Chromatography (I)

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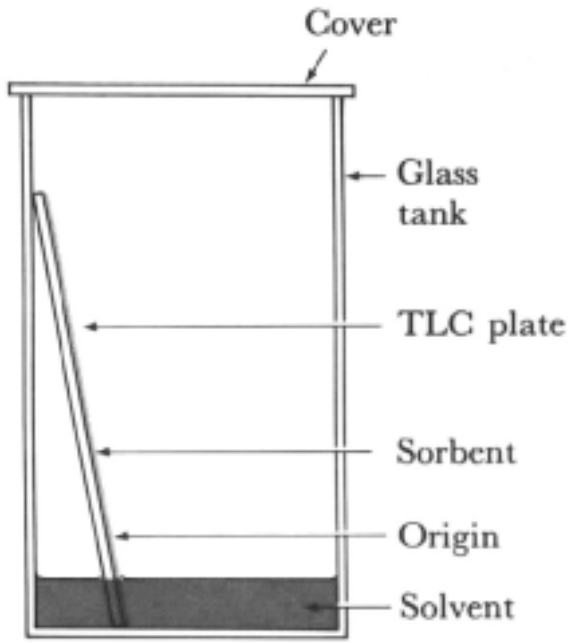
10/28/2004

Objectives

- The physical basis of chromatography
- The chemical basis of the principal chromatography methods
- Performance criteria for comparing chromatography systems

History

- 1903 Mikhail Tswett (Russian)
  - Separate plant pigments
  - “chroma” – color in greek
  - Example
    - Thin layer chromatography (TLC)
- Chromatography
  - A separation technique
  - Based on the different adsorptive or partitioning properties of the sample molecules.

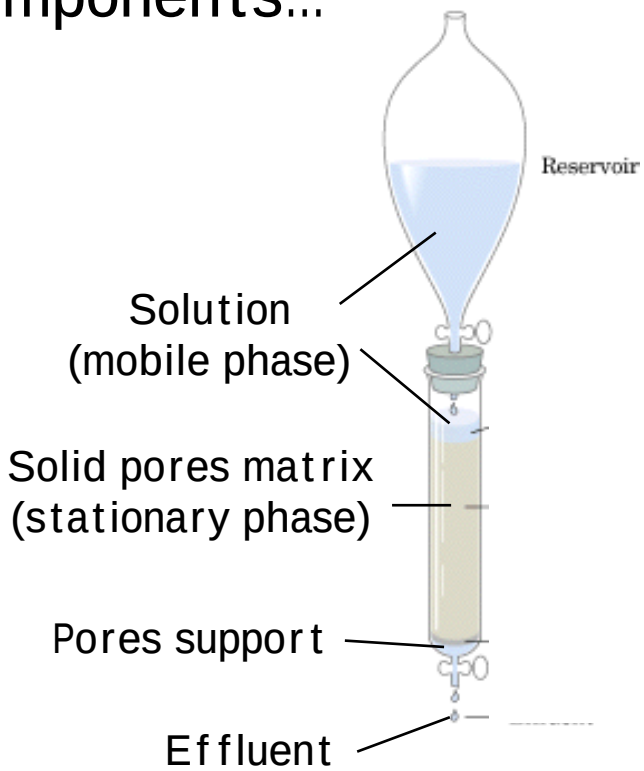


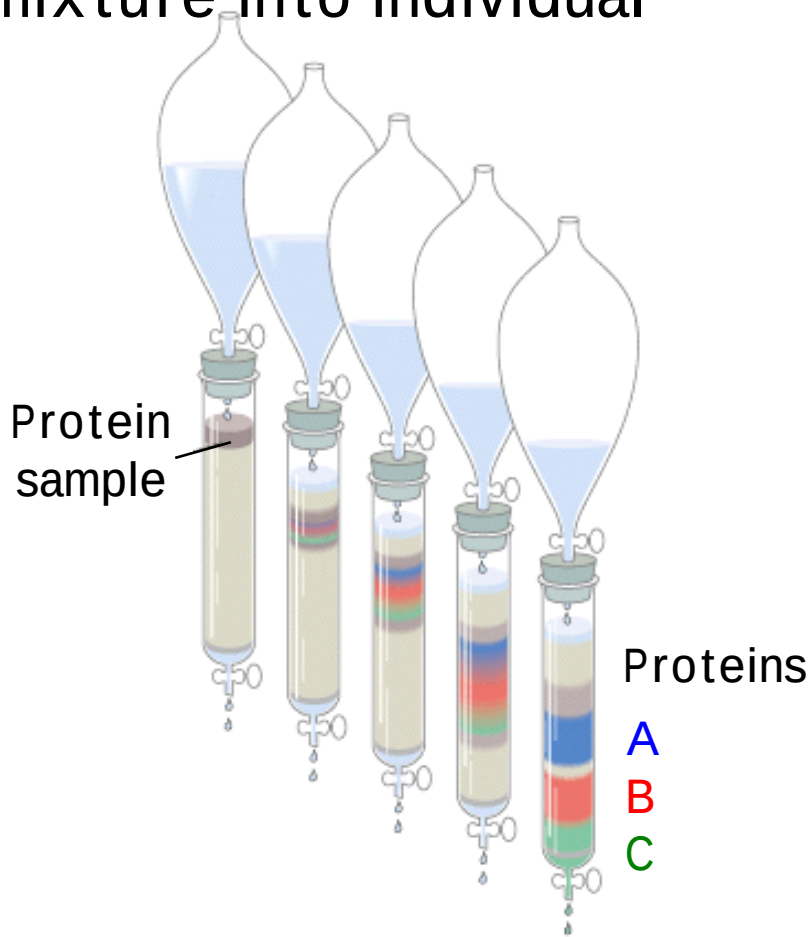
Basic principle

- Chromatography system
  - Stationary phase
    - The stationary adsorbant (immobilized)
  - Mobile phase
    - The fluid moving through the chromatographic column
- Molecule partition between two phases
  - Partition coefficient, K_d
    - $K_d = C_s / C_m$
      - C = [sample] in the S and M phases
      - K is affected by temperature, solvent polarity, etc.
- Separate a mixture into its components

Column Chromatography

- Stationary phase + mobile phase
- By charge, size, binding affinity difference
- Fractionation: separate mixture into individual components…





From Lehninger, Prin. Biochem.

