

Fundamentals, Optimization and Practical Aspects of UHPLC Part - 4

Effect of Ultra-High Pressure on Solvent, Solute and Column Properties in UHPLC, especially the effects of frictional heating, requirements for UHPLC equipment in particular regarding ext. band broadening, detection for UHPLC

Important Insights from Part 1+2+3

- UHPLC delivers extended abilities for HPLC through
 - New column technologies (in particular, sub-2-micron and superficially porous particles)
 - Next generation HPLC equipment with extended operating pressure and reduced extra-column band broadening
- The premier goal in UHPLC is to obtain the best possible resolution in the shortest possible time. Besides chromatographic selectivity, the column plate number is the best handle in achieving highest resolution.
- Kinetic optimization according to Knox and Saleem, showed how to obtain the plate number N_{req} required for best resolution in the shortest possible time by choosing the proper particle size and column length. Though unpractical, the K&S approach delivered as a rule of thumb:
 - For $N_{req} \leq 50000$ (which satisfies 95% of cases) this is achieved by using columns with smallest particles and shortest length at maximum pressure achievable with your next generation HPLC system.
 - Very large N_{req} is obtained by using columns with larger particle size and long length at maximum pressure but at the cost of time.
- Kinetic plot theory is an unbiased methodology to help decide which column (dimensions, particle size/morphology) delivers N_{req} fastest.
- In high temperature UHPLC one obtains lower H (higher N) when $u_0 > u_{0,min}$ at lower back pressure.
- Superficially porous particles ($\approx 2 \mu\text{m}$) outperform totally porous particles because of lower A-term (eddy dispersion) and B-term (axial diffusion) of the HETP vs. u_0 equation.

Effect of Ultra-High Pressure on Solvent, Solute and Column Properties in UHPLC*

- Solvent density (ρ) resp. specific volume, viscosity (η), compressibility (χ) and melting point change

*M. Martin & G. Guiochon, J. Chrom. A, 1090, 16 , (2005)

Pressure Dependence of the Solvent Melting Point.

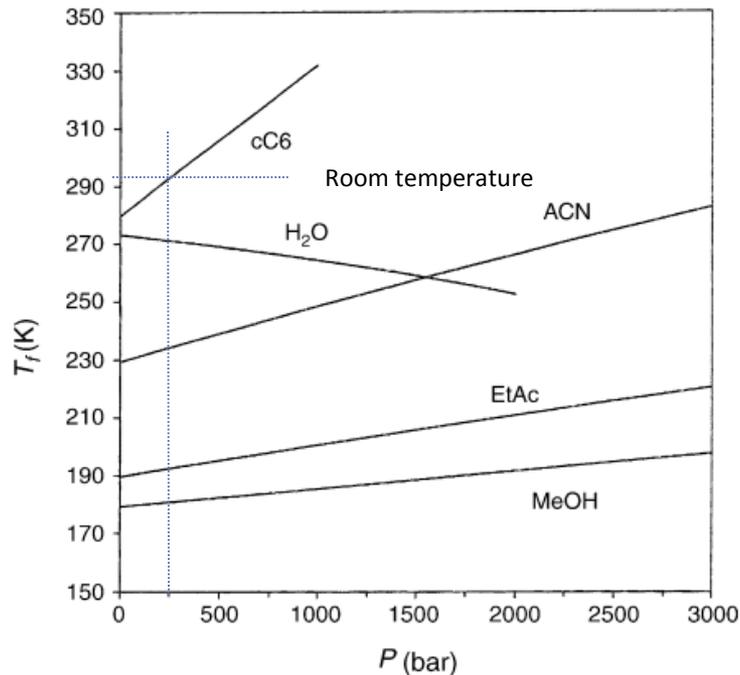


Fig. 1. Plot of the temperature of fusion, T_f , of various compounds vs. the local pressure, P . Symbols: cC6, cyclohexane; H₂O, water; ACN, acetonitrile; EtAc, ethyl acetate; MeOH, methanol. The curves are limited to the pressure

Taken from: M. Martin & G. Guiochon, J. Chrom. A, 1090, 16, (2005)

- Melting point of water decreases with pressure increase
- For organic solvents, the melting point increase with pressure
- Cyclohexane becomes a solid at room temperature and approx. 250 bar

Pressure Dependence of the Solvent Viscosity

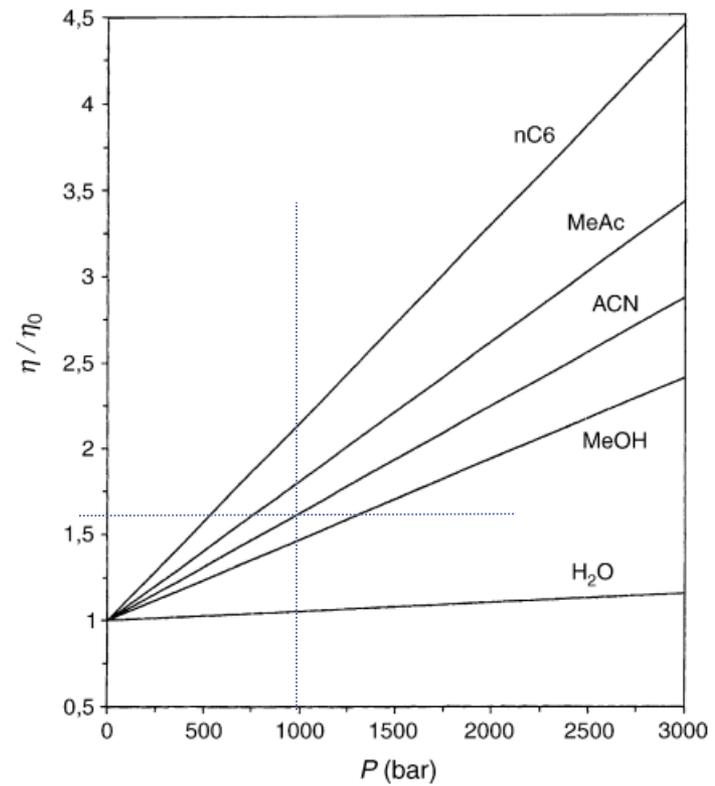


Fig. 3. Ratio of the viscosity under pressure P , η , to the viscosity under atmospheric pressure, η_0 , vs. the pressure, P , for various liquids at 30 °C. Symbols: nC6, *n*-hexane; MeAc, methyl acetate; ACN, acetonitrile; MeOH, methanol; H₂O, water.

Taken from: M. Martin & G. Guiochon, J. Chrom. A, 1090, 16, (2005)

- E.g. the acetonitrile viscosity increases by 60% from 0 → 1000 bar
- Increase of flow rate raises back pressure over proportionally!!
- For dependence of viscosity and compressibility of solvent mixtures on pressure and temperature see: J. Billen et al., J. Chrom. A, **1210**, 30 (2008)

$$\Delta P_{\max} = u_0 \cdot \eta \cdot L \cdot \frac{\Phi_0}{d_p^2}$$

↑

Effect of High Pressure on Solvent and Column Properties in UHPLC*

- Solvent density (ρ) resp. specific volume, viscosity (η), compressibility (χ) and melting point change
- Retention factors (k') and chemical equilibriums

*M. Martin & G. Guiochon, J. Chrom. A, 1090, 16, (2005)

