Chromatography

Physical-chemical methods of separation,
based on different partitioning
of the target compounds between a stationary
and a mobile phase.

What is Chromatography?

Separation of similar molecules from complex mixtures

- The analytes are solubilized in a mobile phase, and transported in a stationary phase
- The two phases are chosen so that the analytes partition differently between them
- The differences in mobility that are created by this partition difference are then the basis for this sample components

Mechanisms

1. Adsorption Equilibrium
   (solid stationary phase, liquid mobile phase): e.g., hydrophobic interaction chromatography, adsorption-chromatography

2. Partition Equilibrium
   (liquid stationary phase, liquid or gaseous mobile phase): e.g., reversed phase chromatography, gas-liquid chromatography

3. Ion-Exchange Equilibrium
   (ion exchanger as stationary phase, electrolyte as mobile phase): e.g., ion exchange chromatography

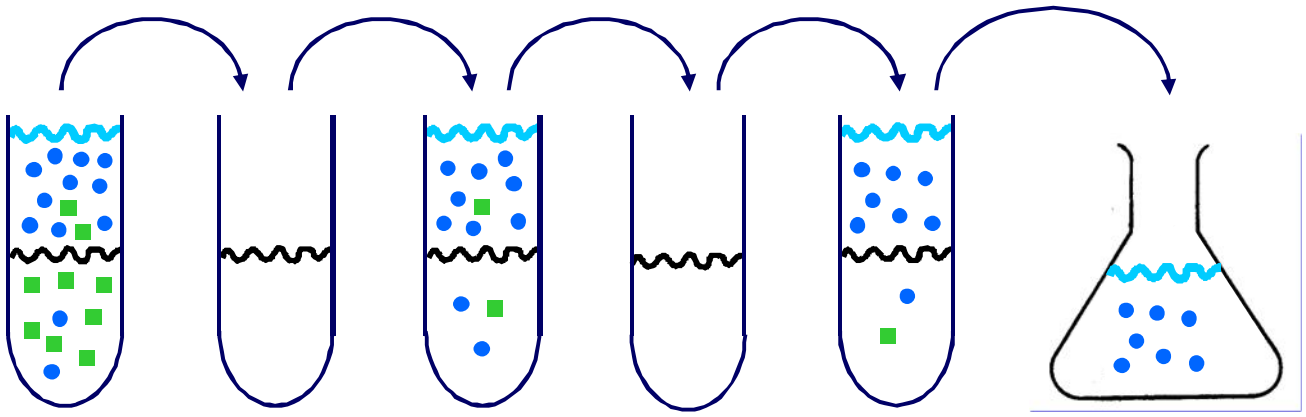
4. Equilibrium between a mobile and stagnant liquid phase : e.g., gel permeation chromatography, size-exclusion chromatography

5. Equilibrium between an immobilized ligand and a liquid mobile phase: e.g., affinity chromatography

Partition

The Partition Coefficient K_d describes how a substance partitions between two, non-mixable phases

$$K_d = \frac{\text{Concentration in phase A}}{\text{Concentration in phase B}}$$



Capacity Factor

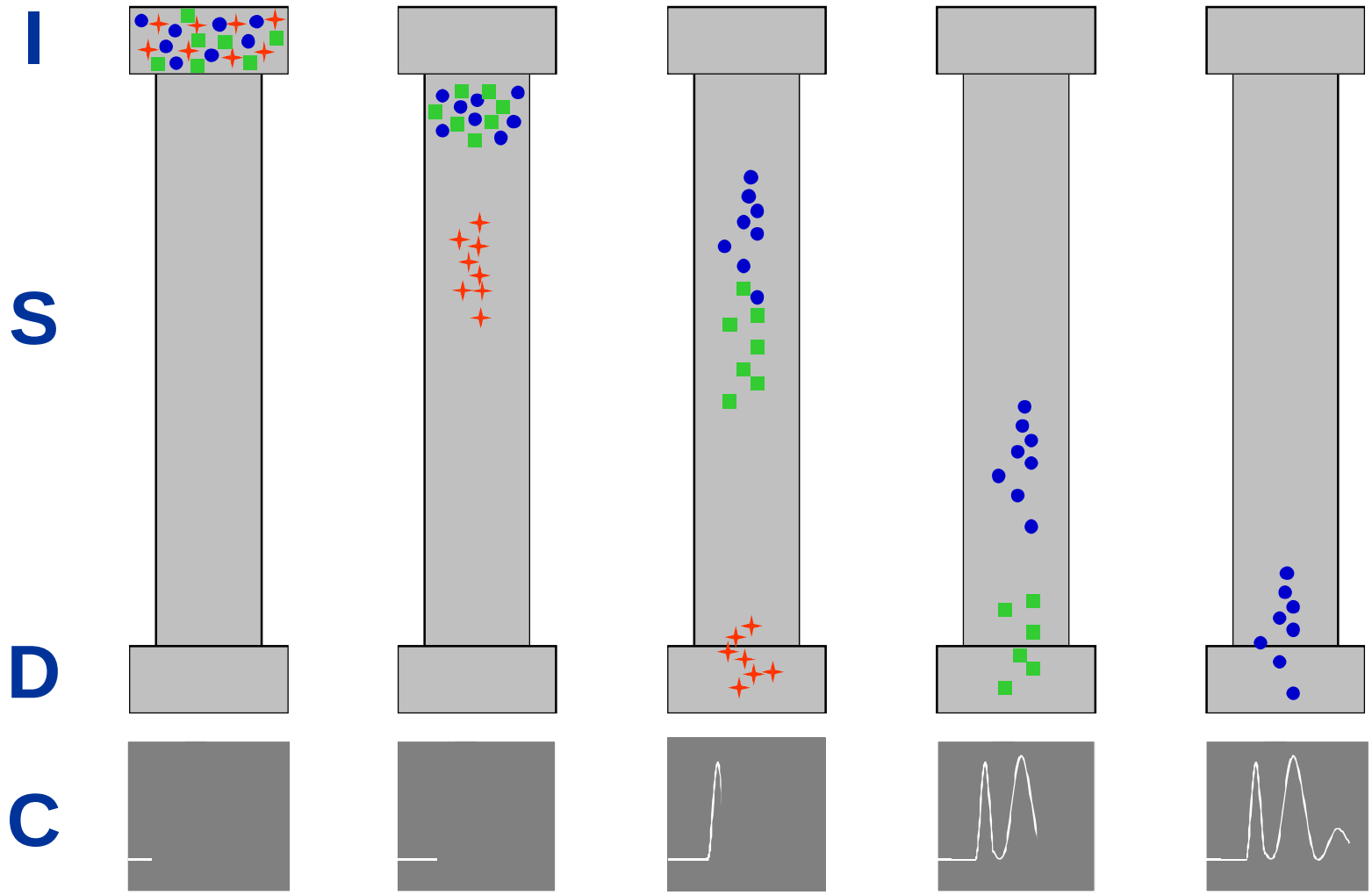
- The Capacity Factor denotes the effective partitioning. It describes which amount of substance stays at any time in the a specific phase.

- $K_x = \frac{\text{Amount of Mol in stationary phase}}{\text{Amount of Mol in mobile phase}}$

