Separation tools for electrospray mass spectrometry

- Liquid Chromatography

Introduction to Chromatography

Definition

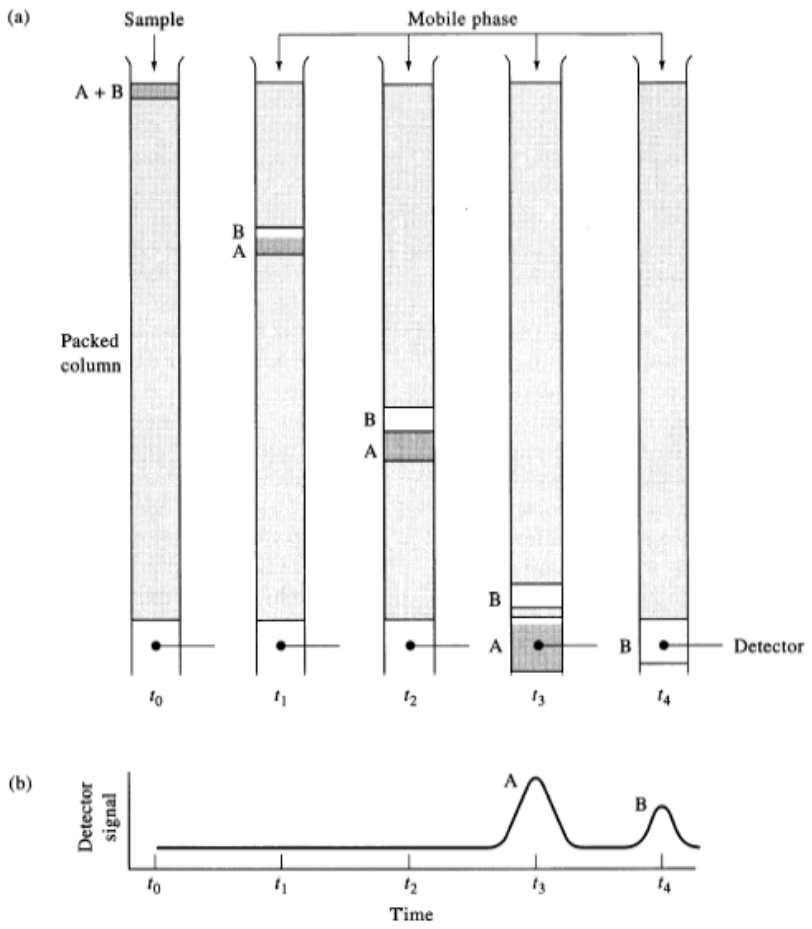
Chromatography is a separation technique based on the different interactions of compounds with two phases, a *mobile phase* and a *stationary phase*, as the compounds travel through a supporting medium.

Components:

mobile phase: a solvent that flows through the supporting medium

stationary phase: a layer or coating on the supporting medium that interacts with the analytes

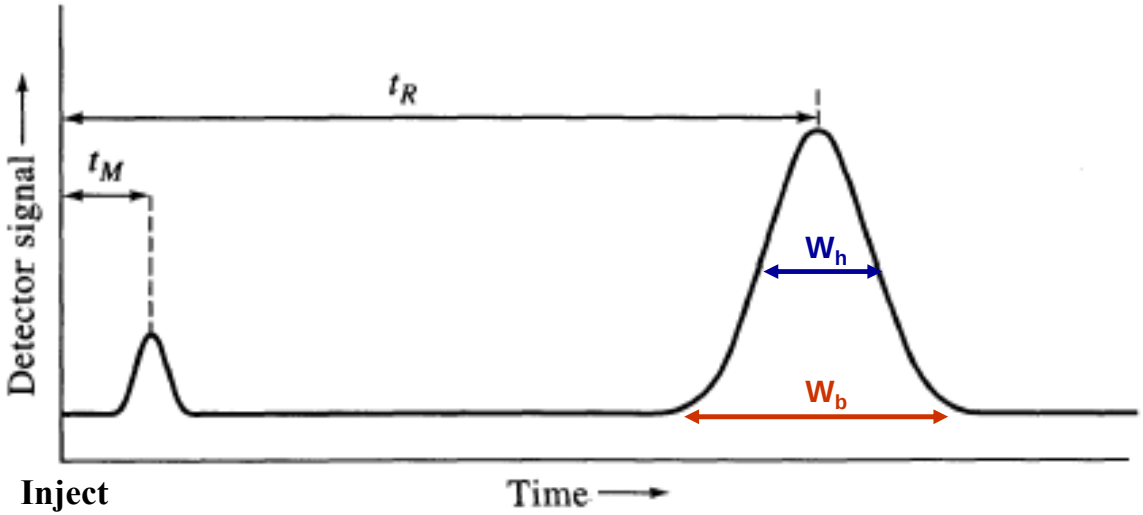
supporting medium: a solid surface on which the stationary phase is bound or coated


(a)
Sample
Mobile phase
A + B
Packed column
B
A
B
A
B
A
B
Detector
t_0
t_1
t_2
t_3
t_4
(b)
Detector signal
A
B
t_0
t_1
t_2
t_3
t_4
Time

Theory of Chromatography

I. Typical response obtained by chromatography (i.e., a chromatogram):

chromatogram - intensity vs. elution time



Where:

t_R = retention time
t_M = void time
W_b = baseline width of the peak in time units
W_h = half-height width of the peak in time units

II. Solute Retention:

Capacity factor (k'): more universal measure of retention, determined from t_R or V_R.

$$k' = (t_R - t_M) / t_M \qquad \text{or}$$

$$k' = (V_R - V_M) / V_M$$

$$k' = \frac{\text{moles A}_{\text{stationary phase}}}{\text{moles A}_{\text{mobile phase}}}$$

k' is directly related to the strength of the interaction between a solute with the stationary and mobile phases.

A simple example relating k' to the interactions of a solute in a column is illustrated for partition chromatography:

$$\text{A (mobile phase)} \underset{}{\overset{K_D}{\rightleftharpoons}} \text{A (stationary phase)}$$

where: K_D = equilibrium constant for the distribution of A between the mobile phase and stationary phase

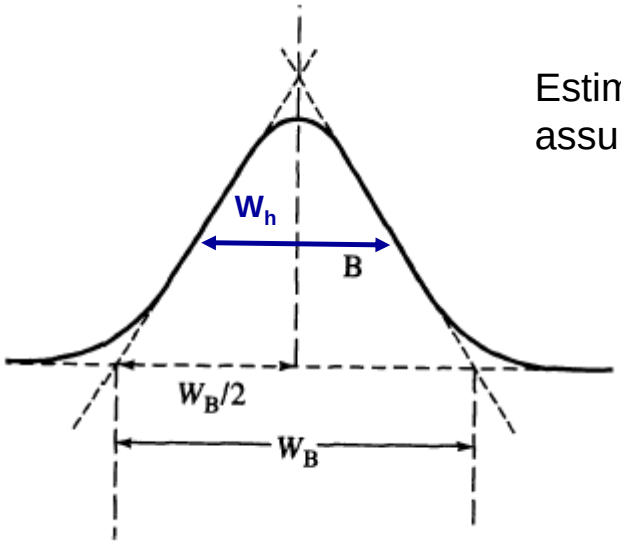
Assuming local equilibrium at the center of the chromatographic peak:

$$k' = \frac{[A]_{\text{stationary phase}}\ \text{Volume}_{\text{stationary phase}}}{[A]_{\text{mobile phase}}\ \text{Volume}_{\text{mobile phase}}}$$

$$k' = K_D \frac{\text{Volume}_{\text{stationary phase}}}{\text{Volume}_{\text{mobile phase}}}$$

As K_D increases, interaction of the solute with the stationary phase becomes more favorable and the solute's retention (k') increases

III. Efficiency: is related experimentally to a solute's **peak width**.



Estimate σ from peak widths, assuming Gaussian shaped peak:

$$W_b = 4\sigma$$

$$W_h = 2.354\sigma$$

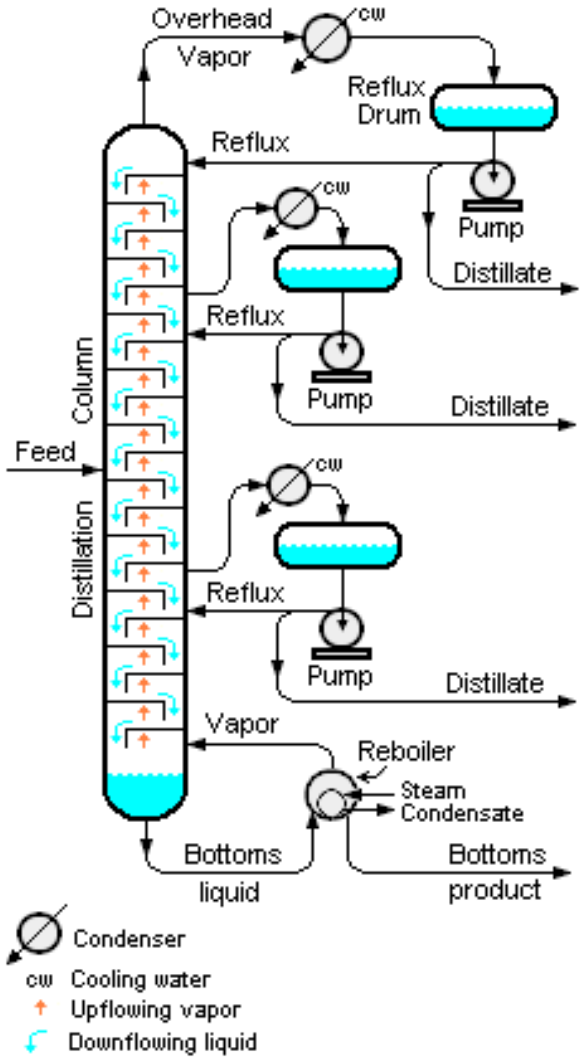
Number of theoretical plates (N)

$$N = (t_R / \sigma)^2$$

or for a Gaussian shaped peak

$$N = 16\ (t_R / W_b)^2$$

$$N = 5.54\ (t_R / W_h)^2$$



The larger the value of N is for a column, the better the column

- the better the ability to resolve solutes that have small differences in retention
- N is independent of solute retention
- N is dependent on the **length** of the column

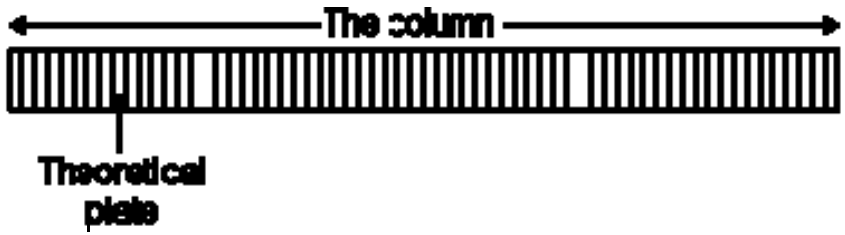


Plate height or height equivalent of a theoretical plate (H or HETP)

$$H = L / N$$

where: L = column length, N = number of theoretical plates for the column

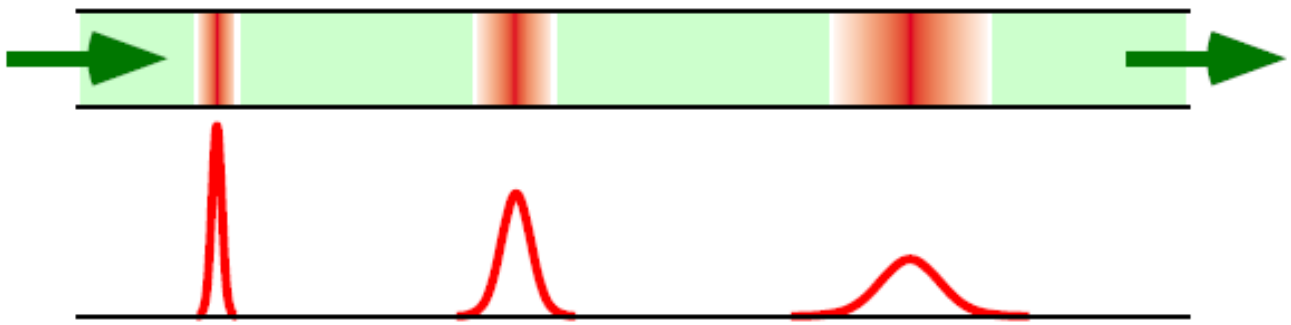
H can be also used to relate various chromatographic parameters (e.g., flow rate, particle size, etc.) to the kinetic processes that give rise to peak broadening

Why Do Bands Spread?