Relative Retention Factor vs. Pressure*

Insuline

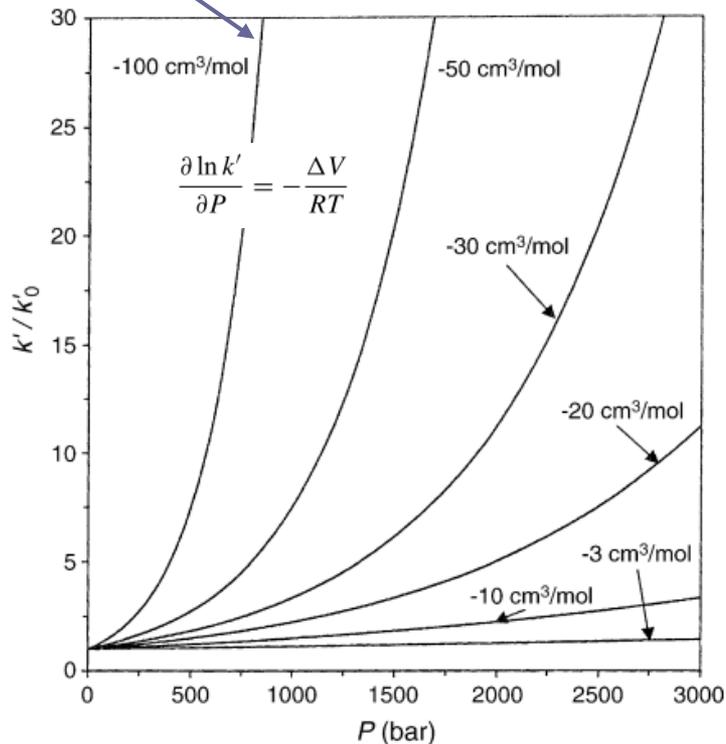
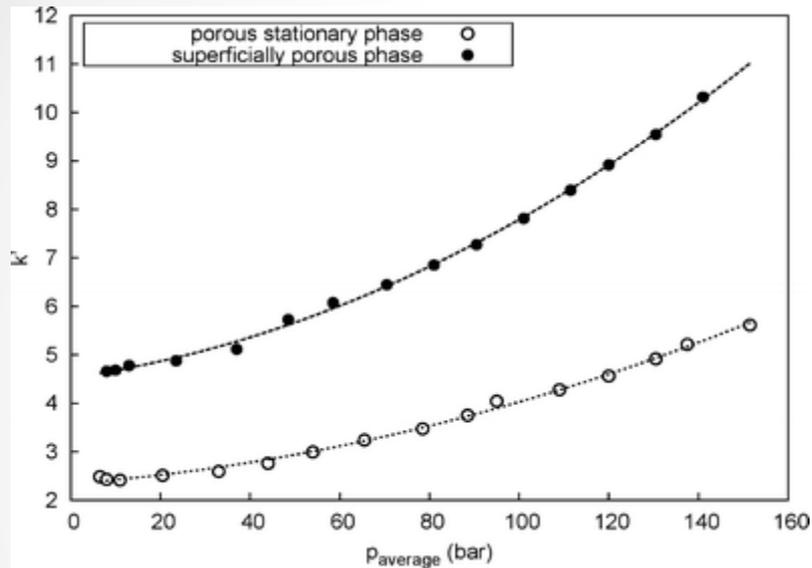


Fig. 5. Ratio of the retention factor, k' , under pressure P to the retention factor under atmospheric pressure, k'_0 . Plot of k'/k'_0 versus P , according to Eq. (16) for various values of the change in partial molar volume, ΔV , associated to the transfer of one mole of solute from the mobile to the stationary phase at 25 °C.

- Solute retention increases with pressure
- High molecular weight solutes are more strongly affected
- E.g. retention of insulin doubles when the pressure increases from 50-250 bar
- Frictional heating is not accounted for here!

Relative Retention Factor vs. Pressure*



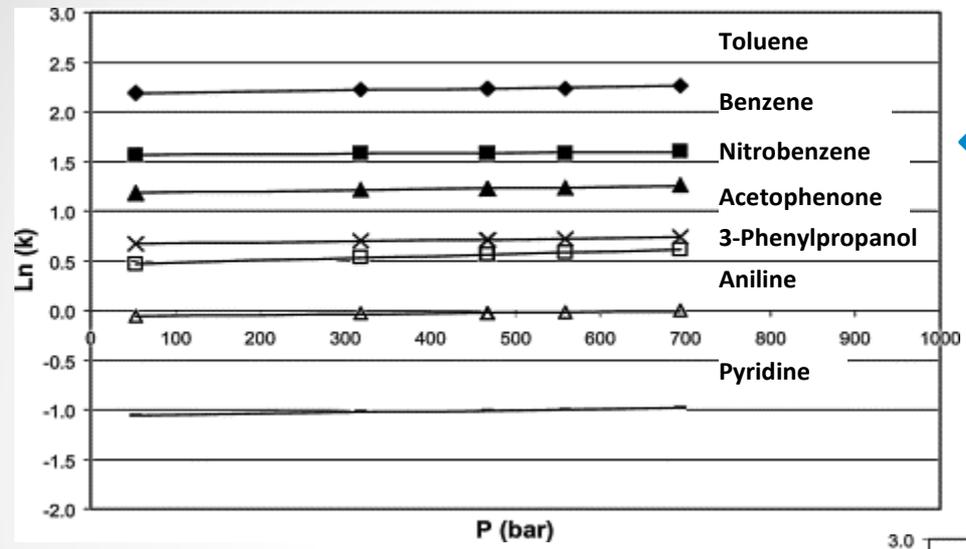
Retention factor of human insulin against the average pressure drop. The average pressure drop was altered by varying the flow rate of the mobile phase

*A. Felinger, Anal. Bioanal. Chem. (2010) 397:1307 – 1314

Relative Retention Factor vs. Pressure

Experimental Results*

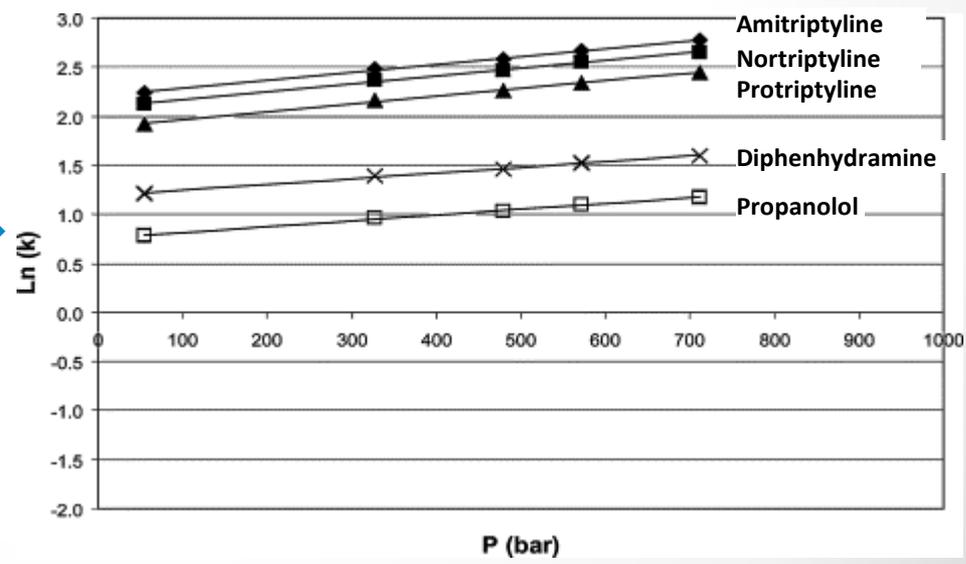
Neutral Solutes



XBridge C 18 BEH, 5 m, 50 mm × 2.1 mm column, 30°C, mobile phase acetonitrile–water (40:60, v/v) at 0.4 mL/min.
 Pressure is applied through narrow capillaries after the column.
 Increase of k/500 bar approx. 5-10%

XBridge C 18 BEH, 5 m, 50 mm × 2.1 mm column, 30°C, mobile phase acetonitrile–water (40:60, v/v) at 0.4 mL/min.
 Pressure is applied through narrow capillaries after the column.
 Increase of k/500 bar approx. 30-40%

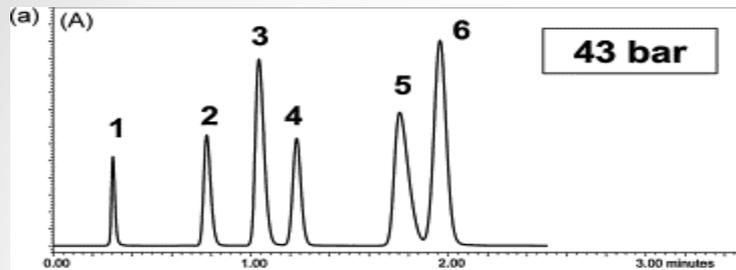
Basic Solutes



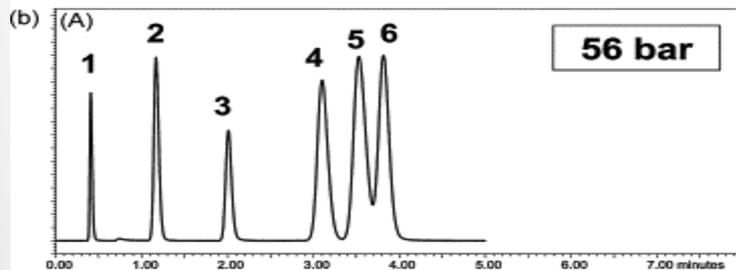
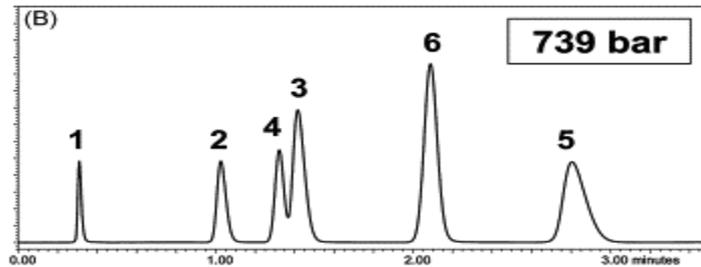
*M.M. Fallas et al., J. Chrom. A, 1209, 195 (2008)

Relative Retention Factor vs. Pressure

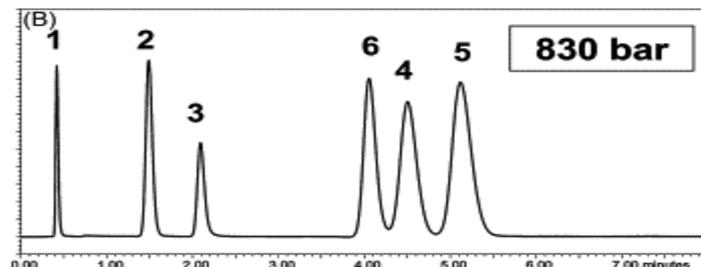
Experimental Results*



- 1, thiourea;
- 2, propranolol;
- 3, diphenhydramine;
- 4, acetophenone;
- 5, protriptyline;
- 6, nitrobenzene.