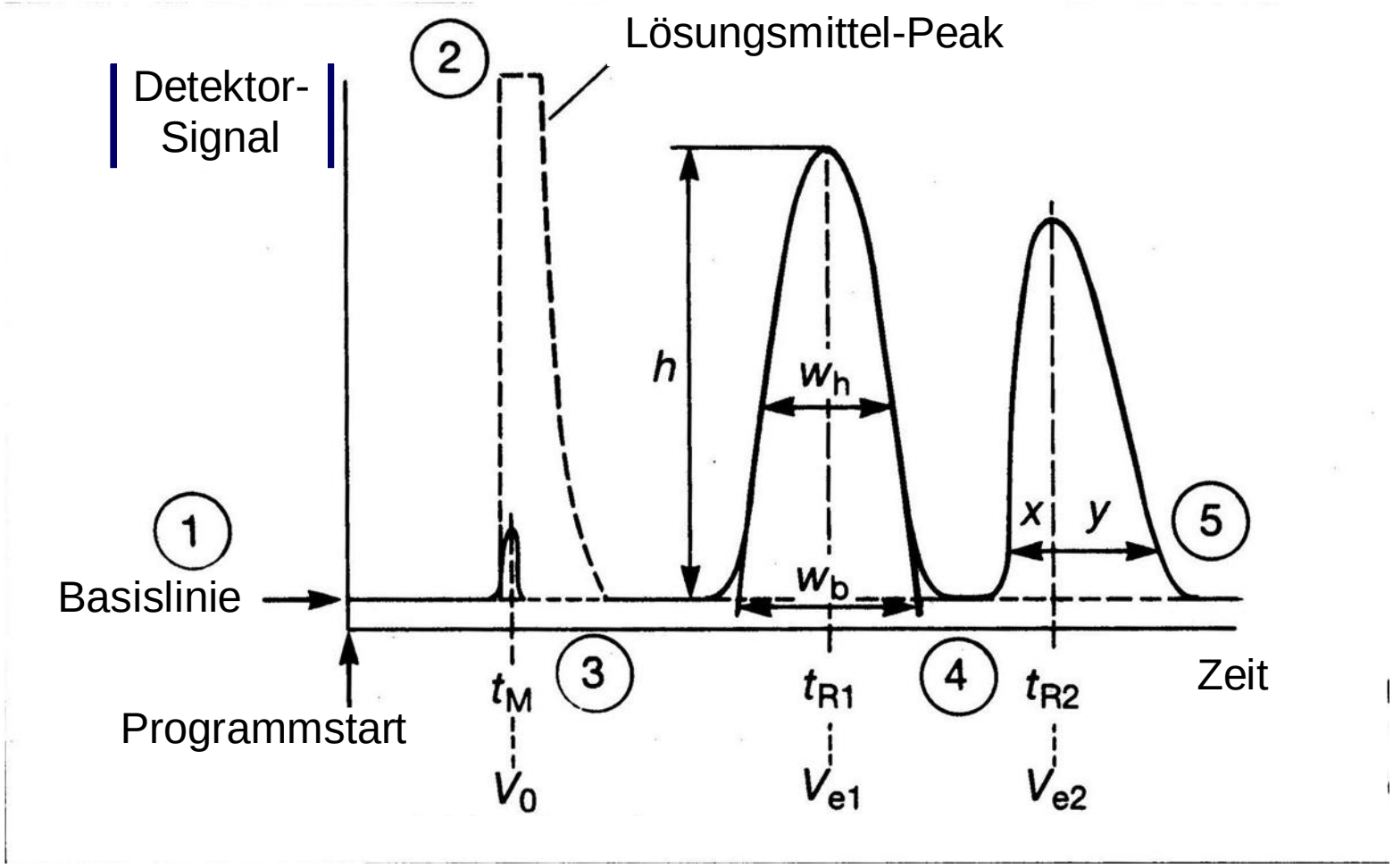
- The capacity factor is independent of the amount of the phases!

  e.g., $K_d = 1$ and stationary : mobile phase = 10 : 1
  $\Rightarrow$ 10 x more in A als in B

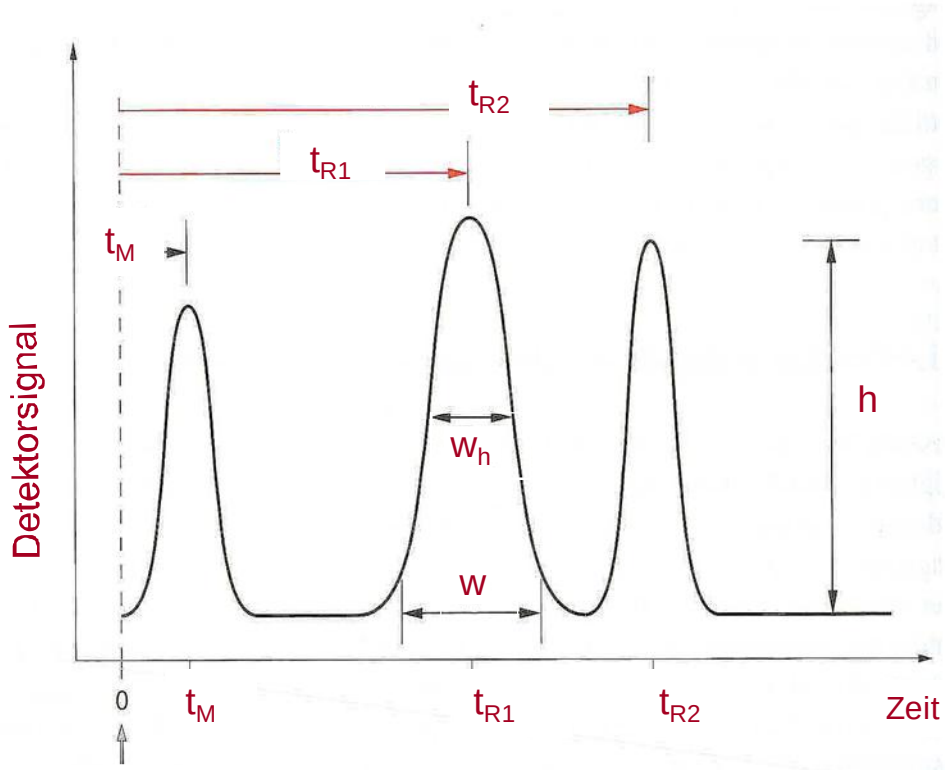
Separation



Chromatogram



Retention factor



t_R ... retention time
t'_R ... net- retention time
t_M ... dead volume
w ... peak width at base

$$k' = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M}$$

Relative Retention Factor α

Relative retention α is a measure for the selectivity of the system

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

α is dependent on the properties of the mobile and of the stationary phase.

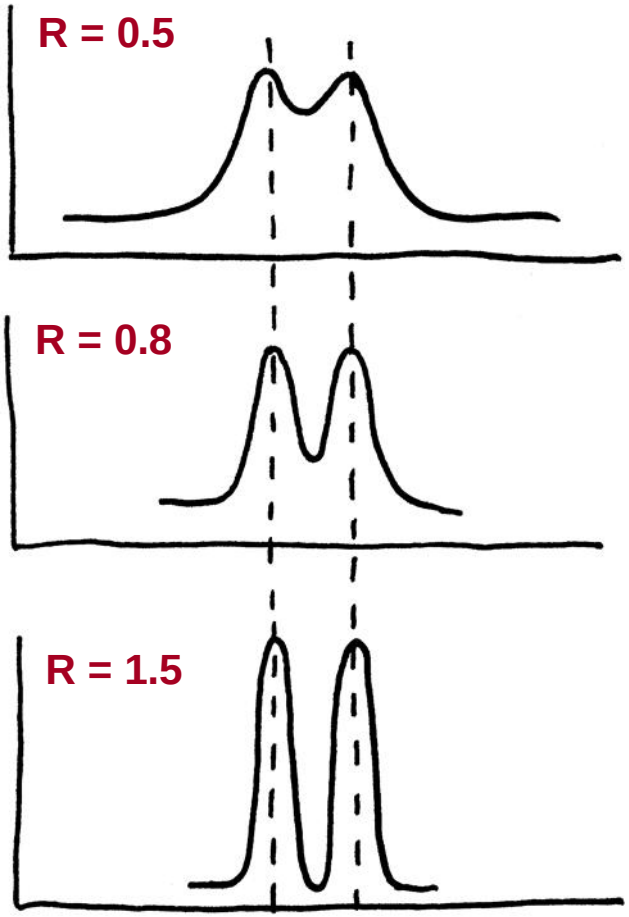
k‘ is independent of the length of the column and of the velocity of the mobile phase!

Substances can only be separated if k_1' und k_2' are different from each other.

Resolution

The resolution R of two neighboring peaks A + B is defined as:

$$R = \frac{2\,(t_{RB} - t_{RA})}{w_A + w_B}$$



Theoretical Plates

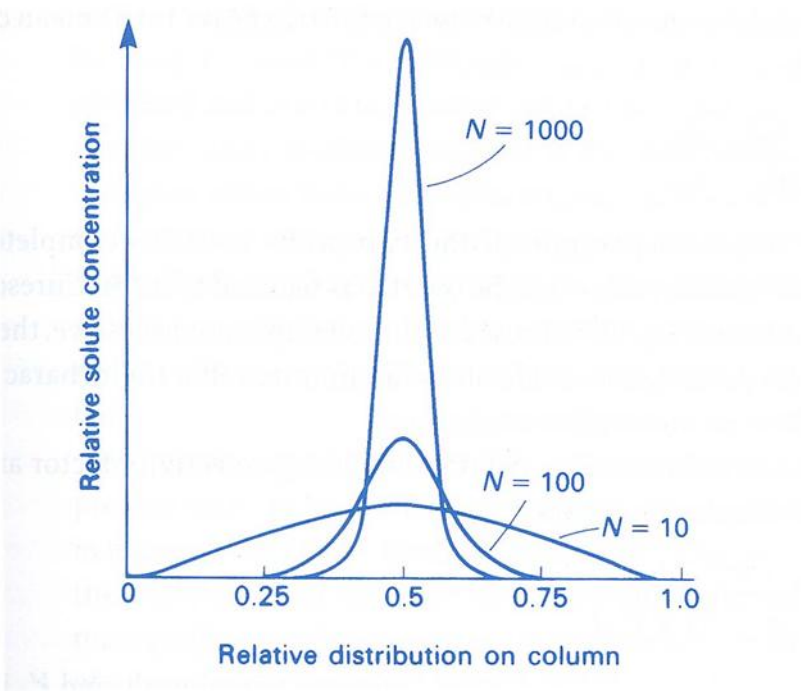
$$N = 16 \cdot \left(\frac{t_R}{W}\right)^2$$

Theoretical Plates

$$H = \frac{L}{N}$$

Height equivalent of a theoretical plate

The theoretical plate N (*theoretische Bodenzahl*) of a column is how often an equilibration of the Sample between the stationary and the mobile phase can occur. The hight equivalent H (*Trenn-stufenhöhe*) is the length of the column (L) in which the equilibrium will occur once (the smaller the better).