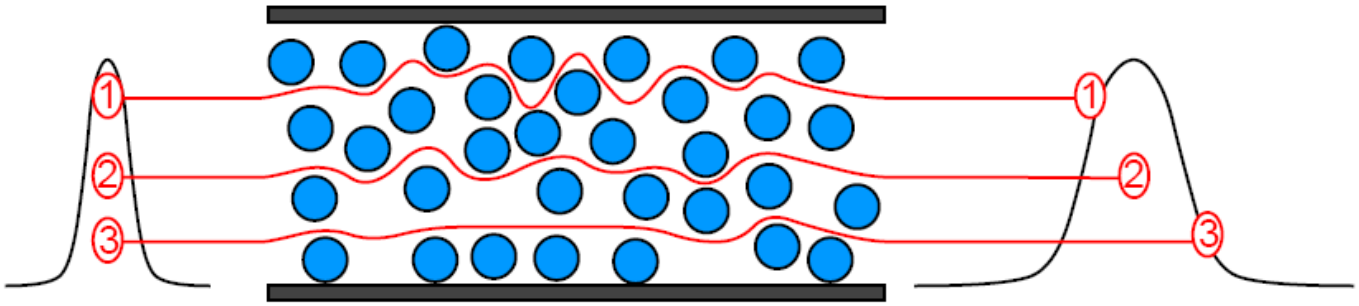
a. Eddy diffusion (multiple Paths)

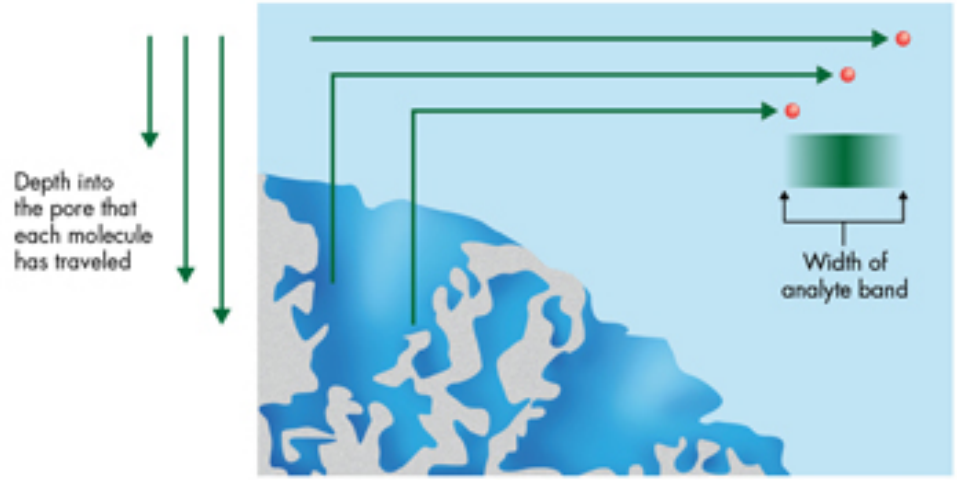
b. Stationary phase mass transfer

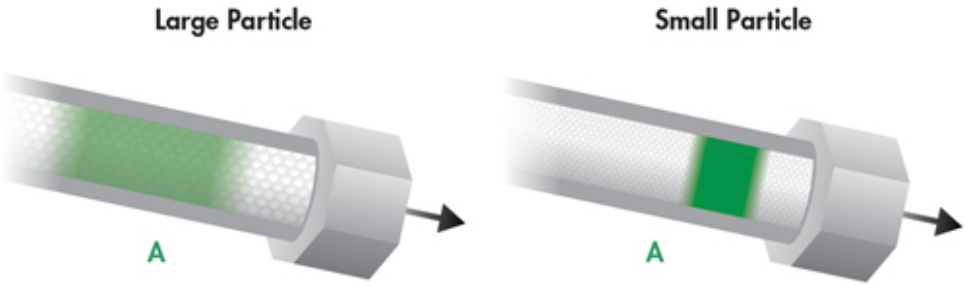
c. Longitudinal diffusion



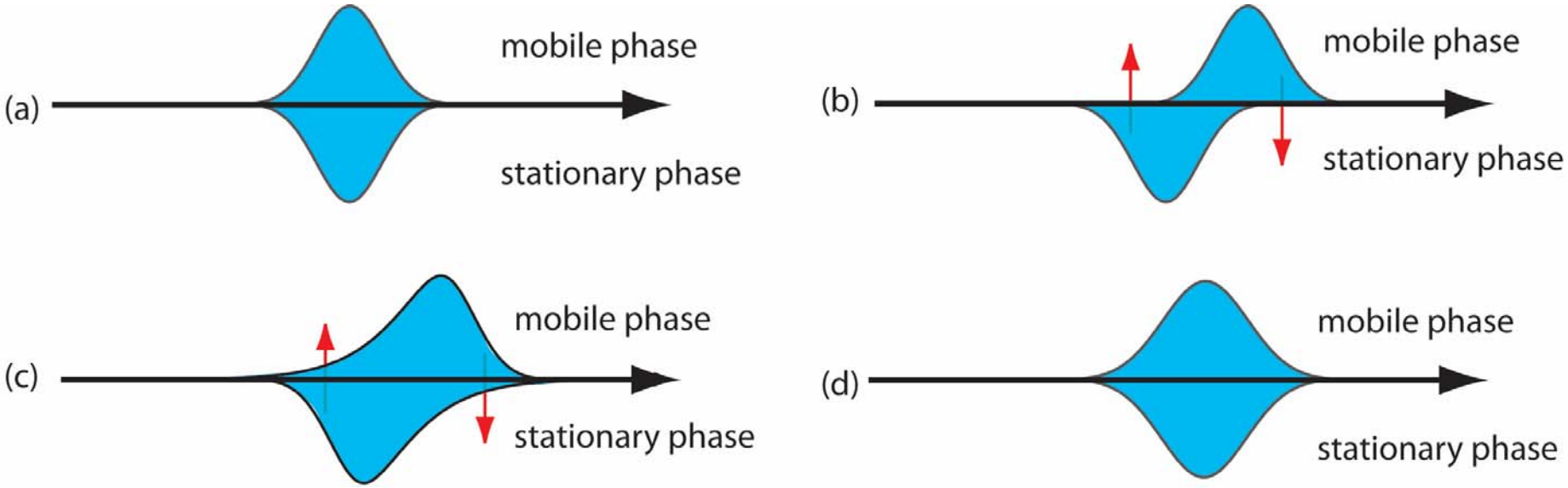
a) *Eddy diffusion* – multiple flow paths through a <u>packed</u> column.

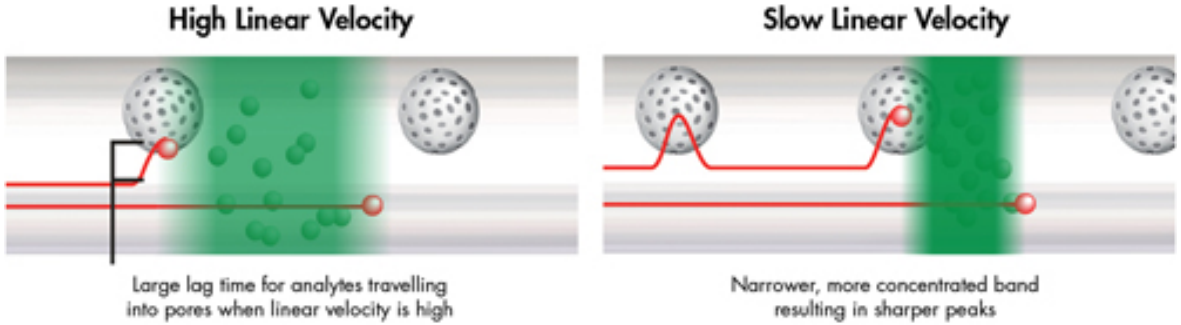




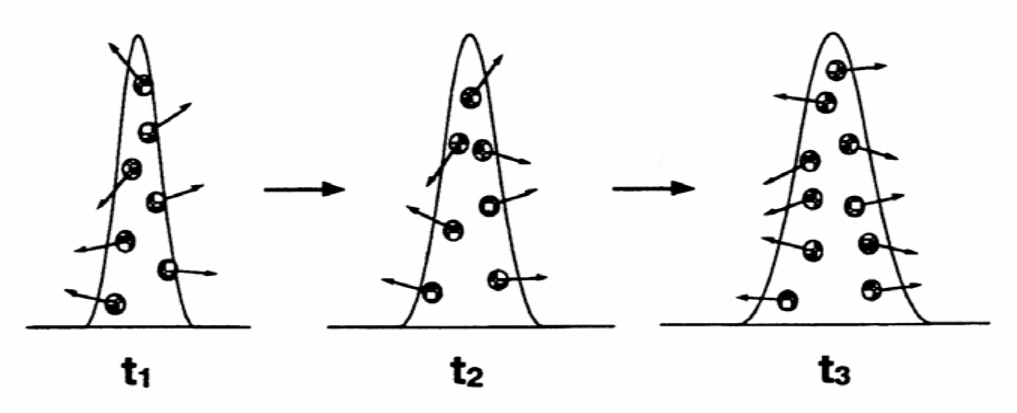


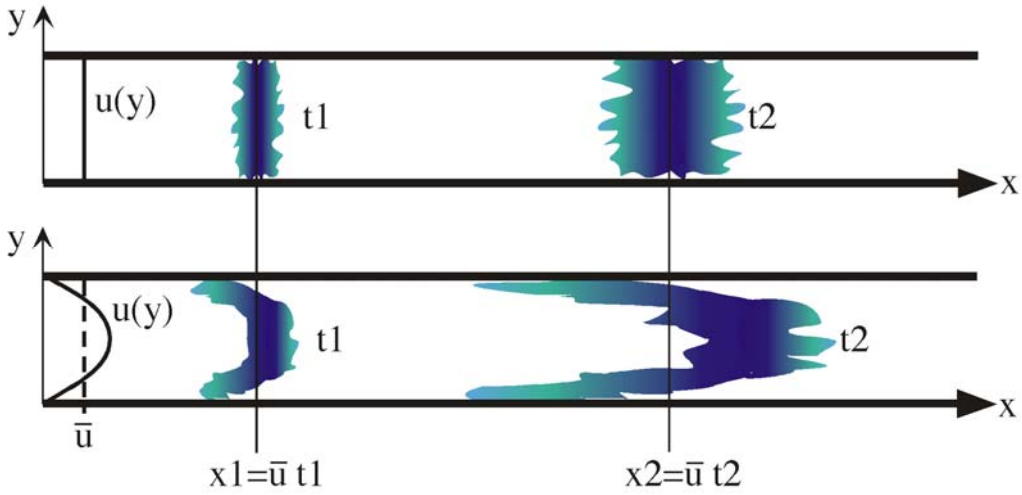
b) Stationary phase mass transfer (equilibrium factor)