- 1, thiourea;
- 2, propranolol;
- 3, benzene;
- 4, amitriptyline;
- 5, nortriptyline;
- 6, toluene.



**Pressure increase after the column →
no frictional heating.**

Effect of High Pressure on Solvent and Column Properties in UHPLC*

- Solvent density (ρ) resp. specific volume, viscosity (η), compressibility (χ) and melting point change
- Retention factors (k') and chemical equilibriums
- Solute diffusion coefficient (D_m) \searrow
- Total porosity of the packed bed (ε_T) \nearrow
- Column dimensions length and diameter (L and d_c)
- Frictional heating causes temperature gradients (longitudinal: ΔT_L and radial: ΔT_R)

*M. Martin & G. Guiochon, J. Chrom. A, 1090, 16, (2005)

First Lesson about the Effects of Backpressure

- Single parameter dependence:
Solvent and solute properties change notably when operating at ultra high back pressure $\gg 400$ bar
- A single parameter change, e.g. flow rate will affect other solvent and solute properties by the simultaneously change in pressure
- Which renders the optimization of separation in UHPLC at pressures $\gg 400$ bar a far more difficult task than in < 400 bar operation like in HPLC*

*M. Martin & G. Guiochon, J. Chrom. A, 1090, 16 , (2005)

Some Thermal Considerations

- Friction Heating
 - Axial Temperature Gradient
 - Radial Temperature Gradient
- Role of column thermostat method

Frictional Heating

Power (P) generated in an HPLC column by frictional heat

$$P = \Delta P \cdot F$$

$$W = J / s = \frac{Nm}{s} = \frac{N}{m^2} \frac{m^3}{s}$$

Equivalent with electrical power $P=V.I$!!!

Frictional Heating*

Power (P) generated in an HPLC column by frictional heat

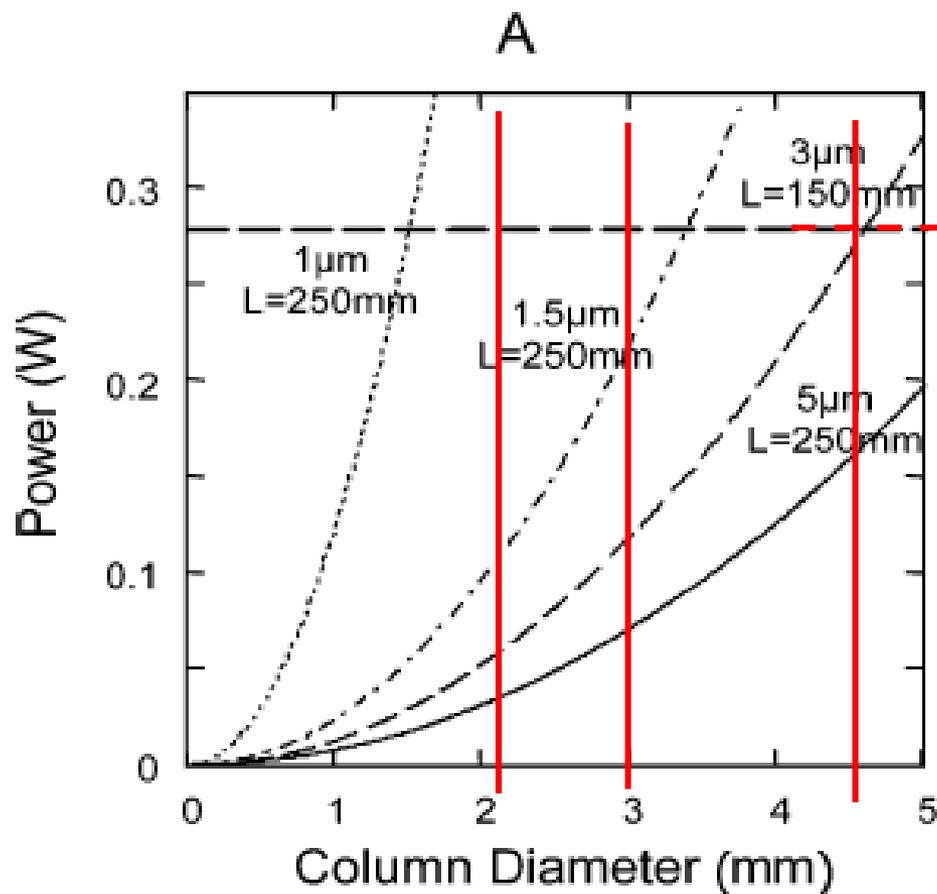
$$P = \Delta P \cdot F$$

E.g. Column: 150 mm x 4.6 mm, $d_p = 3 \mu\text{m}$
F = 1 mL/min (mobile phase velocity = 1.4 mm/s, optimal flow rate)
Water ($\eta = 1.10^{-3} \text{ Pa s}$)
 $K_s = 0.89 \times 10^{-14} \text{ m}^2$
 $\Delta P = 169 \text{ bar} = 1.69 \times 10^7 \text{ Pa}$

Power = 280 mW

*Colón et al., *Analyst*, 129 (2004) 503

Colón Power Plots for various d_p and column I.D.*



$$\text{Power} = \Delta P \times F$$

Colon's Rule of Thumb:

Separation Efficiency (N) assumed to be undisturbed when $P < 280$ mW

All columns operate at the same solvent velocity. Flow rate is scaled with column diameter for constant u_0 (2.5 mm/s)

*L.A.Colón et al., *Analyst*, 129 (2004) 503

Example Exceeding the Colón Limit

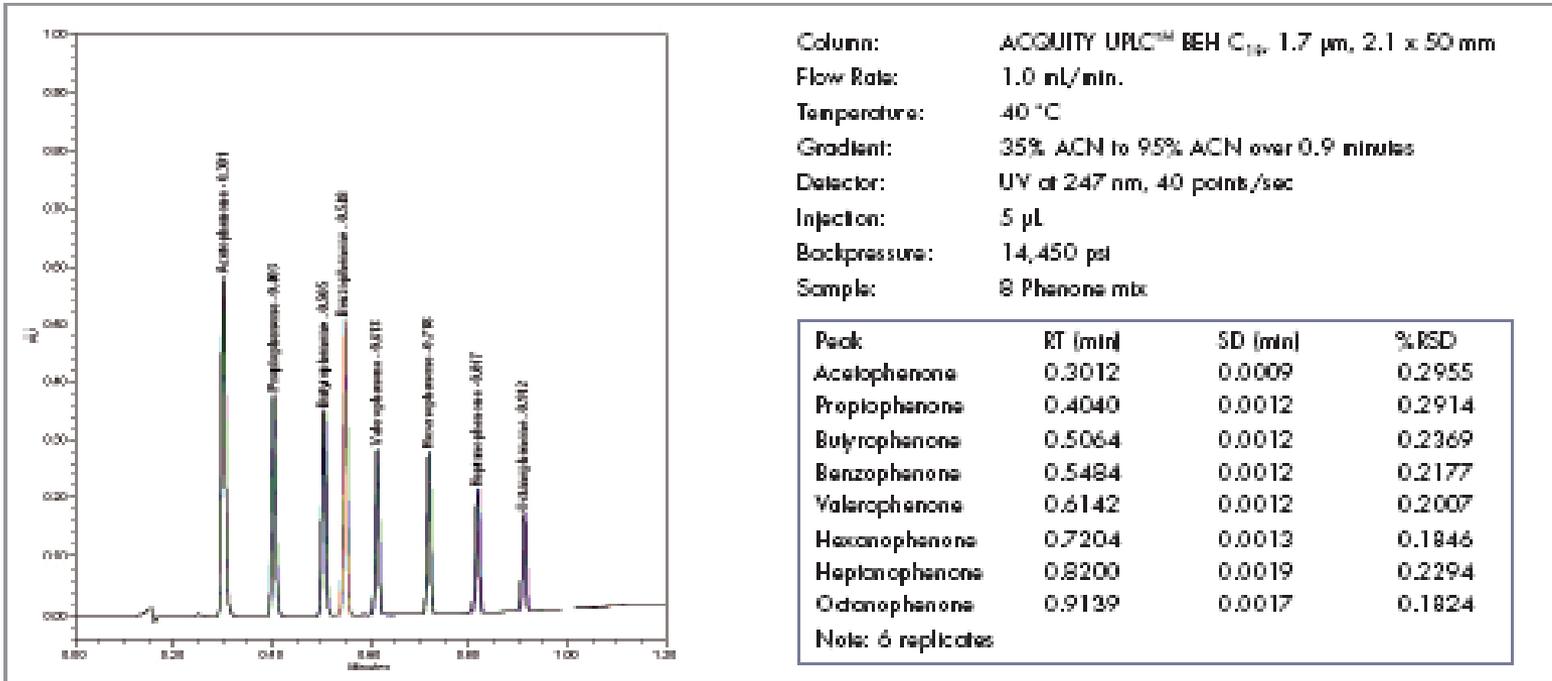


Figure 10. ACQUITY UPLC™ gradient performance is illustrated by this high throughput analysis. Retention time reproducibility is <0.3% RSD for a 1.2 minute total separation time under UPLC™ operating conditions.