Chromatographic Separation

$$R = 0.25 \cdot (\alpha - 1) \cdot \sqrt{N} \cdot \left(\frac{1 + k'}{k'} \right)$$

Selectivity of the System „Interactions“

Performance of the column

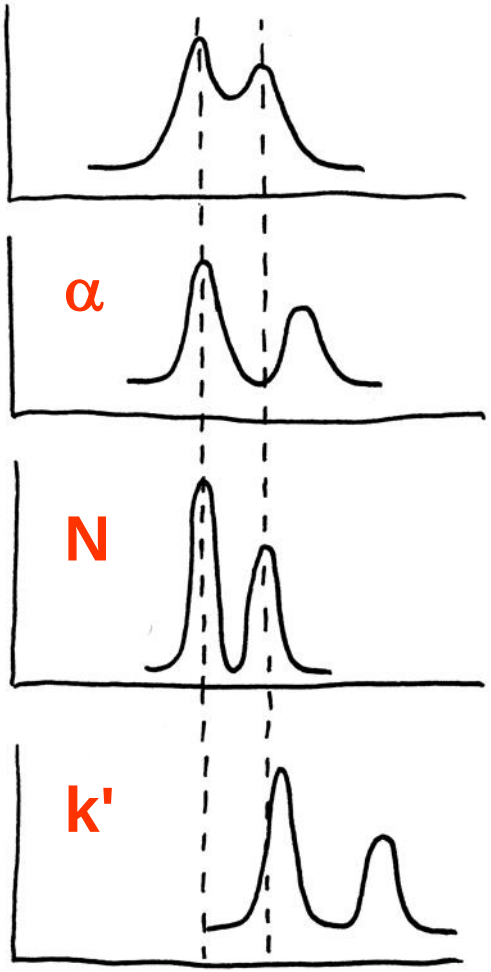
Strength of the eluent

How to improve the separation

N and k'
stay the same

α and k'
stay the same

N and α
stay the same



1. **Relative retention (α)**
   Change the stationary phase
   Interaction with mobile phase
2. **Theoretical plates (N)**
   better or longer column
   ($2L \approx 1.4\ R$); v of mobile phase
3. **Capacity factor (k‘)**
   Change strength of mobile phase

Quantification

- Establish the baseline
- Measure peak area or -height
- Simple calibration
- Use of internal standards

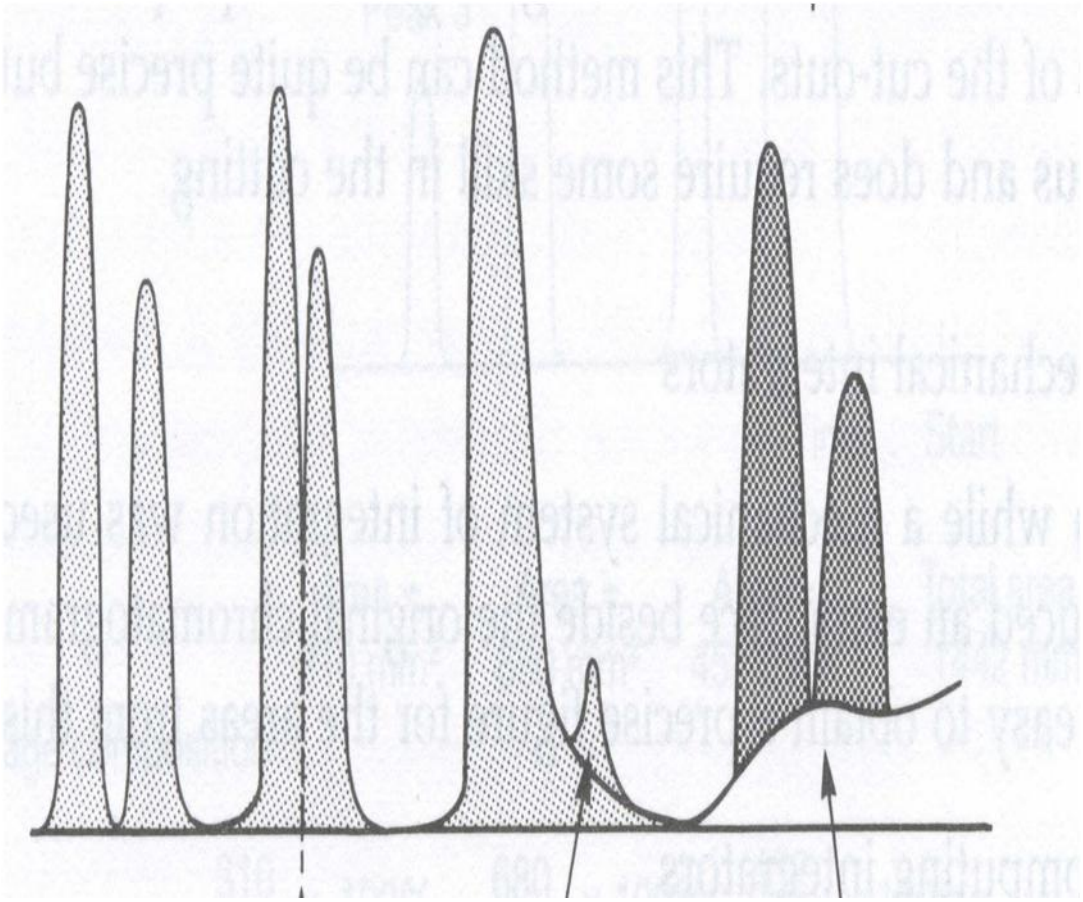
Baseline

The baseline is not (always) flat!

⇒„Drift“ of baseline

⇒ „Tailing“ or „Fronting“

⇒ Column „bleeding“

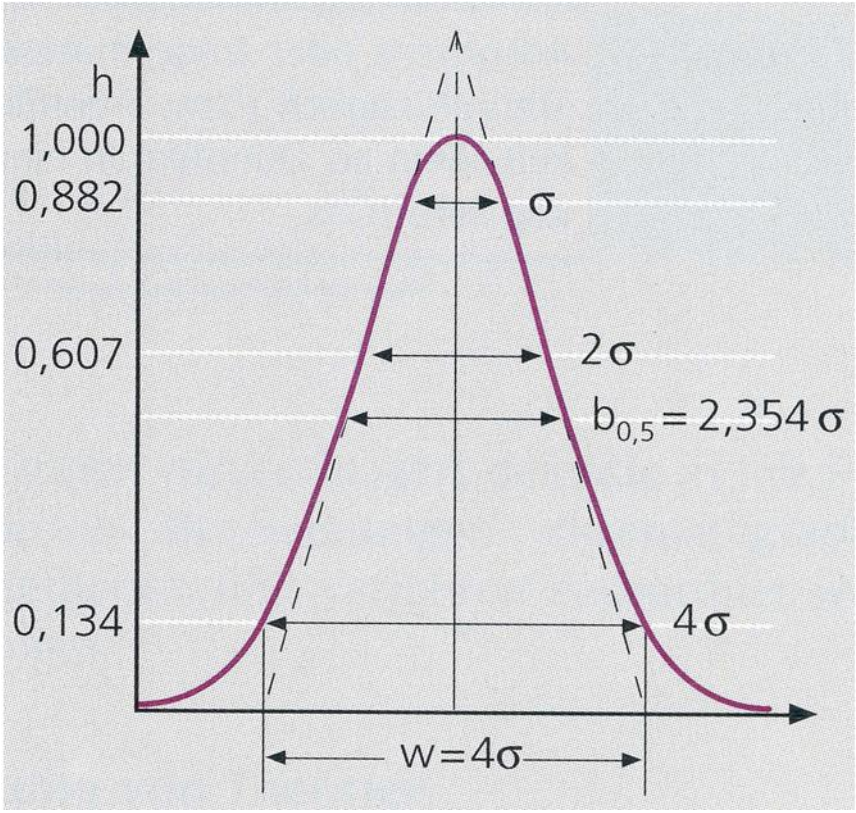


Peak-Area und Peak-Height

Estimation of peak-area A according to peak width at half height ($w_h = b_{0.5}$)

