Fundamentals, Optimization and Practical Aspects of (U)HPLC Part 1

Definitions and basic concepts of Ultra High Performance Liquid Chromatography

Fundamentals, Optimization and Practical Aspects of (U)HPLC

When in this tutorial (U)HPLC is used in descriptions it applies to HPLC and UHPLC; when UHPLC is used is pertains to specifics Ultra High Performance Liquid Chromatography

Overview (U)HPLC Tutorial

- Part 1: Basic Principles of (U)HPLC
  - Fundamental considerations on (U)HPLC for (bio)chemical analysis
  - Definitions, equations and important considerations of (U)HPLC
  - Explain “Van Deemter” (and other) equations and about there role in obtaining a certain plate number in the fastest way
- Part 2: Optimization of (U)HPLC systems
  - Kinetic Plot Theory
- Part 3: (U)HPLC Column Technology
  - The role of column temperature in (U)HPLC
  - About superficially porous particles versus totally porous particle columns
  - And what about monoliths?
  - HPLC Chips for UHPLC
- Part 4: Practical aspects of (U)HPLC
  - Influence of high pressure on UHPLC performance, especially by frictional heating
  - Requirements for (U)HPLC equipment

2004: HPLC evolved to UHPLC

Two key ingredients for this evolution:

- Introduction of new, ultra-high pressure stable, sub-2-µm diameter particles in 2004 followed by the introduction of superficially porous, low diameter particles in 2006

- Next generation HPLC instrumentation capable to deliver solvents at ultra-high pressure and able to conserve the ultra high efficiency separation of columns packed with these new particles

UHPLC versus HPLC

UHPLC has extended abilities compared to HPLC by:

- New Column Technologies:
  - Totally porous (**TP**) sub-2-micrometer particles
  - Superficially porous (**SP**) particles
  - (Monolithic columns)

Mind this nomenclature!

- Next generation HPLC instrumentation:
  - Delivering mobile phases at ultra high pressure (now up to 1500 bar)
  - Systems optimized to conserve peak fidelity before, during and after separation
- Does this mean that UHPLC differs from HPLC?

➔ no!

- Basic theory of HPLC applies as well to UHPLC

Can UHPLC be Marked as an Innovation?*

- Pioneered by Waters Inc. with BEH particle technology and UPLC® system (Acquity) followed by Advanced Materials Technology with HALO particles.

- A plethora of (U)HPLC systems and column technology has followed since.

Waters:	UPLC®, Acquity H-Class, I-Class
Agilent:	Infinity 12XX Series,
Thermo:	Vanquish UHPLC Series
Shimadzu:	Nexera X2

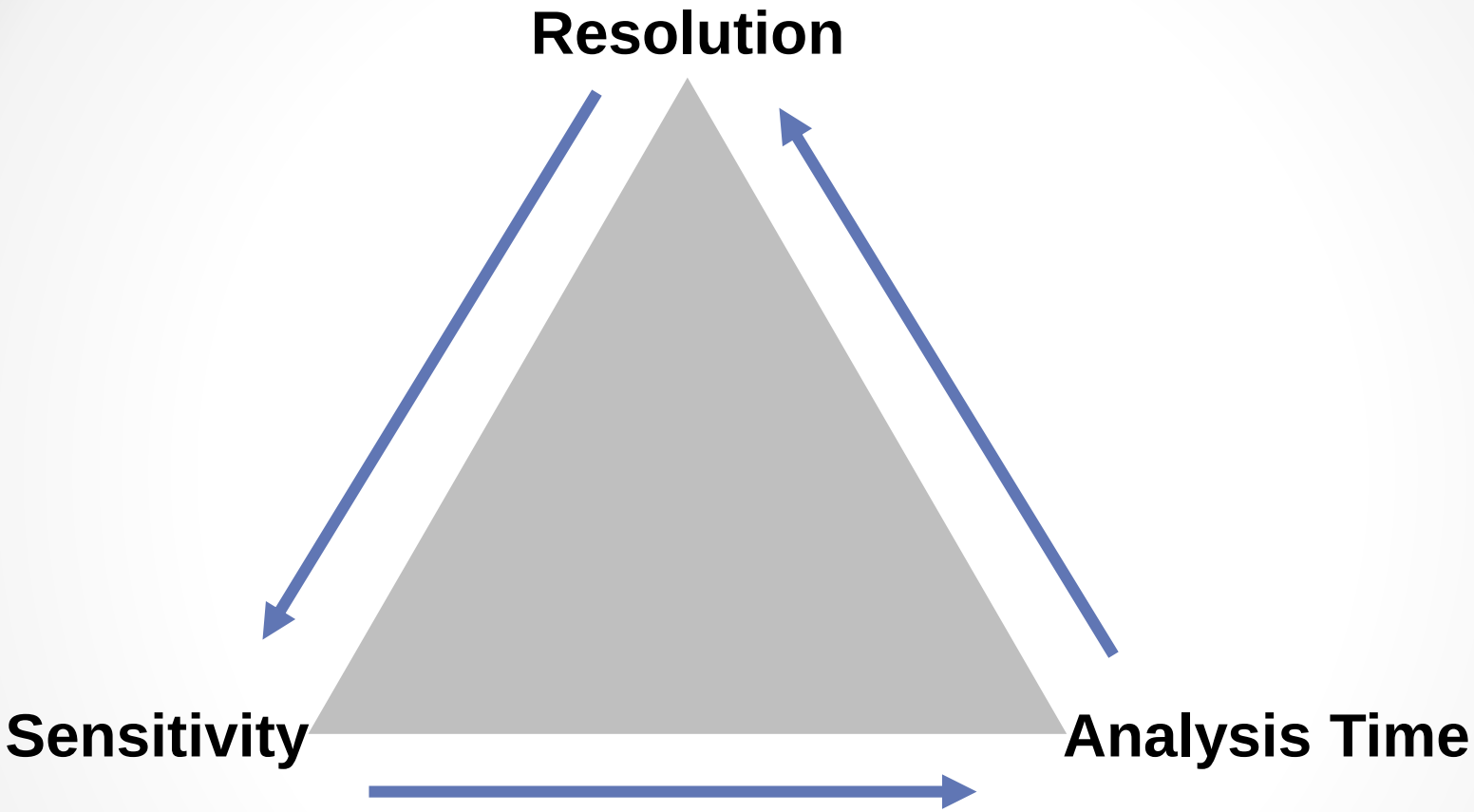
*An innovation is something original, new, and important in whatever field that breaks into a market or society. This is accomplished through more effective products, new processes, services, technologies, or ideas that are readily available to and successful in markets, governments and society.
Source: http://en.wikipedia.org/wiki/Innovation

Considerations Before Optimizing an (U)HPLC Separation

- Should Comprise all Operations of an HPLC Analysis
  - Sample Preparation
  - Sample Handling and Injection
  - Chromatographic Separation
  - Detection
  - Quantitation, Data Evaluation and Information Management

- Should Meet User Requirements
  - Robustness; towards slight change in conditions
  - Reliability, stability and longevity
  - Reproducibility and accuracy
  - Affordable
  - Ease of use; match skill of the operators
- Must Meet Internal and External Compliance and Regulatory Requirements (OQPV, Validation, ISO, GLP, etc.)