Ion Exchange

- By charge difference
- Cation and anion exchanger
  - Refers to the target interested
- Changing pH or salt concentration

Negatively charged beads

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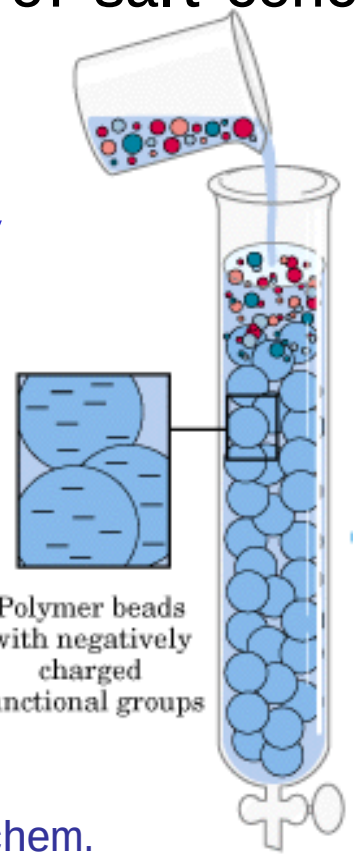
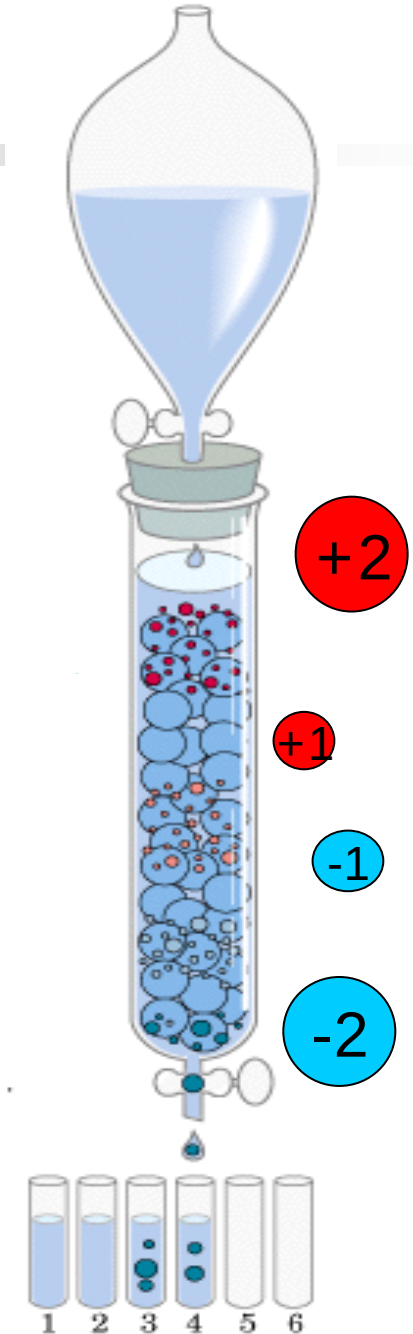
Cation exchanger

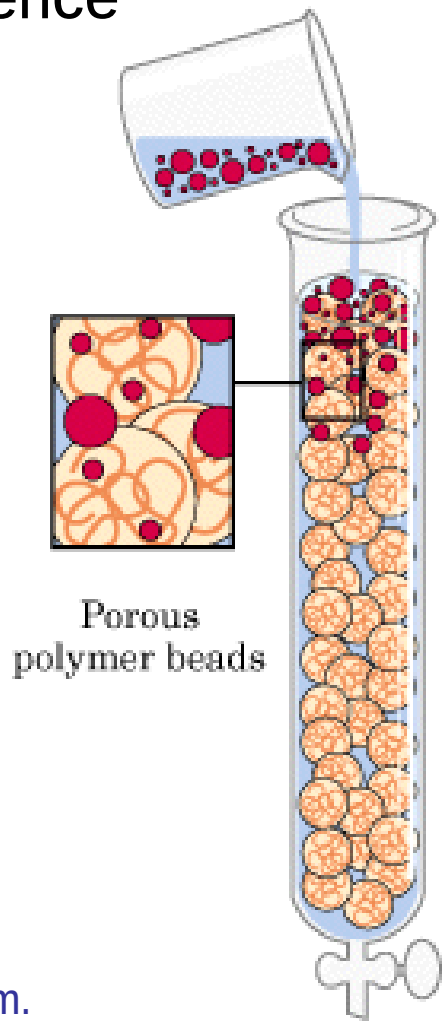


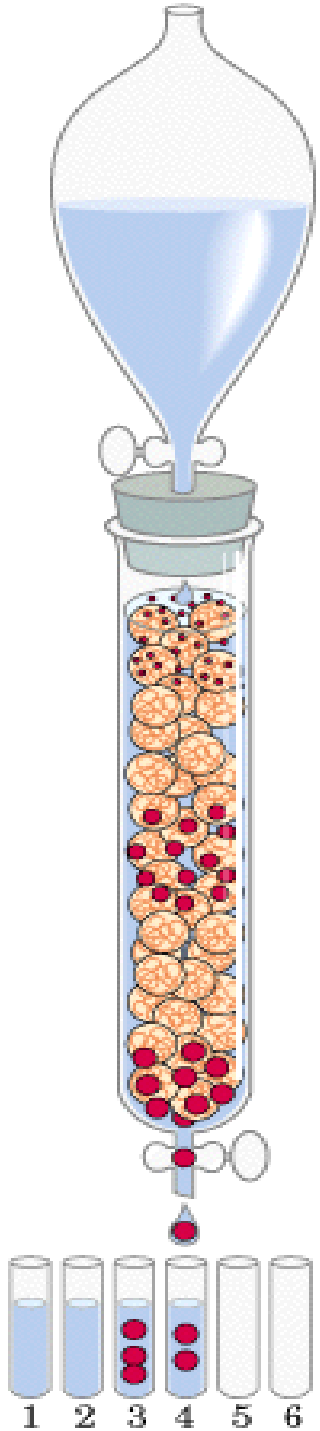


From Lehninger, Prin. Biochem.