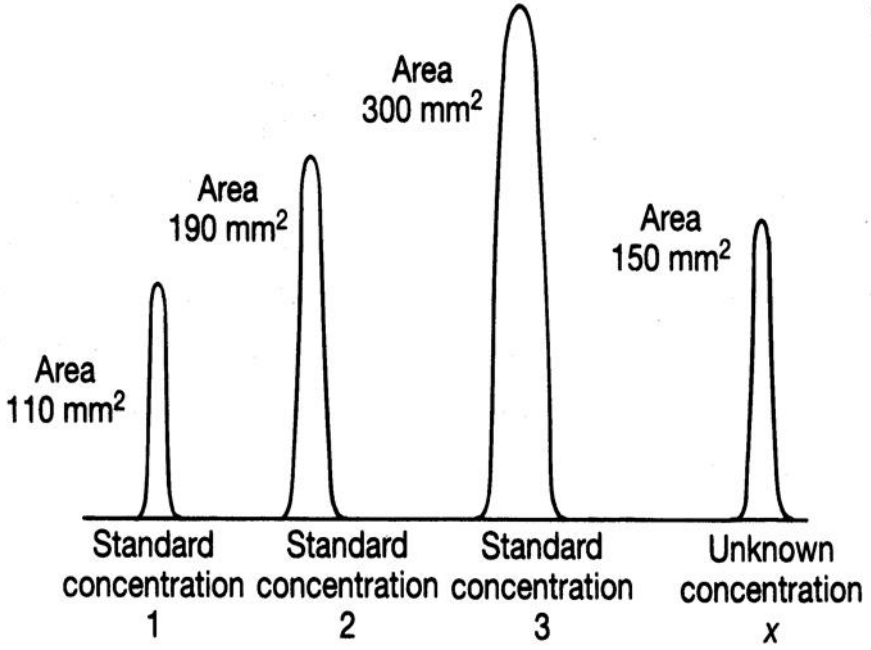
$A = w_h \cdot h$

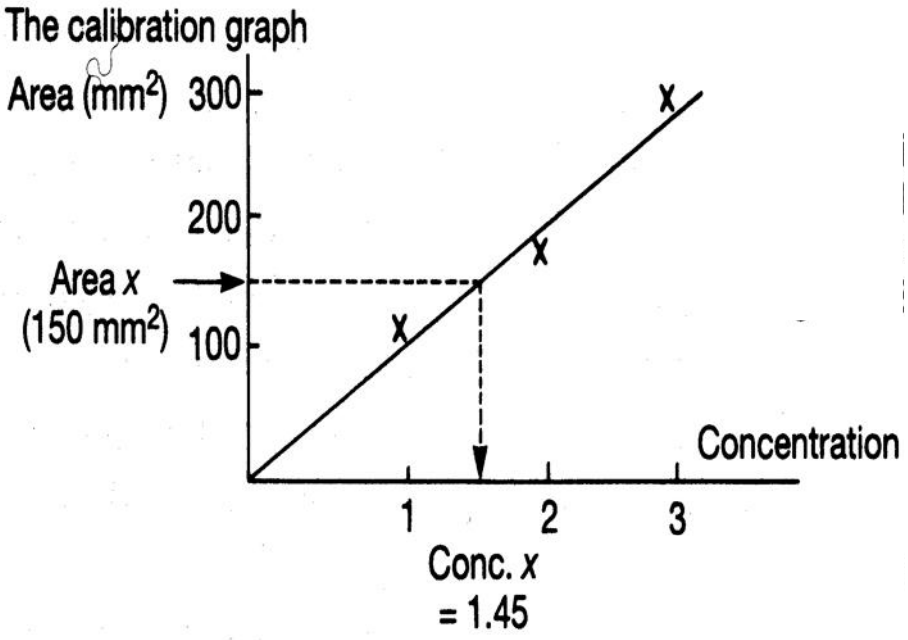
Today done with specific software



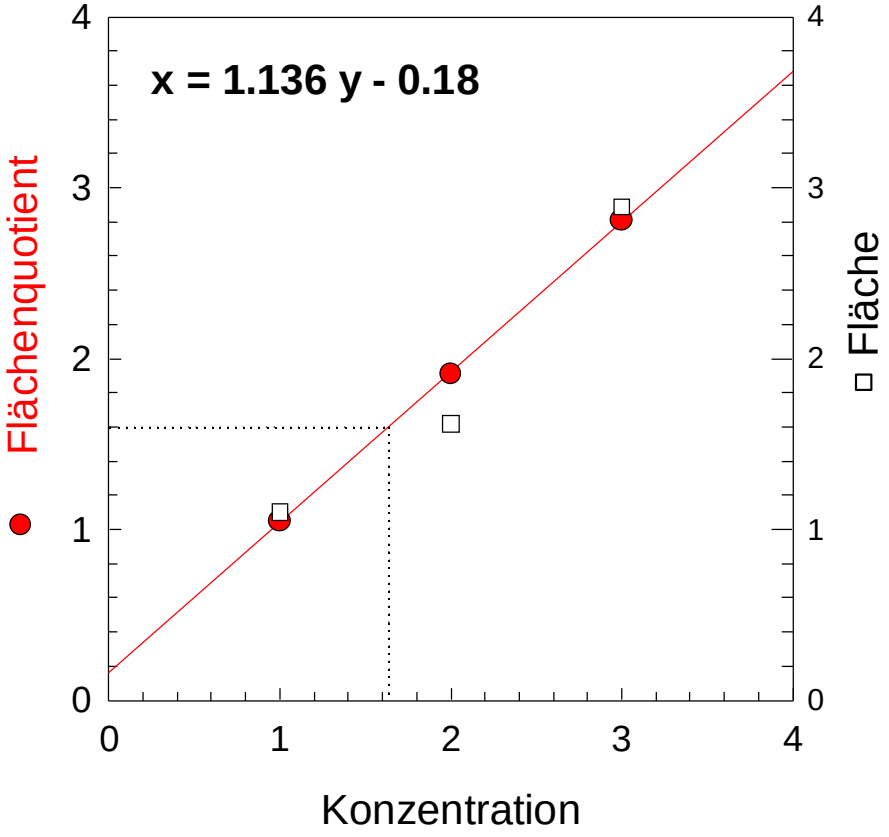
Simple Calibration

For each substance a calibration curve must be constructed. Calibration should be done at at least 4 concentrations and should be done for each sample batch!





Calibration with internal standard



Area quotient

$$FQ1 = \frac{\text{Area Std 1}}{\text{Area IStd1}} = \frac{1{,}05}{1{,}00} = 1.05$$

$$FQ2 = \frac{\text{Area Std 1}}{\text{Area IStd2}} = \frac{1{,}62}{0{,}85} = 1.91$$

$$FQ3 = \frac{\text{Area Std 1}}{\text{Area IStd3}} = \frac{2{,}98}{1{,}03} = 2.81$$

$$PQ1 = \frac{\text{Area Sample}}{\text{Area Istd P}} = \frac{1{,}35}{0{,}84} = 1{,}61$$

Mobile Phase

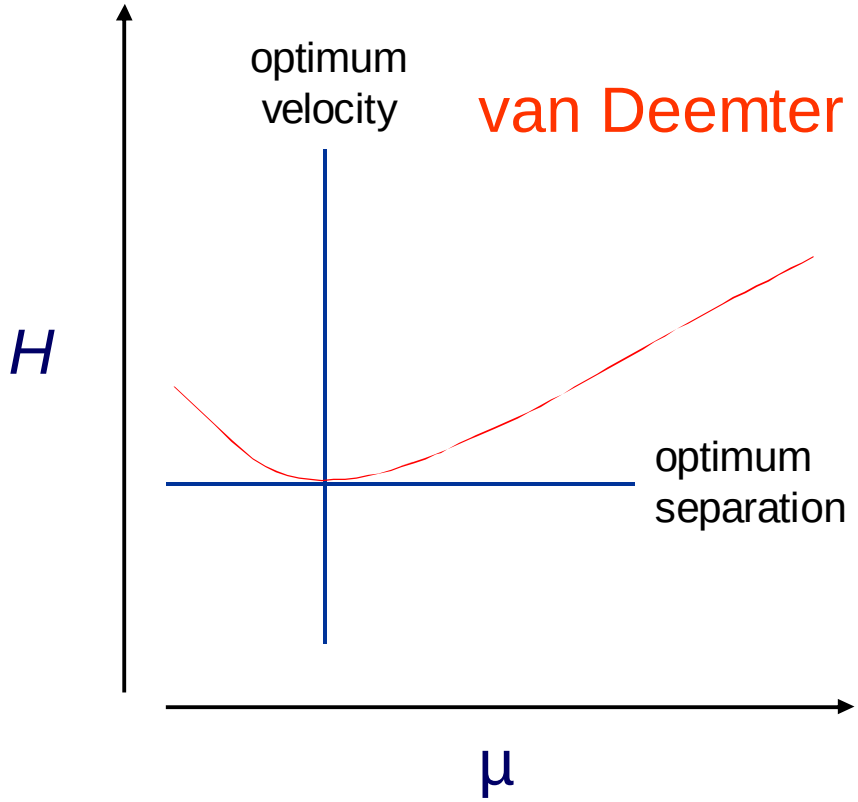
Mobile phase does not (directly) interact with the analyte, but only transports the analyte through the column (the system)!