The Chromatography Optimization "Trilemma"

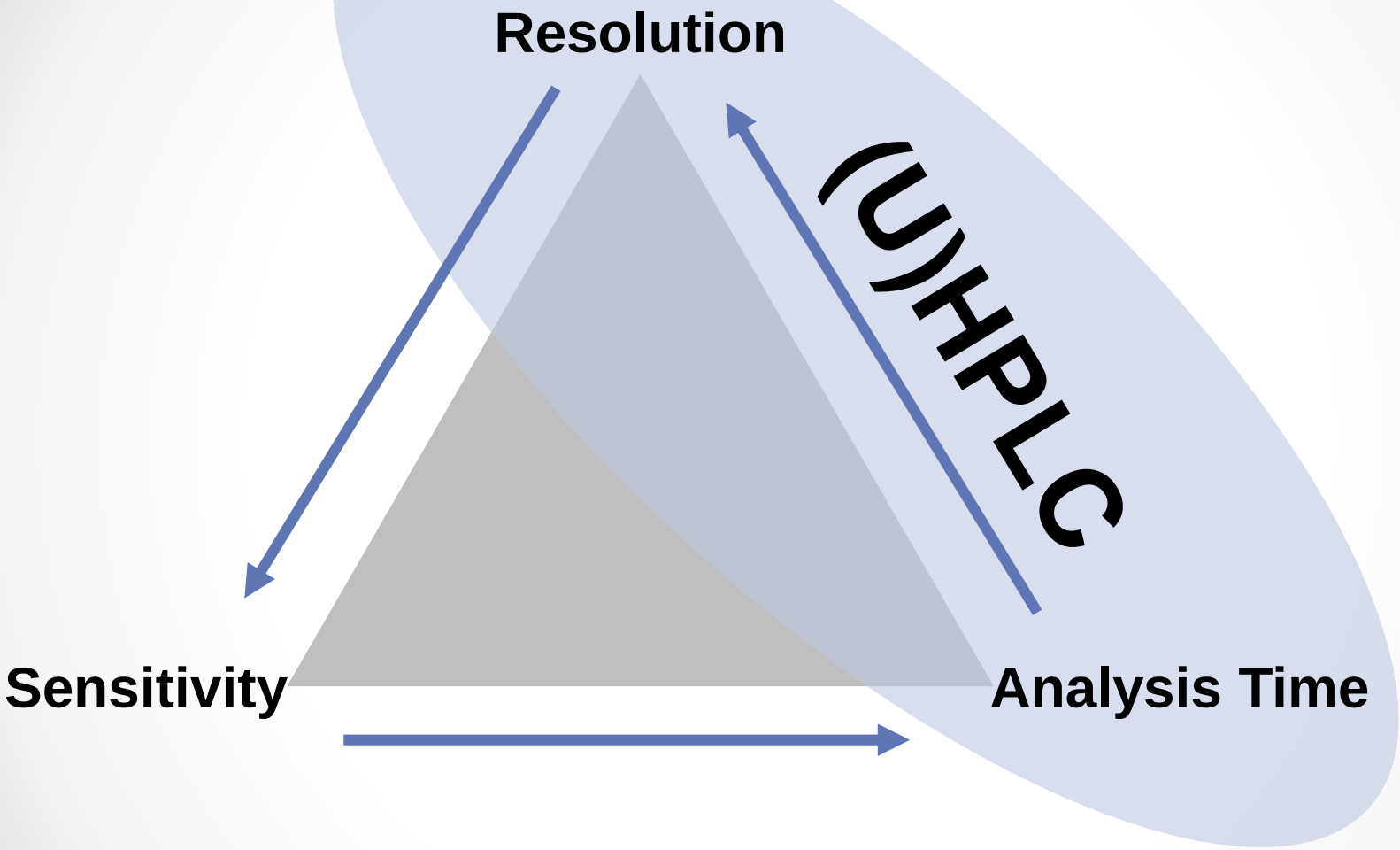


Quoted from Prof. Georges Guiochon*

*"the primary objective of an analysis by HPLC separation is to identify a maximum number of analytes (**resolution**) in the minimum amount of time (t_0) and to derive an accurate quantitative estimate of their concentrations (**sensitivity**).*

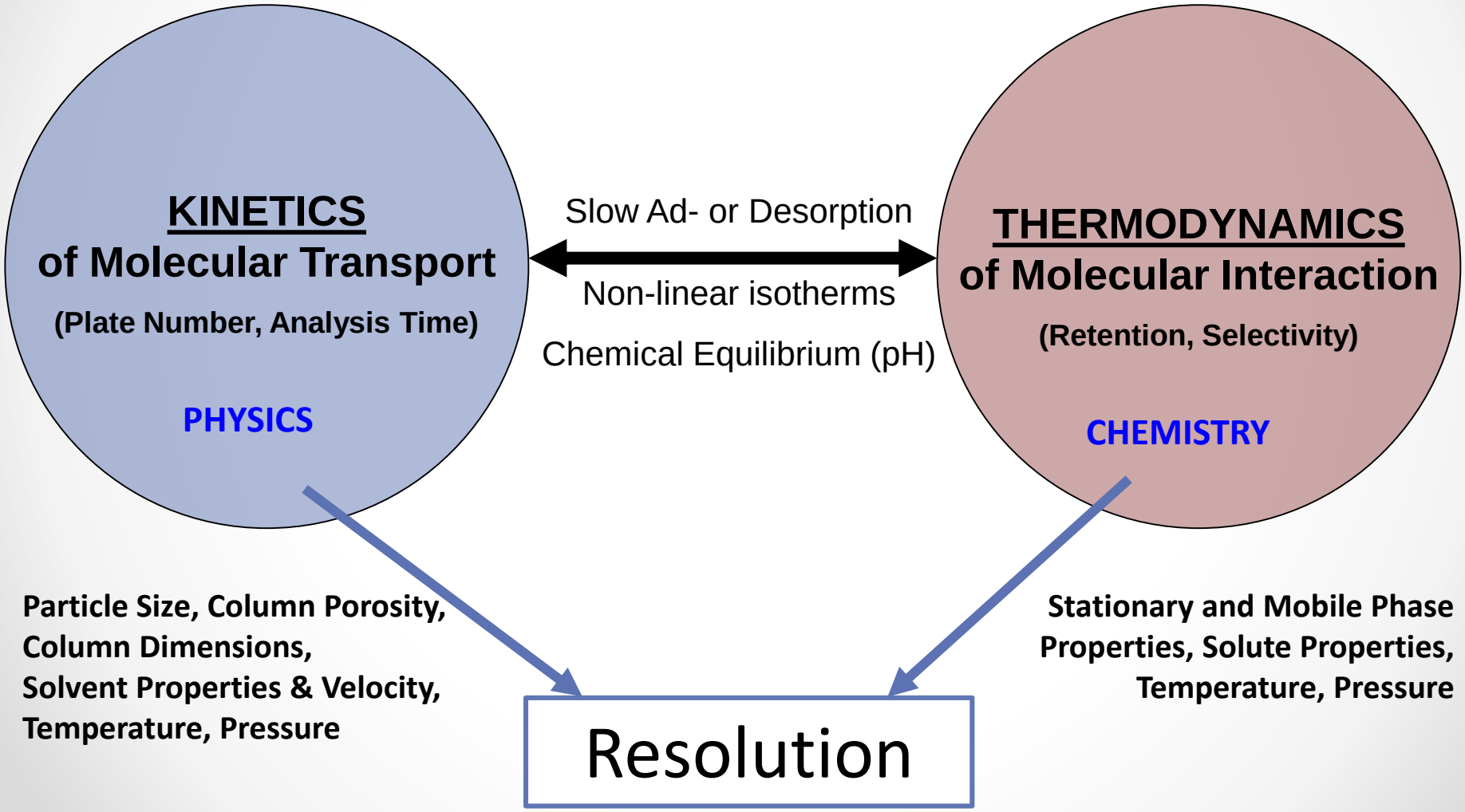
***F. Gritti and G. Guiochon, J. Chromatography A, 1228 (2012) 2–19; Prof. Guiochon passed away, October 21, 2014**

The Chromatography Optimization "Trilemma"



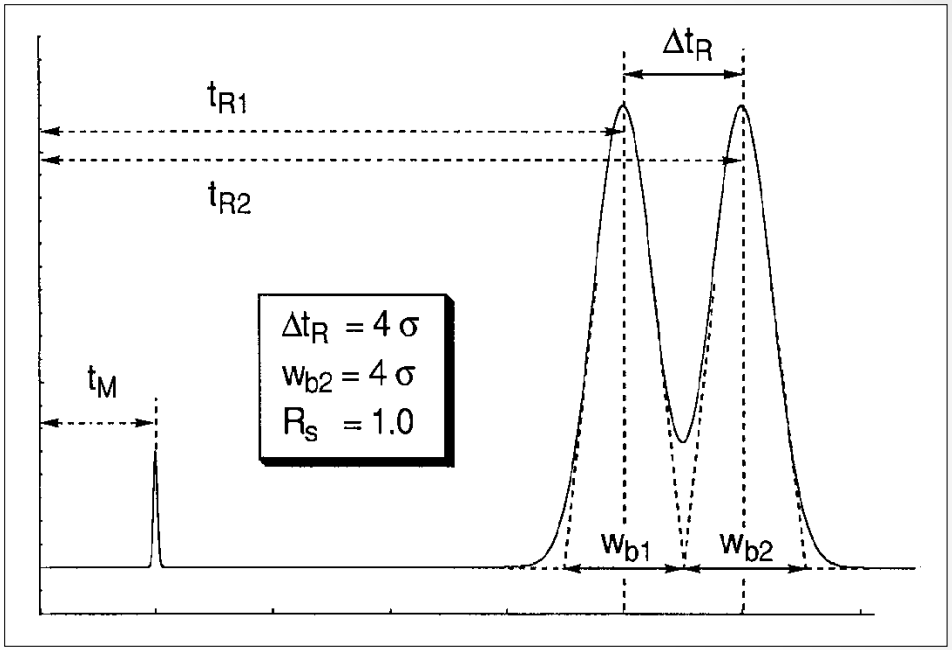
Primary Goal of (U)HPLC:
Obtain the best possible resolution in the shortest possible time

Mechanisms of Separation in HPLC



Resolution of Adjacent Peaks

$$R_s = \frac{2\left(t_{R,2} - t_{R,1}\right)}{\left(w_{b,1} + w_{b,2}\right)}$$



Which can be reformulated*:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

***Purnell equation!**

N_2 plate number of the second peak

α selectivity for two adjacent peaks ($\alpha = k_2 / k_1$)

k_2 retention factor for the 2nd peak

The indices are important!

