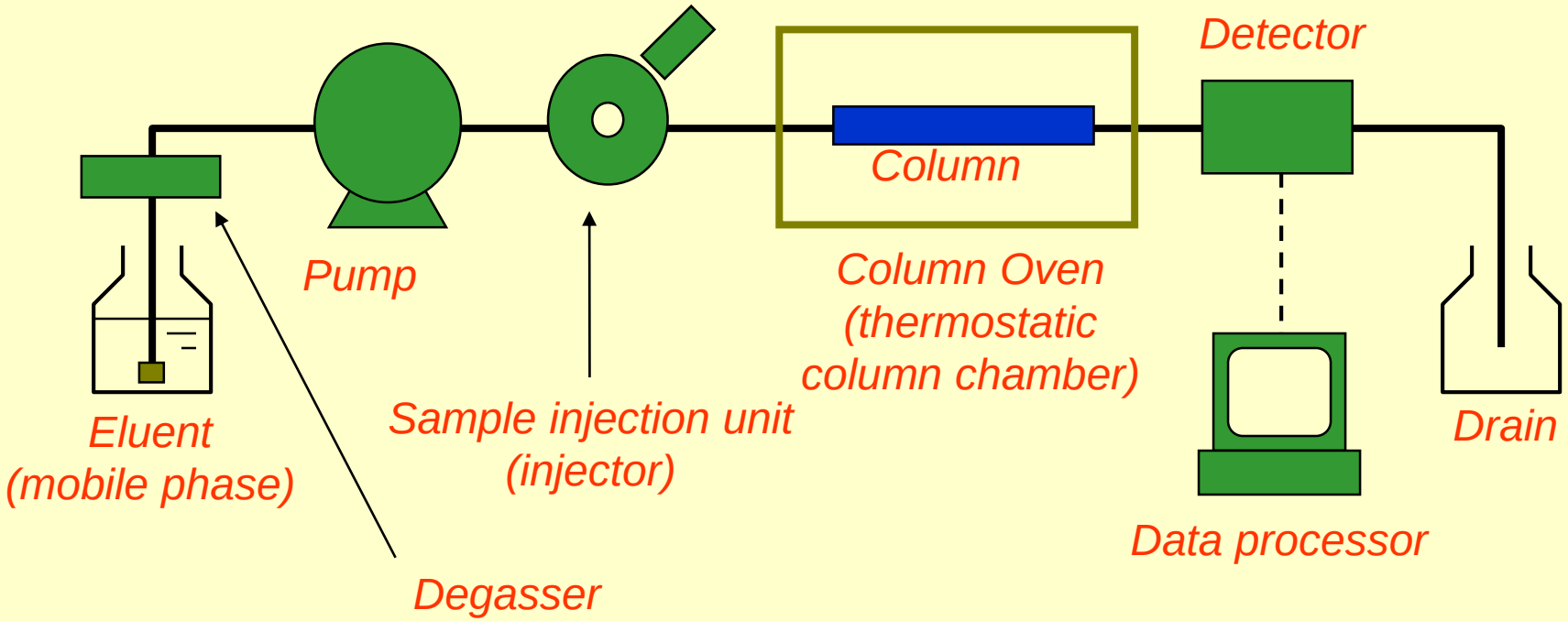
Fields in Which High Performance Liquid Chromatography Is Used

- 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

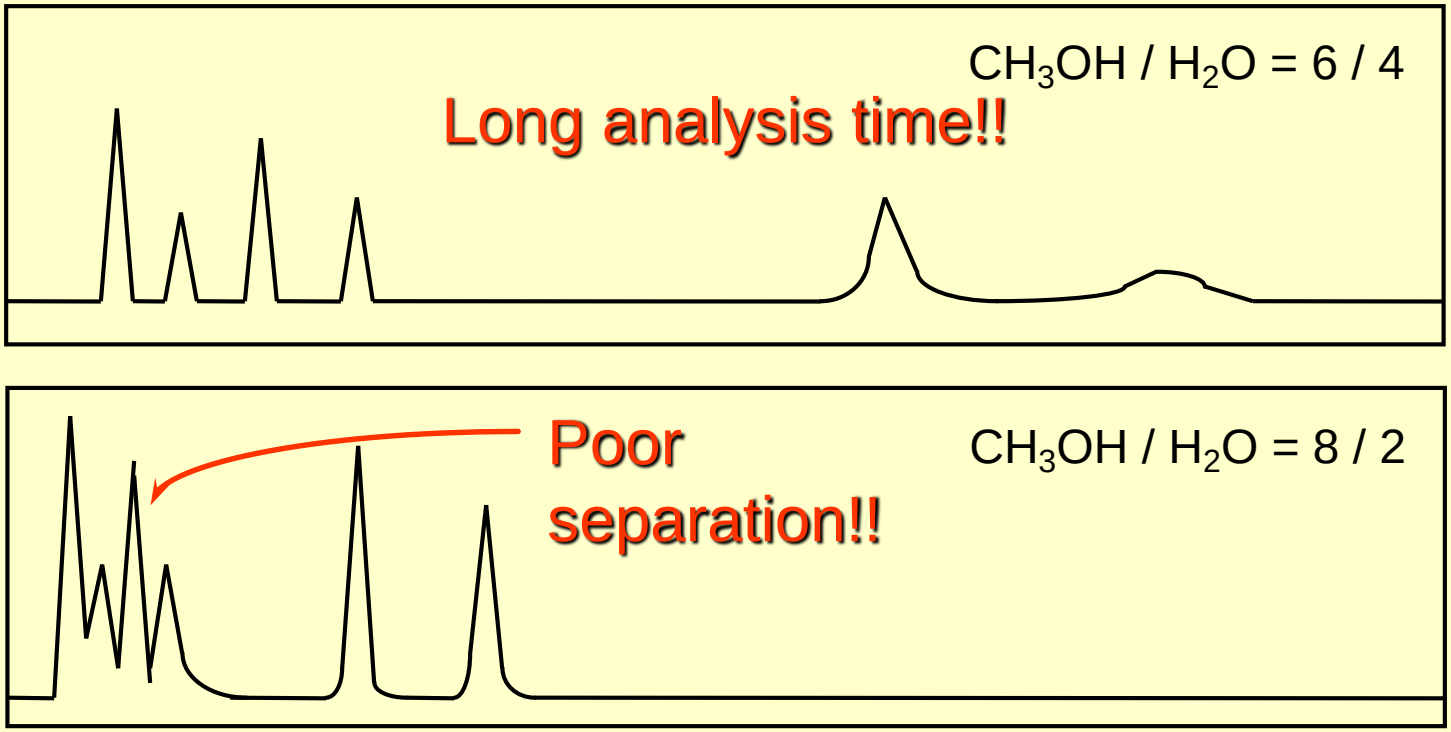
- Syringe pump
- Plunger pump
- Diaphragm pump

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)

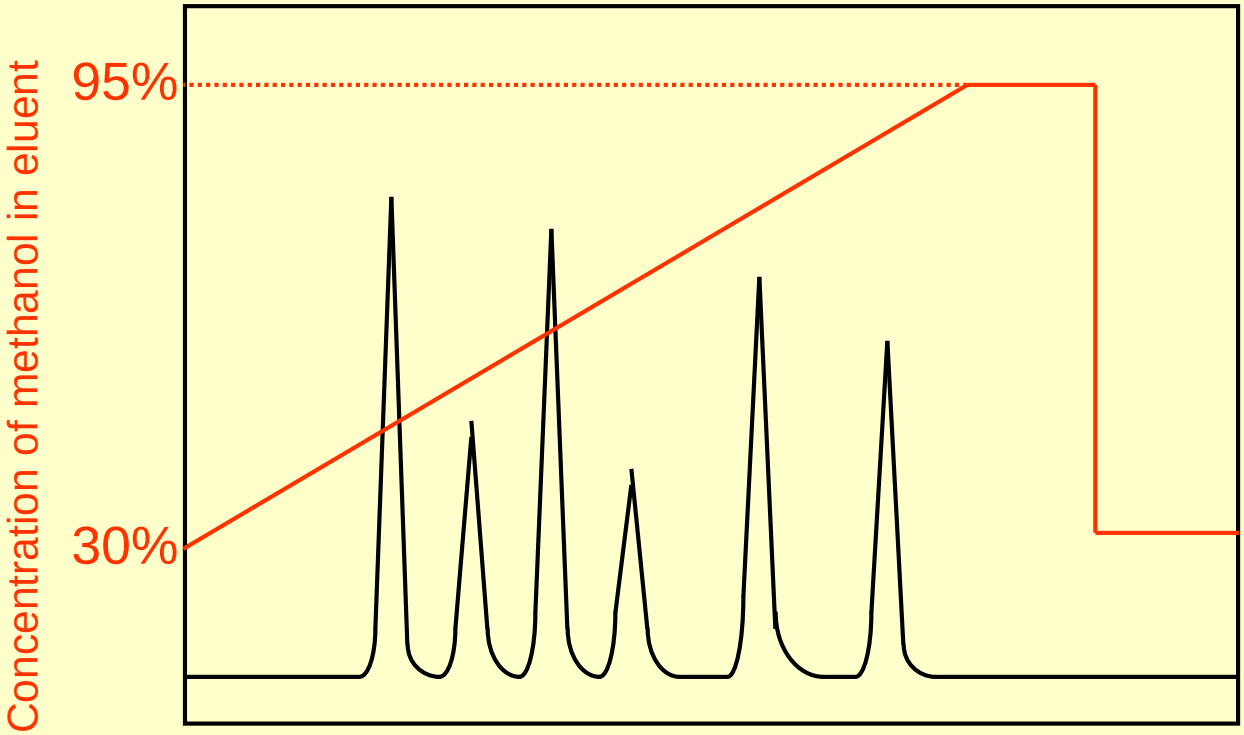
- In isocratic mode



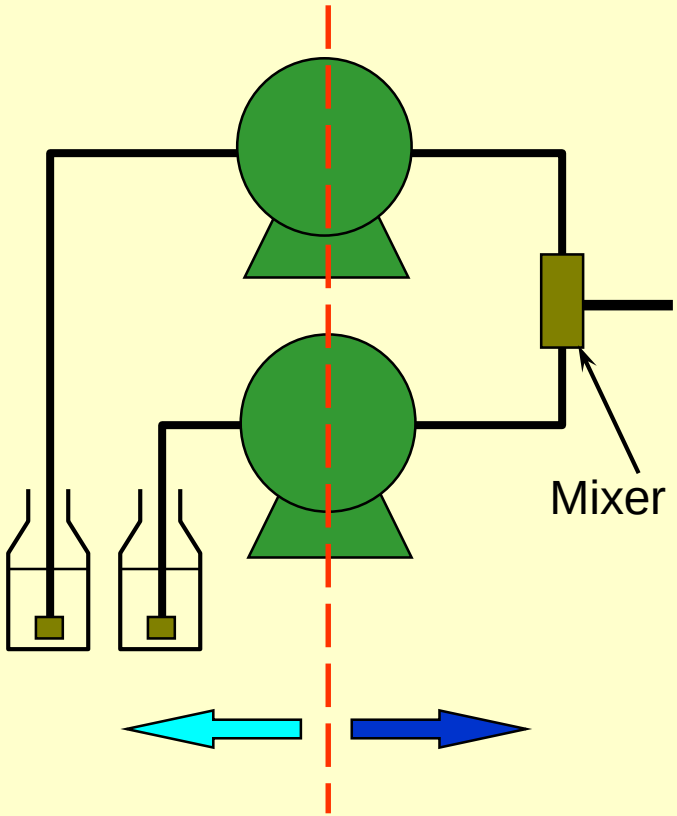
(Column: ODS type)

Aim of Gradient System (2)

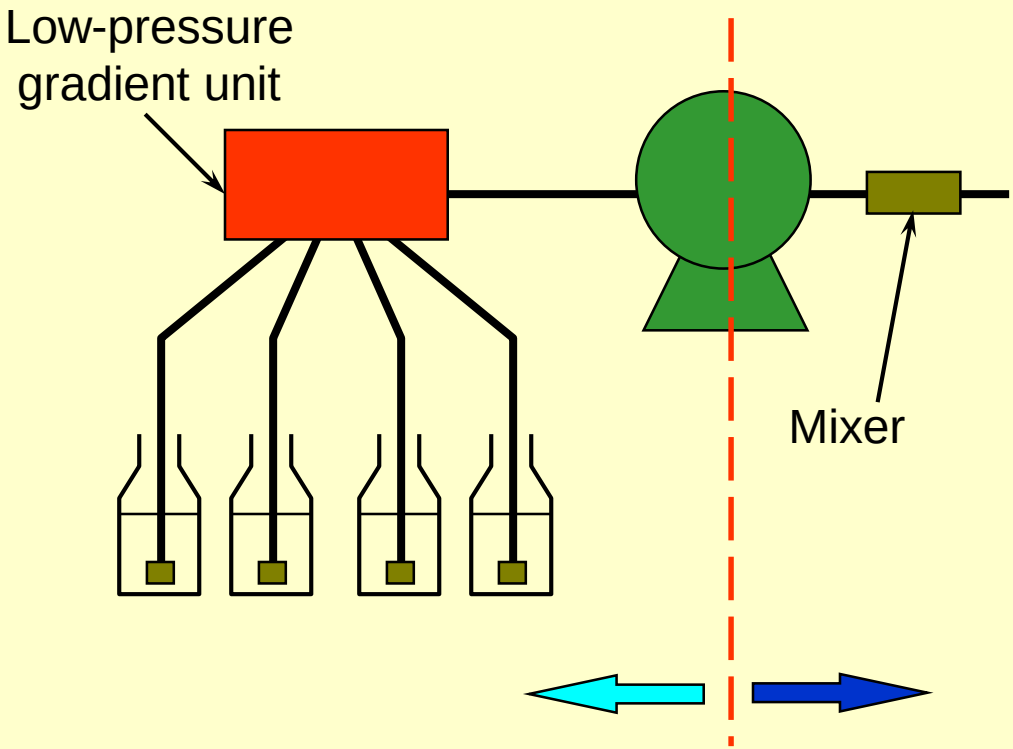
- If the eluent composition is changed gradually during analysis...