Gel filtration

- Size-exclusion, gel permeation
- By size difference
- Pores beads

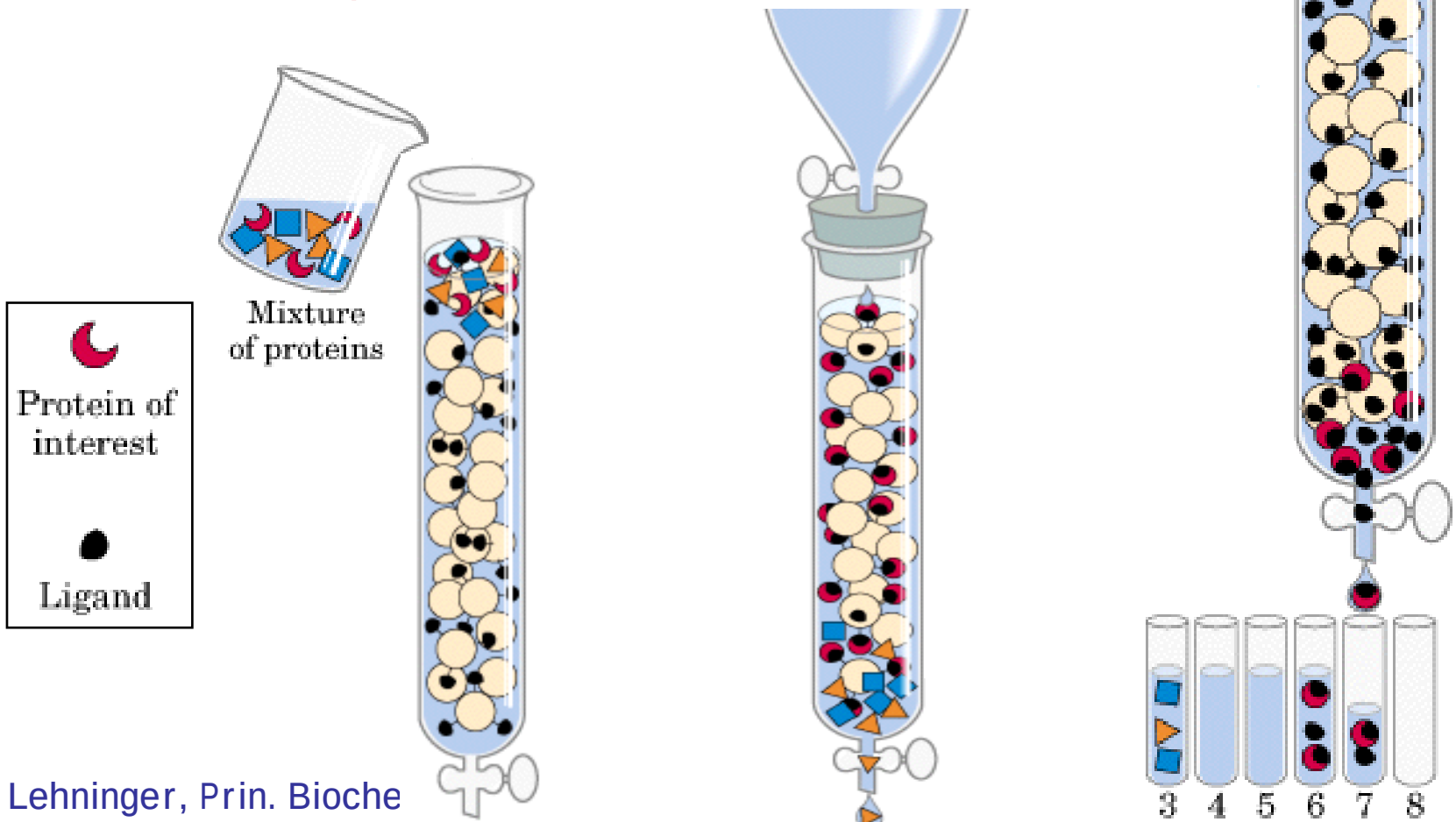




From Lehninger, Prin. Biochem.

Affinity

- By protein-ligand binding specificity
  - His-tag fusion protein and Ni-column
  - GST-fusion protein and GSH-column