* J. H. Purnell, J. Chem. Soc. (1960) 1268.

Other Equations for Resolution[1,2]

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

Pernell equation is obtained when one assumes that the width of the first peak equals the width of the second peak.

$$R_s = \frac{\sqrt{N_1}}{4} \cdot (\alpha - 1) \cdot \left[\frac{k_1}{k_1 + 1}\right]$$

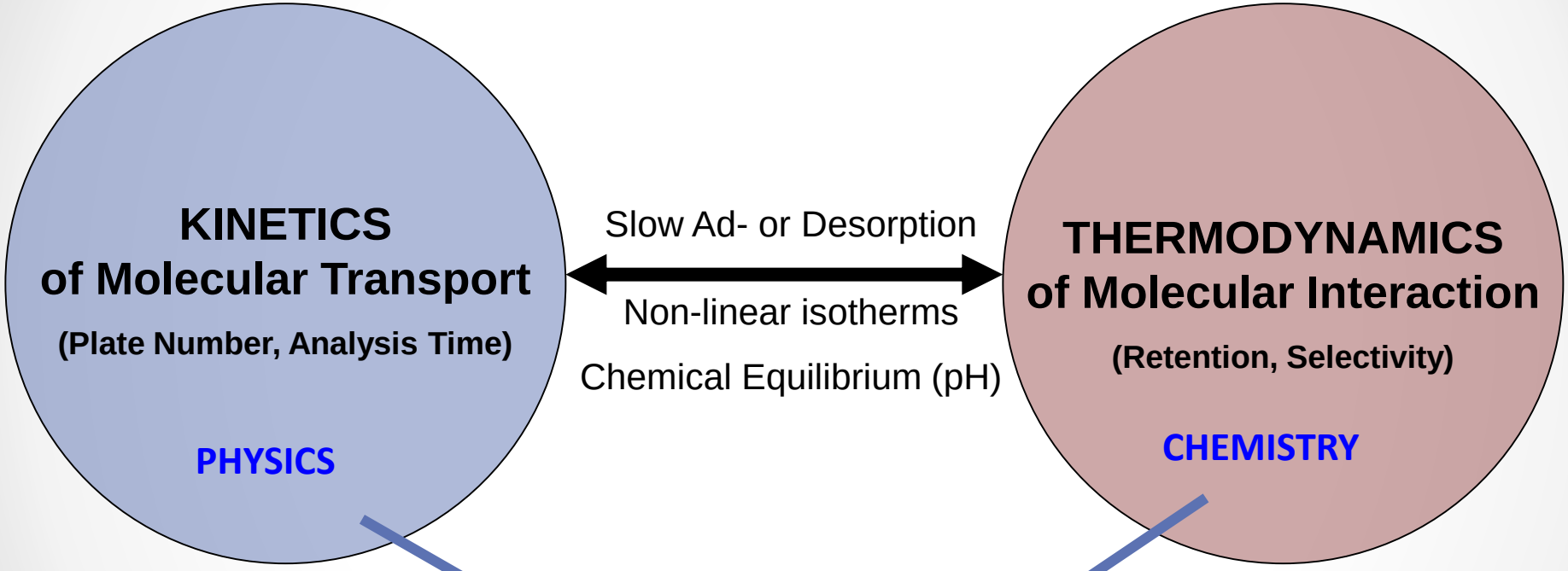
Is obtained when one assumes that the width of the second peak equals the width of the first peak (also called the Knox equation for resolution).

$$R_s = \frac{\sqrt{\overline{N}}}{2} \cdot \frac{(\alpha - 1)}{(\alpha + 1)} \cdot \left[\frac{\overline{k}}{\overline{k} + 1}\right]$$

Is obtained when the average width of the first and second peak is used. In this case k is the average of k_1 and k_2.

[1]A.S. Said, J. of High Resolution Chrom. & Chromatography Comm. 2, 193 (1979)
[2]P. Sandra, J. of High Resolution Chrom. & Chromatography Comm. 12, 82 (1989)

Mechanism of Separation in HPLC



Particle Size, Column Porosity, Column Dimensions, Solvent Properties & Velocity, Temperature, Pressure

Stationary and Mobile Phase Properties, Solute Properties, Temperature, Pressure

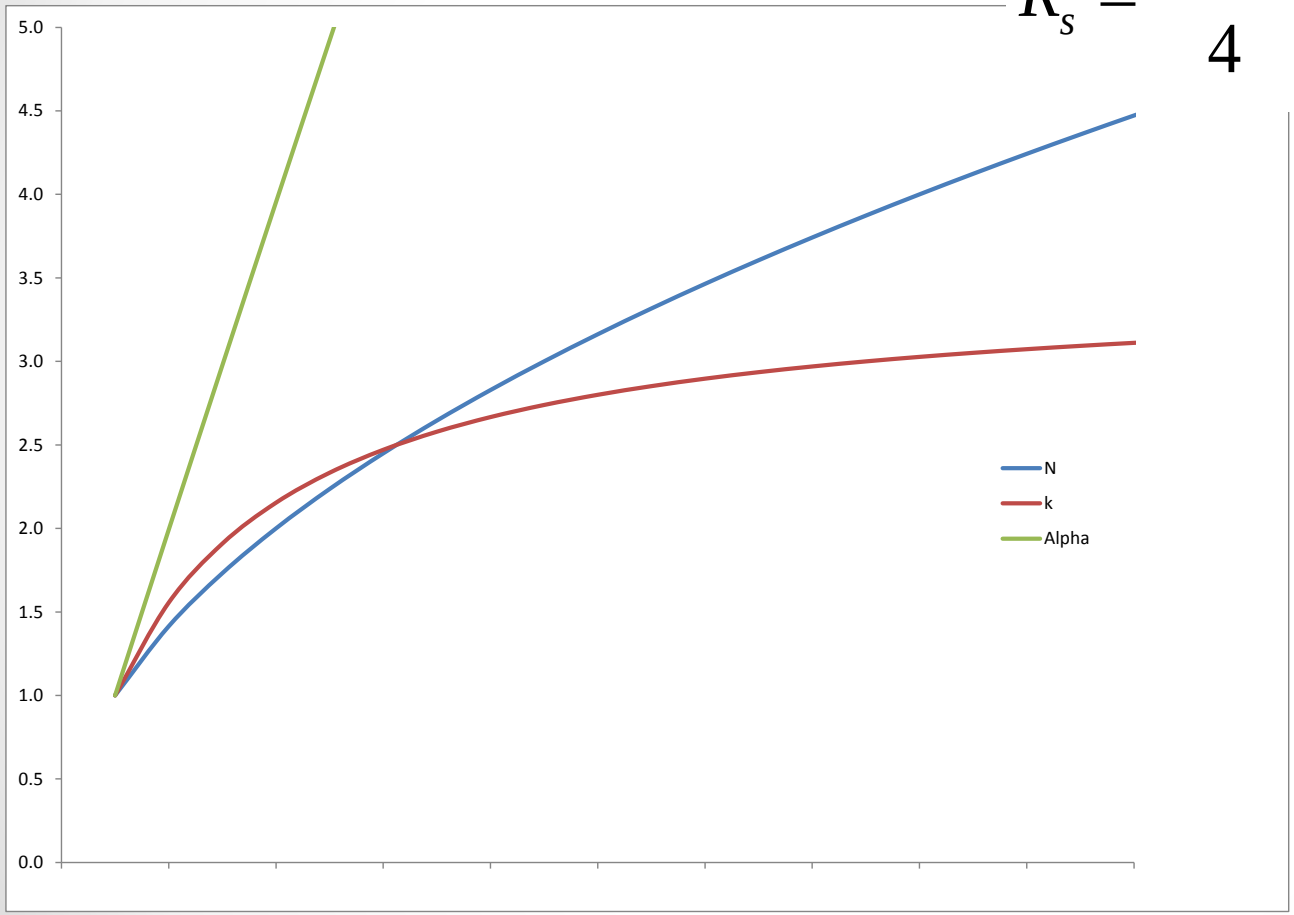
$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