High- / Low-Pressure Gradient System



High-pressure gradient



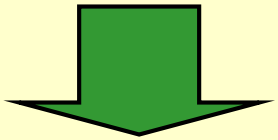
Low-pressure gradient

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

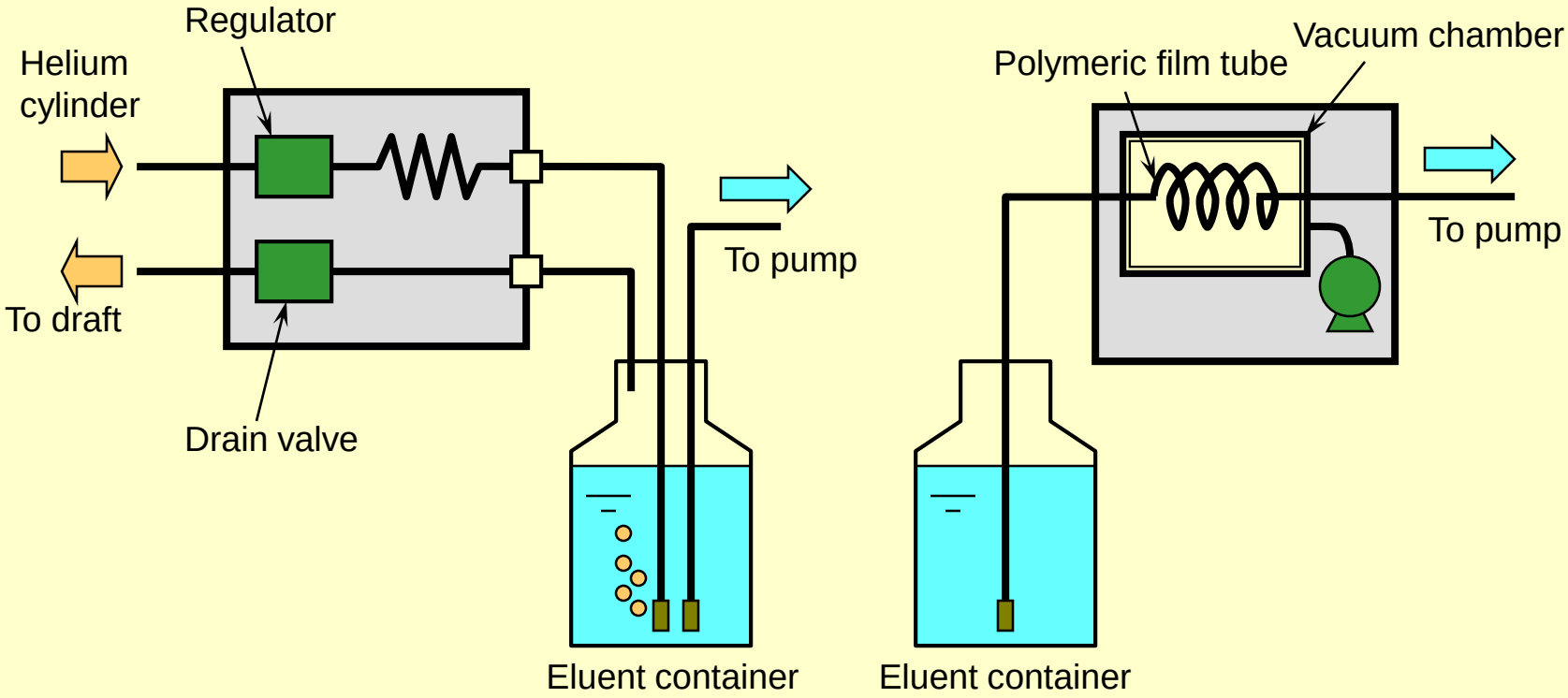
Degasser

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



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

Online Degasser



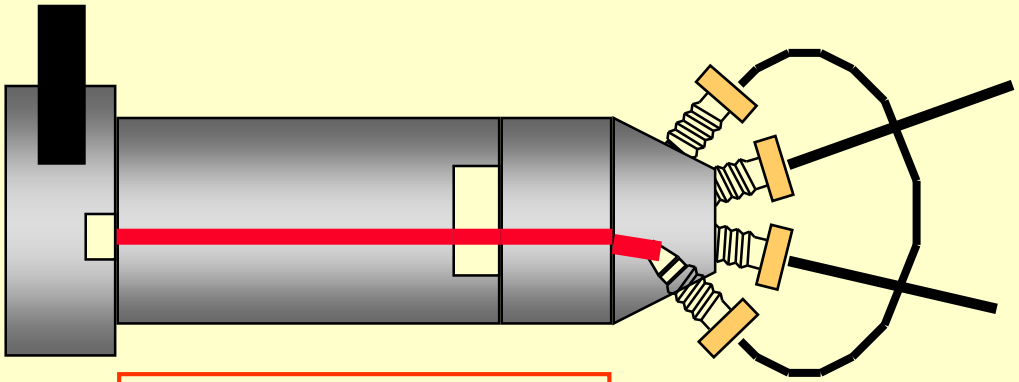
Helium purge method

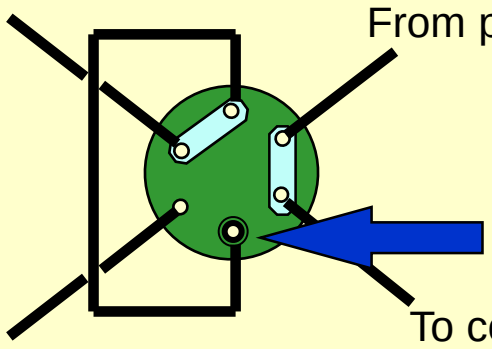
Gas-liquid separation membrane method

Sample Injection Unit (Injector)

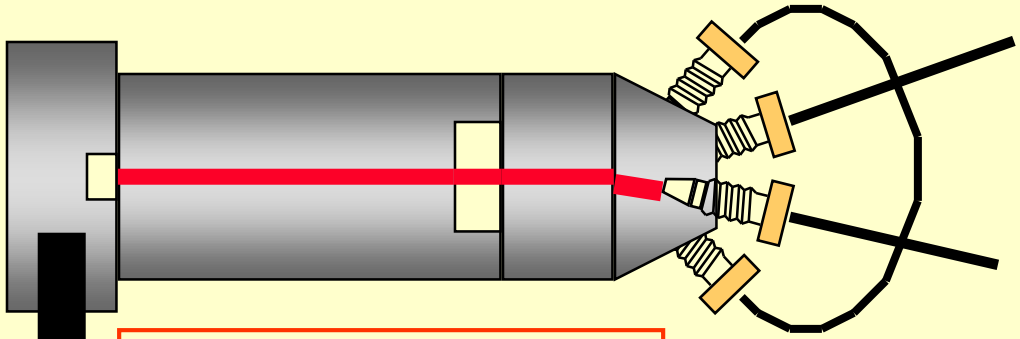
- Performance Requirements
  - No sample remaining in unit
  - Minimal broadening of sample band
  - Free adjustment of injection volume
  - Minimal loss
  - Superior durability and pressure resistance

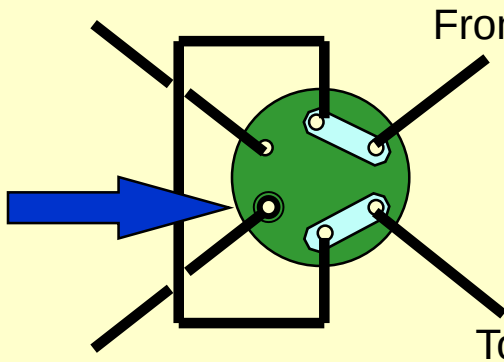
Manual Injector





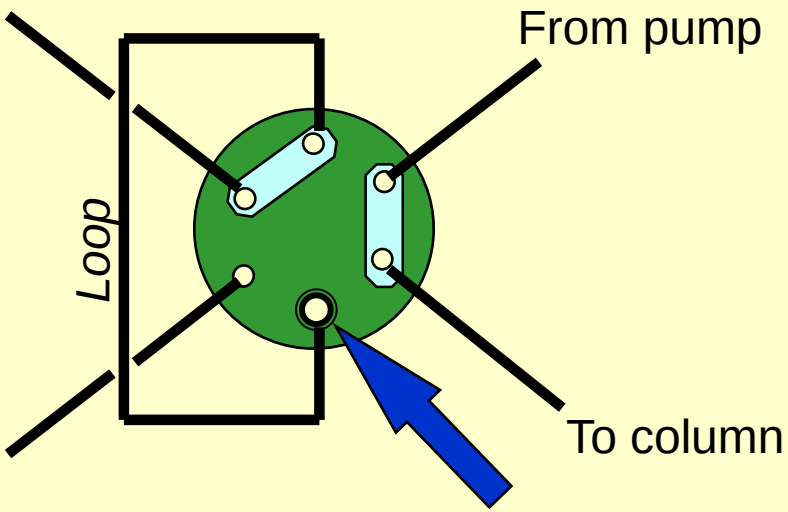
LOAD position





INJECT position

Manual Injector: Operating Principle of Sample Injection



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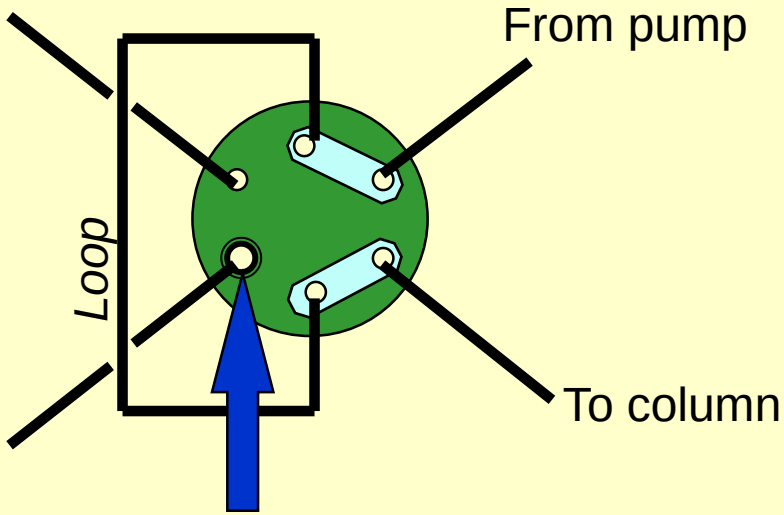
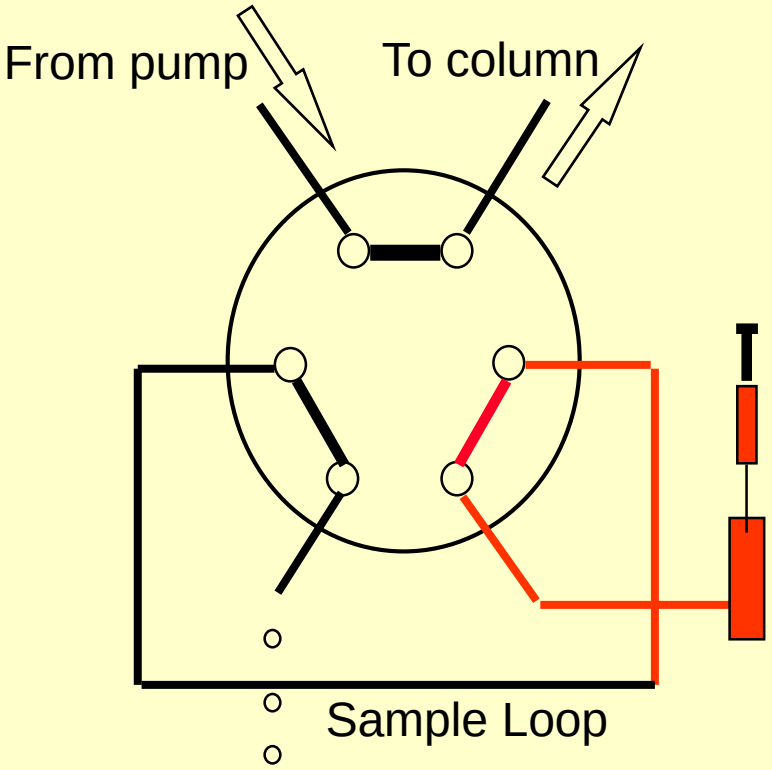