Properties
chemical and physical properties determine elution strength (e.g., ion strength, lipophilicity,…)

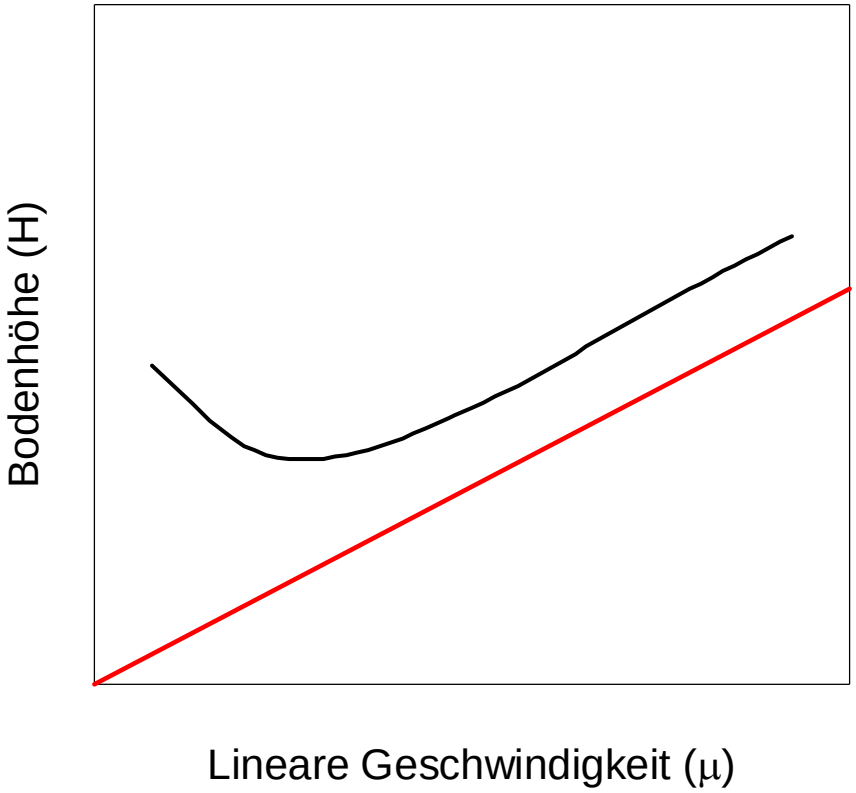
Flow rate
important for the separation
flow: 0.1 - 2.0 mL min^{-1}
linear velocity (μ)

Band broadening in chromatography

Effect of flow rate on separation (band broadening)



Van Deemter Equation 1



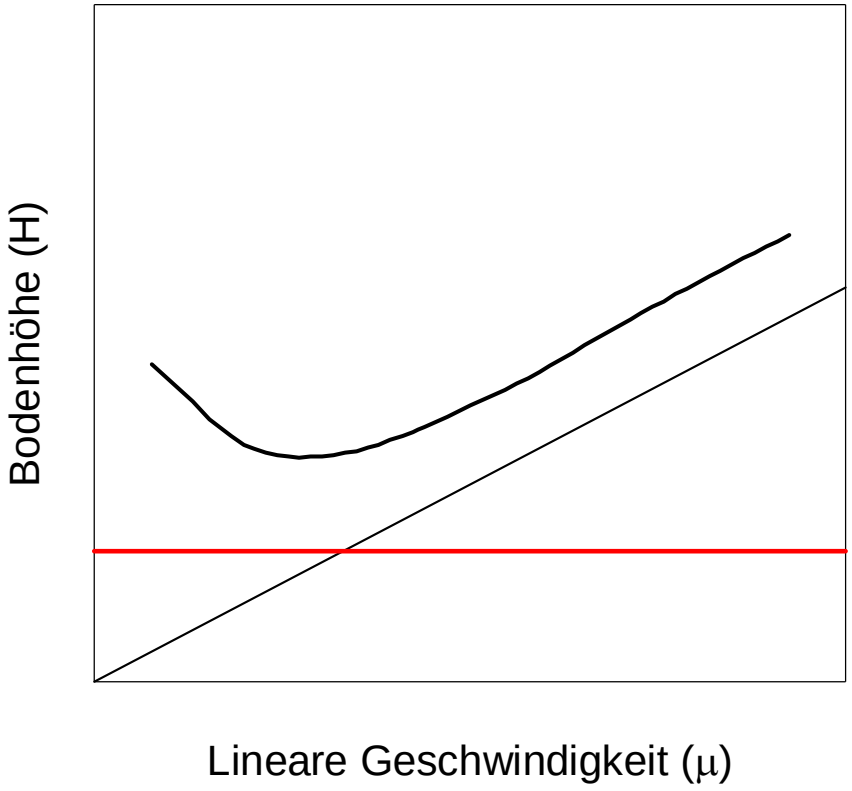
Mass transfer coefficent (C)

Diffusion controlled mass transfer between stationary and mobile phase. ***Linearly dependent on velocity of mobile phase***

Molecules in the mobile phase are transported, while molecules in the stationary phase are stagnant. Leads to

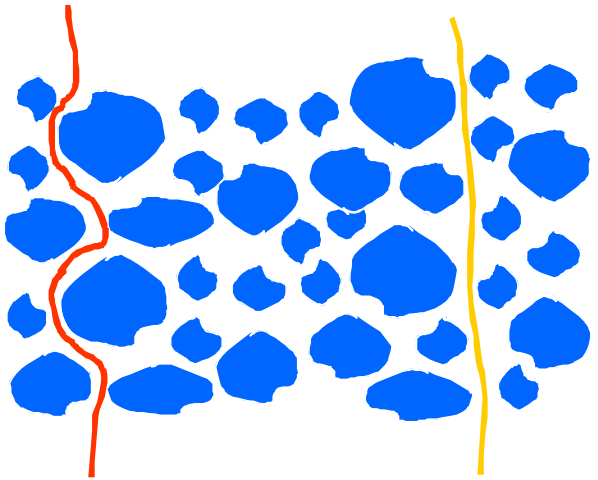
⇒ ***Band broadening***

Van Deemter Equation 2

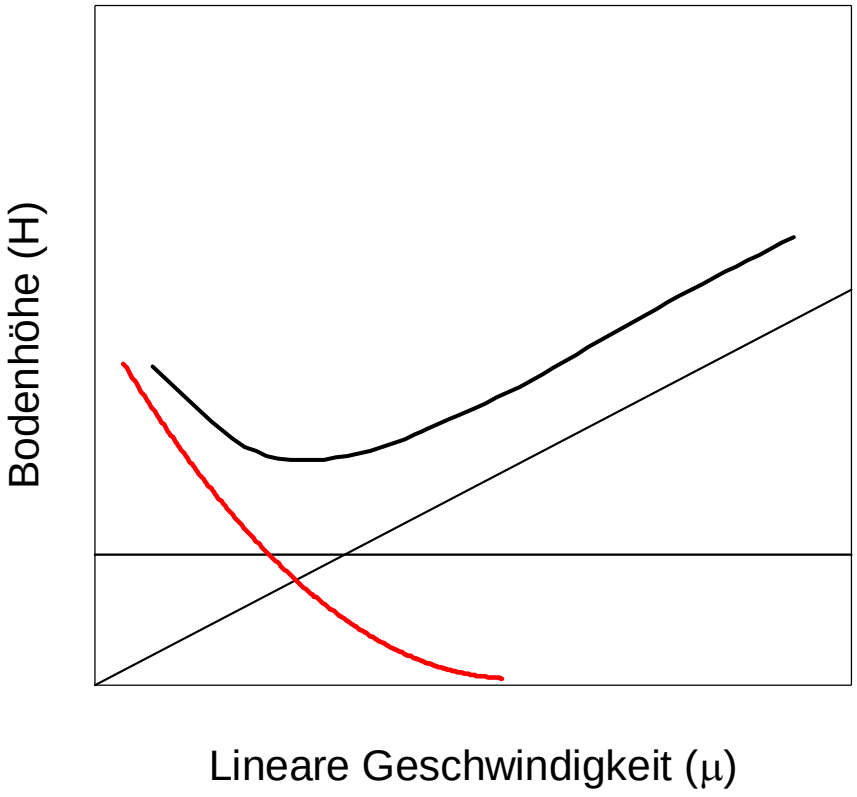


Eddy-Diffusion (A)

Solute molecules will take different paths through the stationary phase at random.
Independent of velocity!