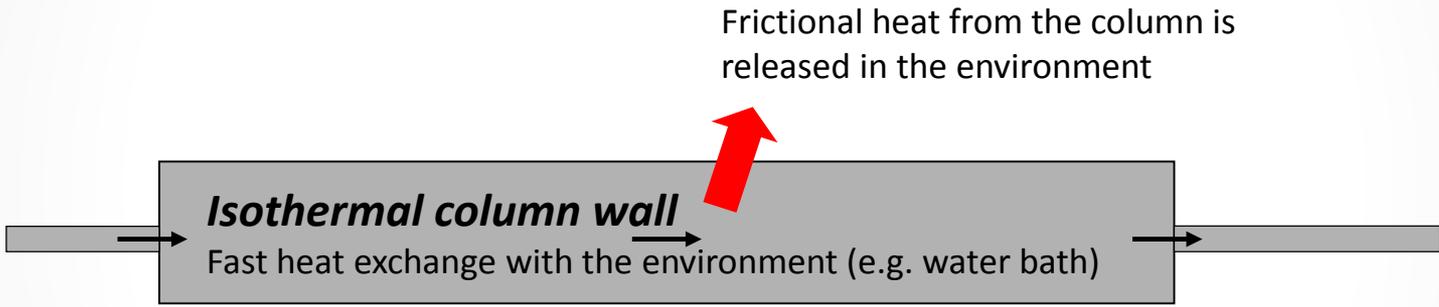
$\Delta P = 996 \text{ bar}$, $F = 1.0 \text{ mL/min}$, $P = 1.66 \text{ W} = 5.9x \text{ Colón limit ! } (= 280 \text{ mW})$
 $\lambda(\text{mix}) \approx 0.33 \text{ W/m}^\circ\text{C}$ (estimated)
 Heat cap.(mix) $\approx 2.6 \times 10^6 \text{ J/m}^3\text{C}$ (estimated)

*Taken from Waters Brochure 720000880 EN, 2004

Frictional Heating

Two Limiting Cases for Thermal Environment

Assume Infinitely Thin Column Wall (no thermal mass)



Frictional heat is released via column effluent

Frictional heating: Adiabatic conditions

Adiabatic conditions: there is no heat transfer to environment

A longitudinal temperature gradient along the column is established, no radial temperature gradient.

The longitudinal temperature gradient is given by

$$\Delta T_L = \frac{\Delta P \cdot (1 - q_{ad})}{c_{p,LM} \rho_{LM}}$$

ΔT_{Long} = longitudinal temperature gradient

Δp = pressure drop across column

q_{ad} = adiabatic expansion coefficient

$c_{p,LM}$ = heat capacity of eluent

ρ_{LM} = density of eluent

Temperature increase along column axis can be very large

Frictional heating: Isothermal conditions

Isothermal conditions: fast heat exchange with environment

a radial temperature gradient in the column is established, no longitudinal gradient.

The radial temperature gradient is given by

$$\Delta T_{radial} = \frac{u_s (\Delta P / L) r_{out}^2}{4\lambda_{rad}}$$

ΔT_{rad} = radial temperature gradient

Δp = pressure drop across column

u_s = superficial linear velocity

l = column length

r = column radius

λ_{rad} = heat transfer coefficient

Temperature Increase with Radial Position*

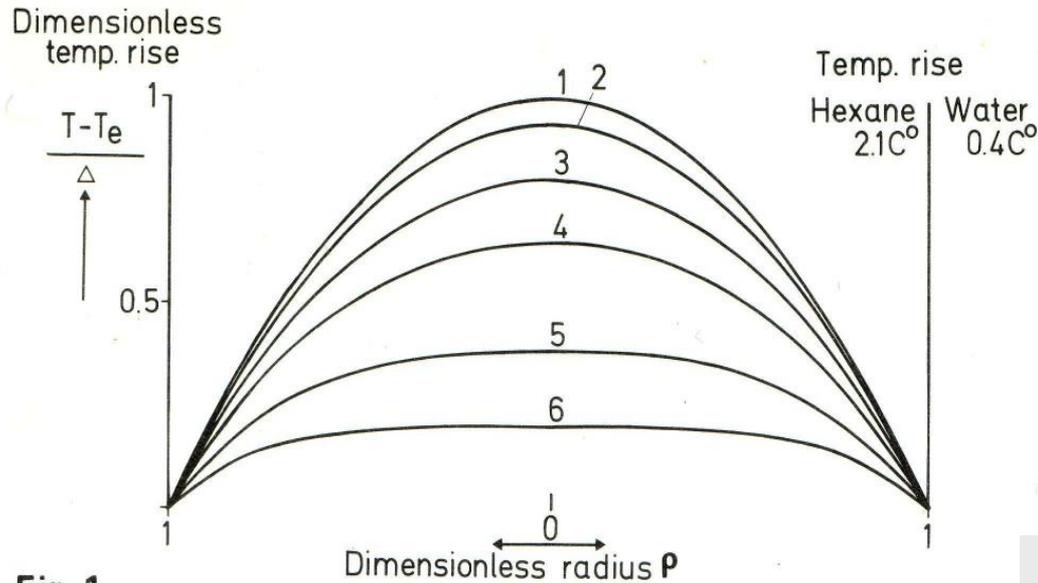


Fig. 1

Column: $R = 2.3 \text{ mm}$; $\Delta P/L = 10 \text{ bar/cm}$; $u_s = 2 \text{ mm/s}$
 $\Delta T_R (\text{hexane}) = 2.1 \text{ }^\circ\text{C}$
 $\Delta T_R (\text{water}) = 0.4 \text{ }^\circ\text{C}$

- 1 56 mm into the column bed
- 2 34 mm into the column bed
- 3 23 mm into the column bed
- 4 11 mm into the column bed
- 5 5.6 mm into the column bed

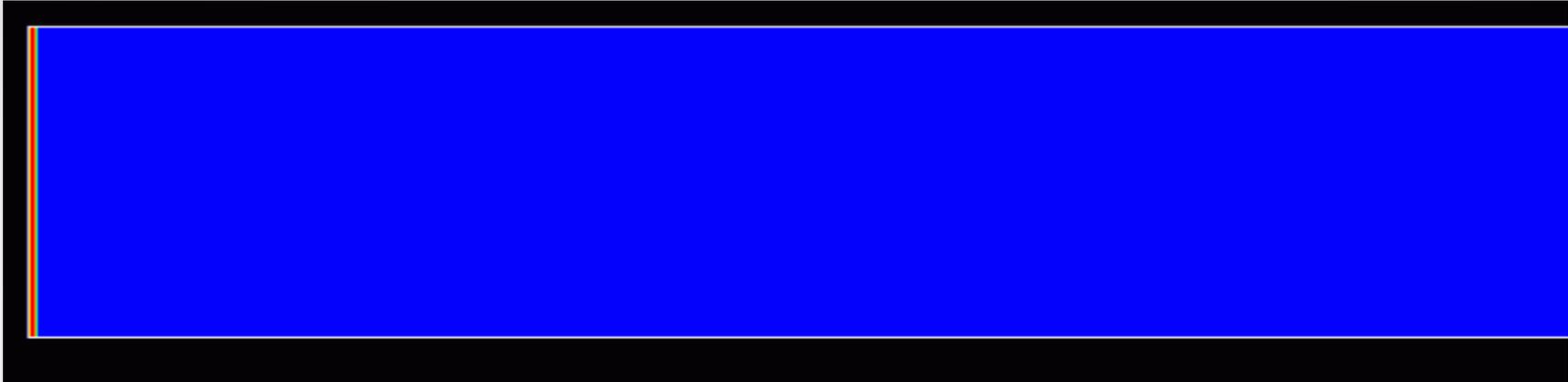
For hexane, for water these values are smaller