Increase Resolution

– Normalized Plot of Individual Terms

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

Normalized R_s



Range used for calculation

N,	2000-40000
k,	0.4 – 8.0
A,	1.004 – 1.2

Normalized for N, k and α

Improve Resolution; Selectivity

- Selectivity (a) helps best but:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

- Is driven by difference in molecular properties (polarity, charge, hydrophobicity, size etc.)
  - "Know your molecules"!!
- Is difficult to predict
  - Experience helps (ask your colleague, check the literature, http://scholar.google.com)
  - Establish retention model for all molecules one is interested in
- Use of optimization software for the separation of multi-component mixtures (ChromSword, DryLab)
  - May become a laborious procedure especially with multicomponent samples
  - Impossible to do with very complex samples >50 components (e.g. tryptic digests, biomedical or environmental samples)

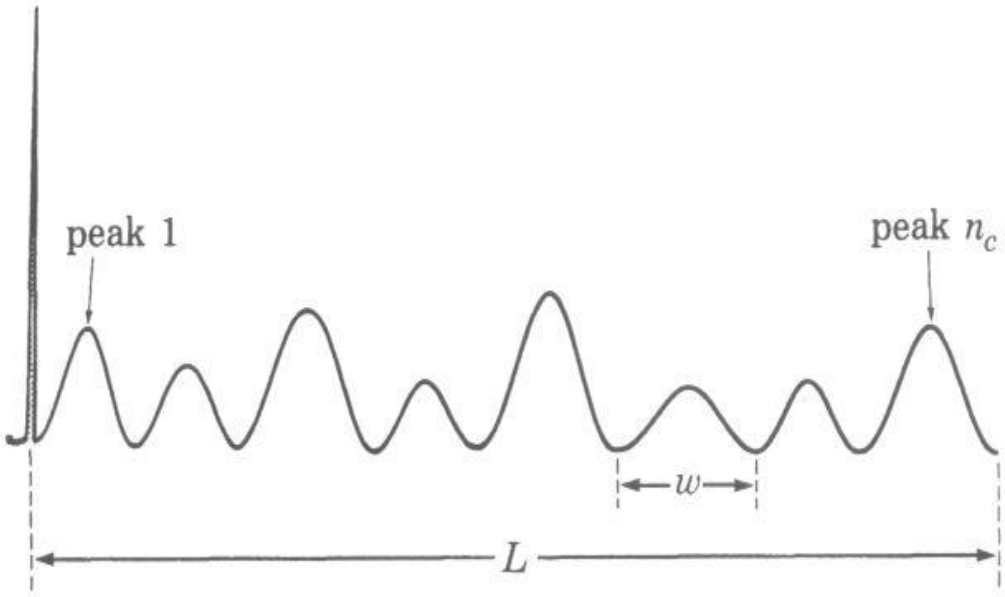
Improve Resolution; Increase Efficiency

High plate number provides:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

- Improved resolution
- Narrower and therefore higher peaks resulting in better signal/noise ratio
- Reduction of the separation time
- Higher peak capacity

Peak Capacity (Z_p) – Ideal Case*



Peak capacity is defined as the number of peaks or zones Z_p that can be separated (at specified R_s) over the "path length" of a chromatogram (or elution volume range V_{max} - V_{min}) in the separation system

***Taken from J. Calvin Giddings, Unified Separation Science, pg. 105 and references cited therein**

Peak Capacity (Z_p) - Isocratic Elution*

$$Z_p = \frac{\sqrt{N}}{6R_s} ln\left(1 + k'_{last}\right) + 1$$

Z_p : peak capacity (for uniform distribution in chromatographic space)
k'_{last} : retention factor of the last peak
R_s : required resolution (base line separation: $R_s \rightarrow 1.5$)

LC column, N = 25,000, k' = 20, Z_p = calculate

***Taken from J. Calvin Giddings, Unified Separation Science, pg. 106 and J.C. Giddings, Anal. Chem. 1967, 39, 1027–1028**

Peak Capacity (Z_p) – Gradient Elution

Assuming linear solvent strength gradient*

$$Z_p = 1 + \frac{t_g}{1/n \Sigma_1^n w} = 1 + \frac{t_g}{w_{av}}$$

t_g = gradient time

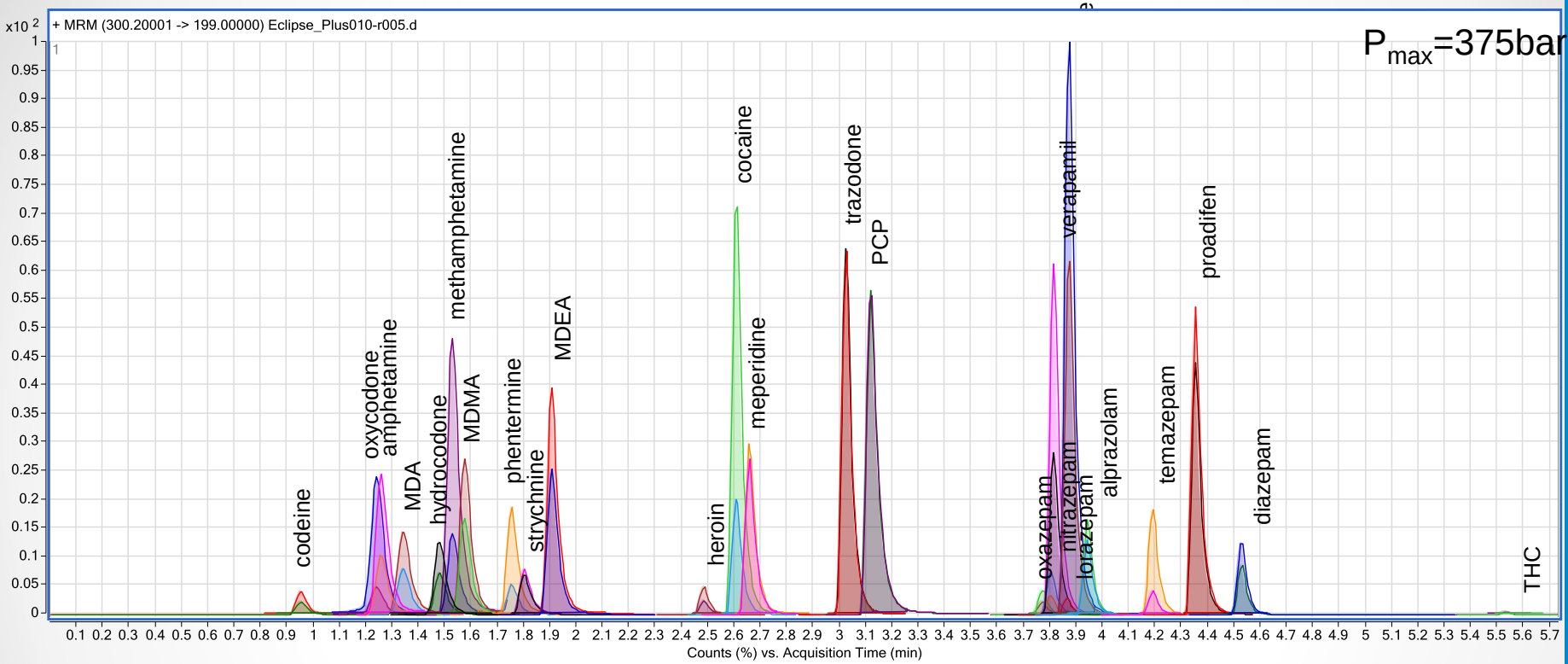
w_{av} = average peak width

***U.D. Neue, Theory of Peak Capacity in Gradient Elution, J. Chrom. A, 1079, 153, 2005 and references cited therein.**

Constraints in Achieving High Peak Capacity

- Solutes do not elute in an equidistant way
- Congestion in the chromatographic space is leading to peak overlap/co-elution which becomes particularly relevant when there is a high number of solutes in the sample

Peak Capacity (Z_p) in Practice



LC/MS Toxicology Test Mixture with 25 components analyzed on Zorbax Eclipse Plus C18, 1.8 µm, 100x2.1 mm*

***Courtesy of Agilent Technologies**

Davis–Giddings Statistical Model of Peak Overlap (SMO)*

Giddings et al. have assumed that the occurrence of a peak in the chromatographic space (from t_0 till t_{last}) is a randomly appearing discrete event (Poisson distribution).