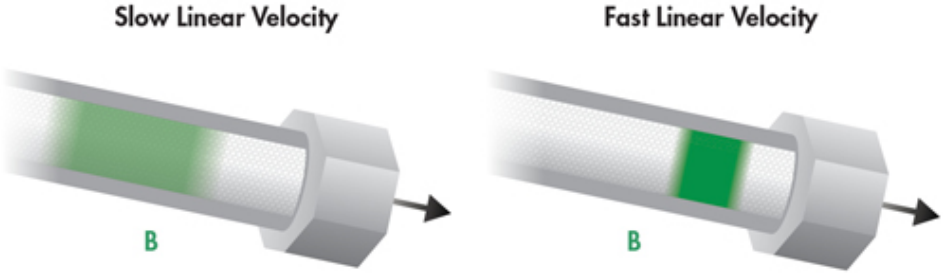




c) Longitudinal diffusion

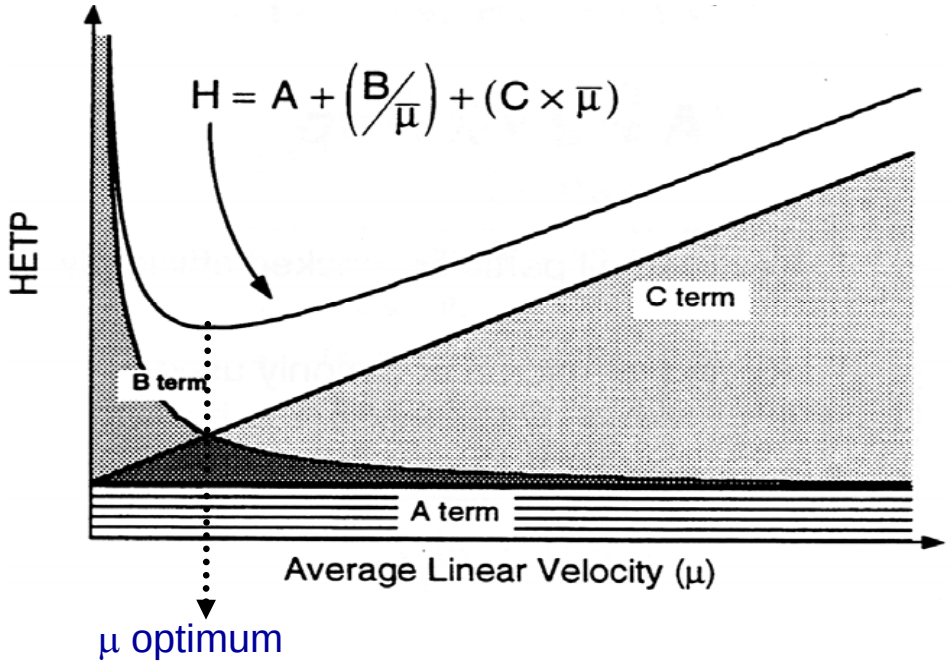






Van Deemter equation:

$$H = A + \frac{B}{u} + Cu$$



Number of theoretical plates (N) = 5.54 $(t_R / W_h)^2$ = L / **H**

Smaller H → better separation

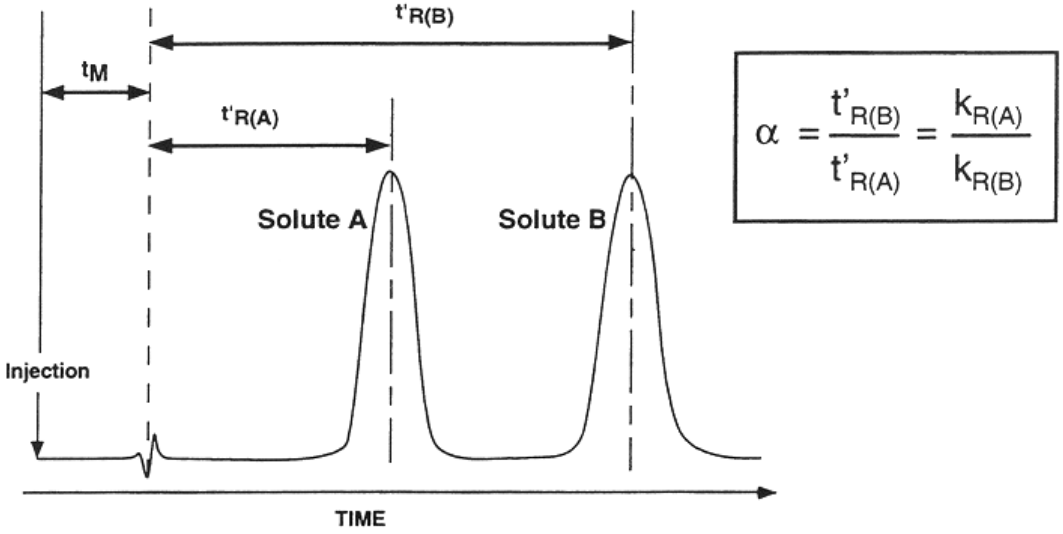
IV. Measures of Solute Separation:

Separation factor (α) = k'_2/k'_1 $\qquad$ $k' = (t_R - t_M)/t_M$

where: k'_1 = the capacity factor of the first solute

k'_2 = the capacity factor of the second solute, with $k'_2 > k'_1$

A value of $\alpha > 1.1$ is usually indicative of a good separation



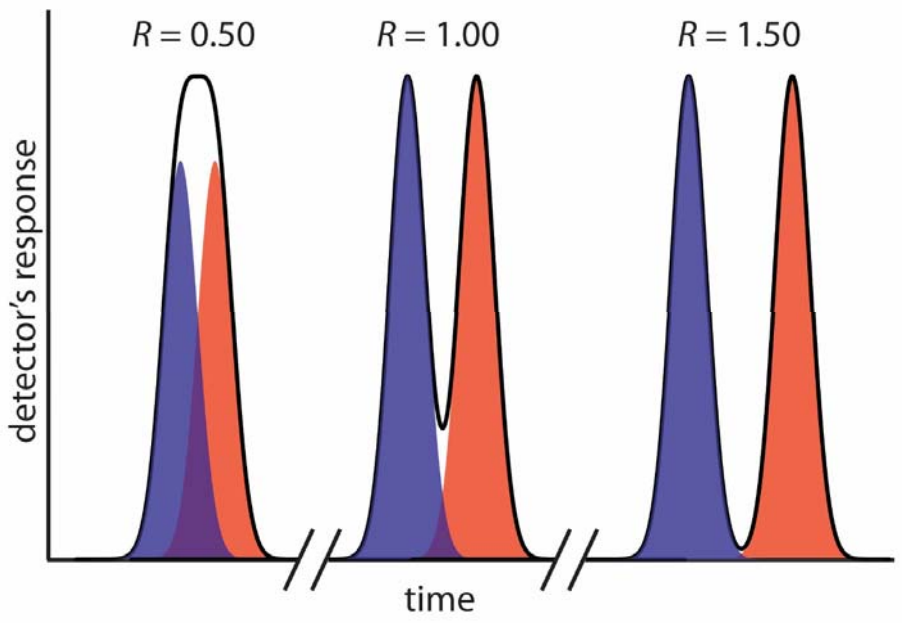
Does <u>not</u> consider the effect of column efficiency or peak widths, only retention.

Resolution (R_S) – resolution between two peaks is how well two peaks are separated:

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)} \qquad \alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

$$\Rightarrow \quad R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right)$$

$$R \propto \sqrt{N} \propto \sqrt{L}\,, \quad \left(N = \frac{L}{H}\right)$$

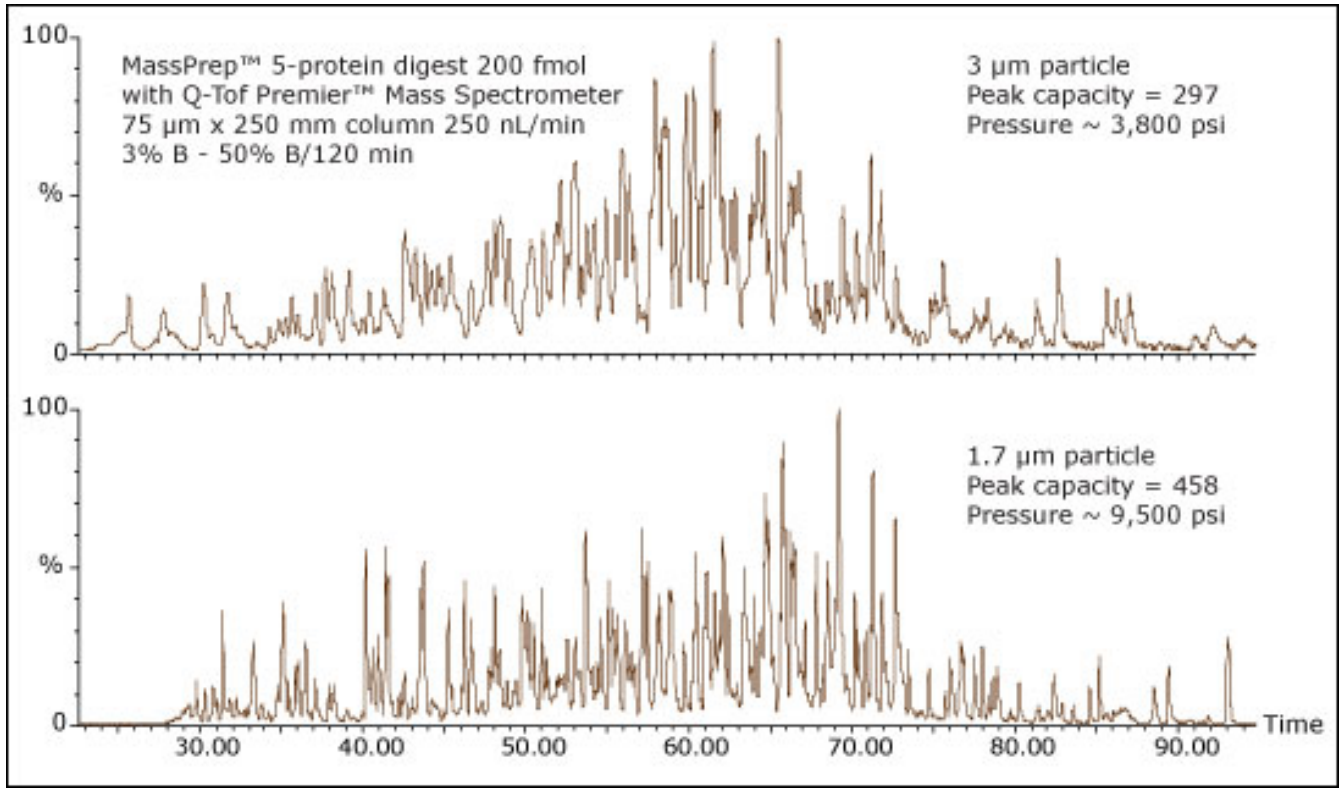


R_s > 1.5 represents *baseline resolution*, or complete separation of two neighboring solutes → ideal case.