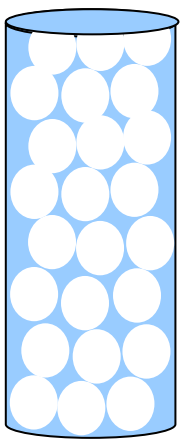
From Lehninger, Prin. Bioche

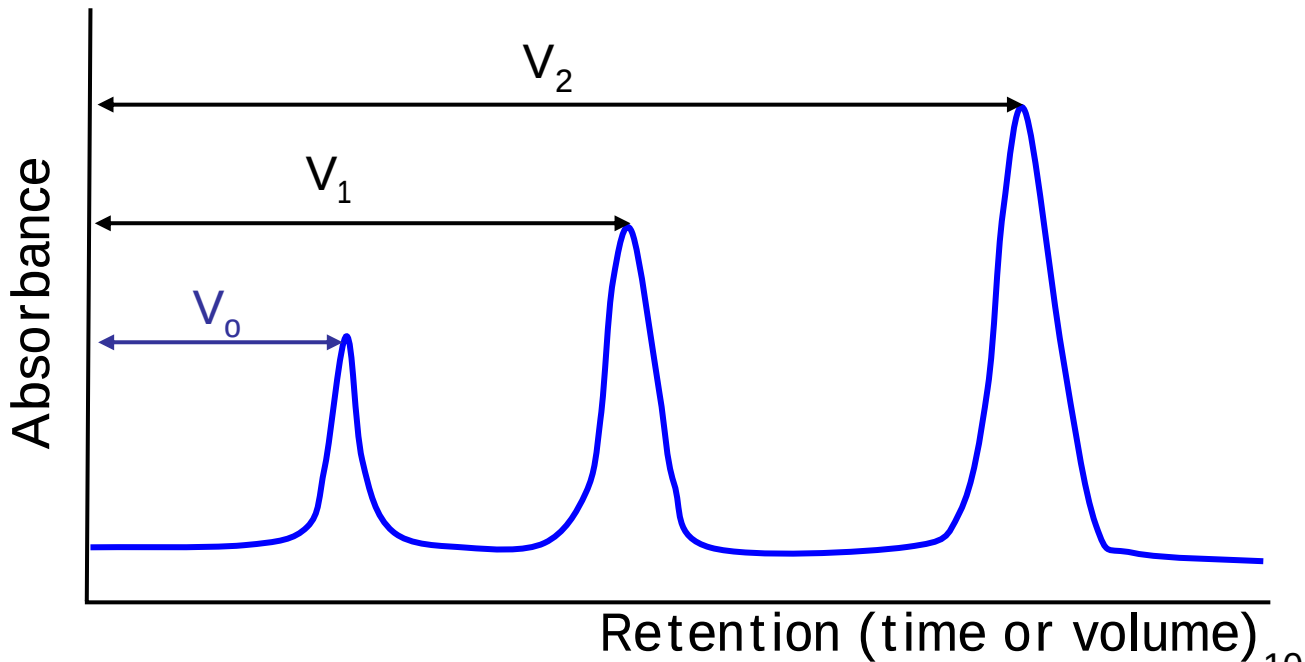
Performance parameters

- For comparison of different systems:
  - Retention
  - Resolution
  - Peak broadening
  - Plate number
  - Peak symmetry
  - Capacity factor

Retention

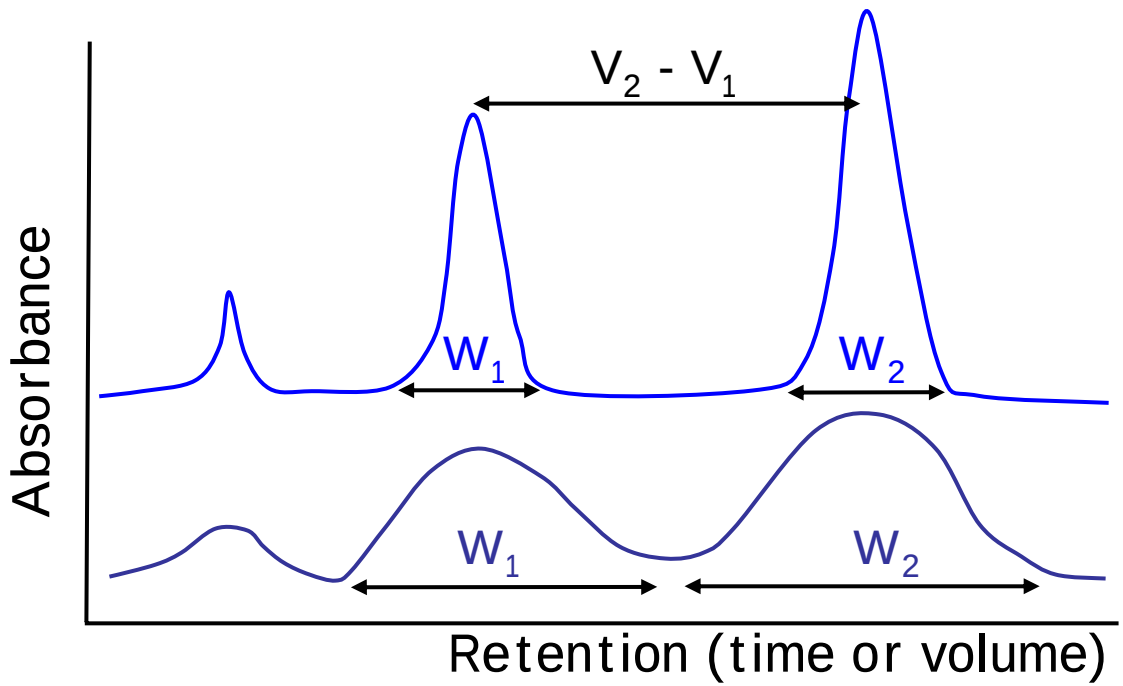
- Samples are retained on column to different extent
  - Retenion time (t_R), retention volume (V_R)
    - $V_R = f\, t_R$, f = flow rate
    - Retention $\propto$ column length, $1/f$
  - Void (excluded) volume, V_o
    - The contained volume of the column – volume of packed matrix