Depending on the width of each peak (which determines the peak capacity) and the number of solutes n_c in the sample, the number of solutes s that elute as a single band is given by:

$$s = n_c \exp\left(\frac{-2n_c}{Z_p}\right)$$

In the example before and n=10

*** J. C. Giddings, Unified Separation Science, pg. 131 and references cited therein**
J. C. Giddings & J. Davis, Anal. Chem., 55, 418 (1983); 57, 2168 (1985)

Improve Resolution ➔ Increase Plate Number

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

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

Action	Constraint
Increase length of the column	Longer analysis time Higher column pressure
Decrease H(ETP) by using smaller particles	Higher column pressure

Relation between Particle Size and Column Backpressure

Permeability of Packed Columns*,**

From chemical engineering:
For an open tube

$$u_s = \frac{F}{A} = \frac{d_c^2}{32} \cdot \frac{\Delta P}{\eta L}$$

u_s = **superficial velocity. This equation is known as the Hagen-Poisseuille equation**

For a packed bed

$$u_s = \frac{F}{A} = K_o \cdot \frac{\Delta P}{\eta . L}$$

K_o = **specific permeability. This equation is known as the Darcy equation*****

How does K_0 relate to particle size?

*Cramers et al, Chromatographia, 14, 439 (1981)
**Desmet et al, Anal. Chem. 2005, 77, 3986-3992
*** http://en.wikipedia.org/wiki/Darcy%27s_law

Relation between Particle Size and Column Backpressure

Permeability of Packed Columns*,**

$$u_s = u_e \varepsilon_e$$

u_e = **interstitial solvent velocity**
ε_e = **interparticle porosity**

$$u_e = \frac{d_p^2}{180} \cdot \frac{\varepsilon_e^2}{(1-\varepsilon_e)^2} \frac{\Delta P}{\eta L}$$

Kozeny-Carman equation based on interstitial velocity (flow through a infinitely wide particulate bed)

$$u_s = \frac{d_p^2}{180} \cdot \frac{\varepsilon_e^3}{(1-\varepsilon_e)^2} \frac{\Delta P}{\eta L}$$

Kozeny-Carmen equation based on superficial velocity which is also written as:

$$u_s = \frac{d_p^2}{\Phi} \cdot \frac{\Delta P}{\eta L}$$

in which Φ is the dimensionless flow resistance factor. With ε_e 0.4 its value is 1012.5

*Cramers et al, Chromatographia, 14, 439 (1981)
**Desmet et al, Anal. Chem. 2005, 77, 3986-3992
http://en.wikipedia.org/wiki/Kozeny%E2%80%93Carman_equation

Relation between Particle Size and Column Backpressure

Permeability of Packed Columns*,**

According to chromatographers:

$$u_0 = \frac{L}{t_0} = B_0 \frac{\Delta P}{\eta L}$$

***u_0* chromatographic solvent velocity with**

$$u_s = \varepsilon_T u_0$$

***B_0* is called chromatographic permeability**

$$u_0 = \frac{d_p^2}{180} \cdot \frac{\varepsilon_e^3}{\varepsilon_T (1-\varepsilon_e)^2} \frac{\Delta P}{\eta L} = \frac{d_p^2}{\varepsilon_T \Phi} \cdot \frac{\Delta P}{\eta L}$$

Kozeny-Carman equation based on chromatographic solvent velocity

$$\varepsilon_T \Phi = \Phi_0 \qquad B_0 = \frac{d_p^2}{\varepsilon_T \Phi} = \frac{d_p^2}{\Phi_0}$$

***Φ_0* chromatographic flow resistance factor (has the value of 700 for a good column)**

$$\Delta P = \frac{\Phi_0}{d_p^2} . u_o \eta L$$

Column pressure increases linearly with column length and with the inverse of the particle size squared !!

Improve Resolution ➔ Increase Plate Number

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$

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

Action	Constraint
Increase length of the column	Longer analysis time Higher column pressure
Decrease H(ETP) by using smaller particles	Higher column pressure

Height Equivalent to a Theoretical Plate (HETP)

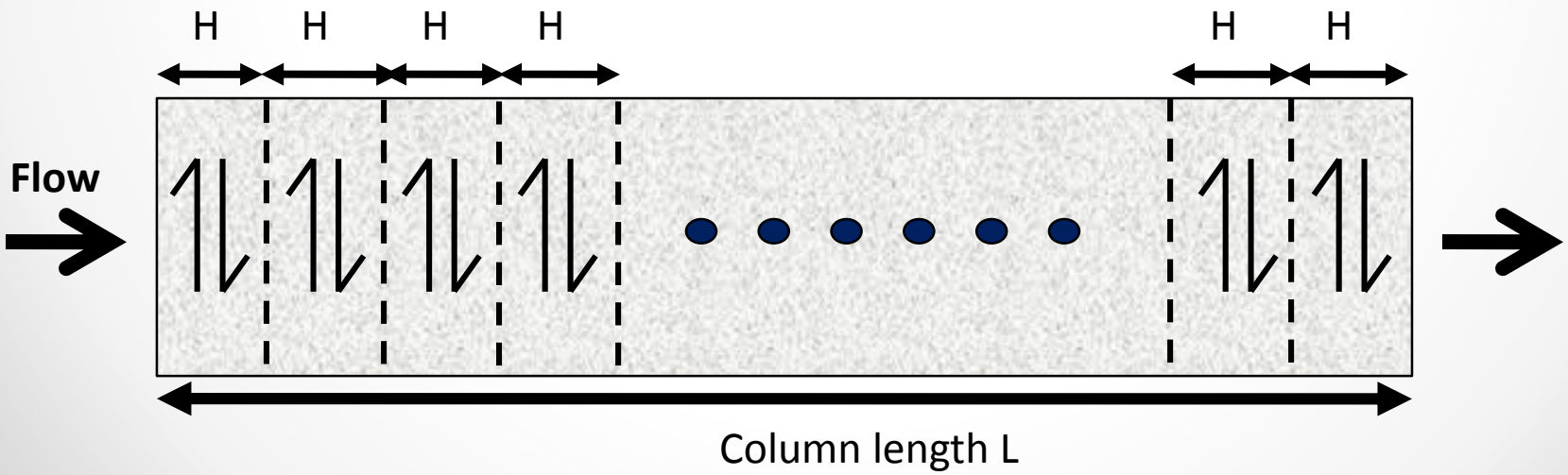
The height equivalent to a theoretical plate (HETP) H, is the hypothetical length in a column which is required to reach equilibrium of the distribution of the solute between a moving mobile and stationary phase.

H depends on:

- Particle size and particle porosity
- Packing bed porosity and structure
- Solute diffusion coefficient
- Solvent flow velocity
- Retention of the solute

Plate Number

$$N = L / H$$



Height Equivalent to a Theoretical Plate (HETP)

(Simplified) Van Deemter Equation*

$$H = A \cdot d_p + \frac{B \cdot D_m}{u} + C \cdot \frac{d_p^2}{D_m} u$$

A-term B-term C-term

A-term describes the solvent velocity inequalities through the bed and depends on the particle size

B-term describes the influence of solute diffusivity and decreases with solvent velocity

C-term describes the mass transport in and out of the particles, depends on particle size and solute diffusivity and increases with solvent velocity

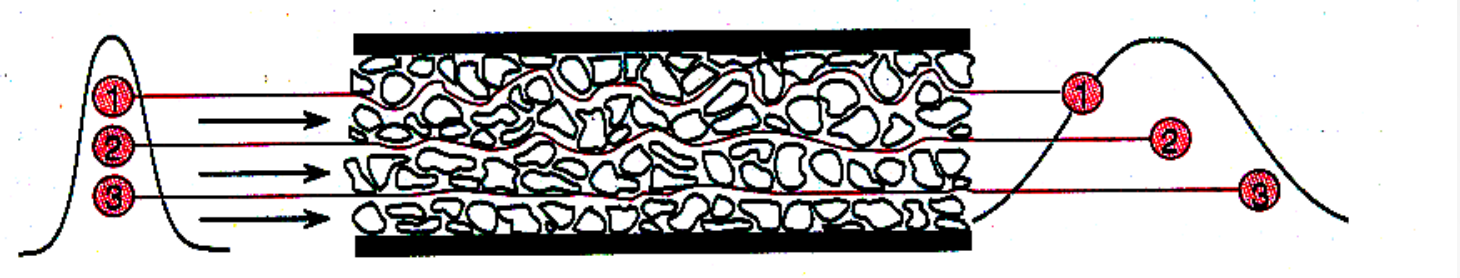
$$u_{opt} = \frac{D_m}{d_p}\sqrt{\frac{B}{C}}$$