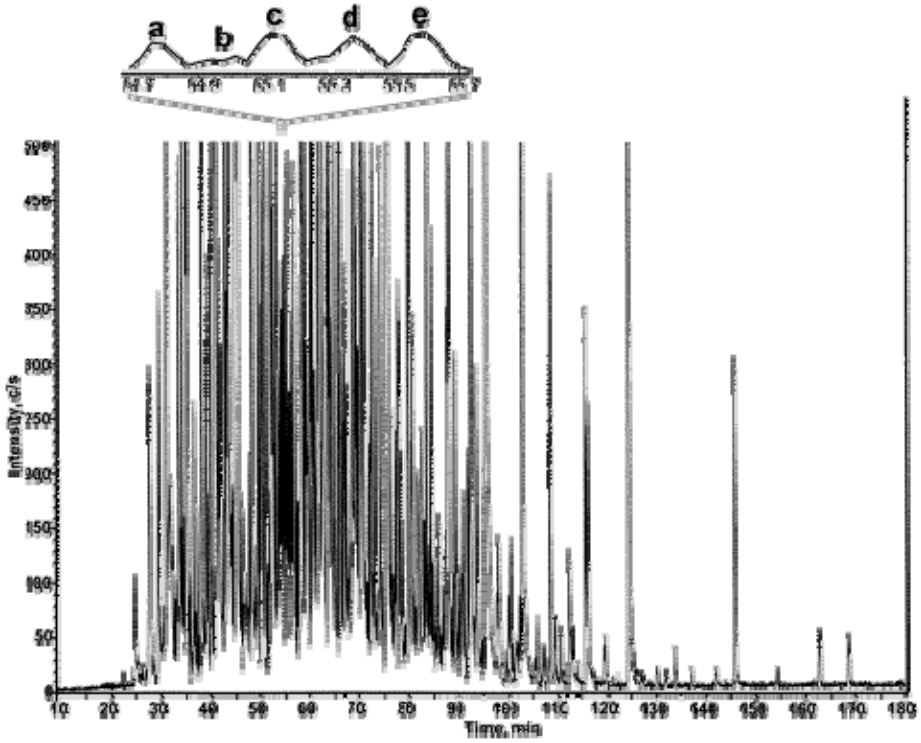
R_s > 1.0 considered adequate for most separations.

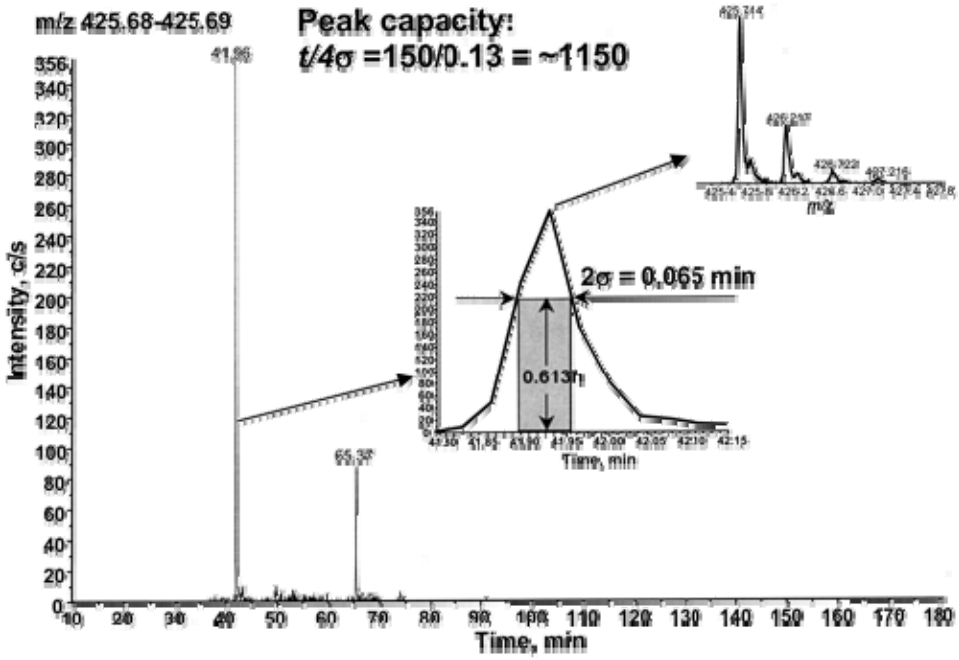
IV. Peak capacity

Defined as number of peaks that can be separated within a retention window; thus peak capacity can be simply the gradient run time divided by the average peak width



IV. Peak capacity



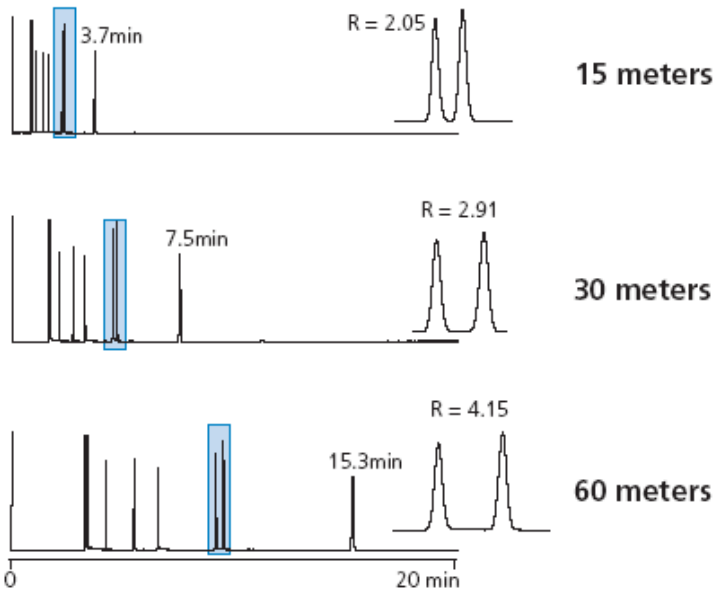


Anal. Chem., **2002**, *74* (16), pp 4235–4249

Summary: how to improve LC separation power ?

$$R \propto \sqrt{N} \propto \sqrt{L}\ , \ (N = \frac{L}{H})$$

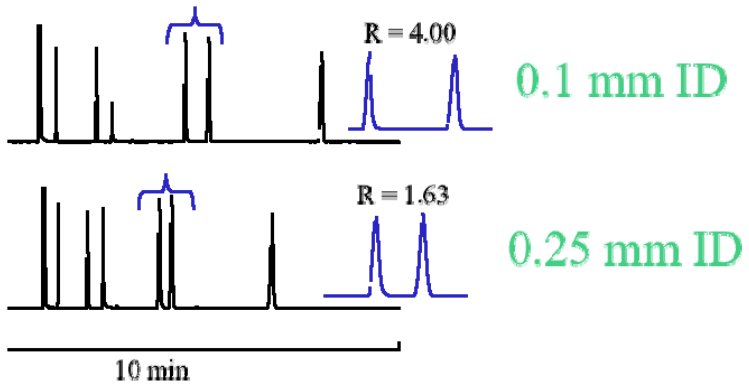
1. Increase column length

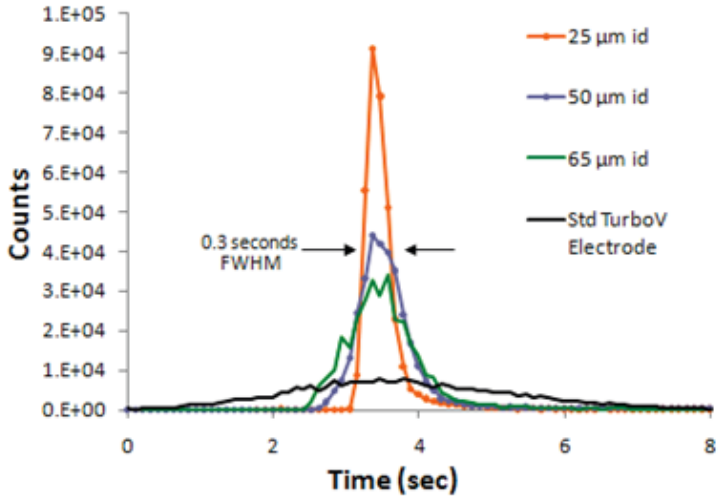


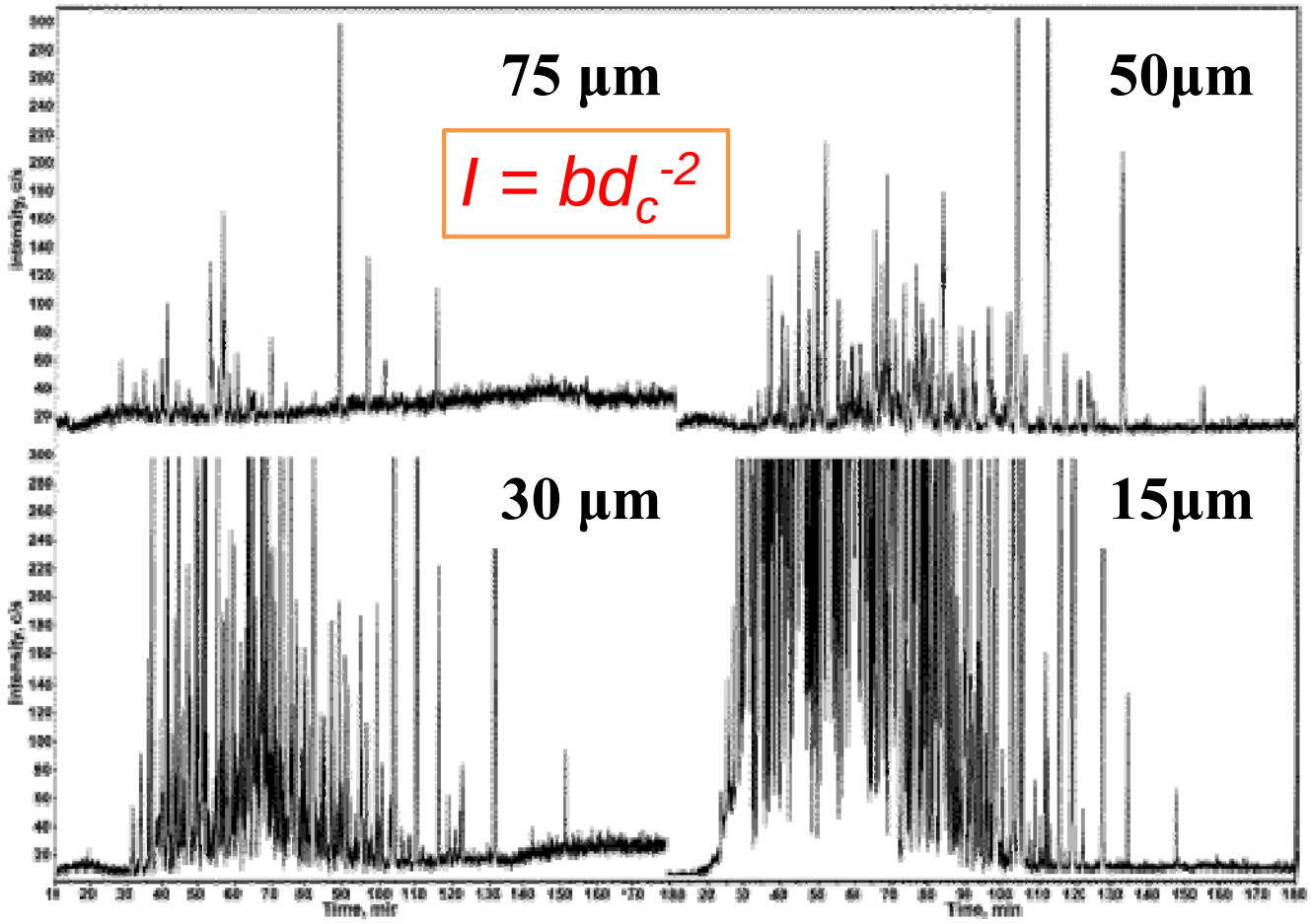
Summary: how to improve LC separation power ?