Van Deemter Equation 3

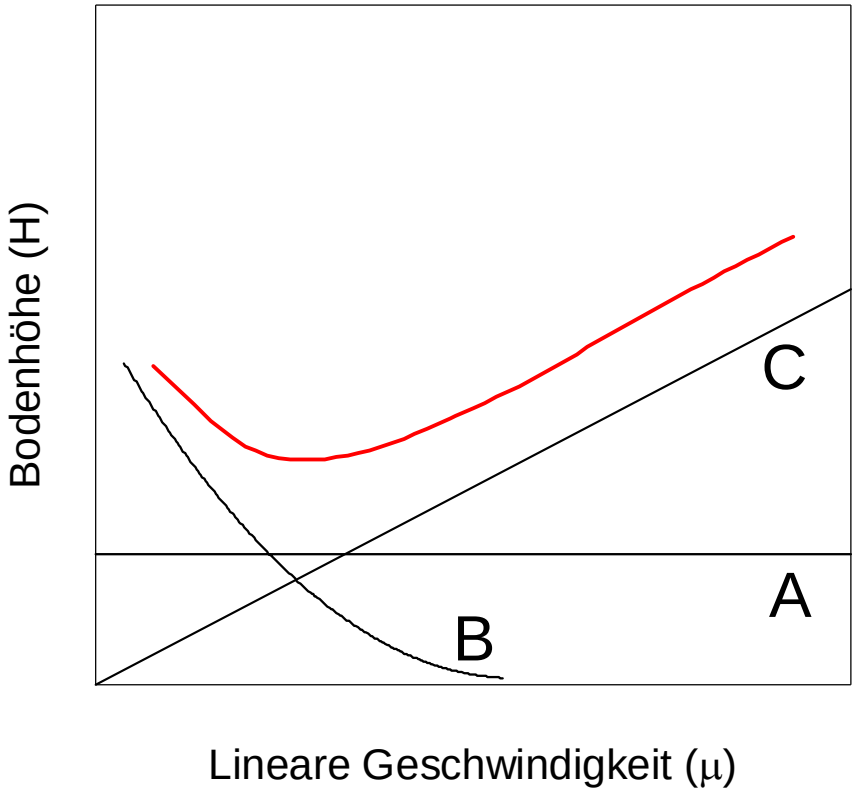


Longitudinal-Diffusion (B)

Diffusion along the length axis of the column (in direction of flow). More important at low velocities

B decreases with higher linear velocities.

Van Deemter Equation 4



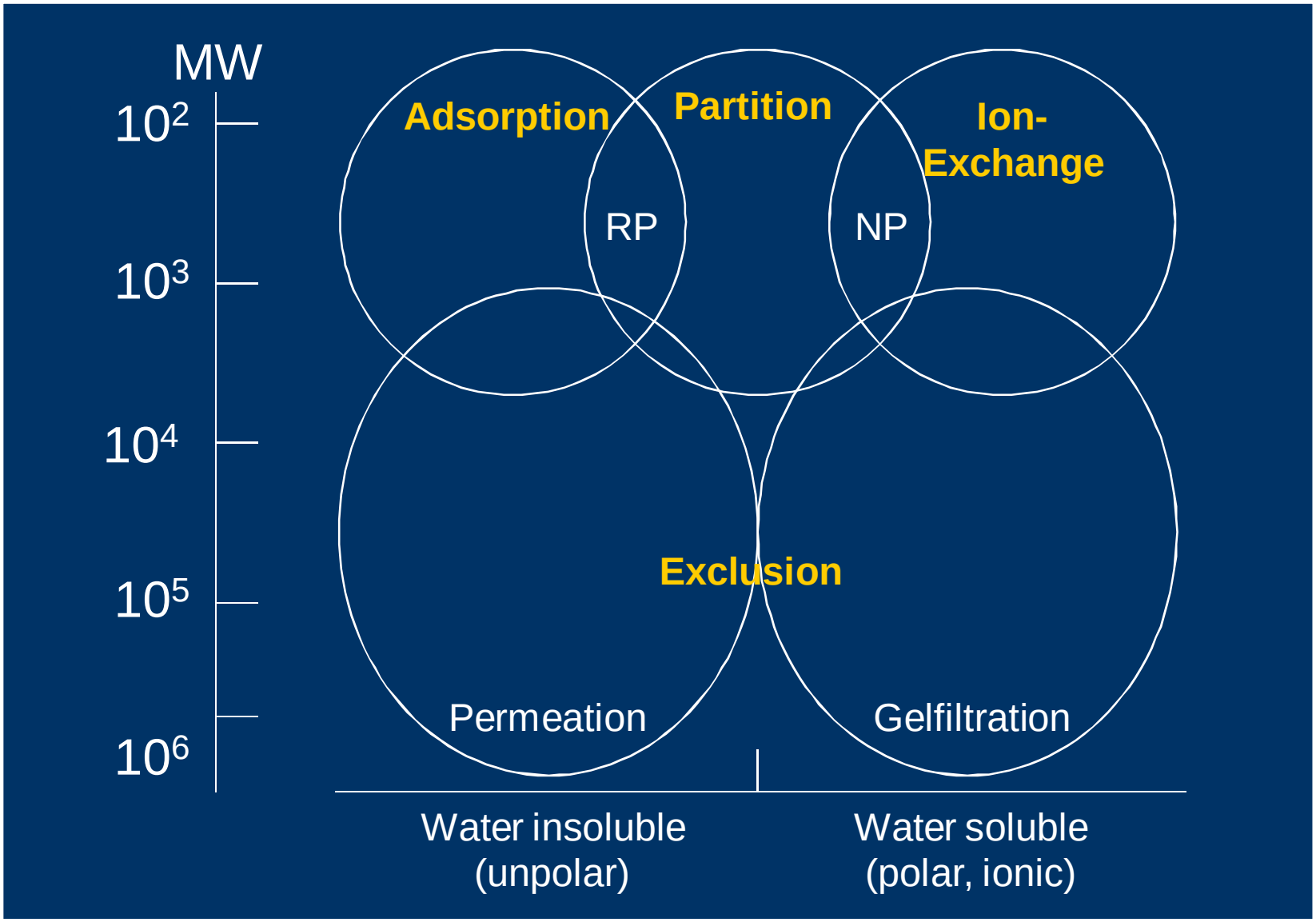
$$H = A + \frac{B}{\mu} + C \cdot \mu$$

Teil 1

HPLC & AEC

High Performance Liquid Chromatography &
Anion Exchange Chromatography

HPLC

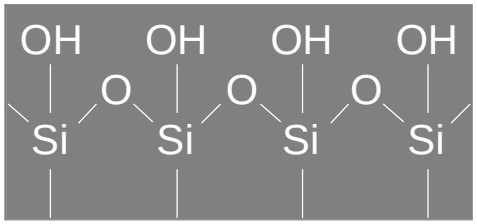

MW
10^2
10^3
10^4
10^5
10^6
Adsorption
Partition
Ion-
Exchange
RP
NP
Exclusion
Permeation
Gelfiltration
Water insoluble
(unpolar)
Water soluble
(polar, ionic)

Partition chromatography

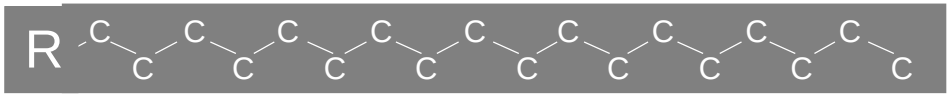
Separation by POLARITY!