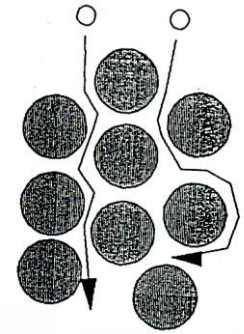
$$H_{\min} = (A + \sqrt{B/C}) \cdot d_p$$

***van Deemter J.J. et al., Chem. Eng. Sc. 5: 271–289 (1956).**

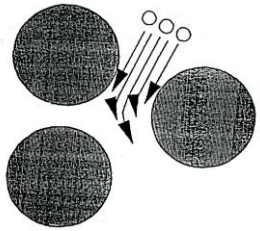
Height Equivalent to a Theoretical Plate (HETP)

Van Deemter Equation: A-Term





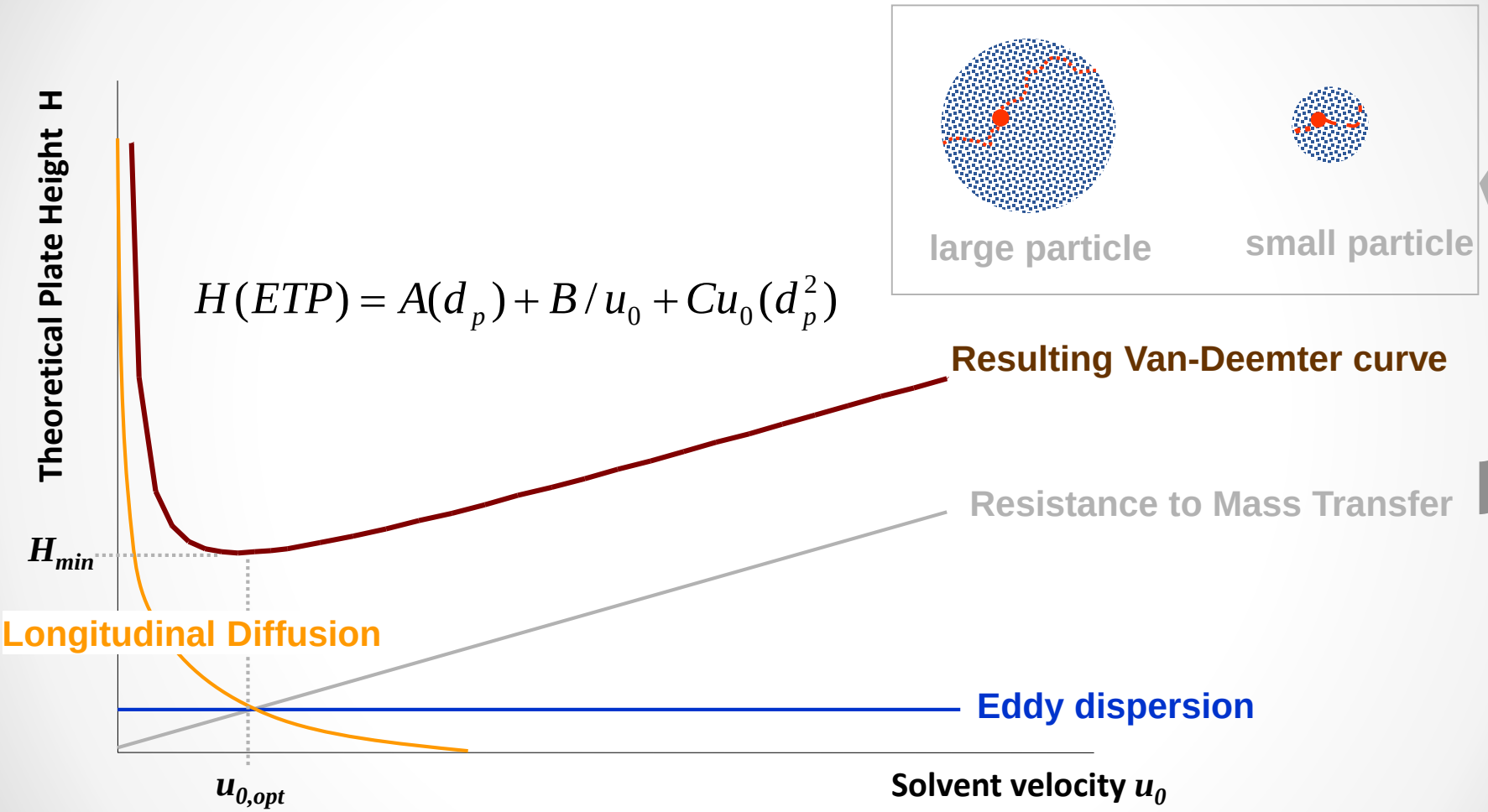
Eddy Dispersion by different length of pathway



Flow velocity profile between particles

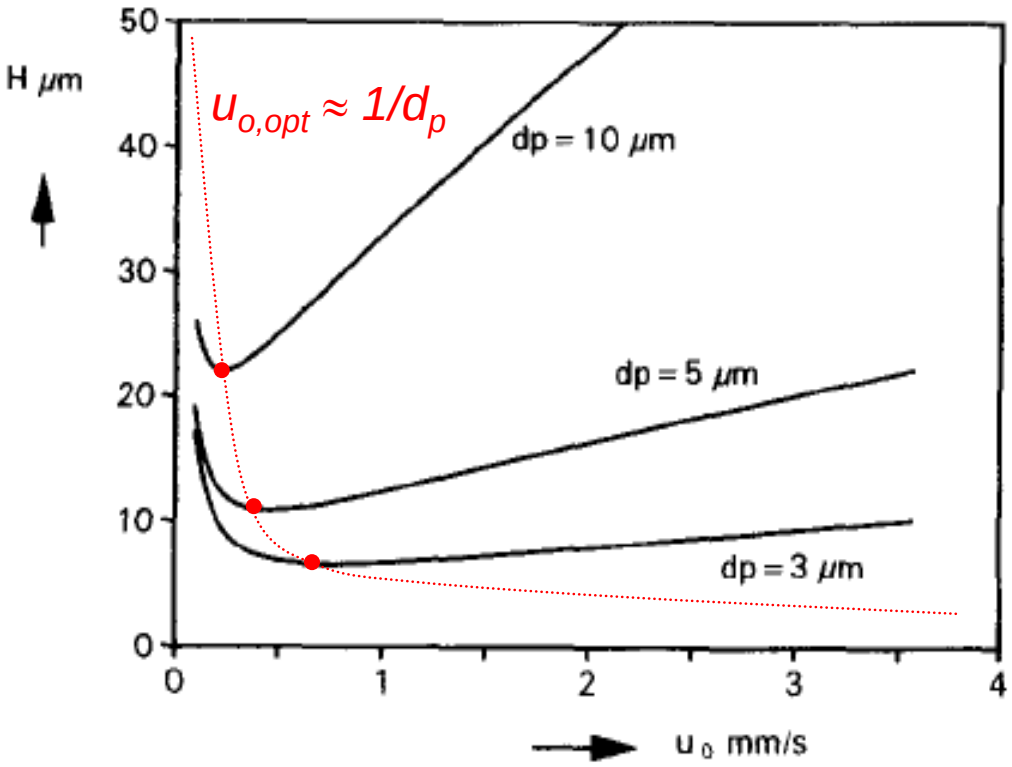
Height Equivalent to a Theoretical Plate (HETP)

Van Deemter Equation: Simplified Version



Height Equivalent to a Theoretical Plate (HETP)

Van Deemter Equation: $u_{o,opt}$



Column pressure increases with inverse particle size square.