$$R \propto \sqrt{N} \propto \sqrt{L}\ ,\ (N = \frac{L}{H})$$

2. Decrease column diameter







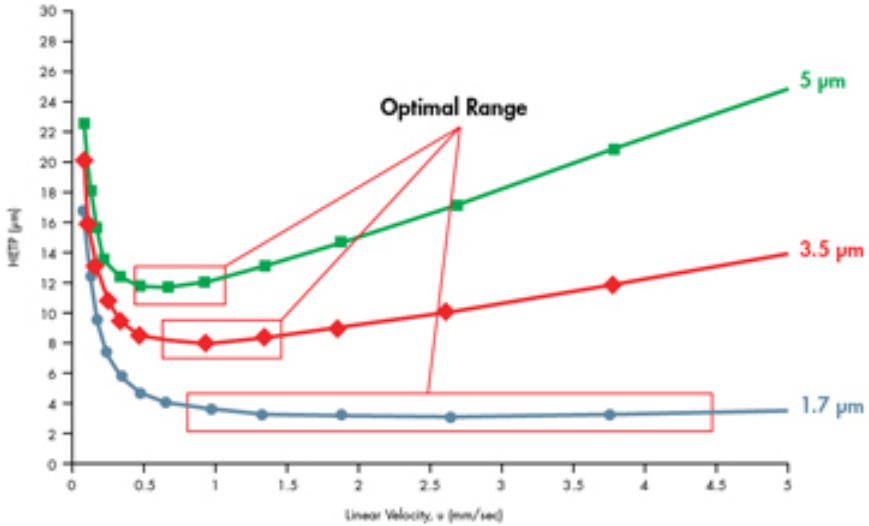
100 ng of a yeast soluble protein tryptic digest

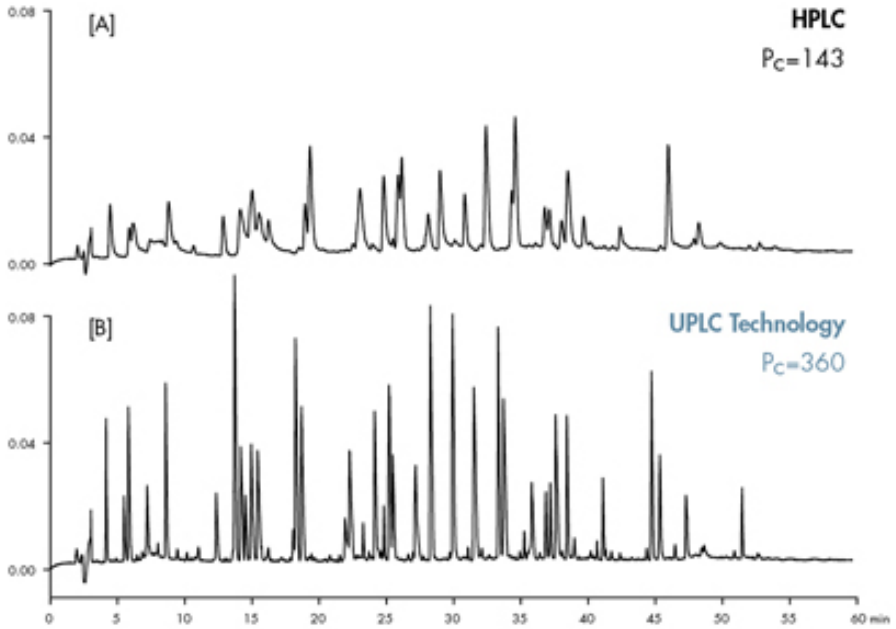
Anal. Chem., **2002**, *74* (16), pp 4235–4249

Summary: how to improve LC separation power ?

$$R \propto \sqrt{N} \propto \sqrt{L}\ ,\ (N = \frac{L}{H}) \qquad H = \mathrm{A} + \frac{B}{u} + Cu$$

3. Smaller particle

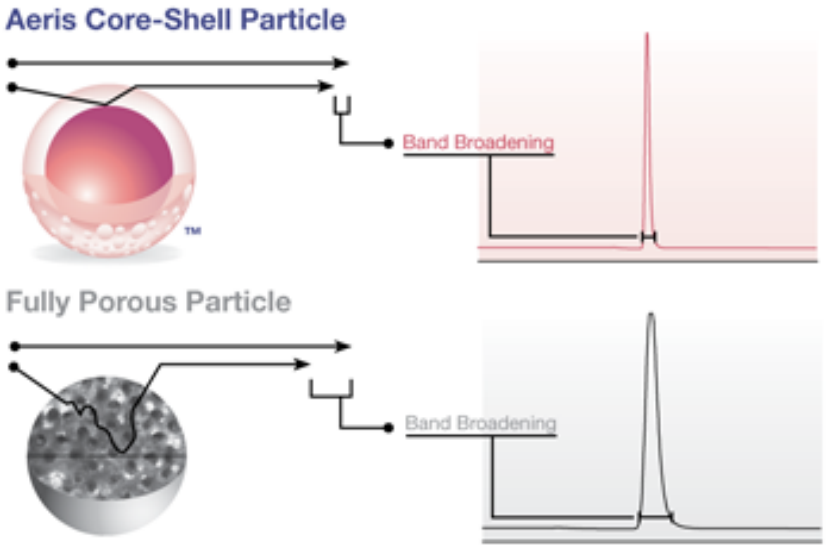


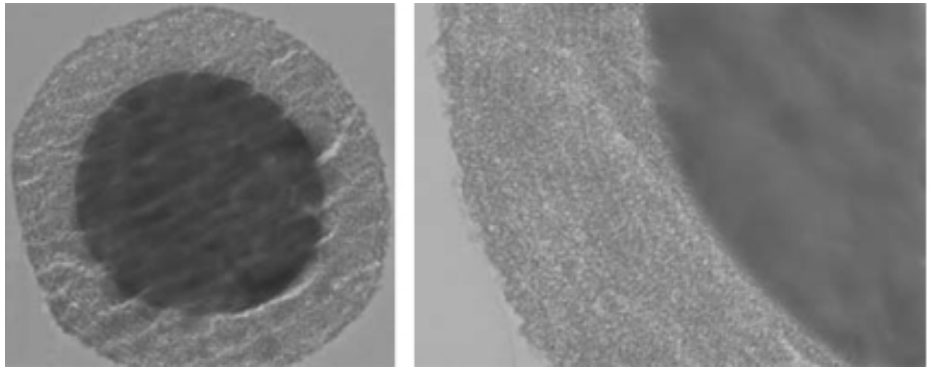


Summary: how to improve LC separation power ?

$$R \propto \sqrt{N} \propto \sqrt{L}\,, \quad (N = \frac{L}{H}) \qquad H = \mathrm{A} + \frac{B}{u} + Cu$$

4. Core-shell particle





Beyond HPLC (high performance LC): Ultra performance LC (UPLC)

High pressure → allow to use smaller particle, long column, narrower column

→ high performance

HPLC =< 400 bar, 6000 psi

UPLC (or UHPLC) >= 690 bar, 10000 psi

Recently, UHPLC w/ 1500 bar (22000 psi) of capacity has been released

History of chromatography

Chromato + graphy = color + writing

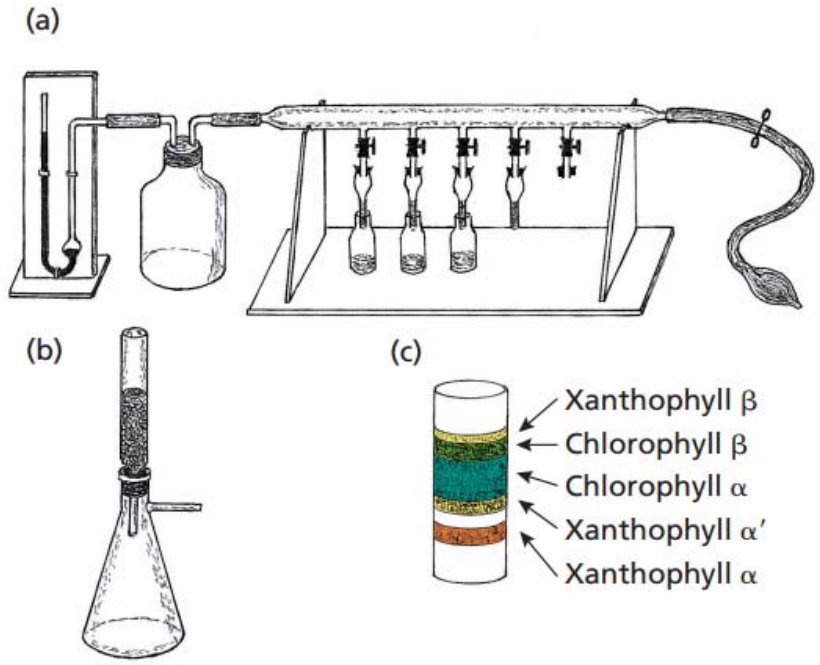
Mikhail Tsvet

Adsorption chromatography

Stationary phase = solid ($CaCO_3$)

Mobile phase = liquid (CS_2)

Figure 2: Illustrations from Tswett's 1906 paper.[6,10,11] (a) Apparatus for the simultaneous use of as many as five columns. The lower part of the small funnel-like glass pieces (2–3 mm i.d. and 20–30 mm length) served as the packed column. (b) Apparatus for larger samples (1–3 cm i.d., packing length: 5–9 cm). (c) Chromatographic separation of plant pigments as drawn by Tswett. Stationary phase: calcium carbonate; eluent: carbon disulphide.