INJECT

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)



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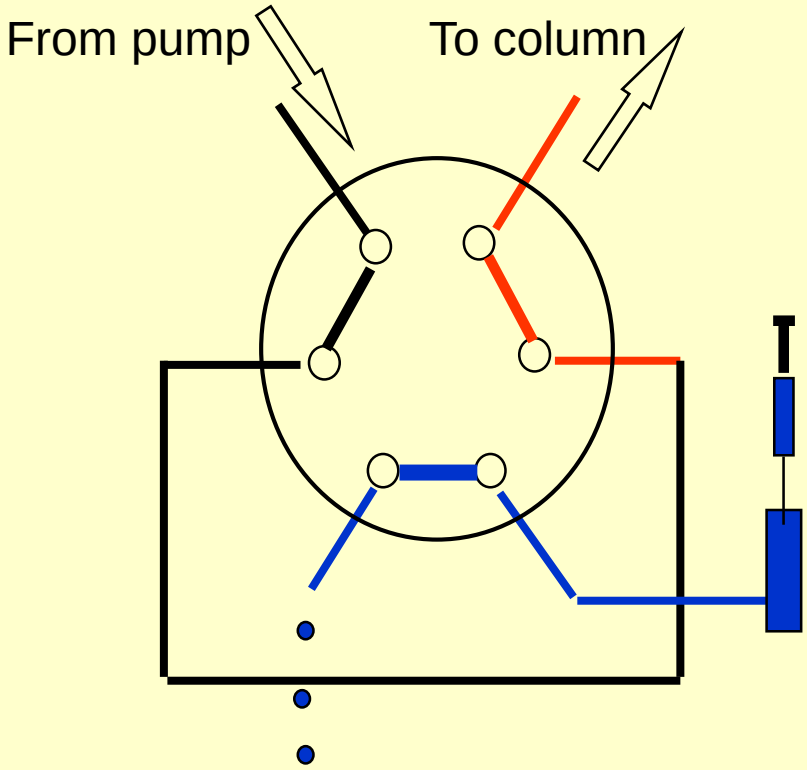


INJECT

HPLC Separation Modes

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

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

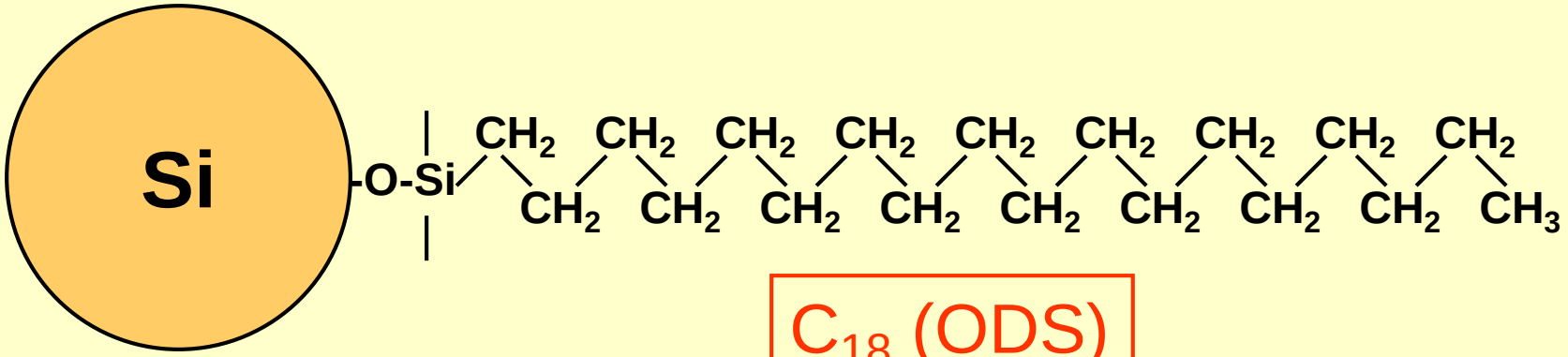
	Stationary phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)

Reversed Phase Chromatography

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

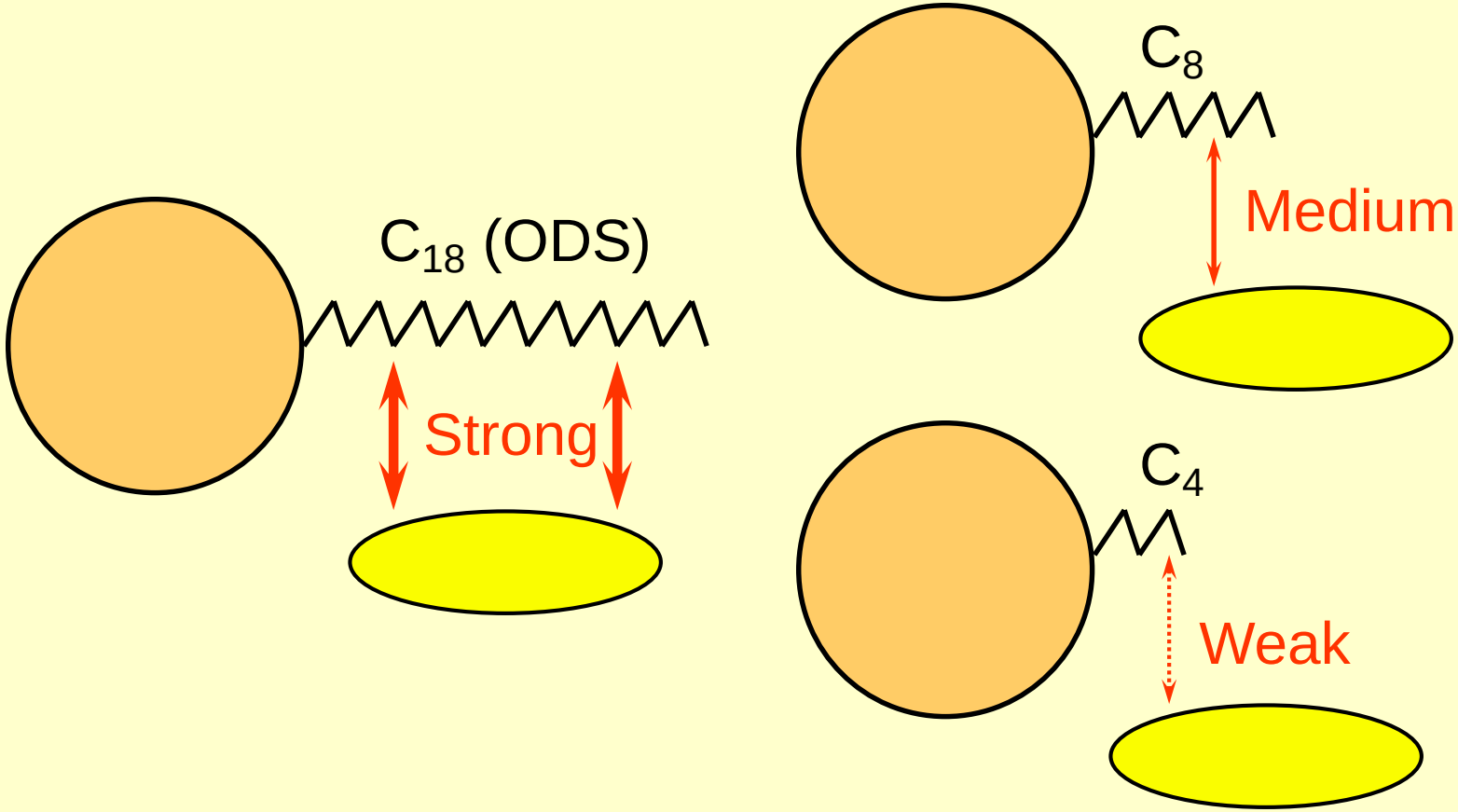
Separation Column for Reversed Phase Chromatography

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

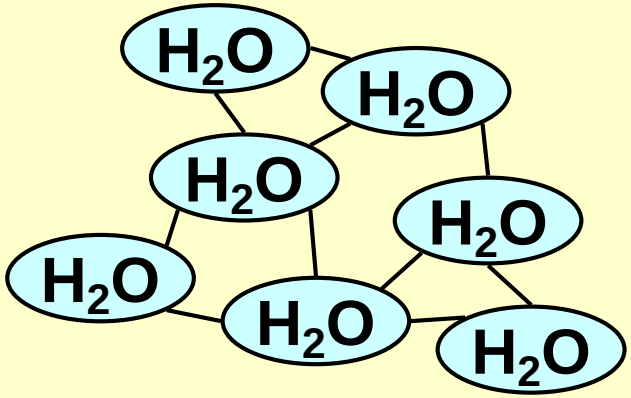


C_{18} (ODS)

Effect of Chain Length of Stationary Phase

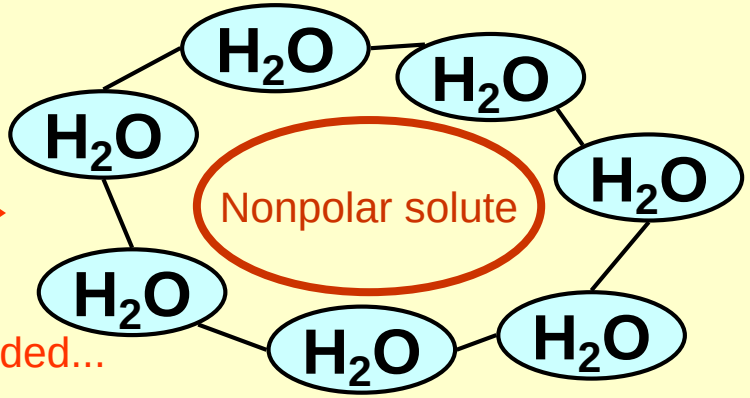


Hydrophobic Interaction

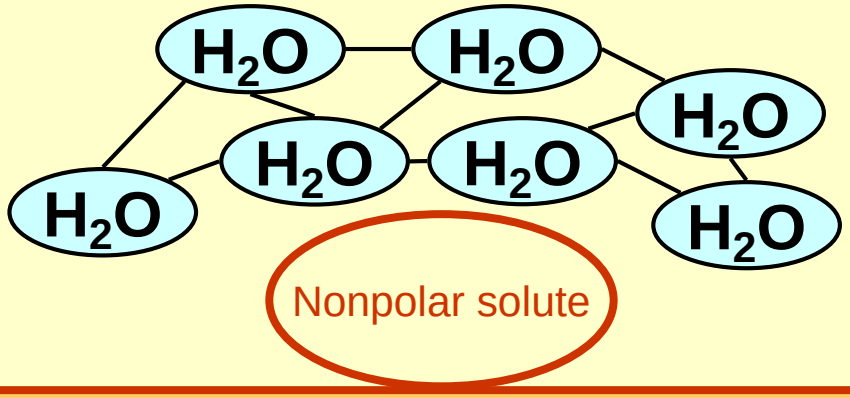


Network of hydrogen bonds

If a nonpolar substance is added...