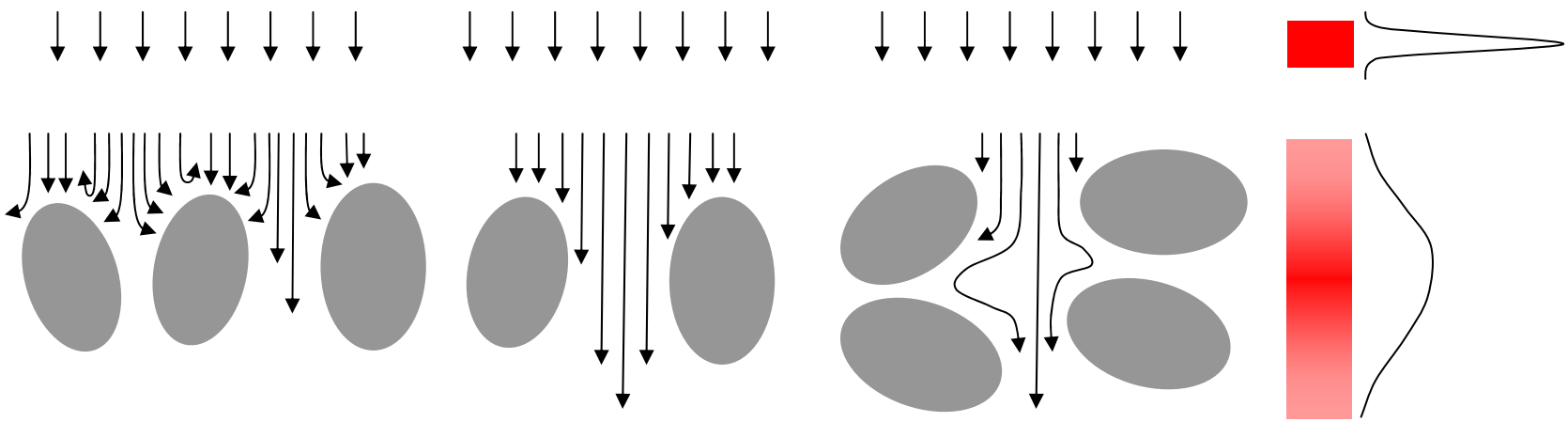
Resolution (R)

- Describe how well peaks are separated
  - $R = (V_2 - V_1) / \frac{1}{2} (W_1 + W_2)$
    - Well separated (large $V_2 - V_1$)
    - Narrow peaks (small W)



Peak broadening

- Eluted volume > applied sample volume
  - Diffusion
    - Sample interact with stationary phase
  - Mass transfer phenomena
    - Within mobile and/or stationary phase



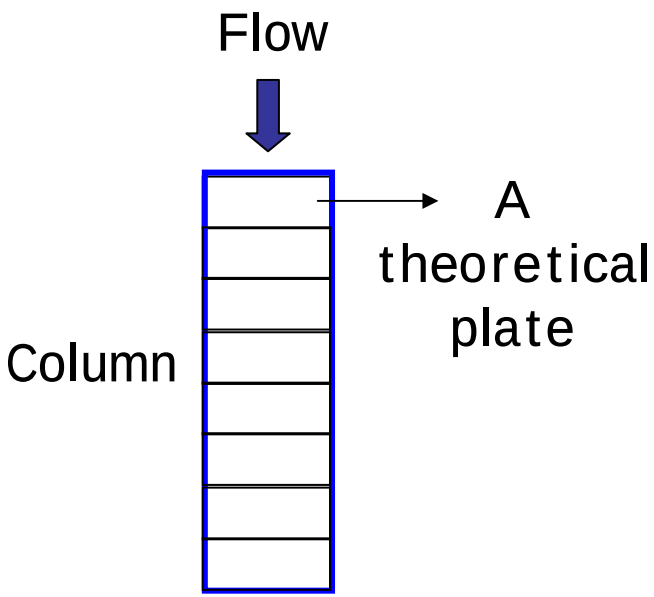
Eddy diffusion

Mobile phase mass transfer

Stagnant mobile phase mass transfer

Theoretical plates

- The column is made of very thin, horizontal plates
  - A very thin section within which molecules can partition between mobile and stationary phases.



When K_d = 1, total of 256 molecules in the sample:

One plate

	Stationary phase	Mobile phase
Plate 1	128	128

Two plates

Plate 1	64	64
Plate 2	64	64

Partition between phases

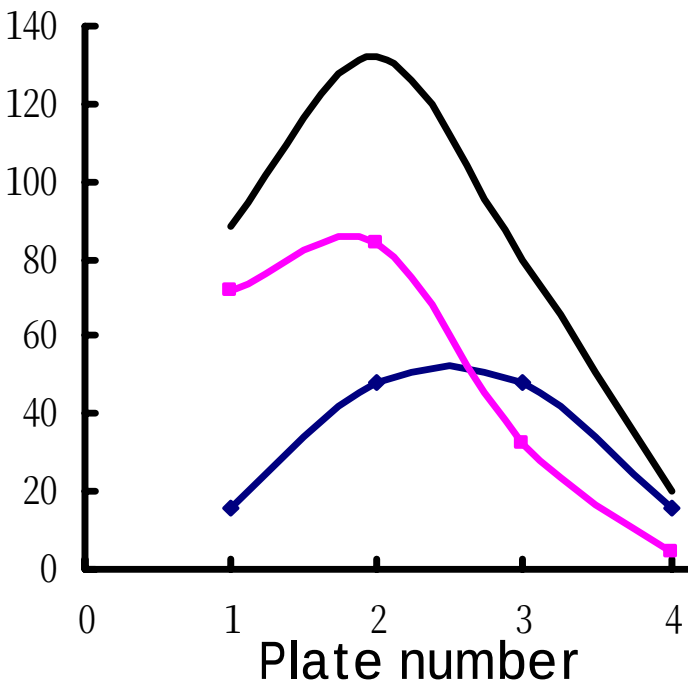
- Redistribution of the molecules between the stationary and mobile phase