Poppe, et al., *Chromatographia*, 14 (1981)515-513

Importance of Operational Conditions

Visualisation of solvent velocity

Adiabatic wall = insulated, no heat exchange with environment



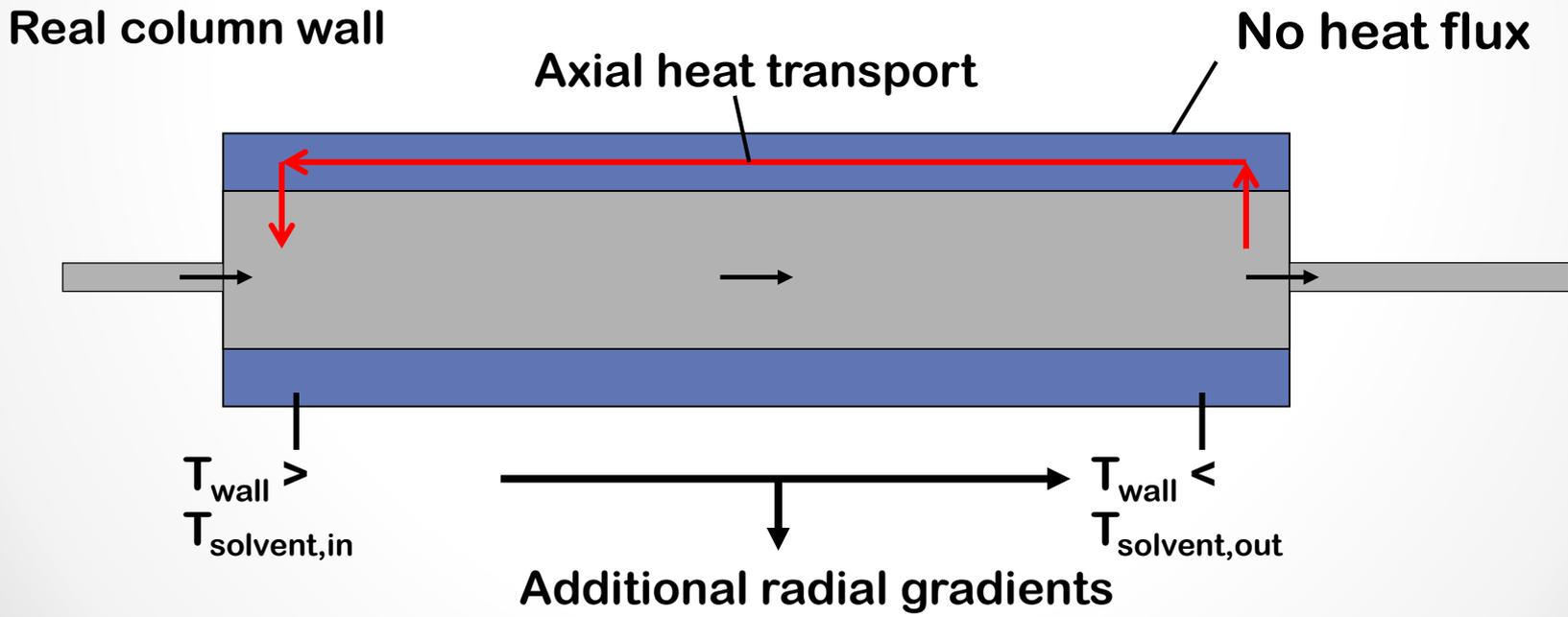
Isothermal case = well thermostatted, fast heat exchange with environment



Simulation courtesy of Desmet et al, Free University of Brussels, Department of Chemical Engineering

Frictional Heating

Practical situation is in between and is called “still air” condition;
The column is in a “thermos bottle”



Magnitude of ΔT_L at high inlet pressures*

T (°C)	F (mL/min)	ΔP bar	P mW	ΔT_L (calculated)	ΔT_L (measured)
25	0.8	670	893	19.3 °C	10 °C
25	1.0	819	1360	23.6 °C	13 °C
40	1.0	749	1250	21.6 °C	13 °C
40	1.1	822	1510	23.7 °C	16 °C

Column: 50 x 2.1 mm; BEH C18; 1.7 μ m; ACN/Water – 30/70

Calculated values differ from measured values:

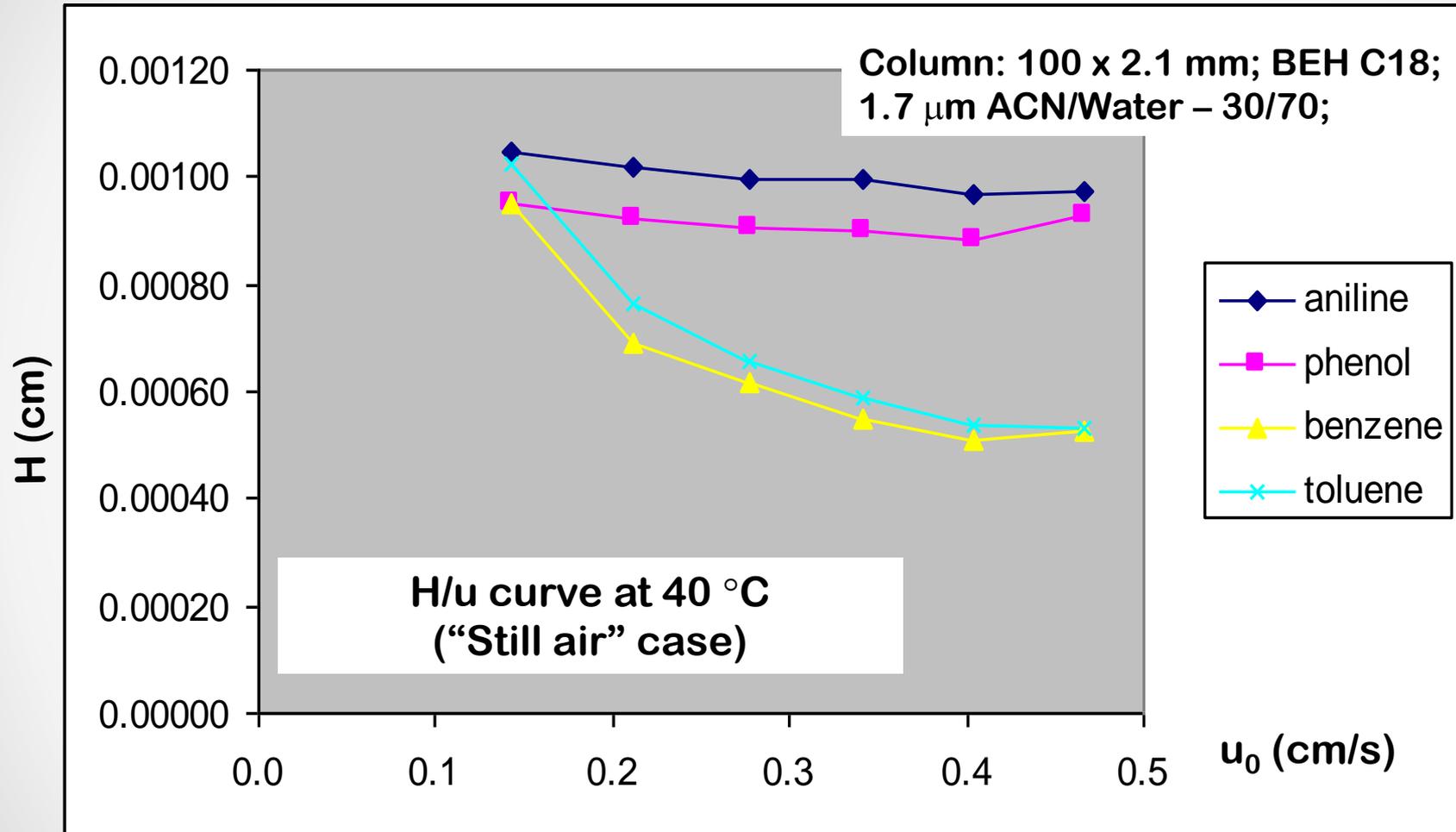
- 1) Heat loss by radiation
- 2) Backflow of heat over column from outlet to inlet

Longitudinal temperature gradient will not be a problem in practical operation provided that the radial temperature distribution is homogenous!