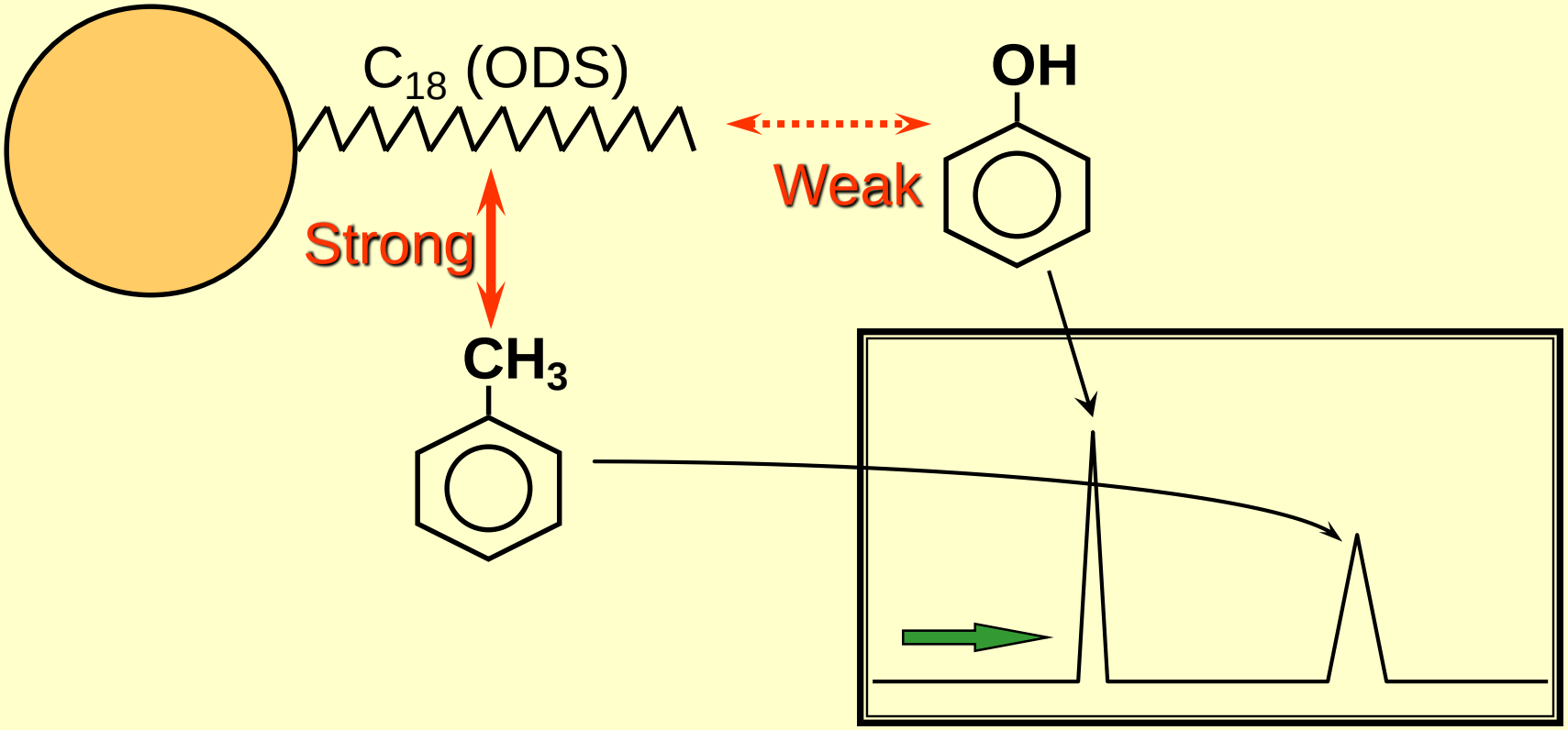
…the network is broken and...



…the nonpolar substance is pushed to a nonpolar location.

Nonpolar stationary phase

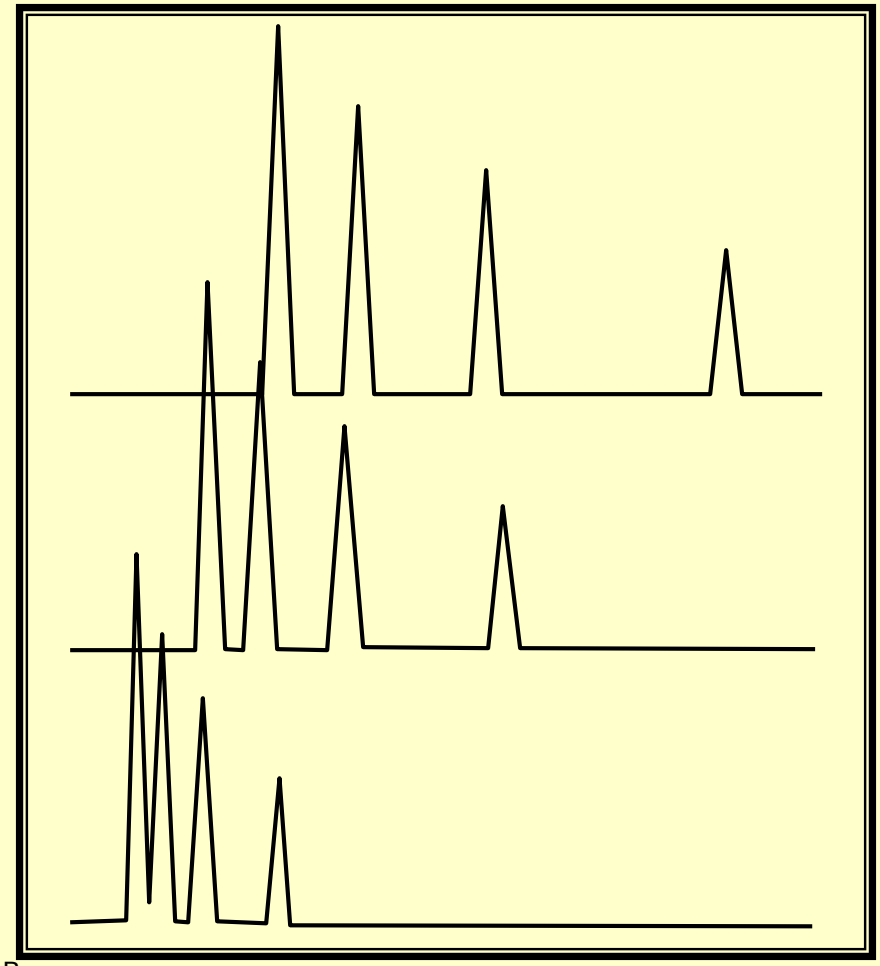
Relationship Between Retention Time and Polarity



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.

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



Eluent: Methanol / Water

60/40

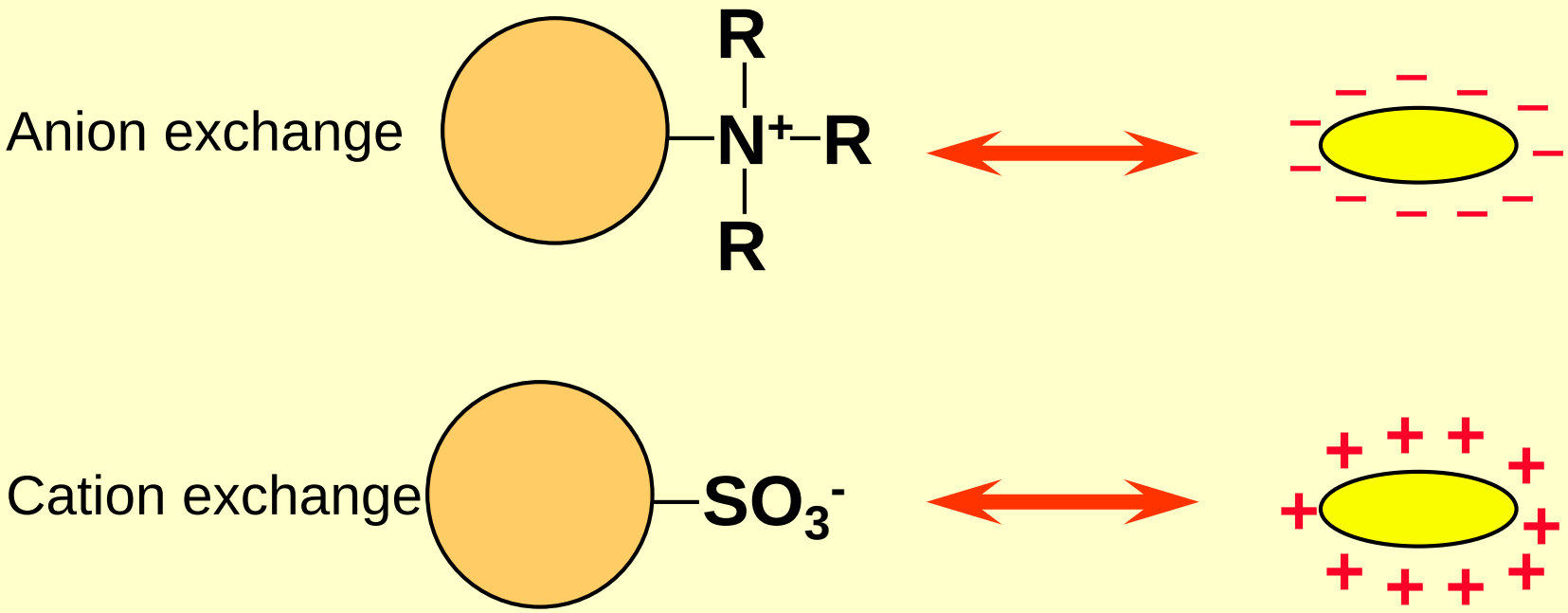
70/30

80/20

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.

Ion Exchange Chromatography



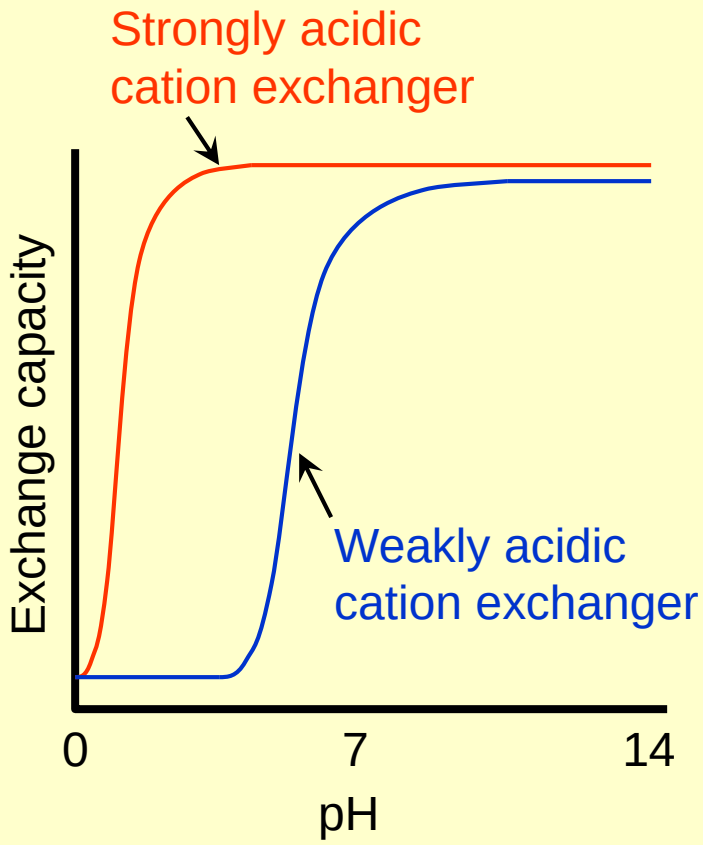
Electrostatic interaction
(Coulomb force)

Stationary Phase Used in Ion Exchange Mode

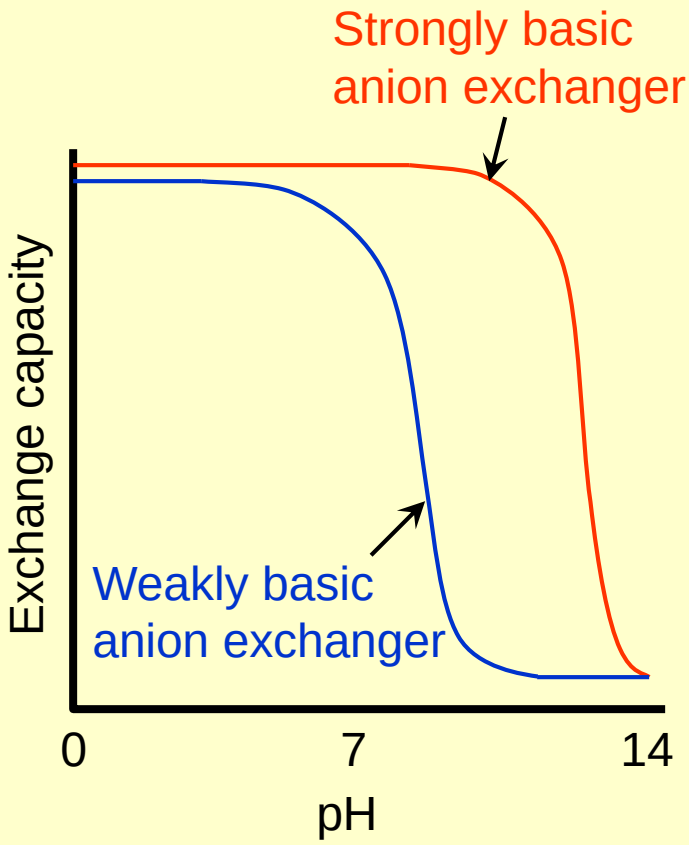
- Base Material
  - Resin is often used.
  - Silica gel is also used.