Liquid-Liquid-Chromatography *or*
Chromatography on chemically bonded phases

Stationary phases:
Silica gel (3-10 µm) modified with:
e.g., C18 (ODS), C4 -, Amino-groups



Silicagel

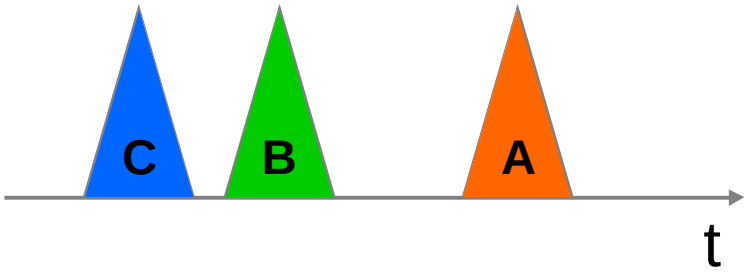


ODS

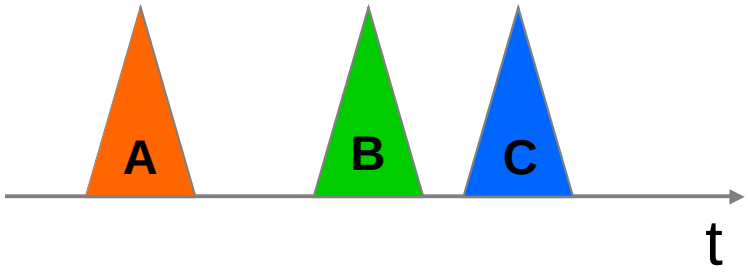
Reversed Phase Chromatography

Normal phase
polar stationary phase +
non-polar mobile phase
(z.B. aluminiumoxid/H_2O +
hexane)

Polarity: A > B > C



Reversed phase
(Umkehrphasen)
non-polar stationary phase +
polar mobile phase
(e.g., C18 +
methanol/acetonitrile)



Adsorption Chromatography

- Silica oder aluminium oxides as stationary phase
- Only the polarity of the mobile phase can be changed to improve the separation
- Used in addition to partition chromatography if substances are strongly non-polar (non-soluble in polar solvents)
- Good for separation of isomers

Ion Chromatography

- Stationary Phases are ion-exchangers (highly cross-linked polystyrene resins) with immobilized, charged functional groups:

  Anion exchanger: [— $N(CH_3)_3^+$ OH^-]

  Cation exchanger : [— SO_3^- H^+]

- Electrolytes as mobile phases
  salt solutions at low concentrations, acids or bases

- Elution by increase of ion strength or by pH changes!

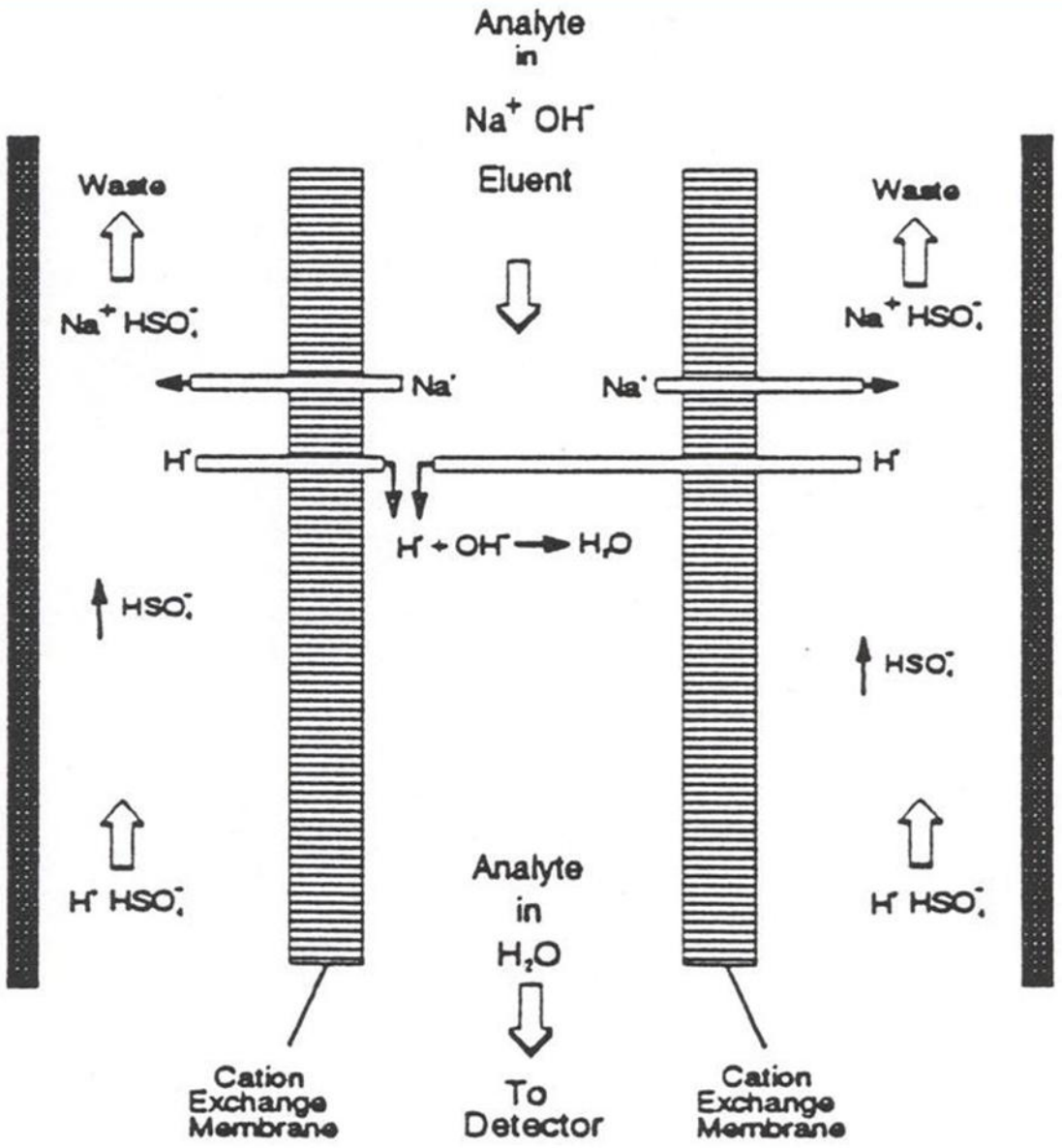
Applications of IC

- Separation of organic and inorganic ions (especially for anions, for which only few other chromatographic methods exist)
- Separation of carbohydrates on anion exchange columns (carbohydrates are charged in alkaline solutions!)
- Determination of amino acids on cation exchanger (amphoteric molecules are cations below pH=6)
- Important methods for the separation of proteins (AEC at weak anion exchangers)

Suppression of eluent conductivity

- In ion chromatography usually conductivity detection
- The eluent, which usually is a salt or a base/acid, inherently has a high conductivity
- This conductivity therefore needs to be suppressed, either electronically or chemically)

Suppression of eluent conductivity



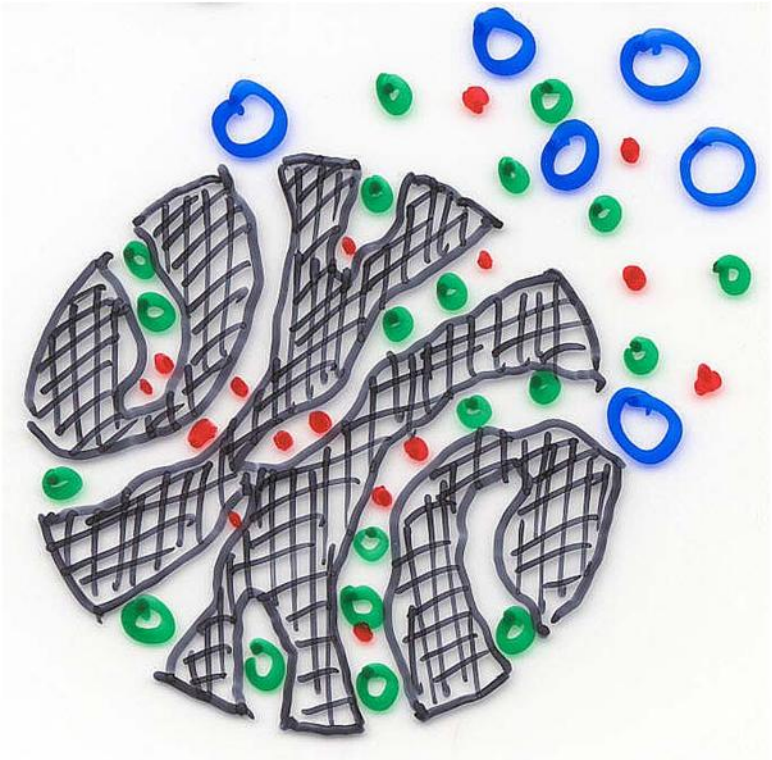
Size exclusion chromatographie

Porous stationary phase (e.g., polymer-particles)