*Sandra et al., J. Chrom. A, 1113, 84 (2006)

Importance of Operational Conditions

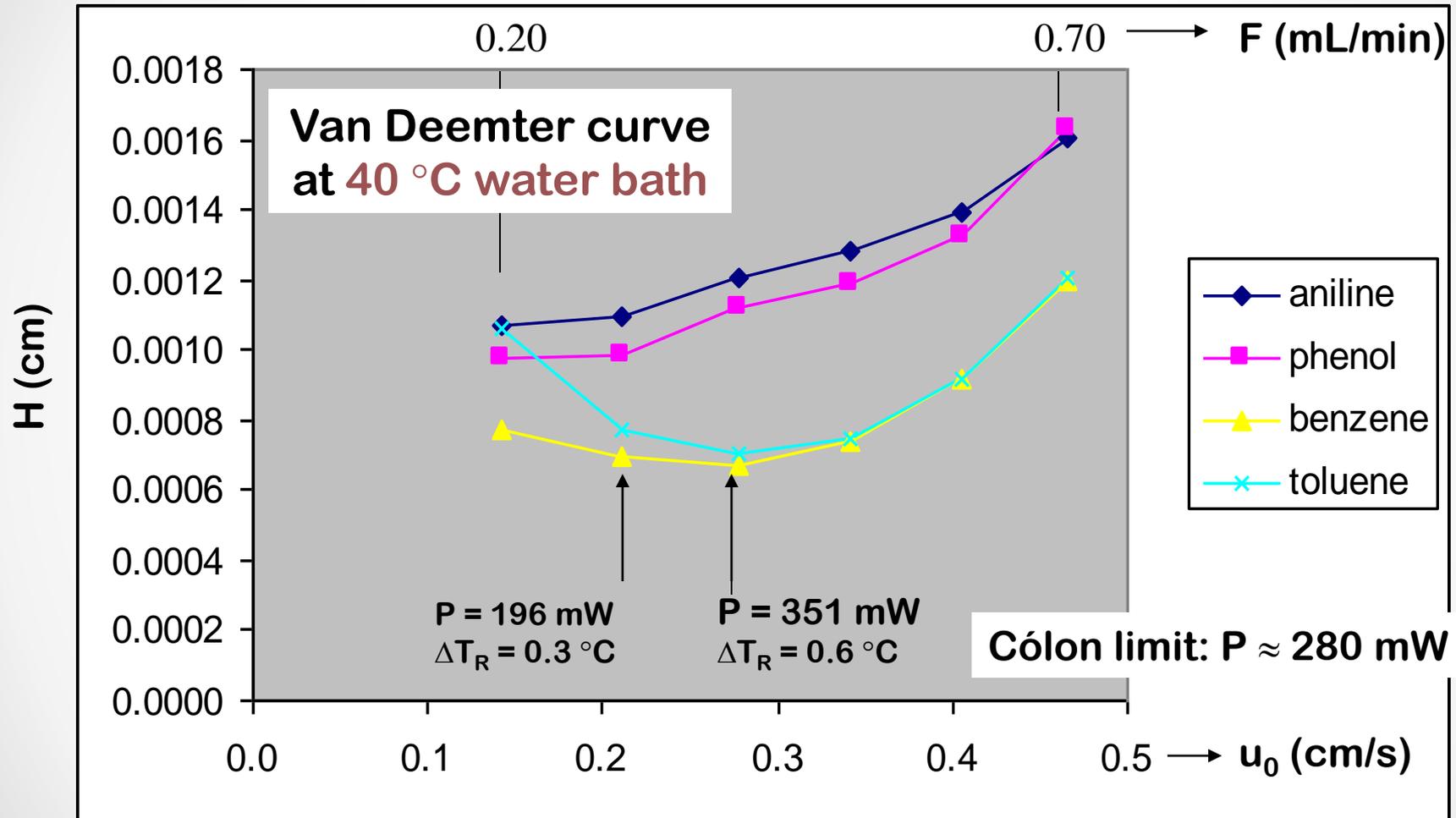
“Still air” Case*



*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

Importance of Operational Conditions

Isothermal Case*

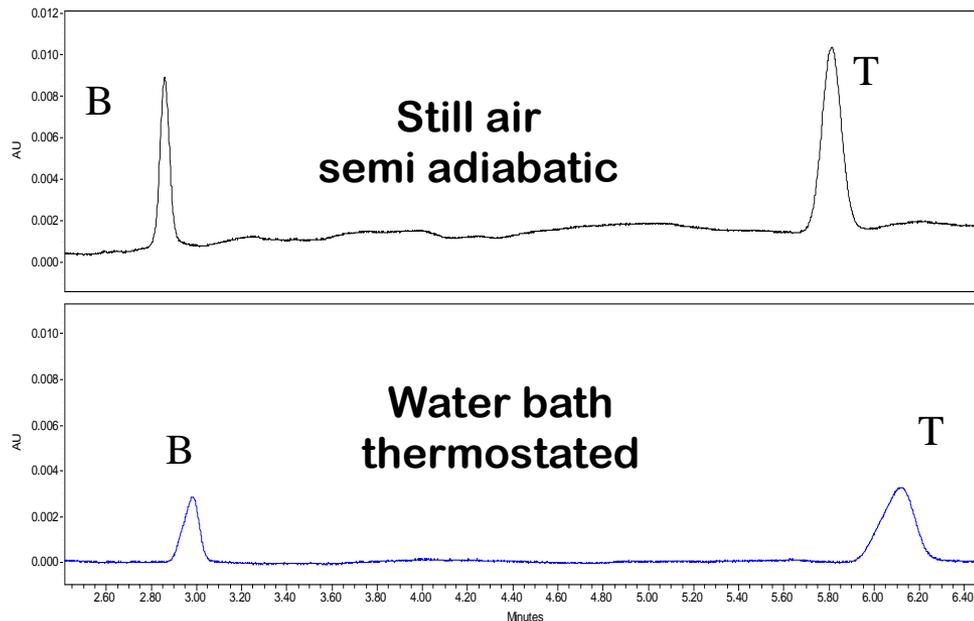


*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

Importance of Operational Conditions

“Still air” Case vs Isothermal Case*

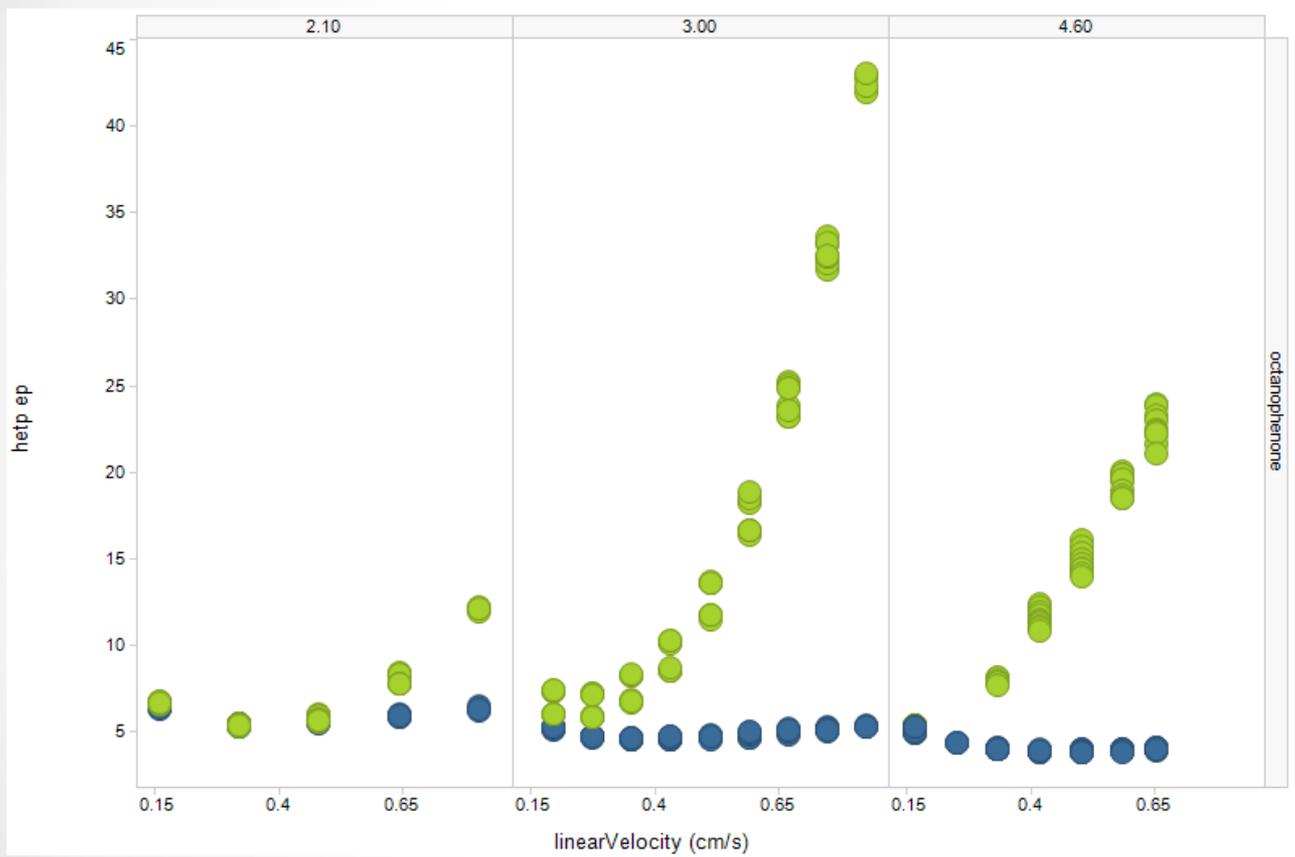
Column: 50 x 2.1 mm; BEH C18; 1.7 μm ; ACN/Water – 30/70, flow rate 0.7 ml/min



Marginal influence of ΔT_L on peak shape and plate number
Major influence of ΔT_R !