M. Tswett, *Ber. Dtsch. Botan. Ges.*, 24, 384–393 (**1906**)

History of chromatography : partition chromatography

The Nobel Prize in Chemistry 1952
Archer J.P. Martin, Richard L.M. Synge

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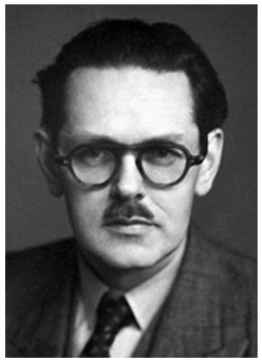
The Nobel Prize in Chemistry 1952



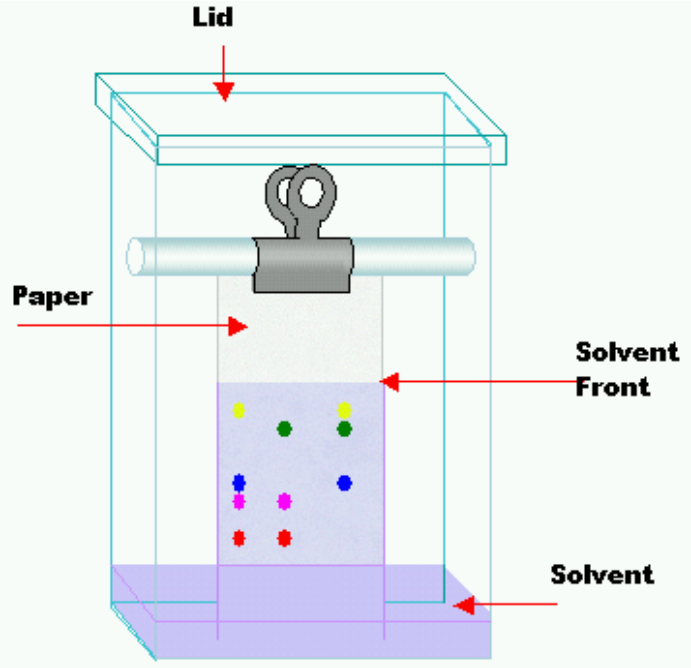
Archer John Porter Martin
Prize share: 1/2

Richard Laurence Millington Synge
Prize share: 1/2

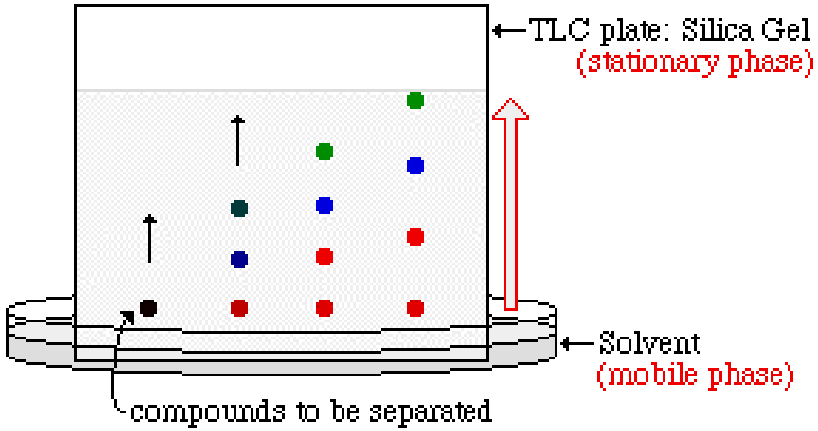
The Nobel Prize in Chemistry 1952 was awarded jointly to Archer John Porter Martin and Richard Laurence Millington Synge *"for their invention of partition chromatography"*

History of chromatography : partition chromatography

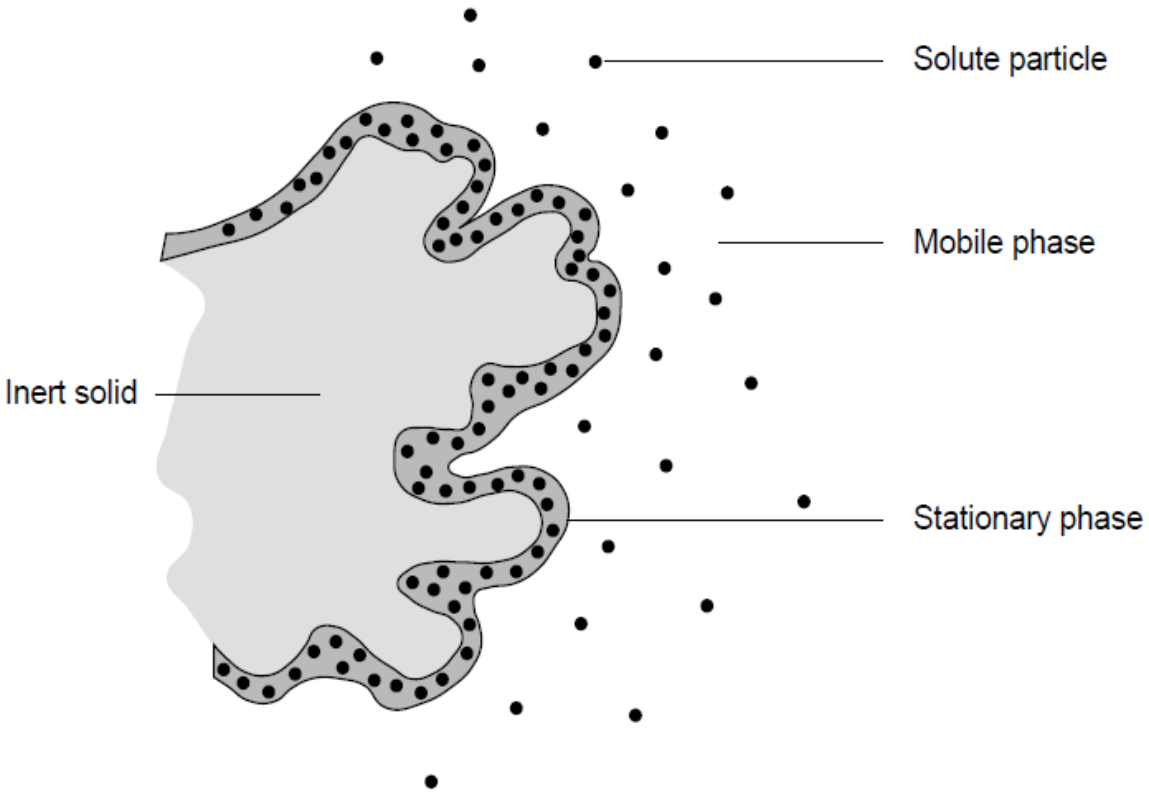
1. Paper chromatography



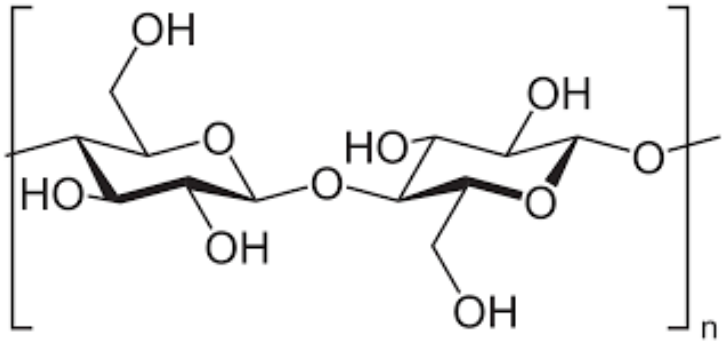
2. Thin layer chromatography

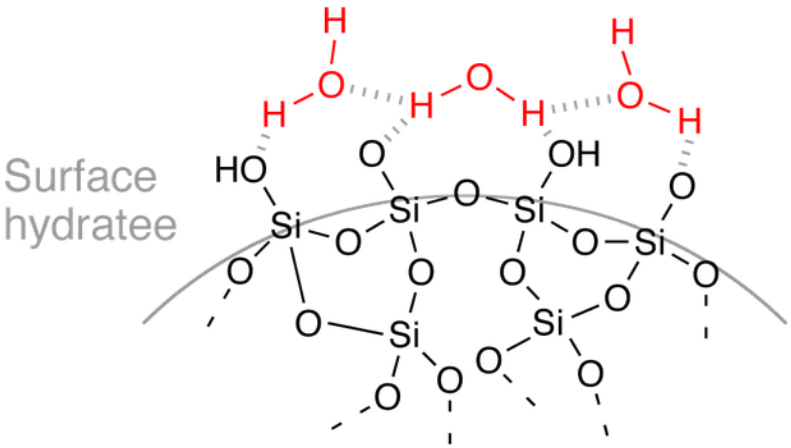


Partition chromatography

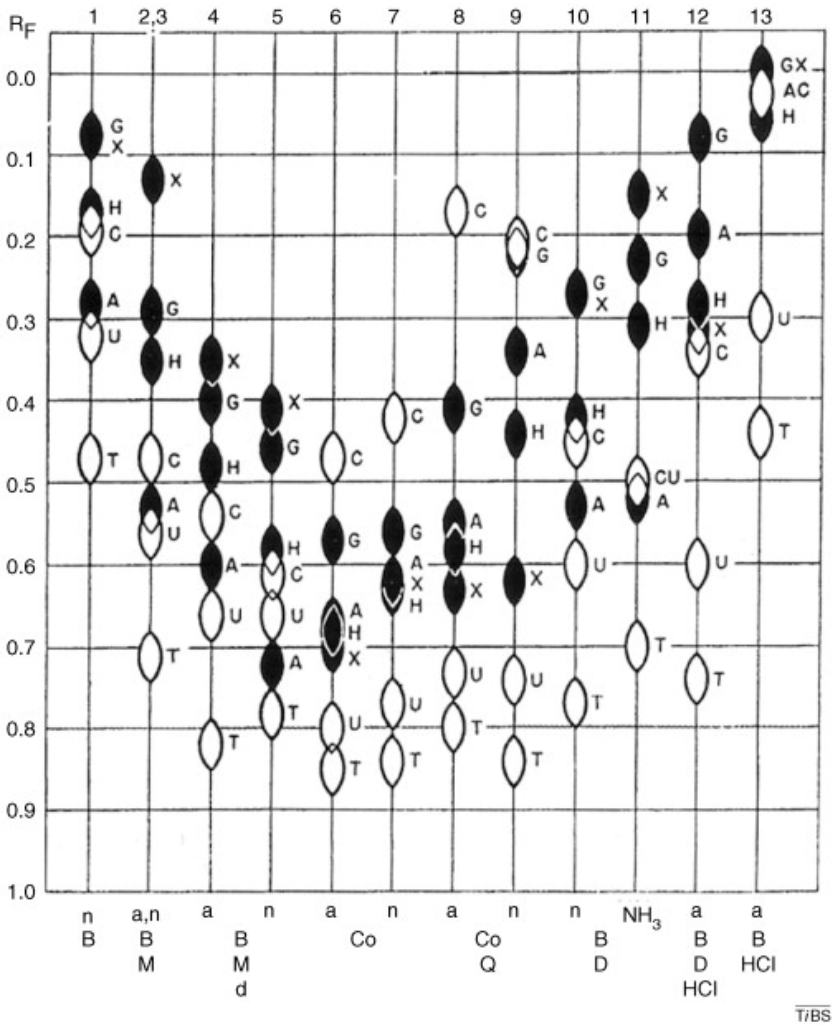


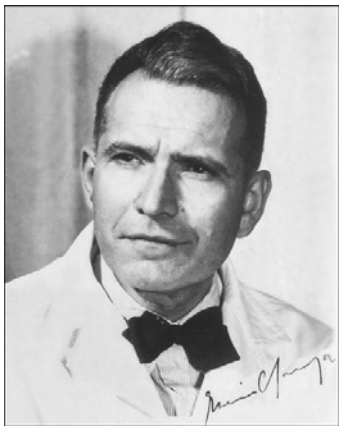
Each solute partitions itself between the stationary phase and the mobile phase





Schematic representation of the position of the purines and pyrimidines





Erwin Chargaff

Table 1. Molar proportions of purines and pyrimidines in DNA from different species and organisms[a]

Species	A/G	T/C	Pu/Py
Ox *Bos taurus*	1.29	1.43	1.1
Man *Homo sapiens*	1.56	1.75	1.0
Wheatgerm *Triticum vulgare*	1.22	1.18	1.0
Yeast *Saccharomyces cerevsiae*	1.72	1.9	1.0
Avian tubercle bacillus (*Mycobacterium avium*)	0.4	0.4	1.1
Haemophilus influenzae, Type C	1.74	1.54	1.0
Escherichia coli, K-12	1.05	0.95	1.0
Serratia *Marcescens*	0.7	0.7	0.9
Hydrogen organism *Bacillus Schatz*	0.7	0.6	1.0

Chargaff's rule

King's College, London. One of us (J. D. W.) has been aided by a fellowship from the National Foundation for Infantile Paralysis.

J. D. WATSON
F. H. C. CRICK

Medical Research Council Unit for the
Study of the Molecular Structure of
Biological Systems,
Cavendish Laboratory, Cambridge.
April 2.

[1] Pauling, L., and Corey, R. B., *Nature*, **171**, 346 (1953); *Proc. U.S. Nat. Acad. Sci.*, **39**, 84 (1953).