- Cation Exchange Column
  - Strong cation exchange (SCX) $-SO_3^-$
  - 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

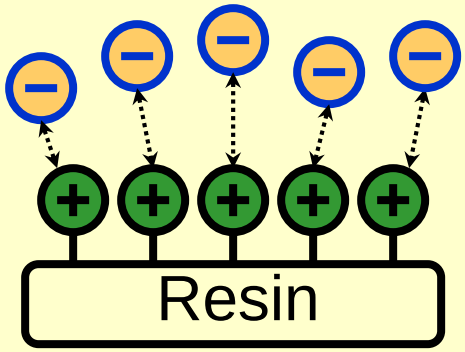


Cation exchange mode

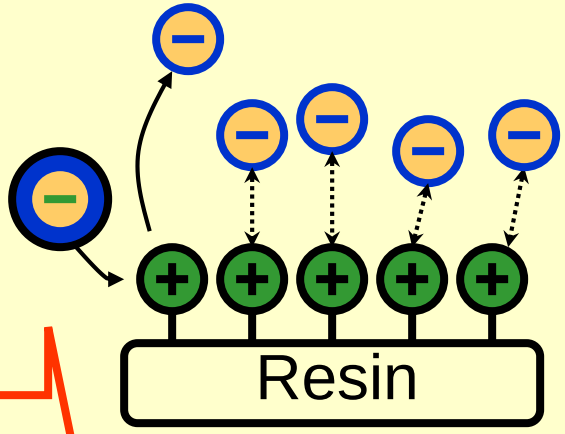


Anion exchange mode

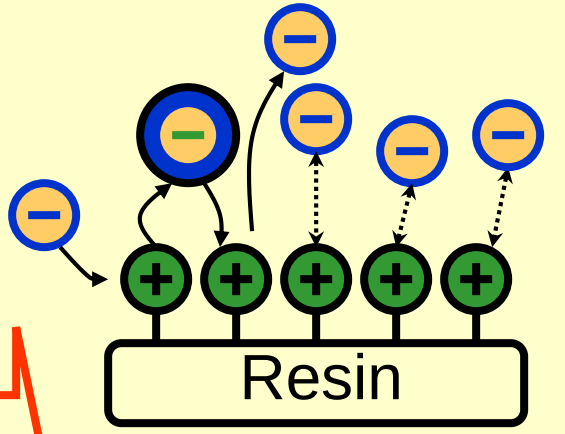
Relationship between Retention Time and Salt Concentration of Eluent in Ion Exchange Mode



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



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

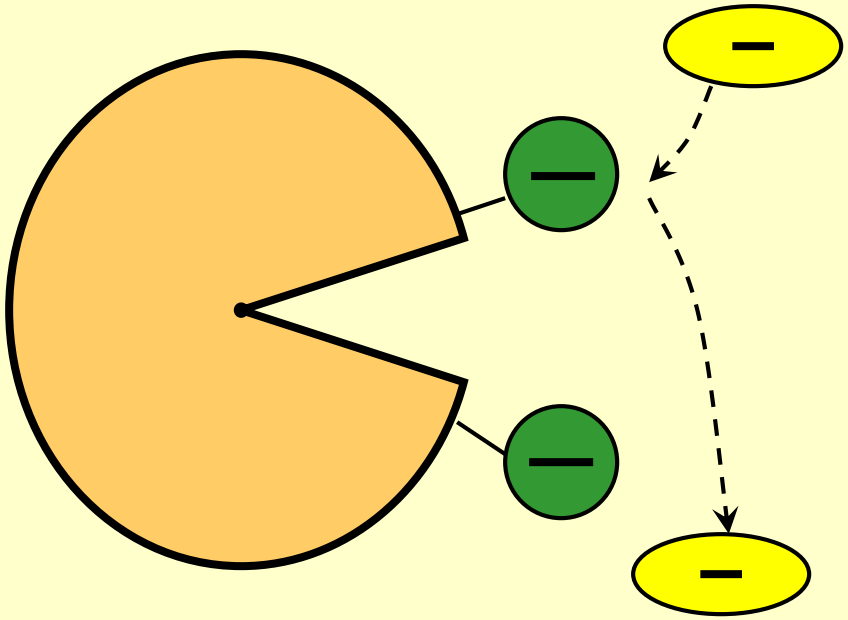


The solute ion is driven away by an eluent ion and is adsorbed by the next exchange group.

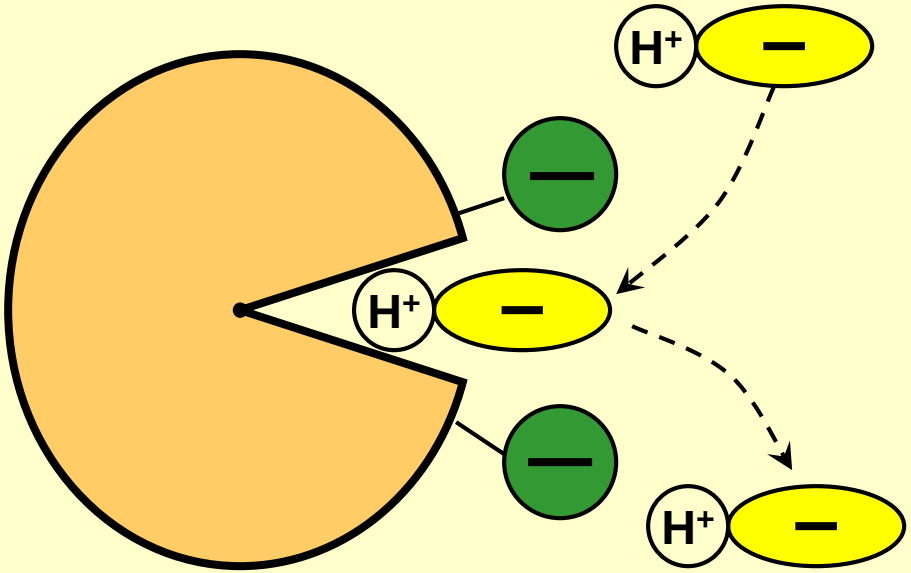
Solute ions and eluent ions compete for ion exchange groups.

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.

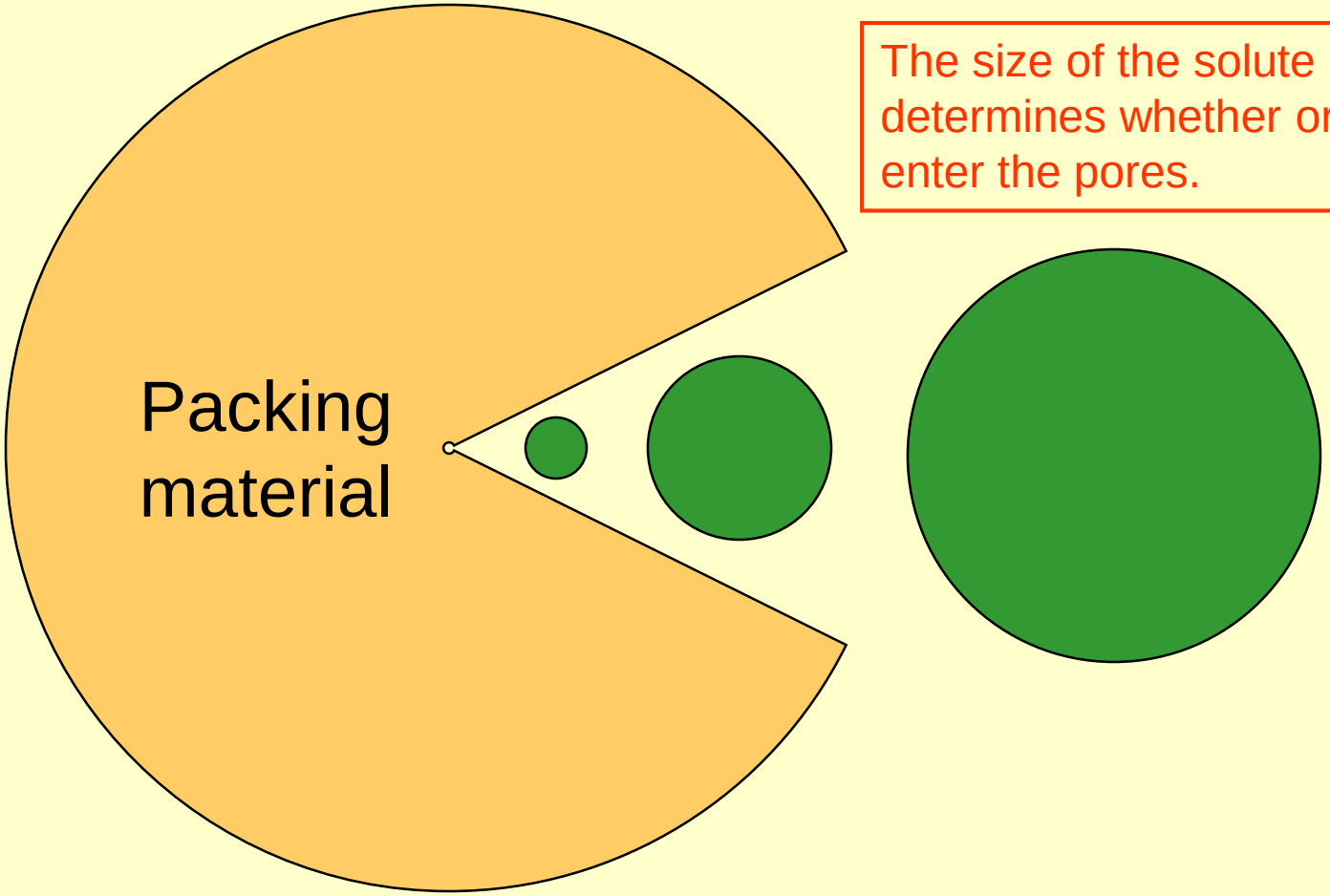


Depending on the level of dissociation, some weak acid ions can enter the pore.

Size Exclusion Chromatography

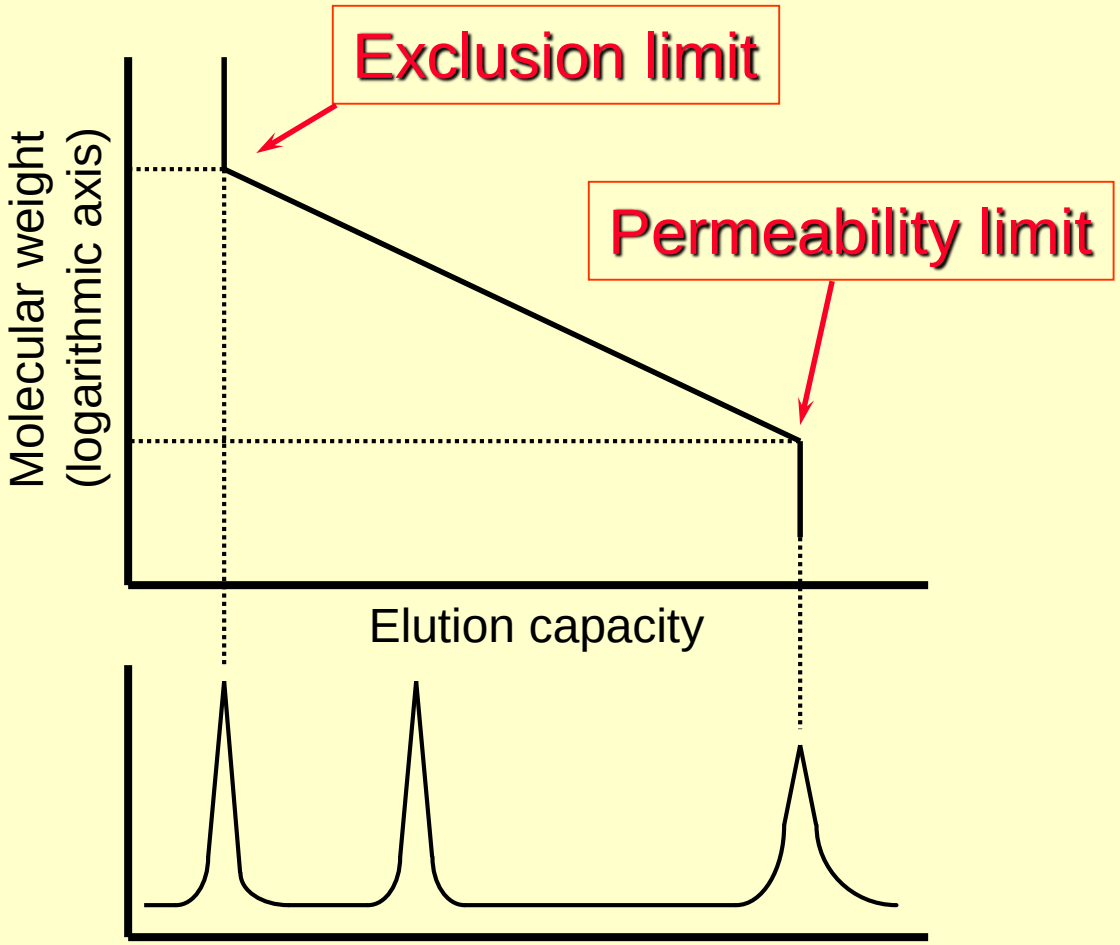
- Separation is based on the size (bulkiness) of molecules.
- The name varies with the application field!
  - Size Exclusion Chromatography (SEC)
  - Gel Permeation Chromatography (GPC)
    - Chemical industry fields, synthetic polymers, nonaqueous systems
  - Gel Filtration Chromatography (GFC)
   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



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.