*P.Sandra et al., J. Chrom. A, 1113, 84 (2006)

Column Efficiencies under Quasi-Adiabatic and Isothermal Conditions (experimental data)



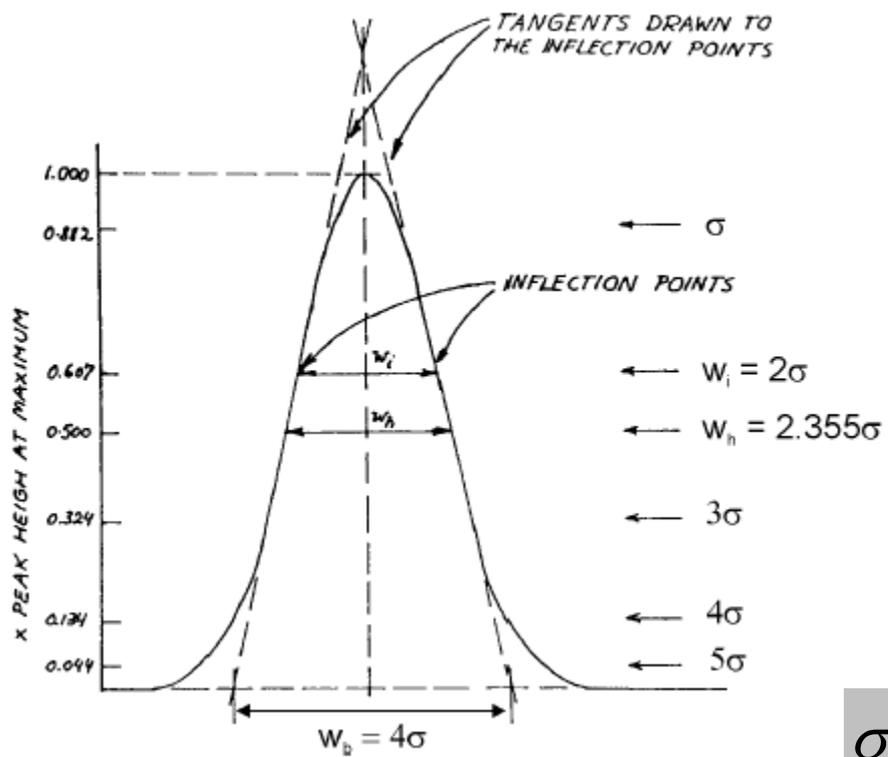
- Column in still air
- Column in water bath

ACN/water 60:40
 Octanophenone
 $k' = 10$

Implications of Frictional Heating

- In a true adiabatic mode, there will be no radial temperature gradient. There will be however a large axial temperature gradient. Expect a long pre- equilibration time
- In a true adiabatic mode, a flow rate changes causes a power change and therefore “chromatographic” temperature change in the column. Column re-equilibration will take very long
- In a true isothermal mode (water bath) with high pressure drop there will be a large radial temperature gradients independent of the actual temperature causing significant broadening of the zones
- In practice, UHPLC columns must be operated in a semi-adiabatic (still air) mode rendering the actual temperature poorly defined

Description of a Chromatographic (Gaussian) Peak*



$$N = \frac{L}{H} = \frac{V_r^2}{\sigma_v^2} = \frac{t_r^2}{\sigma_t^2}$$

$$\sigma_{V,column}^2 = \frac{V_R^2}{N}$$

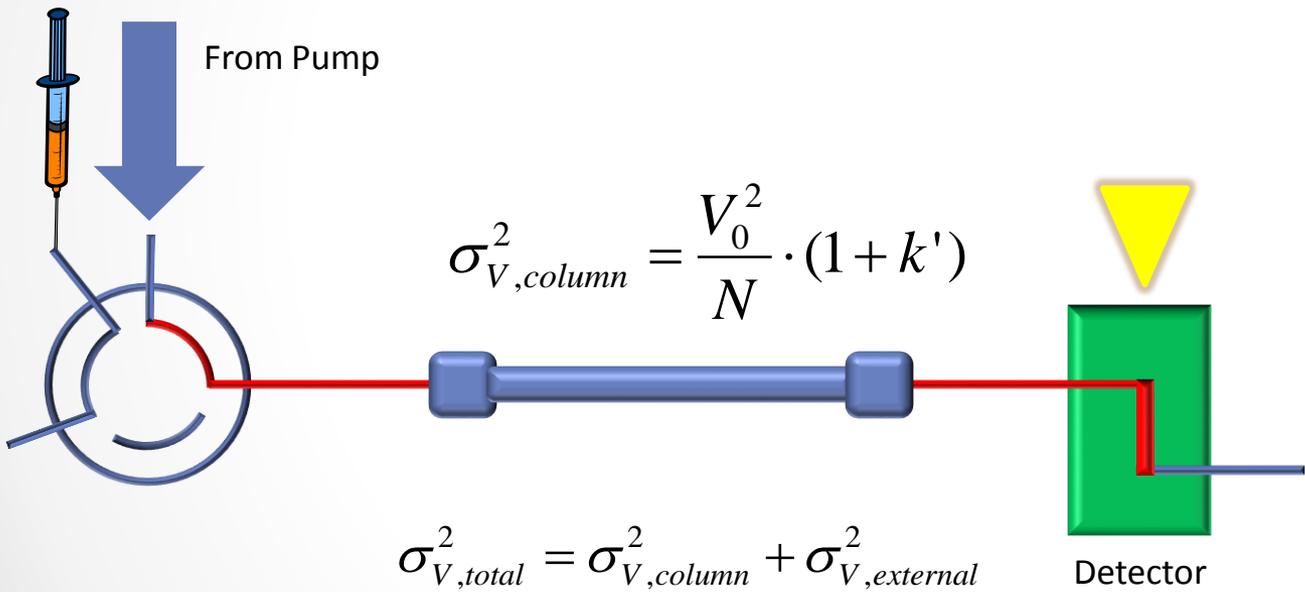
$$\sigma_{V,total}^2 = \sigma_{V,column}^2 + \sigma_{V,external}^2$$

↑
↑
 $H-u_0$
 V_{ext}

*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

Sources of External Band-Broadening

All parts of the system between the point of sample introduction and the point of injection contribute to dispersion.



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

(U)HPLC – Extra Column Band-Broadening

$$\sigma_{v,tot}^2 = \sigma_{v,col}^2 + \sigma_{v,cap}^2 + \sigma_{v,inj}^2 + \sigma_{v,cell}^2$$

$$\sigma_{V,inj}^2 = \frac{V_{inj}^2}{12} \quad (\text{without dispersion in the injector})$$

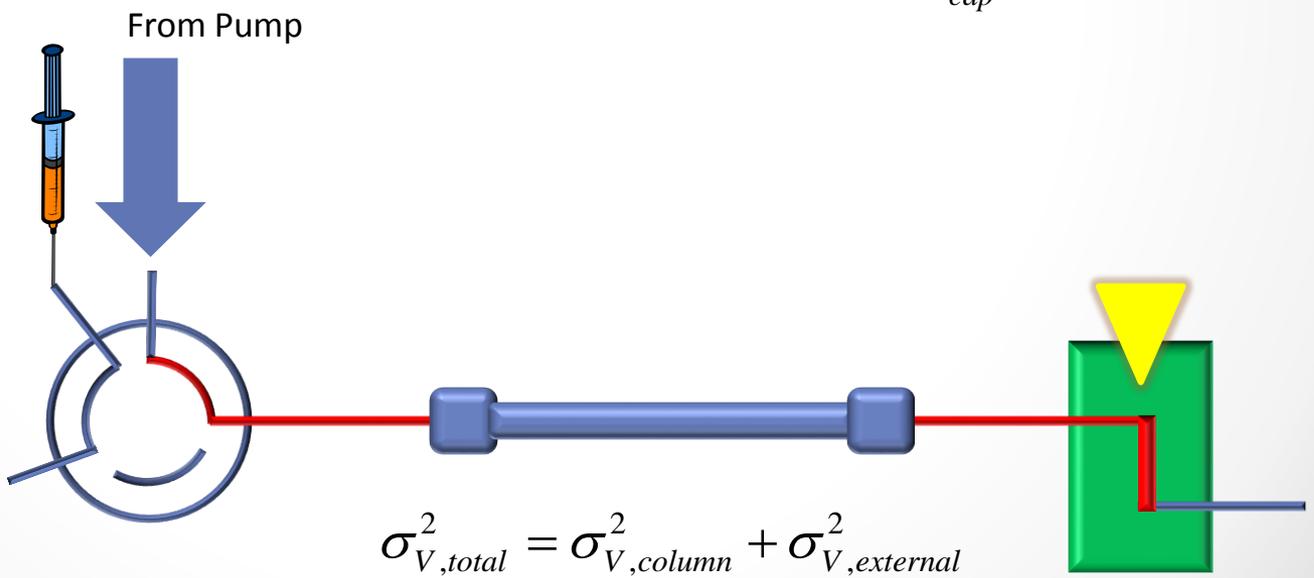
$$\sigma_{V,cap}^2 = \frac{V_{cap}^2}{L} \cdot \frac{r^2 u}{24D_m} \quad (\text{Golay equation})$$

$$\sigma_{v,cell}^2 = \frac{V_{cell}^2}{X}$$

Typical values for variance :