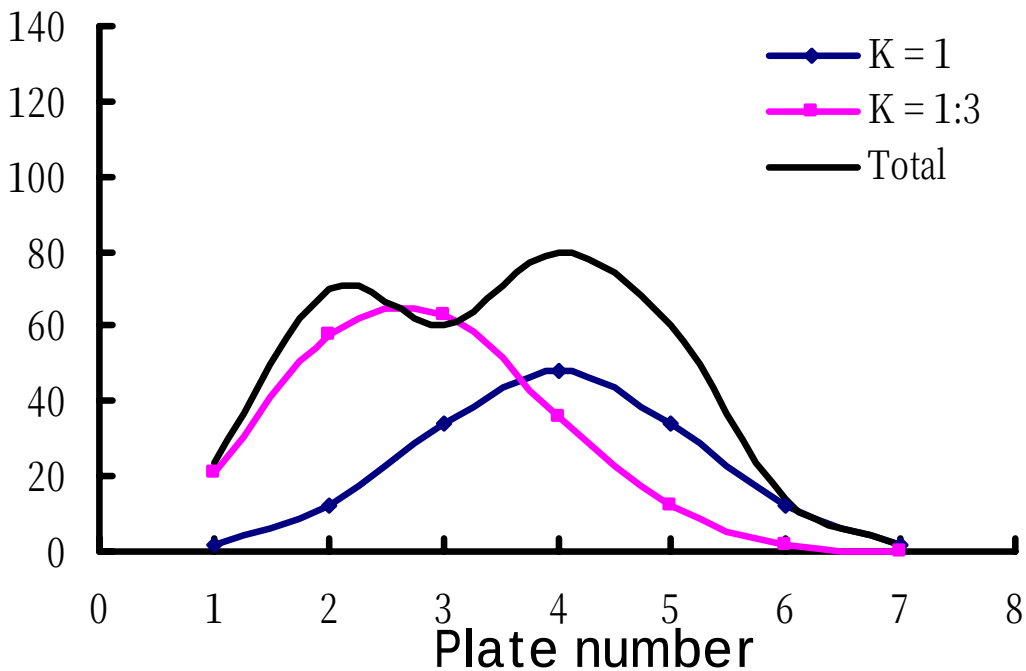
When K_d = 1, total of 128 molecules in the mobile phase

	N=1	N=2	N=3	N=4	N=5	N=6	N=7	N=8
Plate 1	128	64	32	16	8	4	2	1
Plate 2		64	64	48	32	20	12	6
Plate 3			32	48	48	48	34	23
Plate 4				16	32	48	48	41
Plate 5					8	20	34	41
Plate 6						4	12	23
Plate 7							2	6
Plate 8								1

Good resolution

- Large plate number and small plate height
  - Plate number (N)
    - The column is made of very thin, horizontal plates
  - Plate height (H)
    - H = column length/N



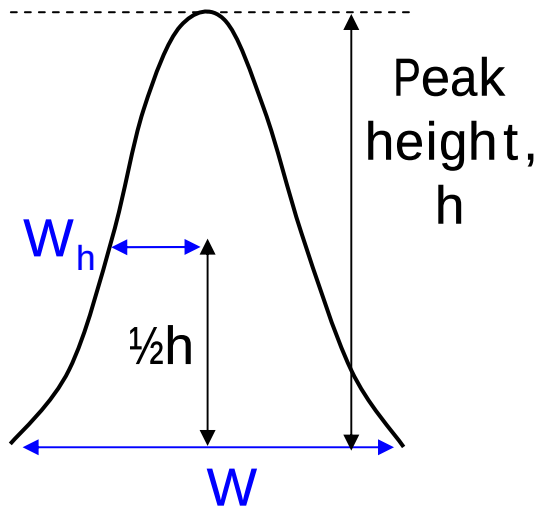


Theoretical plate number, N

- Related to the surface area of the particles of which the stationary phase is composed

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

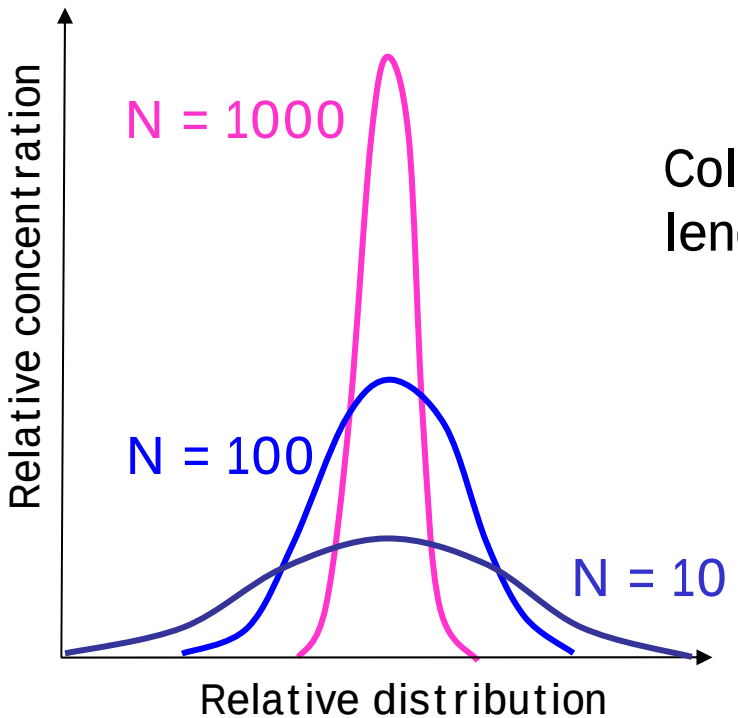
$$\text{or} \quad N = 5.54 \left(\frac{t_R}{W_h}\right)^2$$