HPLC Hardware: Part 2

Detectors and Their Ranges of Application

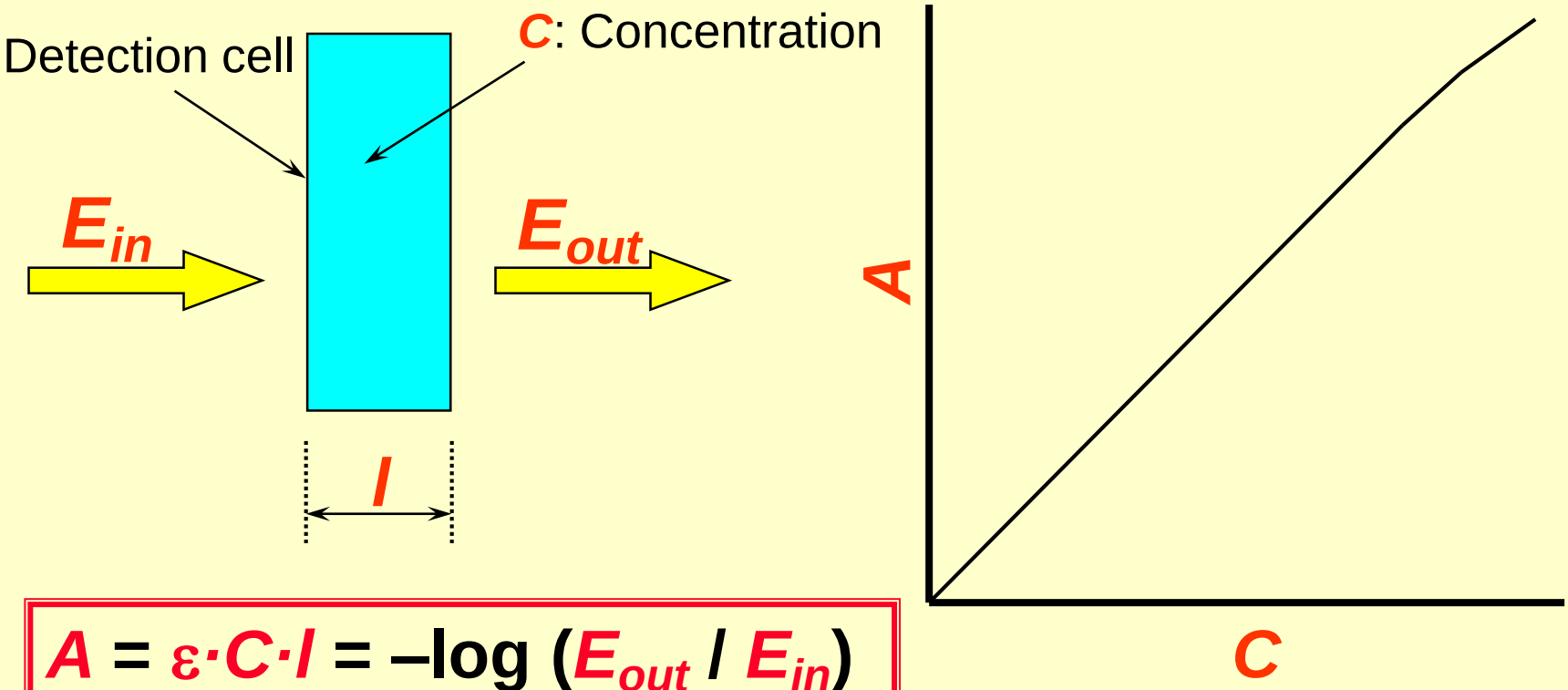
Detection Condition Requirements

- 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

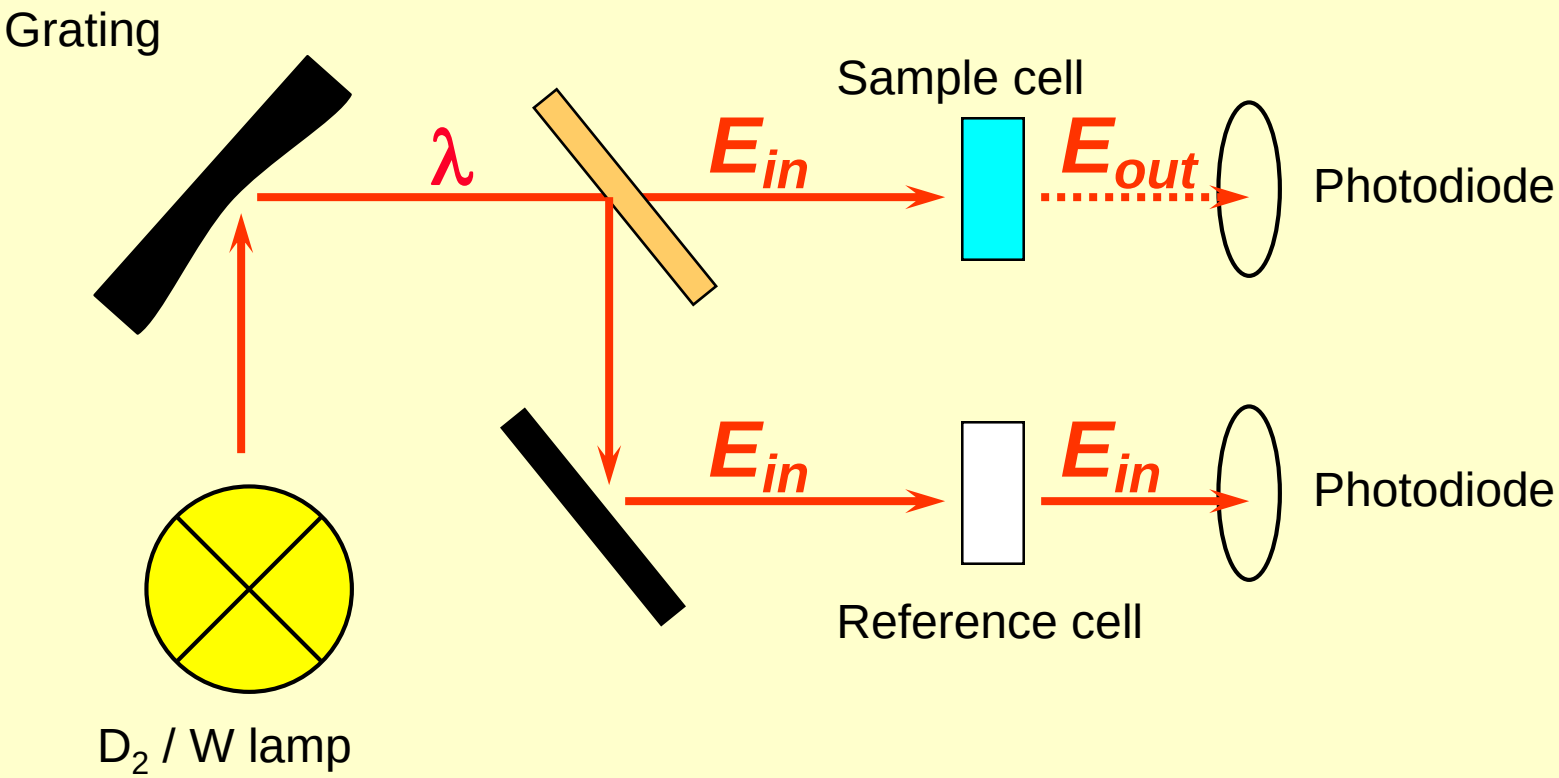
UV-VIS Absorbance Detector



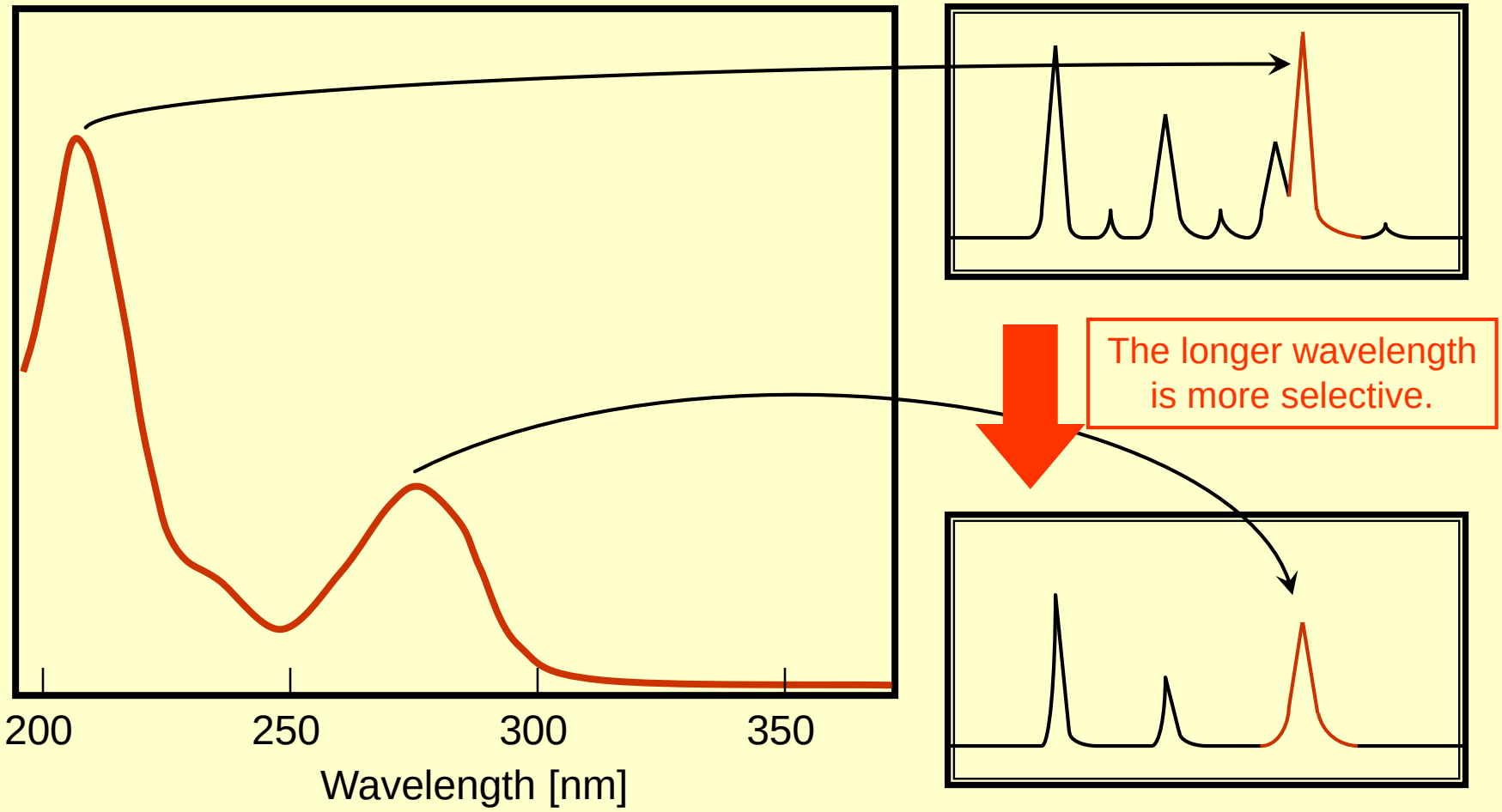
$$A = \varepsilon \cdot C \cdot l = -\log\left(E_{out} / E_{in}\right)$$