Mobile phase is present in 2 forms:

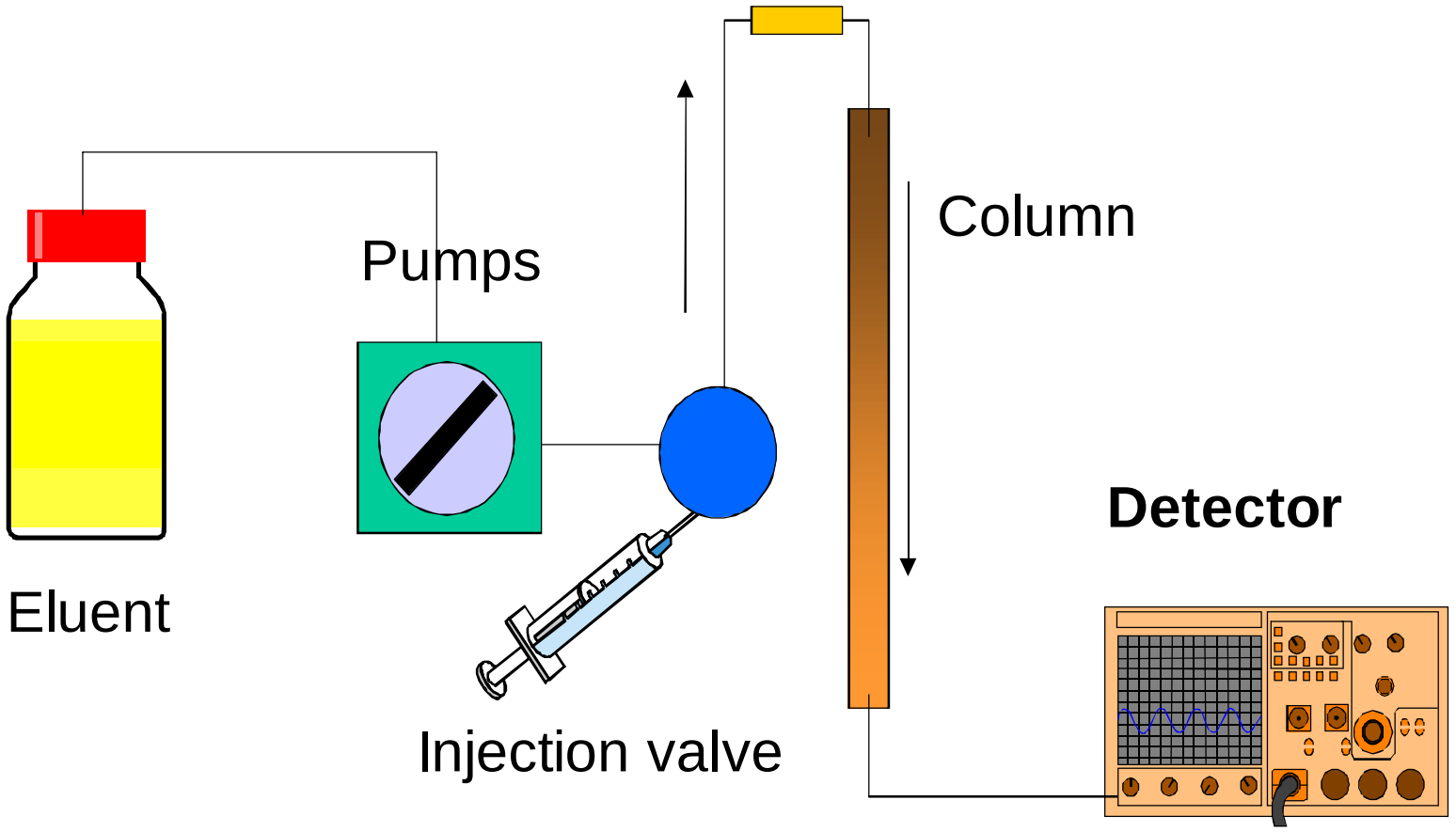
+ flowing, between the particles
+ stagnant, within the pores

Bigger molecules are excluded from the pores and are therefore eluting first; smaller molecules can penetrate the pores and are retarded according to their size (deepness of penetration)



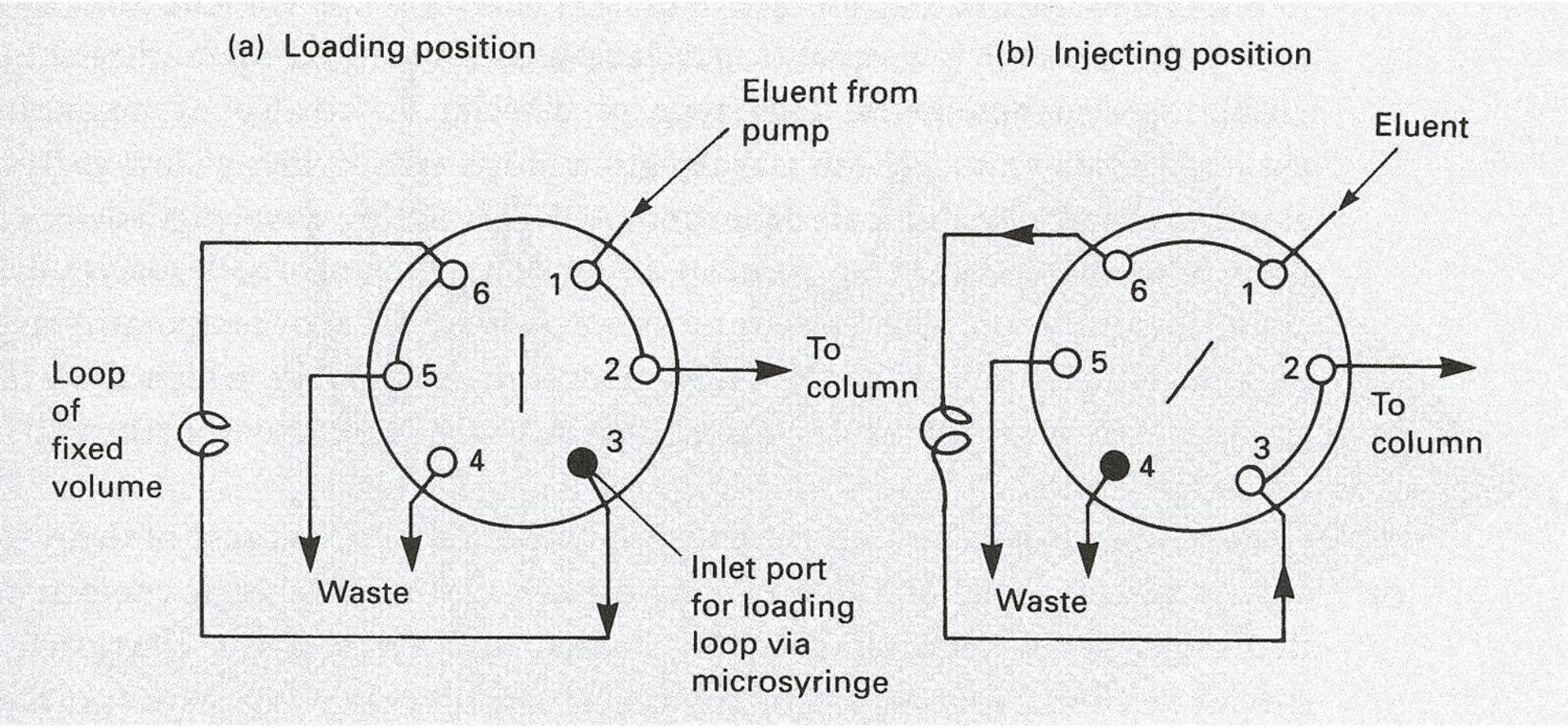
HPLC Systems

...modular systems!



Injectors

Rheodyne



Detectors

	Limit of detection
• UV-Vis detector	1 ng
• Diode-array detector (DAD)	1 ng
• Refractive index-detector (RI)	1 µg
• Electrochemical detector (AD, PAD)	10 pg
• Conductivity detector (CD)	1 ng
• Fluorescent-detector	10 pg
• Light scattering-detector	10 µg
• Mass-selective detectors (HPL-MS)	1 ng