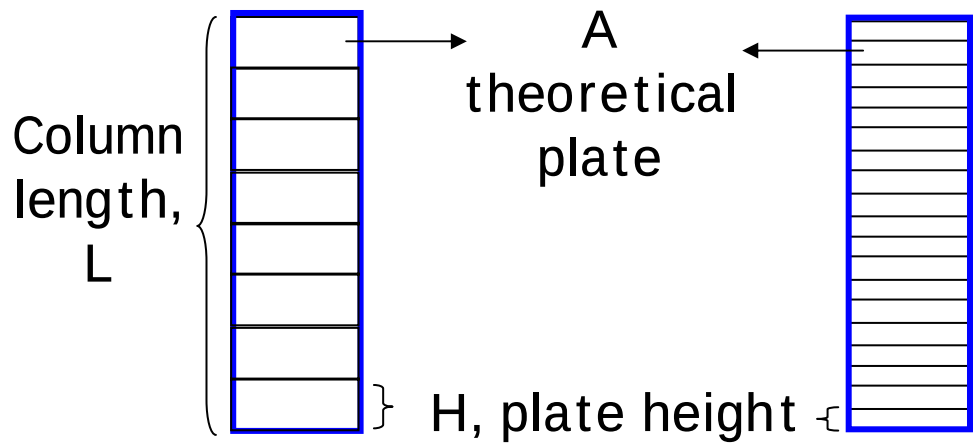


- Two columns of different length (e.g. 15 and 30 cm) containing the same number of theoretical plates (e.g. a typical 15 x 0.46 cm C-8 reverse phase column has N ~ 13000). Which one has better chromatographic performance ?

Plate height number, H

- Height equivalent to a theoretical plate (HETP)
  - H = L/N
- Broadening (W) is correlated with the plate number (N)
  - Large N and small H, small W
  - Small N and large H, large W



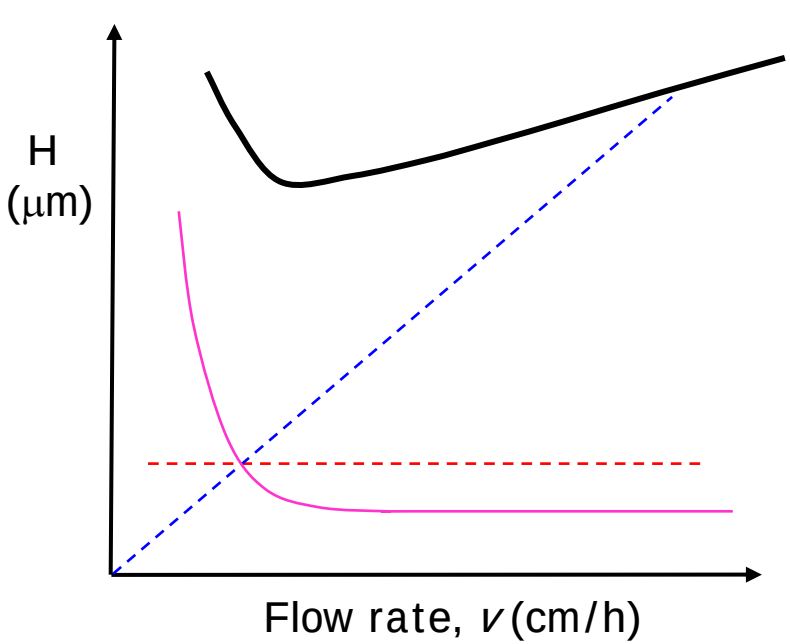


Broadening again…

- $W \propto H$
  - Causes of broadening (in terms of H)
    - Eddy diffusion: $H_1 = C_e d_p$
    - Mobile phase mass transfer: $H_2 = C_m d_p^2 \nu / D_m$
    - Stagnant mobile phase MT: $H_3 = C_{sm} d_p^2 \nu / D_m$
      - C: coefficients for the particular class of particle used as stationary phase
      - d_p: particle diameter
      - ν: flow rate of mobile phase
      - D_m, D_s: diffusion coefficients of a sample in the mobile and stationary phase
  - $H = H_1 + H_2 + H_3$
  - $H = C_e d_p + C_m d_p^2 \nu / D_m + C_{sm} d_p^2 \nu / D_m$
- To reduce broadening (small W), choose…
  - Particle type (material)
  - Small d_p (particle diameter)
  - Small ν (flow rate of mobile phase)
  - Large D_m, D_s (diffusion coefficients of a sample)

Optimum flow rate

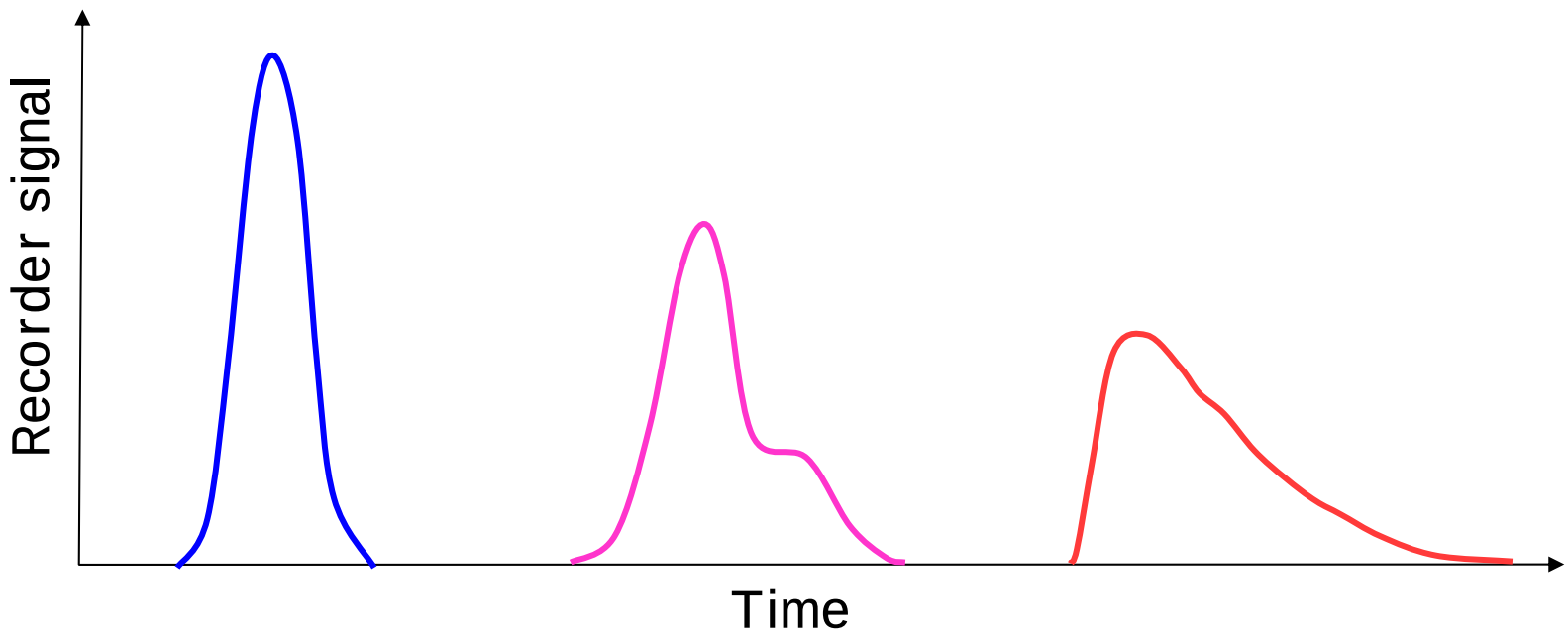
- The van Deemter curve
  - A plot of H versus the flow rate (v)
  - $H = A + B/v + C_v$
    - A: Eddy diffusion
    - B: longitudinal diffusion (along the direction of flow)
    - C: the mass transfer effect