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

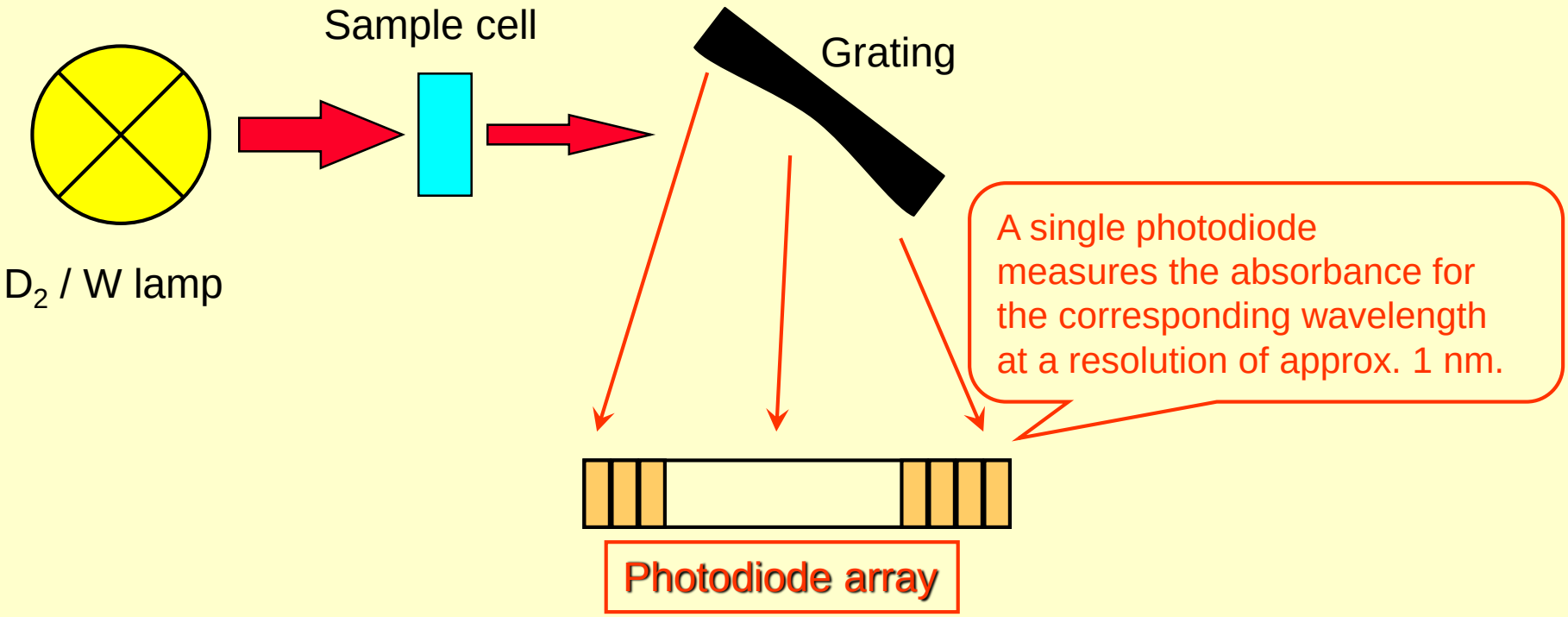
Optical System of UV-VIS Absorbance Detector



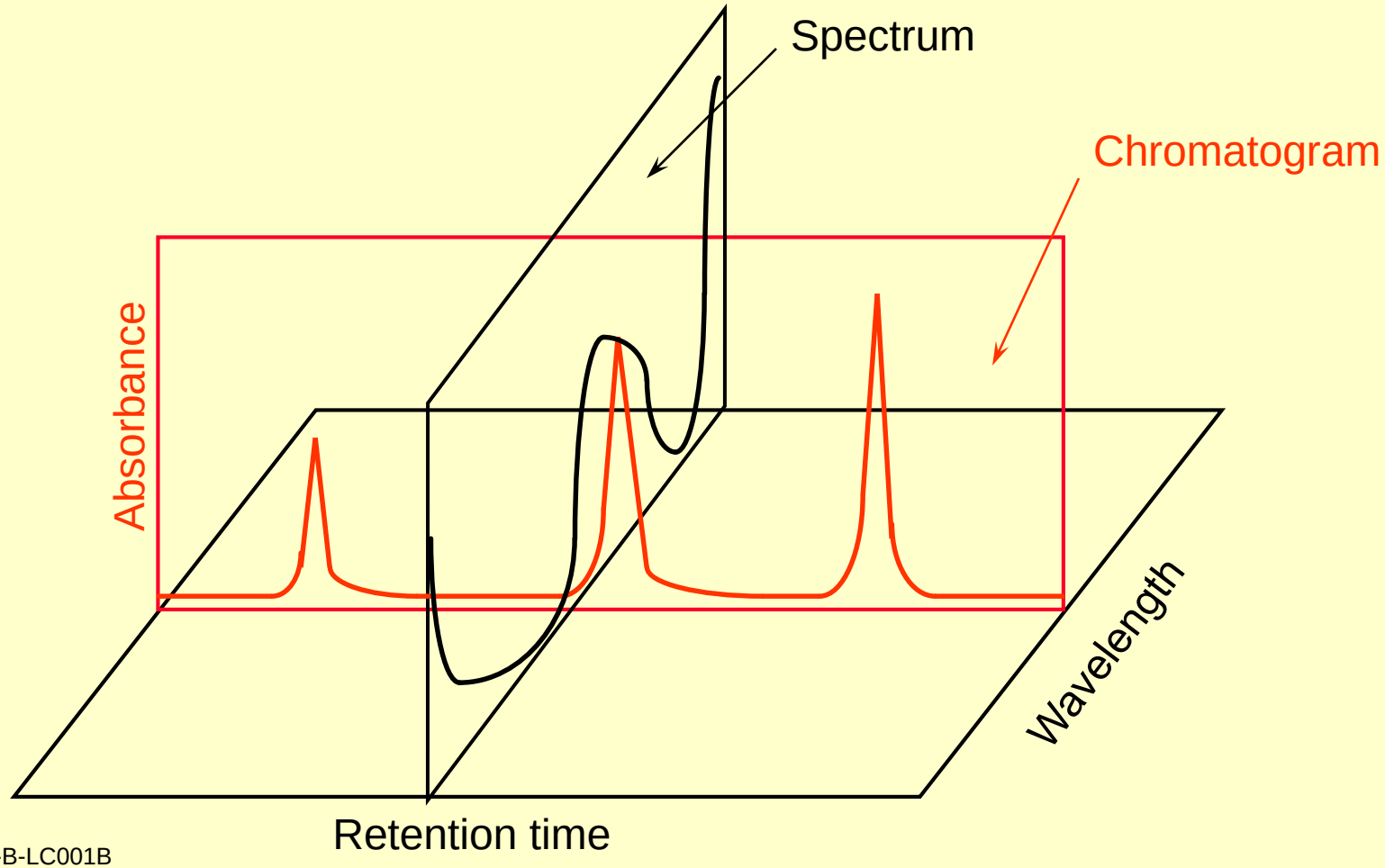
Spectrum and Selection of Detection Wavelength



Optical System of Photodiode Array Detector



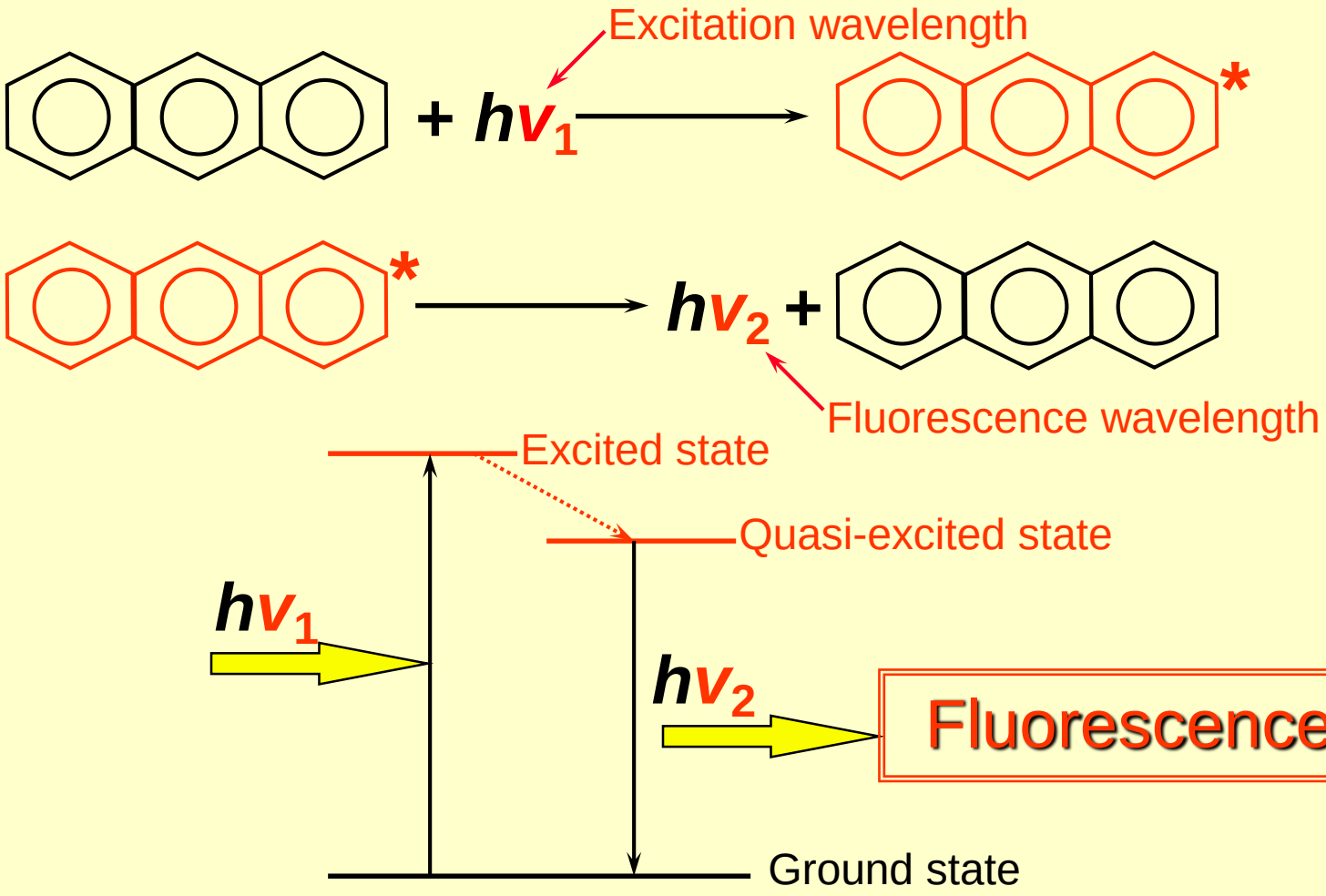
Data Obtained with a Photodiode Array Detector



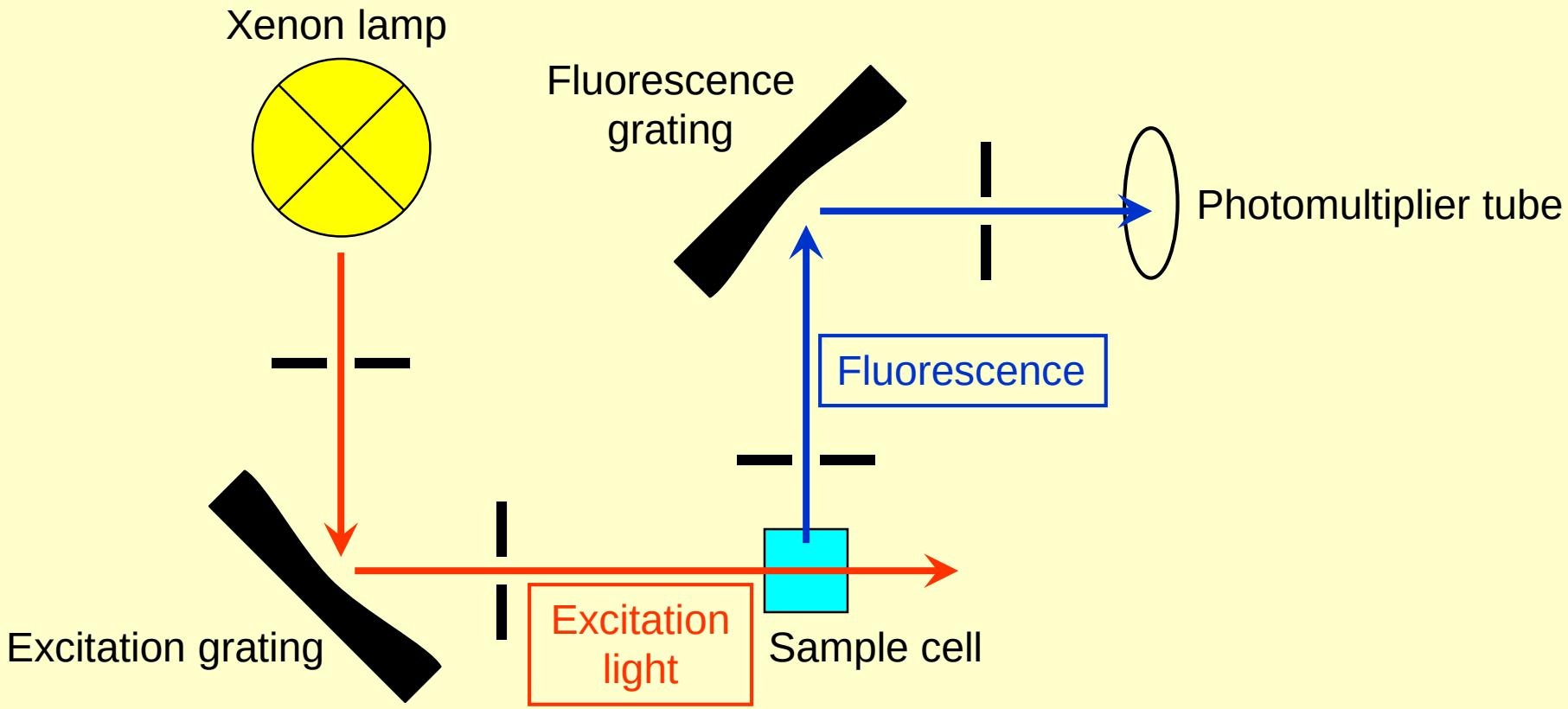
Advantages of Photodiode Array Detectors