Velocity at the minimum increases with the inverse of the particle size

$u_{o,opt}.d_p \approx$ constant

The pressure required to operate a column at $u_{o,opt}$ is inversely proportional with the cubic power of d_p

$$H(ETP) = f(u_o, d_p, D_m, k', ...)$$

Height Equivalent to a Theoretical Plate (HETP)

Knox Equation: $u_{o,opt}$

In the Knox equation, it is assumed that the eddy dispersion (A-term) depends on the solvent velocity

$$H(ETP) = Au_0^{1/3} + B/u_0 + Cu_0$$

The Knox equation is preferably used in a dimensionless formulation with:

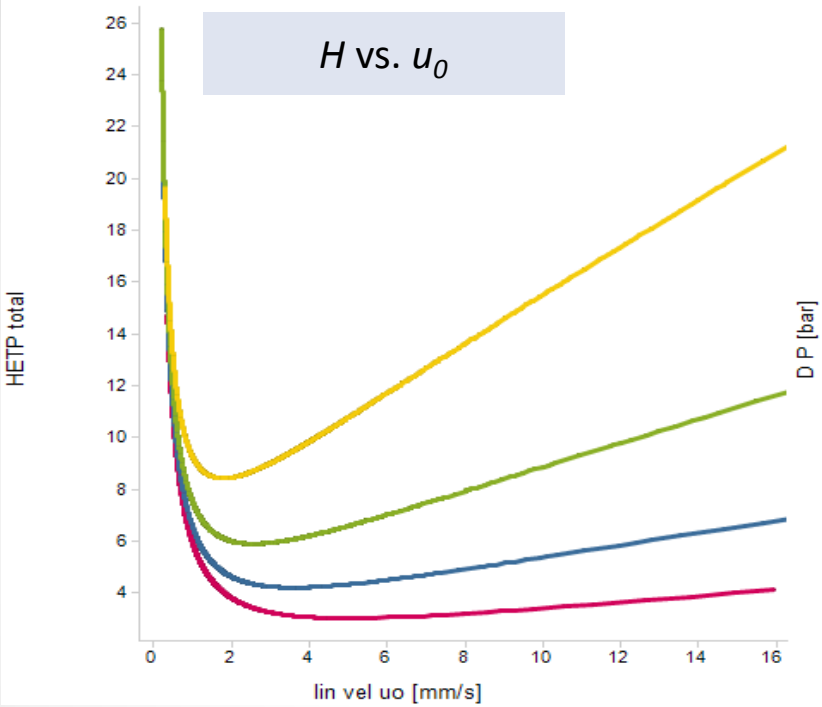
$$h = \frac{H}{d_p} \quad \text{and} \quad \nu = \frac{u_0 d_p}{D_m}$$

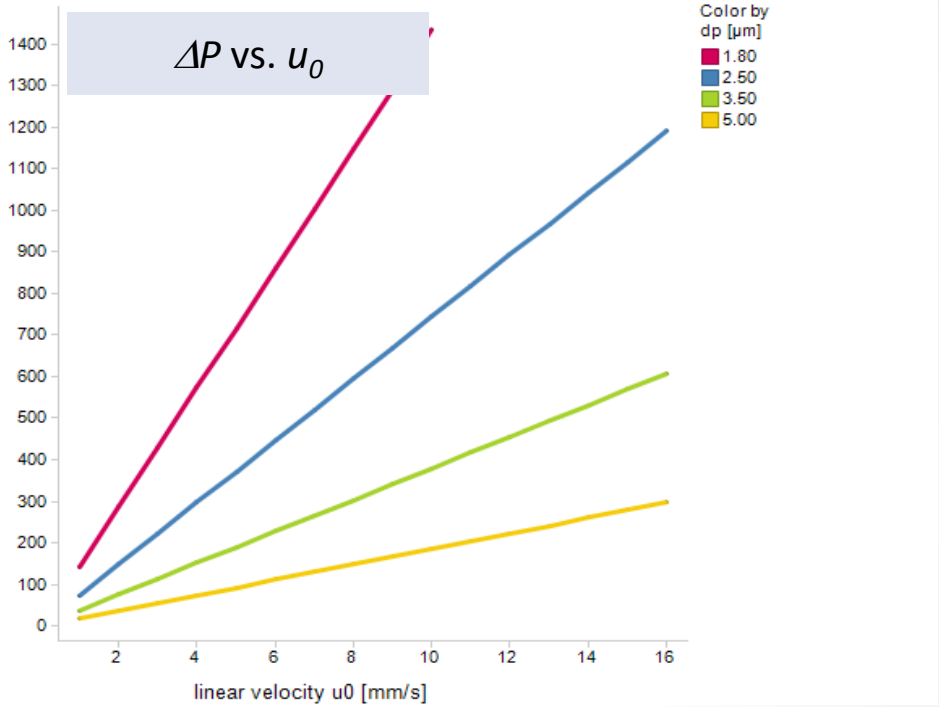
h is called reduced plate height, ν is called the reduced velocity. They are dimensionless parameters

$$h = a\nu^{0.33} + \frac{b}{\nu} + c\nu$$

Knox equation is non-linear

HETP and Pressure Drop vs. Solvent Velocity





$$t_0 = \frac{L}{u_0}$$

$N = L/H$ → Classical Dilemma ← $\Delta P_{max} = u_0 \cdot \eta \cdot L \cdot \frac{\Phi_0}{d_p^2}$

Column length = 100 mm, viscosity = 1 cP

Classical Dilemma of HPLC

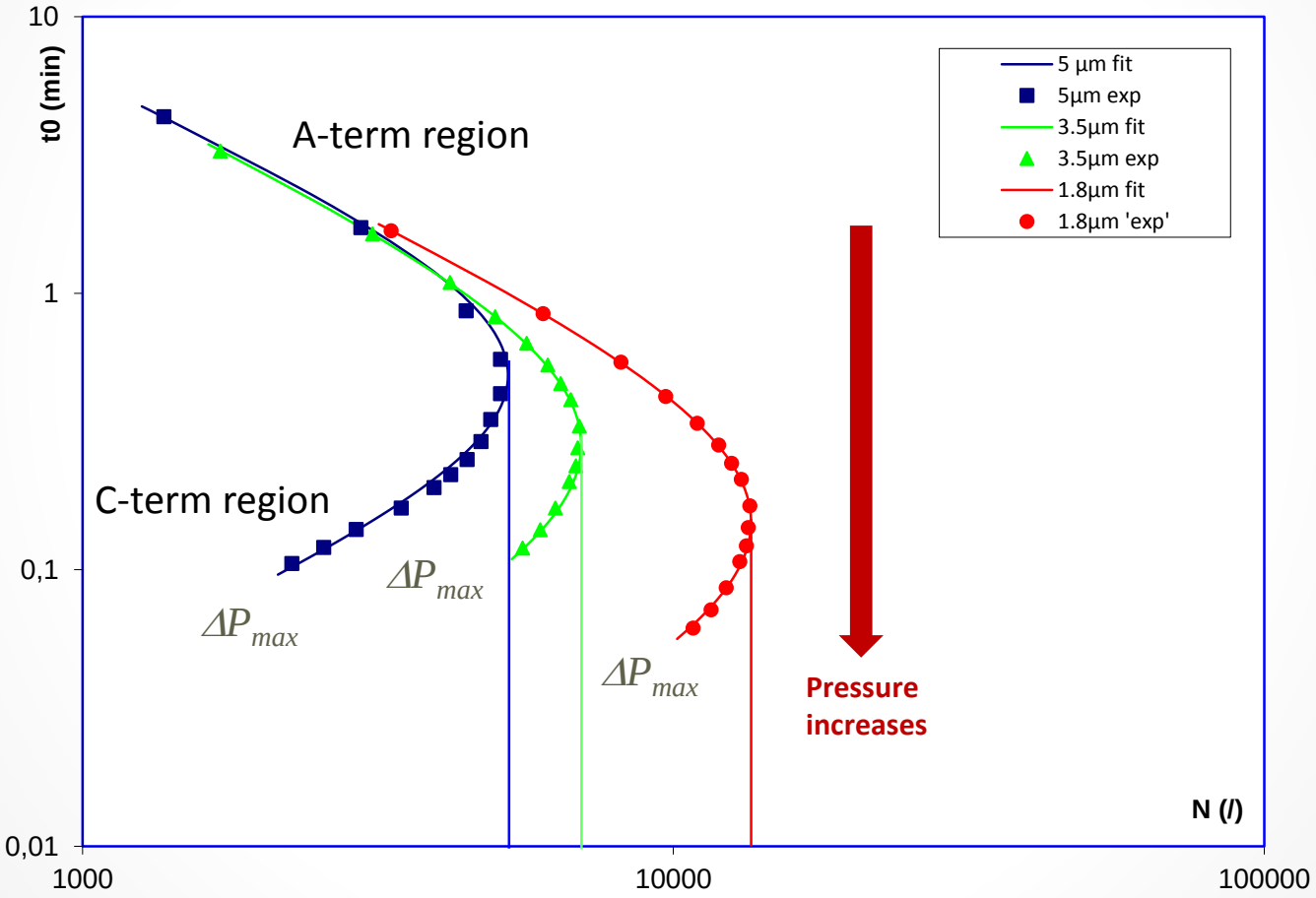
How to achieve a particular plate number $N_{required}$ (N_r), say 20,000 plates, in the shortest time possible at a given maximal column pressure available?