[2] Furberg, S., *Acta Chem. Scand.*, **6**, 634 (1952).

[3] Chargaff, E., for references see Zamenhof, S., Brawerman, G., and Chargaff, E., *Biochim. et Biophys. Acta*, **9**, 402 (1952).

[4] Wyatt, G. R., *J. Gen. Physiol.*, **36**, 201 (1952).

[5] Astbury, W. T., Symp. Soc. Exp. Biol. 1, Nucleic Acid, 66 (Camb. Univ. Press, 1947).

[6] Wilkins, M. H. F., and Randall, J. T., *Biochim. et Biophys. Acta*, **10**, 192 (1953).

Insulin sequencing

The Nobel Prize in Chemistry 1958

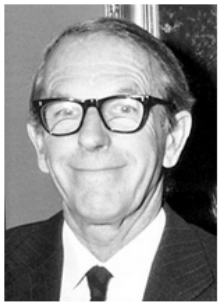
Frederick Sanger
Prize share: 1/1

The Nobel Prize in Chemistry 1958 was awarded to Frederick Sanger *"for his work on the structure of proteins, especially that of insulin"*.

The Nobel Prize in Chemistry 1980

Paul Berg
Prize share: 1/2

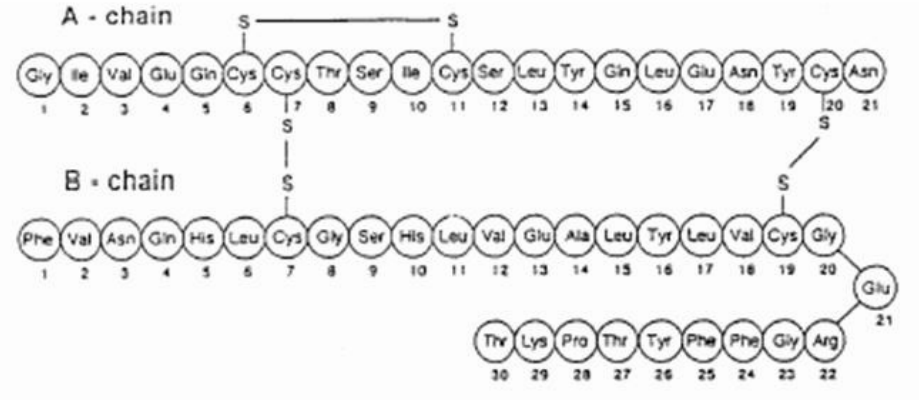
Walter Gilbert
Prize share: 1/4

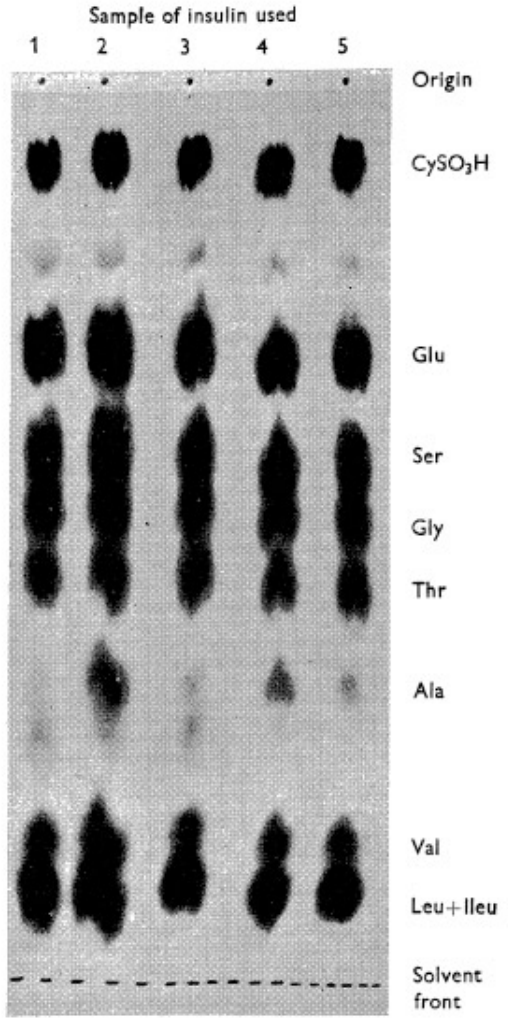


Frederick Sanger
Prize share: 1/4

The Nobel Prize in Chemistry 1980 was divided, one half awarded to Paul Berg *"for his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant-DNA"*, the other half jointly to Walter Gilbert and Frederick Sanger *"for their contributions concerning the determination of base sequences in nucleic acids"*.

Insulin sequencing





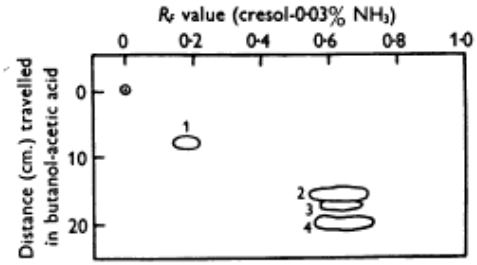


Fig. 3. Chromatogram of the neutral fraction of the chymotryptic hydrolysate (*Bc*) (see Table 2).

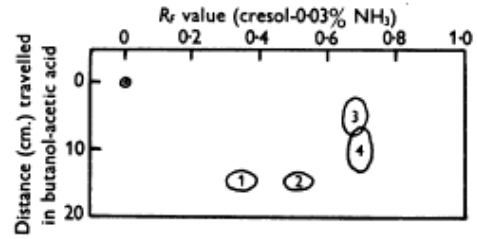


Fig. 5. Chromatogram of peptic hydrolysate of fraction *Bc* 2 (see Table 2).

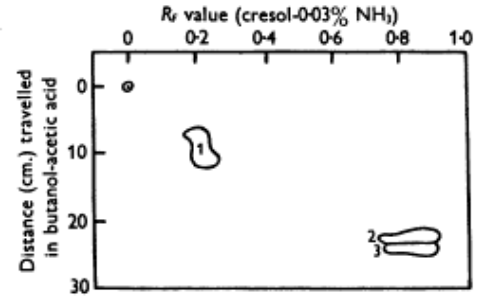
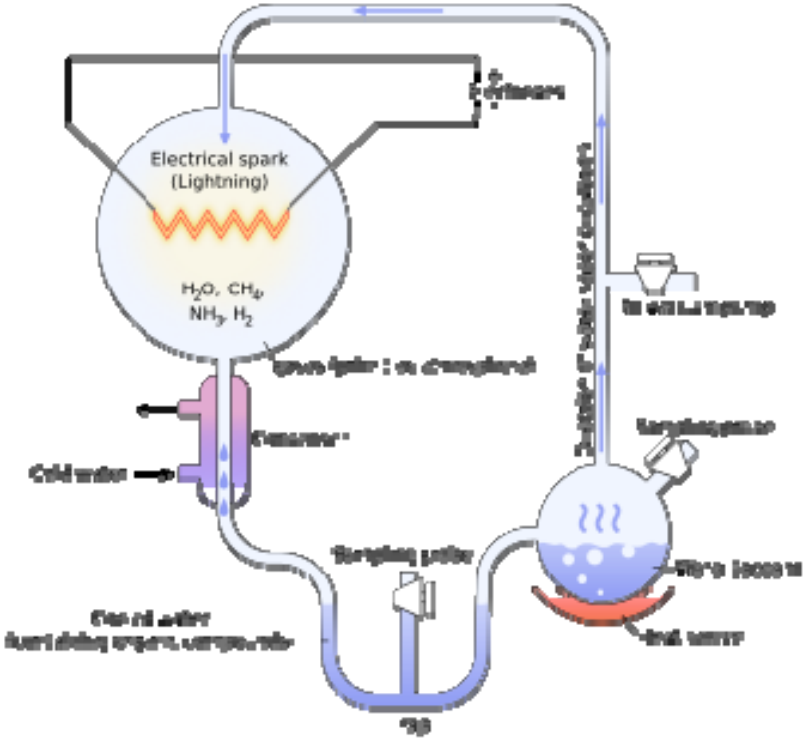


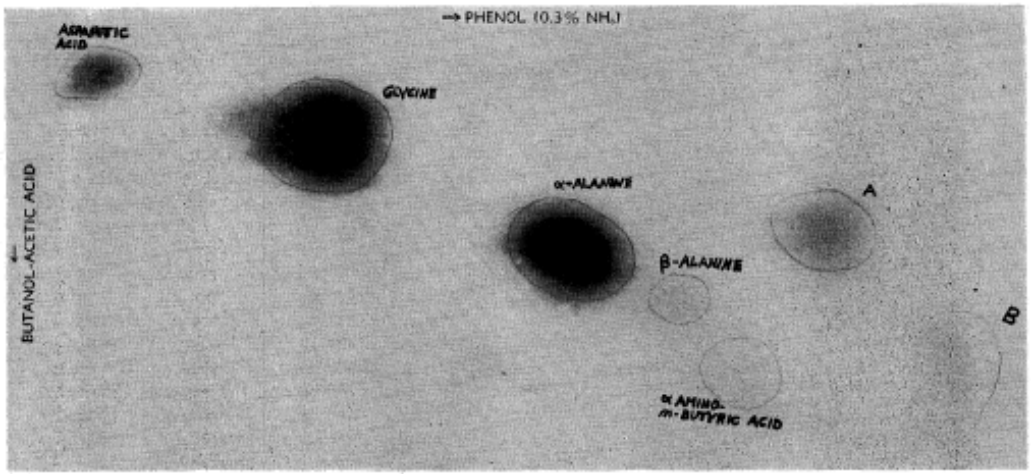
Fig. 6. Chromatogram of the tryptic hydrolysate (*Bt*) (see Table 2).

Biochem J. **1951** Sep; 49(4): 481–490

Miller–Urey experiment



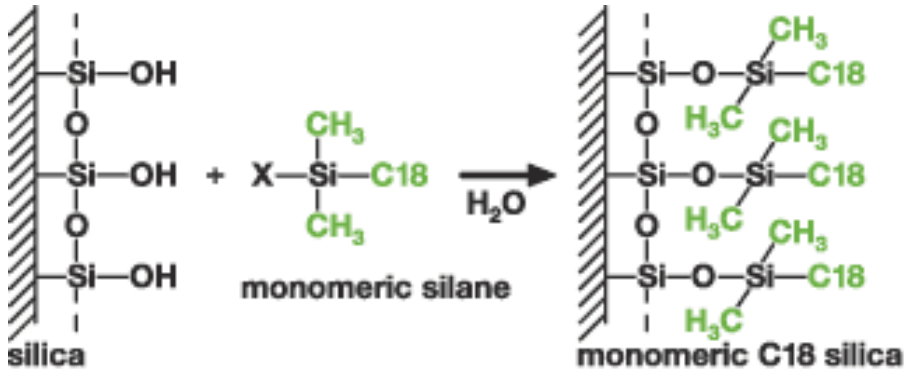
Stanley L Miller

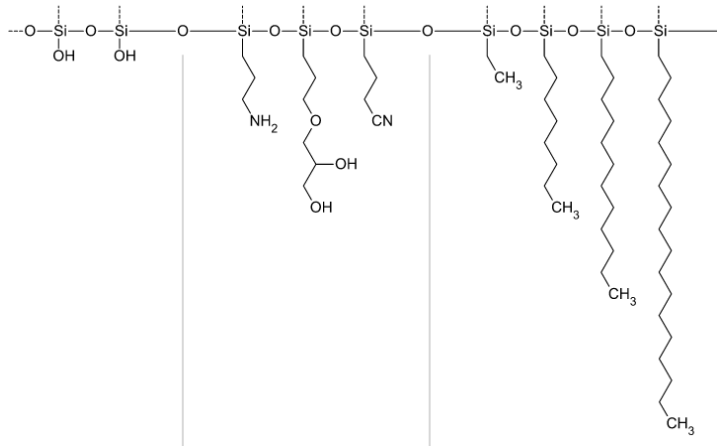


Science **117** (3046): 528–9, **1953**

History of chromatography : reversed phase chromatography

- Until ~ 1970s, a hydrophilic solid support (silica or alumina) was used as a stationary phase → called "normal phase chromatography"
- A technique using alkyl chains covalently bonded to the solid support created a hydrophobic stationary phase → reversed phase chromatography