Chromatography efficiency:
Resolution vs. Analysis time

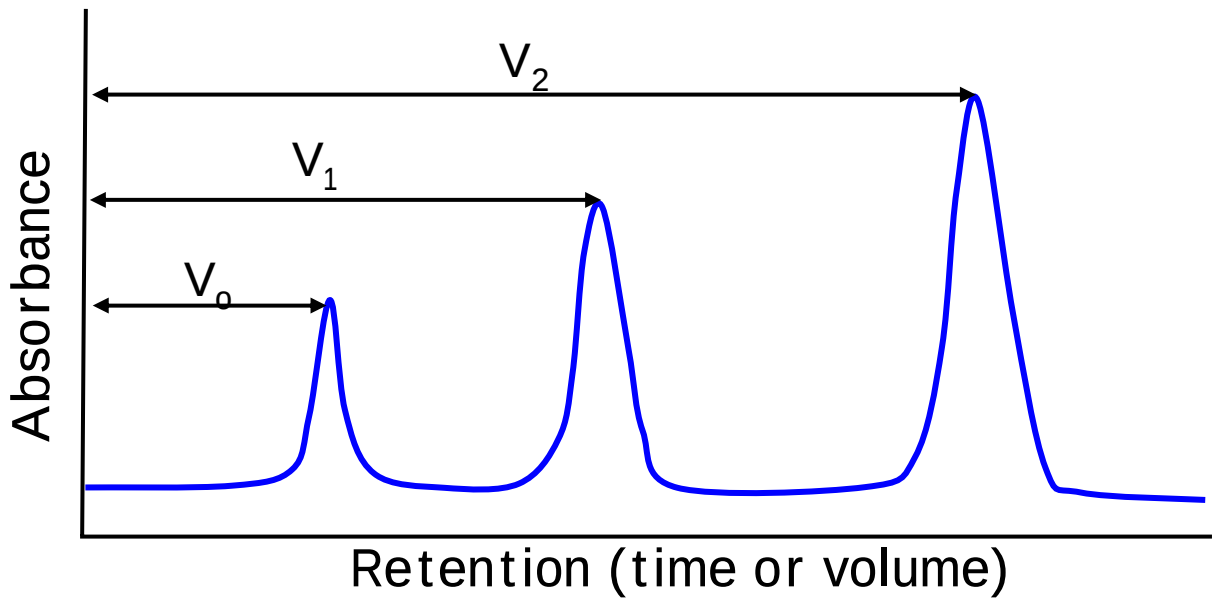
Peak symmetry

- Gaussian shape (normal distribution)
  - Cause of distortion
    - Non-linear flow rates
    - Incomplete separation of peaks (shoulder)
    - Trailing



Capacity (retention) factor, *k*

- For each component in the sample
  - $k_1 = (V_1 - V_o)/V_o$, V_o = void volume
  - or $k_1 = (t_1 - t_o)/t_o$, t_o = dead time
  - $k = K_d (V_s/V_m)$, V = volume of stationary or mobile phase
- Separation (selectivity) factor, α
  - $\alpha = k_2/k_1$
    - $\alpha \geq 1$, species 1 elutes faster than species 2



Exercise 1

- Calculate the capacity factor (k) for an analyte in a column in which the volume of the stationary phase is one-fifth of that of the mobile phase if:
  1) k (partition coefficient) = 1
  2) k (partition coefficient) = 50

Exercise 2

- An analyte eluted from a chromatographic column as a Gaussian peak with a retention time of 7 min 45 sec and a base peak width of 30 sec.
- Calculate:
  1) The number of theoretical plates in the column;
  2) The plate height if the column was 7.5 cm long.

Exercise 3

- Two compounds (1 and 2) with distribution coefficients of 10 and 12 are to be separated on a column in which the volume of the stationary phase is one-fifth that of the mobile phase.
- Calculate
  - The number of theoretical plates required to give a resolution of 1.5.

Exercise 4

- Two compounds A and B were separated on a 25 cm long column. The observed retention times were 7 min 20s and 8 min 20s, respectively. The base peak width for analyte B was 10s.
- When a reference compound, which was completely excluded from the stationary phase under the same elution conditions, was studied, its retention time was 1 min 20s.
- Calculate
  1) The adusted retention time for A and B;
  2) The capacity factor for A and B;
  3) The selectivity factor for the two compounds;
  4) The number of theoretical plates in the column;
  5) The resolution of the two compounds;
  6) The required column length to double the resolution.