According to J.H. Knox and M. Saleem,
J. Chromatogr. Sci., 7 (1969), p. 614

UHPLC - Essentials

van Deemter Plot reordered*

***L* (50 mm) and d_p fixed, select optimal u_o**



***Slide courtesy of Prof. Ken Broeckhoven, Free University of Brussels**

How Obtain N_r in the Shortest Time Possible*

The optimization can be achieved by defining:

1. The maximum pressure capability: ΔP^{max}
2. The number of plates that one wants to obtain
3. A set of columns for which L, d_p and linear velocity is continuously varied until ΔP^{max} pressure is reached.

Equations that allow to achieve this goal:

$L_r = N_r h d_p$ length required to achieve N_r (h is reduced plate height)

$$u_0 = L_r / t_0 = \left(N_r h d_p\right) / t_0$$

$$\Delta P = \left(u_0 \eta L_r\right) / B_0 = \left(u_0 \eta L_r \Phi_0\right) / d_p^2 = \left(h^2 \Phi_0 N_r^2 \eta\right) / t_0$$

*J.H. Knox and M. Saleem. J. Chromatogr. Sci., 7 (1969), p. 614

How Obtain Nr in the Shortest Time Possible*

Solving for t_0:

$$t_0 = \frac{h^2 \Phi_0 N_r^2 \eta}{\Delta P^{\max}} = \frac{E\, N_r^2 \eta}{\Delta P^{\max}}$$

Shortest time to generate a required plate number constrained by a maximum back pressure is obtained when $E = h^2\, \Phi$ is minimal (E is the so-called separation impedance) . This is the case (with Φ_0 is constant) when one works in the minimum of the *H-u* curve and thus at $u_{0,opt}$.

For ΔP^{max} an optimum particle size, $d_{p,opt}$ can extracted:

$$d_p(opt) = \left(\frac{\Phi_0 \eta\, u_{0,\min} h_{\min} N_r}{\Delta P^{\max}} \right)^{1/2}$$

Particle size has to increase in order to obtain a higher N_r in the shortest time!!

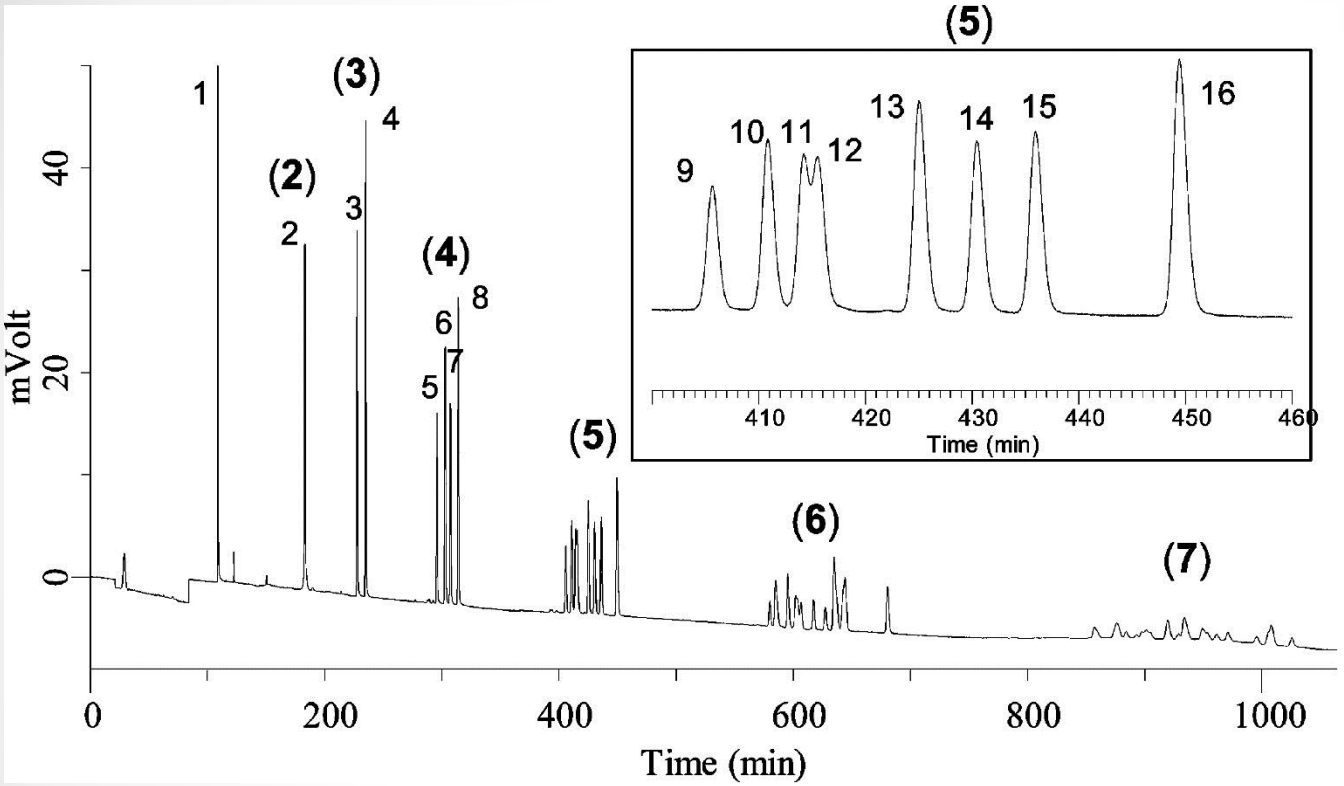
*J.H. Knox and M. Saleem. J. Chromatogr. Sci., 7 (1969), p. 614

How to Obtain the Required Plate Number in the Shortest Time?

200 bar			
N_r	t_0	d_p	L
1000	0.2 s	0.5 µm	1.1 mm
10000	20 s	1.7 µm	35 mm
100000	2000 s	5 µm	1100 mm
1000000	2.3 days	17 µm	35 m
400 bar			
1000	0.1 s	0.39 µm	0.78 mm
10000	10 s	1.22 µm	24.4 mm
100000	1000 s	3.87 µm	774 mm
1000000	1.16 days	12.25 µm	24.5 m
1000 bar			
1000	0.04 s	0.25 µm	0.5 mm
10000	4 s	0.77 µm	15.5 mm
100000	400 s	2.45 µm	490 mm
1000000	0.46 days	7.75 µm	15.5 m

Unretained retention, *optimal particle size* and column length at pressure limited situations in HPLC

How to Achieve very Large Plate Numbers*



N_r > 1,000,000

*Separation of styrene oligomers (molecular weight standard for MW = 580). The numbers in parentheses indicate the number of styrene units in the oligomer. Column: **monolithic silica, effective length: 1130 cm**. Mobile phase: acetonitrile–water (95/5). **ΔP = 39.5 MPa**. u = 1.73 mm/s. Detection: 210 nm. Temperature: 30 °C. The inset is a magnification of the chromatogram for the pentamers.

***Nobuo Tanaka et al., *Anal. Chem.* 2008, 80, 8741-8750.**

Lessons learned so far

- In (U)HPLC separations, keep in mind that selectivity works best in obtaining resolution for samples with few compounds, but may require a laborious optimization procedure.
- The objective in (U)HPLC is to obtain the best resolution in the shortest possible time.
- Peak capacity is of limited value as a performance parameter. Statistical peak overlap is unavoidable and a practical constraint.
- Permeability is a crucial column property and depends on the inverse square of particle diameter. Its magnitude differs by the kind of solvent velocity used.
- Very high efficiencies (N > 50,000) require bigger particles and longer columns at the cost of time
- Medium efficiencies (N < 50,000) are obtained in shorter time with very small particles and shorter column lengths at the cost of pressure

Acknowlegdements & Hand-out

- Dr. Monika Dittmann, Agilent Technologies, Germany
- Dr. Ken Broeckhoven, Free University of Brussels, Belgium

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