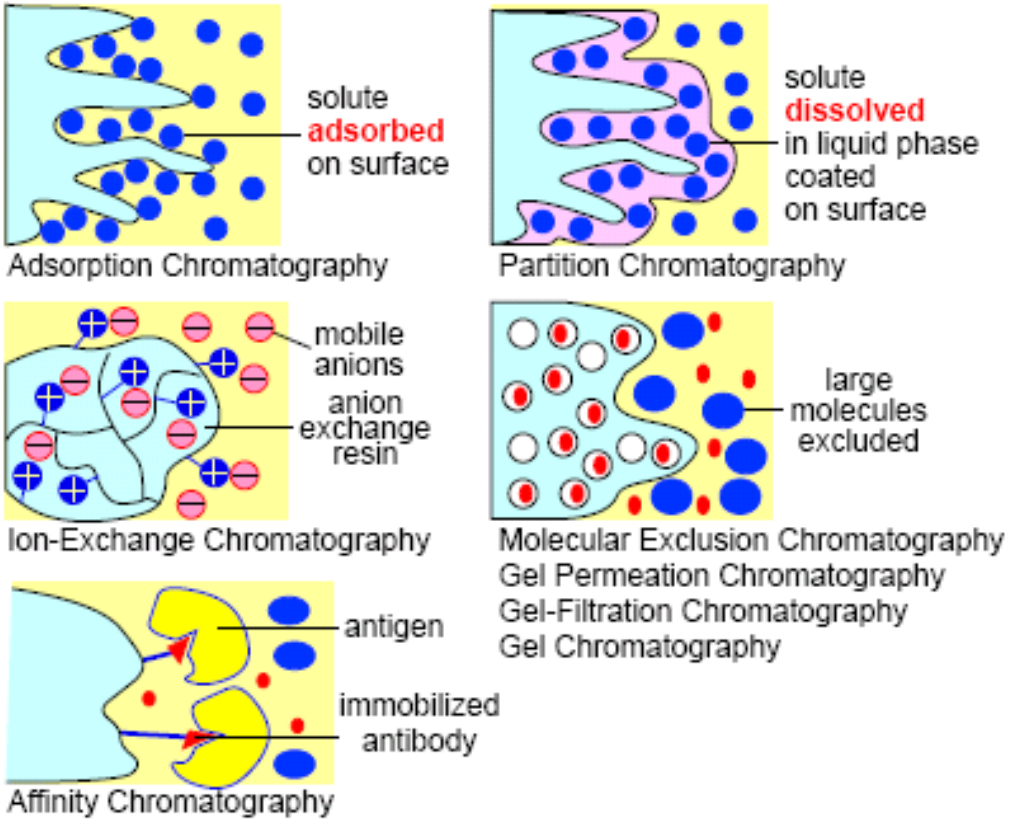
Reversed phase chromatography : impact !

- Mobile solvent: organic → aqueous !! ($$$ → $)
- Amphiphilic molecules can be easily separated: PEPTIDE
- Electrospray-friendly (volatile organic solvent)

Other types of Liquid Chromatography:

②Adsorption chromatography
②Partition chromatography
②Ion-exchange chromatography

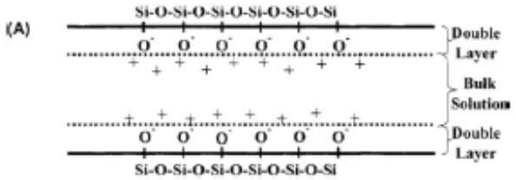
② Affinity chromatography
②Size-exclusion chromatography

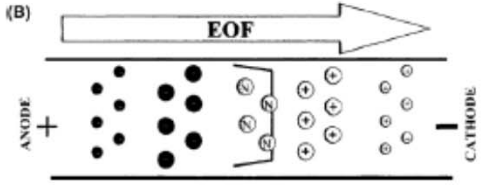


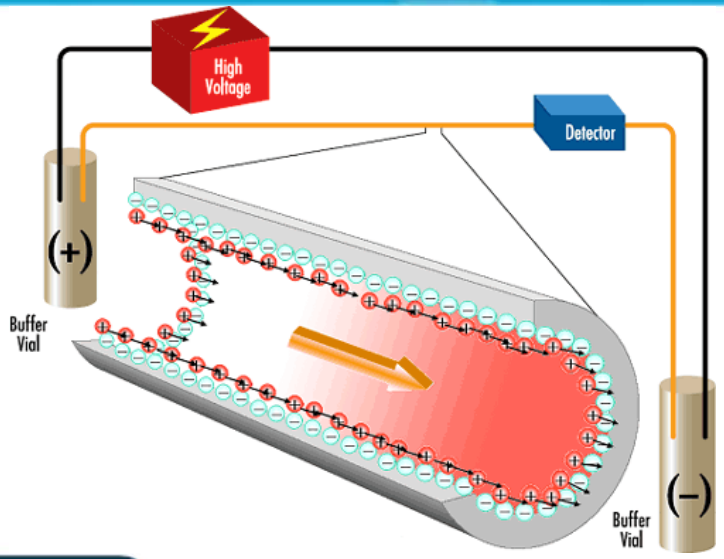
Separation tools for electrospray mass spectrometry

- Capillary Electrophoresis

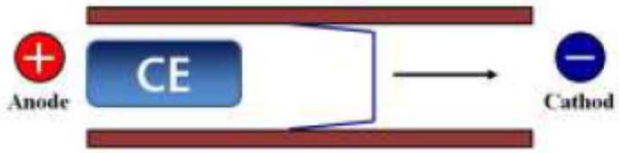
ElectoOsmotic Flow (EOF)





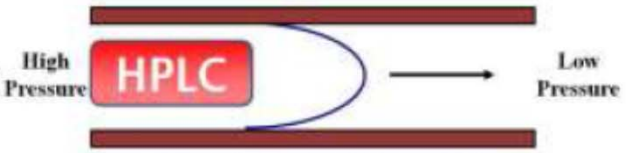


High Efficiency
(높은 분리효율)



Electroosmotic velocity profile

Flat flow

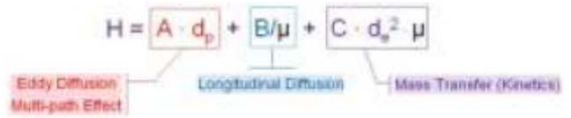


Hydrodynamic velocity profile

Laminar flow

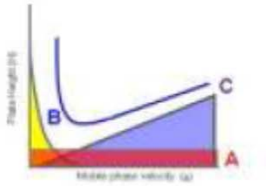
Open Tubular Capillary: How high voltage enhances separation

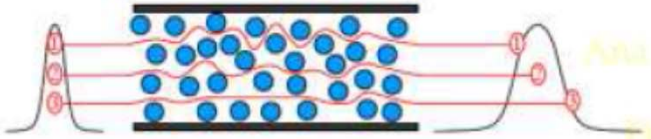
Minimize sample pretreatment



van Deemter equation:

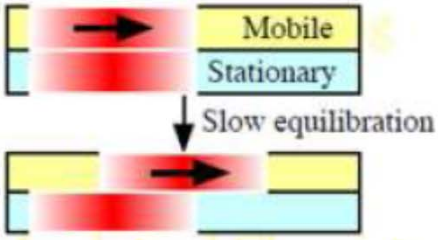
$$H = A + \frac{B}{u} + Cu$$



A 

Multiple Path
다통로

B **Longitudinal Diffusion**
확산

C 

Mass Transfer
질량이동

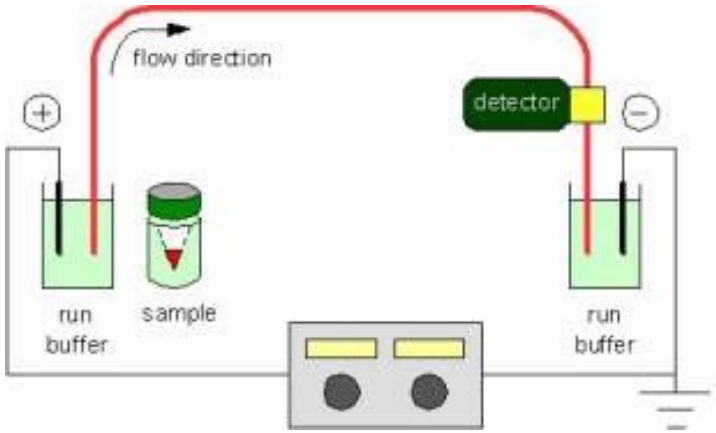
Open Tubular Column

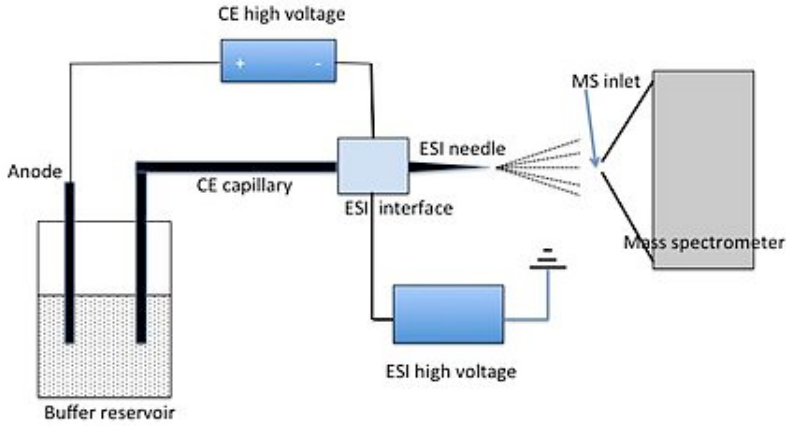
van Deemter equation:

$$H = \cancel{A} + \frac{B}{u} + \cancel{Cu}$$

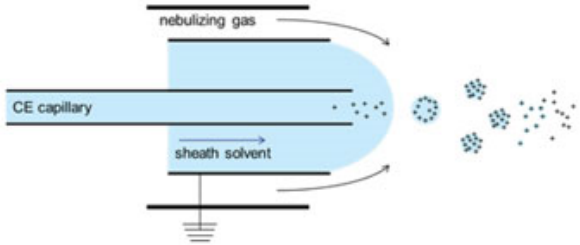
\+ **Flat Flow** → **Sharp Peaks**

Capillary electrophoresis mass spectrometry (CE-MS)

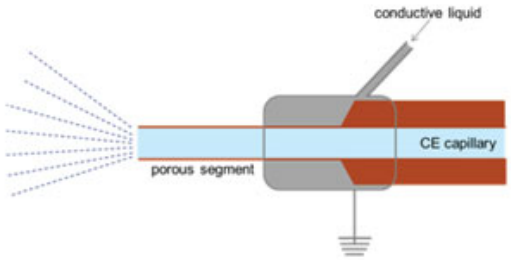




Sheath flow interface



Sheathless interface



Sheath liquid-less CE-MS