- 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

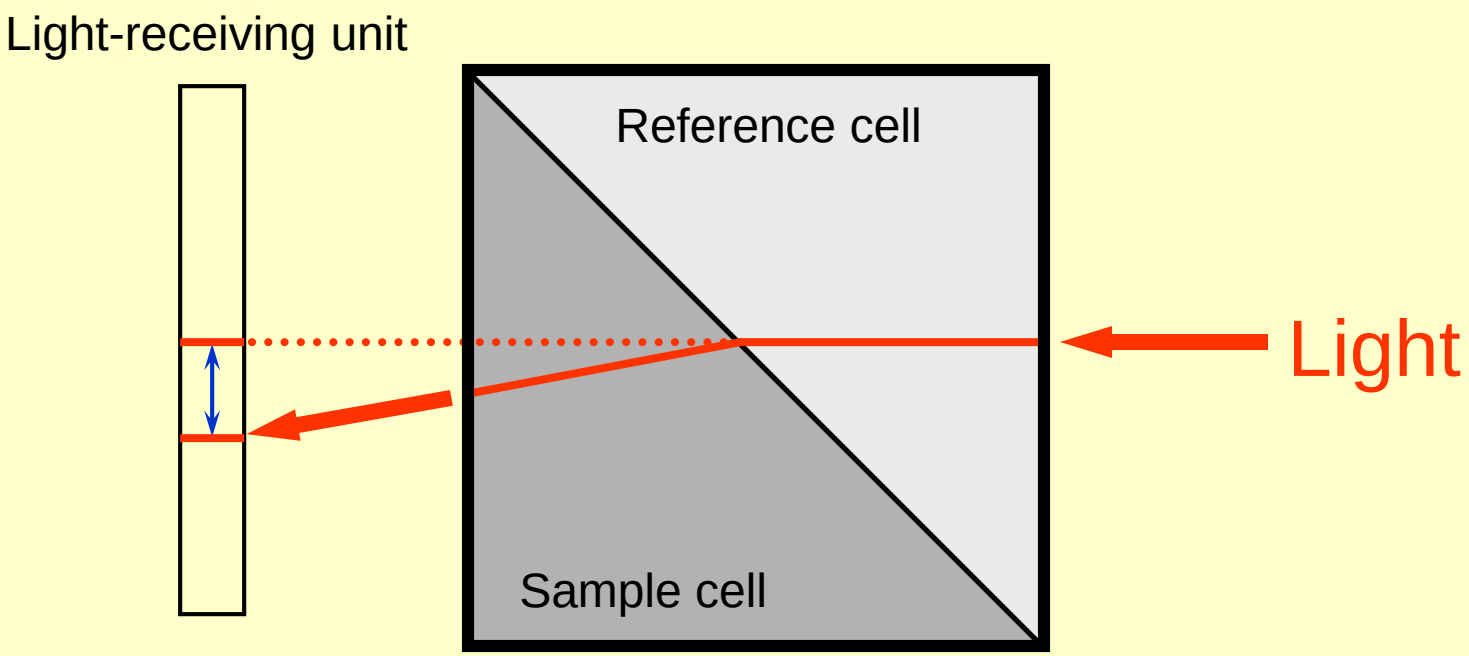
Fluorescence Detector



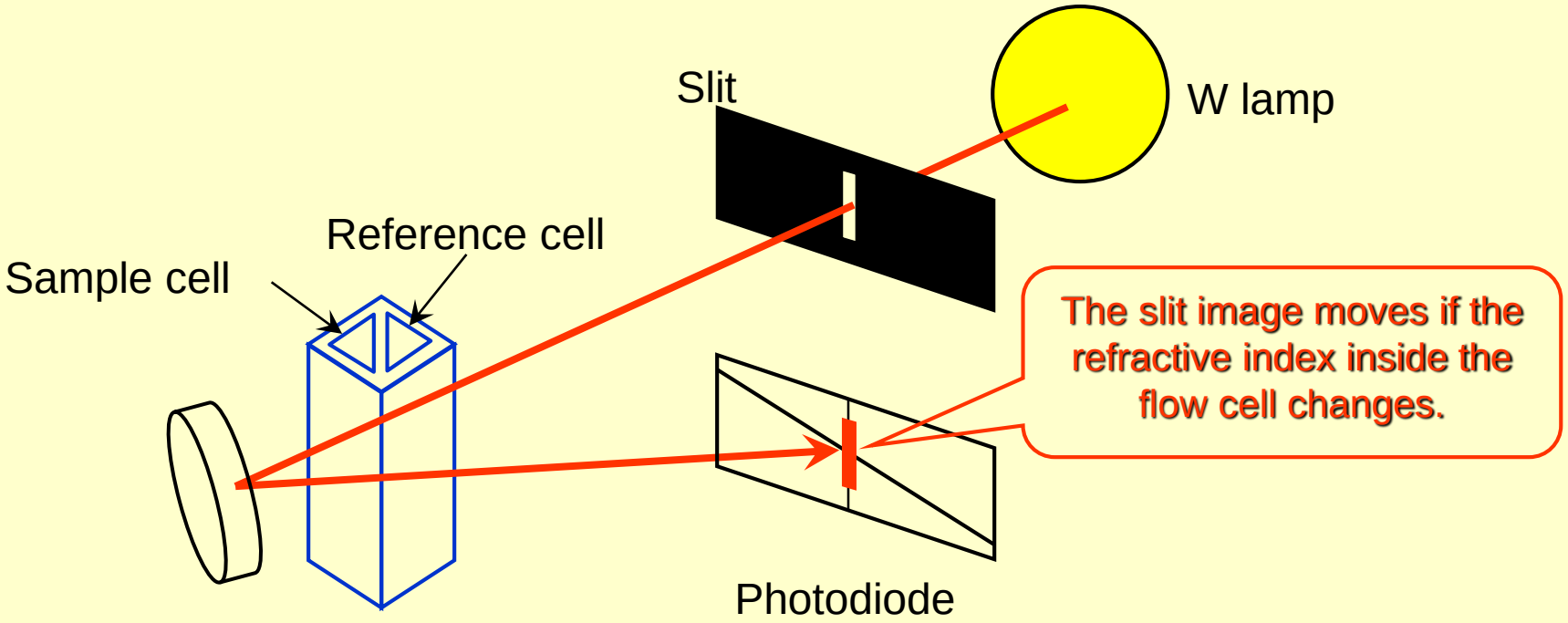
Optical System of Fluorescence Detector



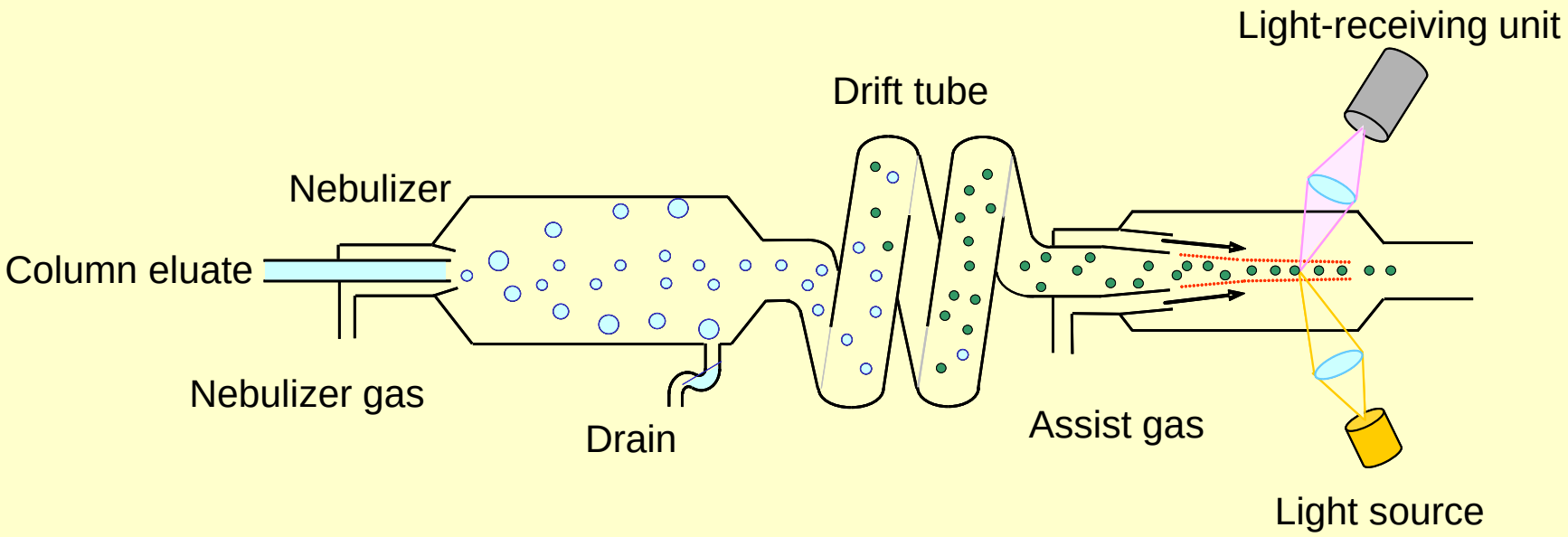
Differential Refractive Index Detector (Deflection-Type)



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

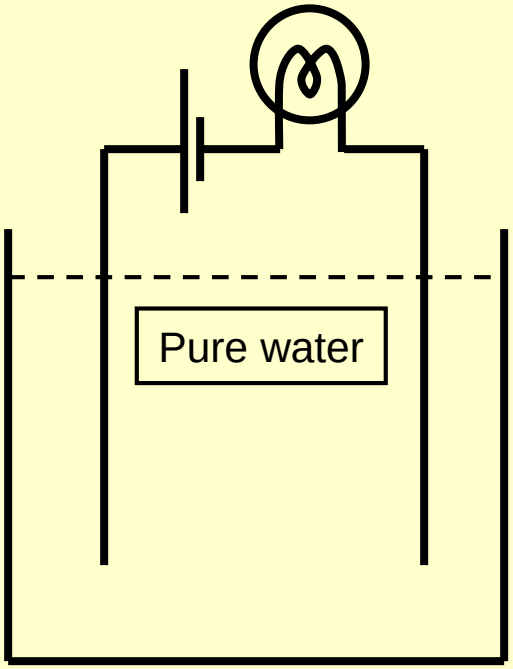


Evaporative Light Scattering Detector

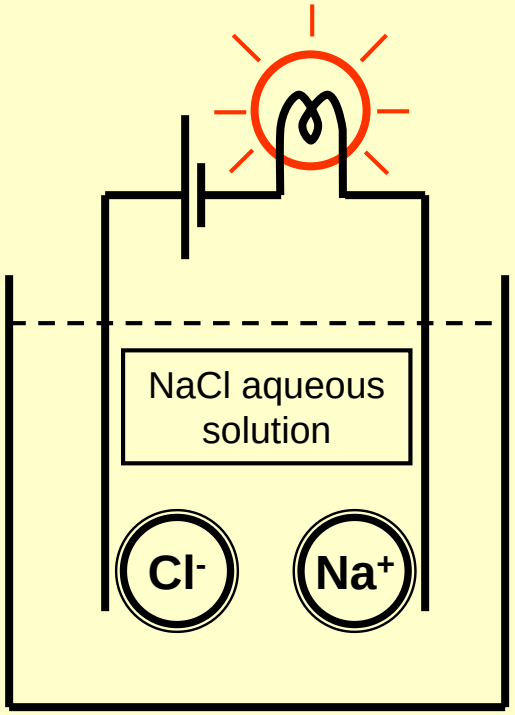


The column eluate is evaporated and the light scattered by the particles of nonvolatile substances is detected.

Electrical Conductivity Detector



The bulb does not light with water.



The bulb lights if there are ions.