Injector	1 μl^2
Capillaries (500x0.12 mm)	7 μl^2
Capillaries (500x0.08 mm)	4 μl^2
Column (2.1x50 mm, k' =1)	3 μl^2
Column (2.1x50 mm, k' =5)	33 μl^2

$$\Delta P_{cap} = \frac{F \cdot 8\eta \cdot L_{cap}}{\pi \cdot r_{cap}^4}$$

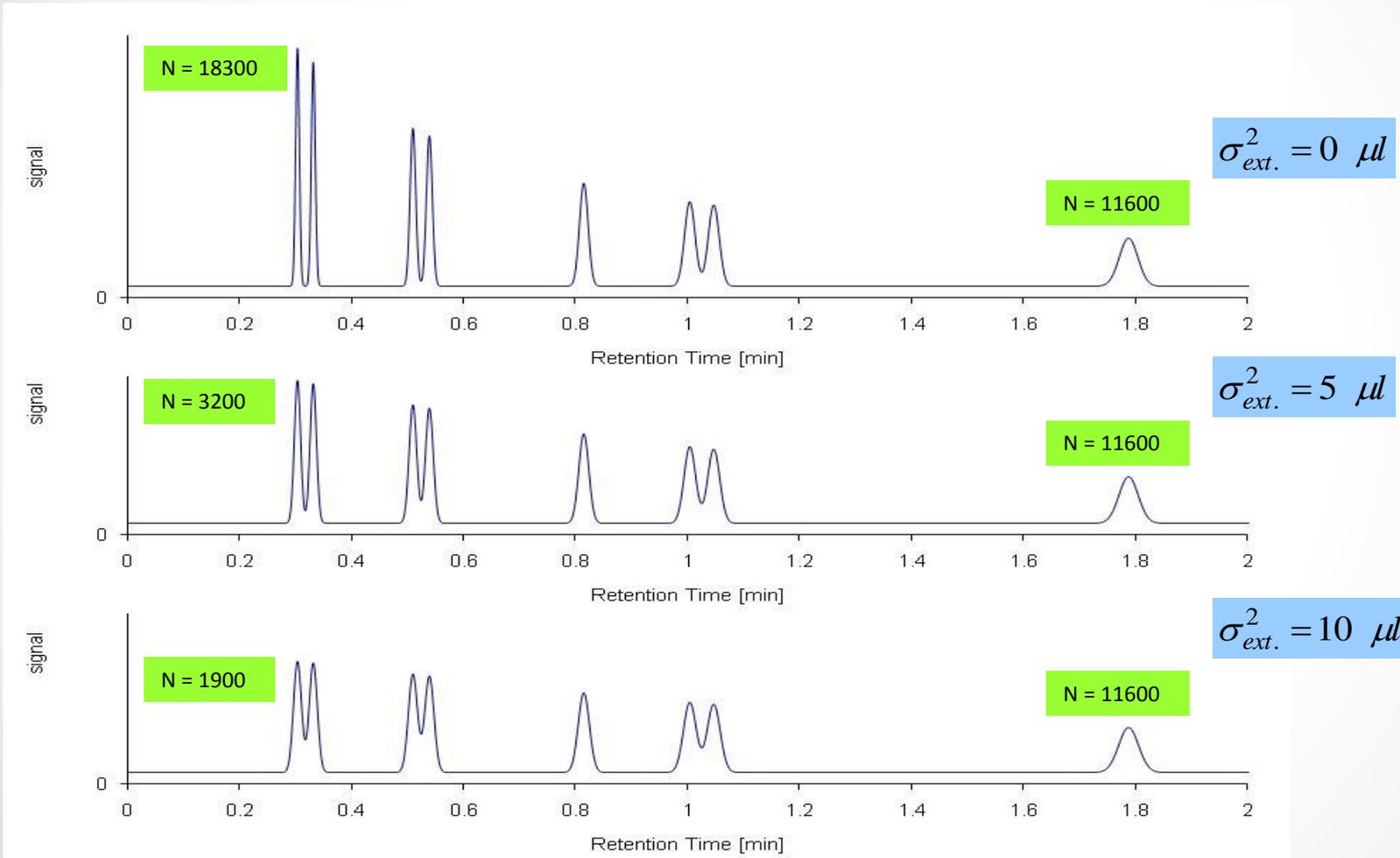


$$\sigma_{V,total}^2 = \sigma_{V,column}^2 + \sigma_{V,external}^2$$

*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

Effect of External Band-Broadening on N

Effect on 2.1 x 50 mm column with 1.8 μm particles; isocratic elution, calculated example



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

Experimental Determination of Ext. Band Broadening Chromatographic Method*

$$\sigma_{v,total}^2 = \sigma_{v,column}^2 + \sigma_{v,external}^2$$

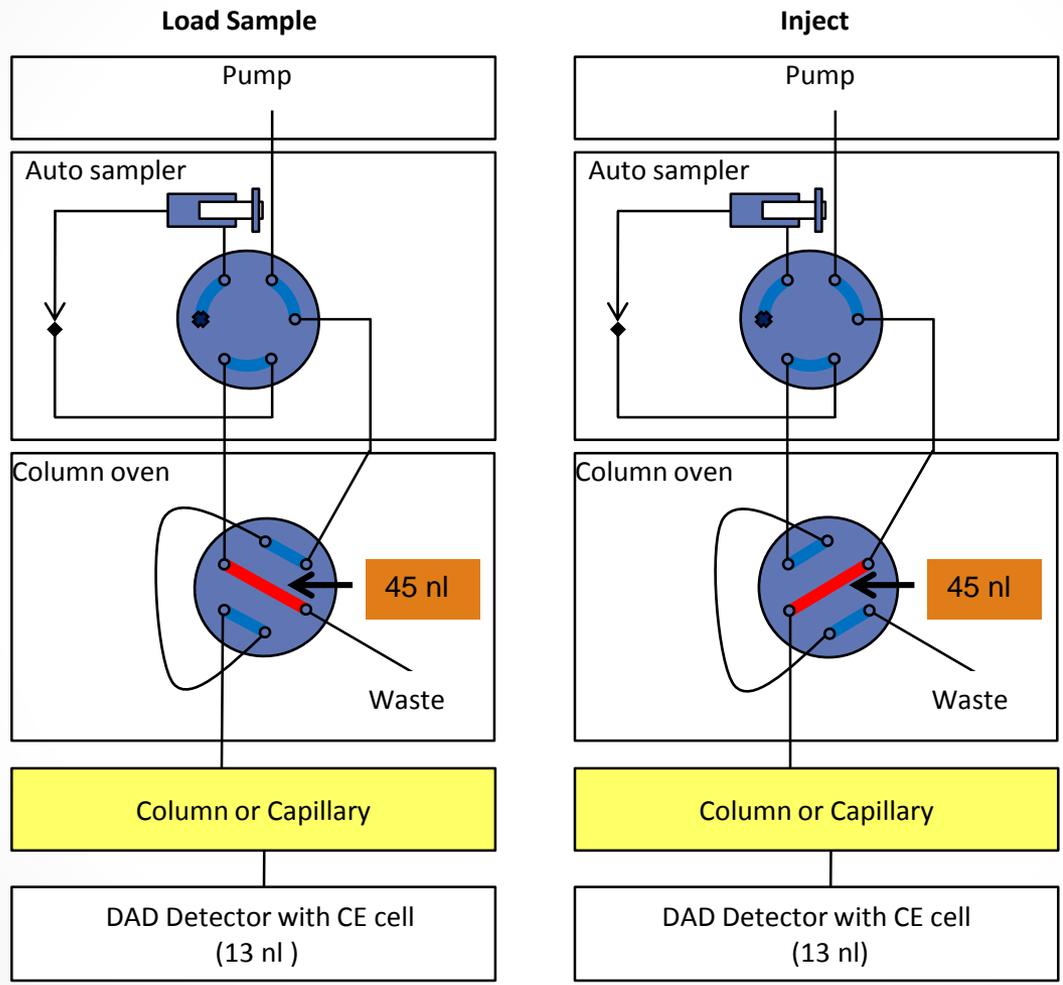
$$\sigma_{v,total}^2 = \frac{V_{R,col}^2}{N} + \sigma_{v,external}^2$$

Linear regression of measured $\sigma_{v,total}^2$ versus $V_{R,col}^2$ results in a linear plot with $1/N$ as the slope and $\sigma_{v,external}^2$ as the y-axis intercept

*H. Lauer & G. Rozing, *Chromatographia*, 11, 640, 1981

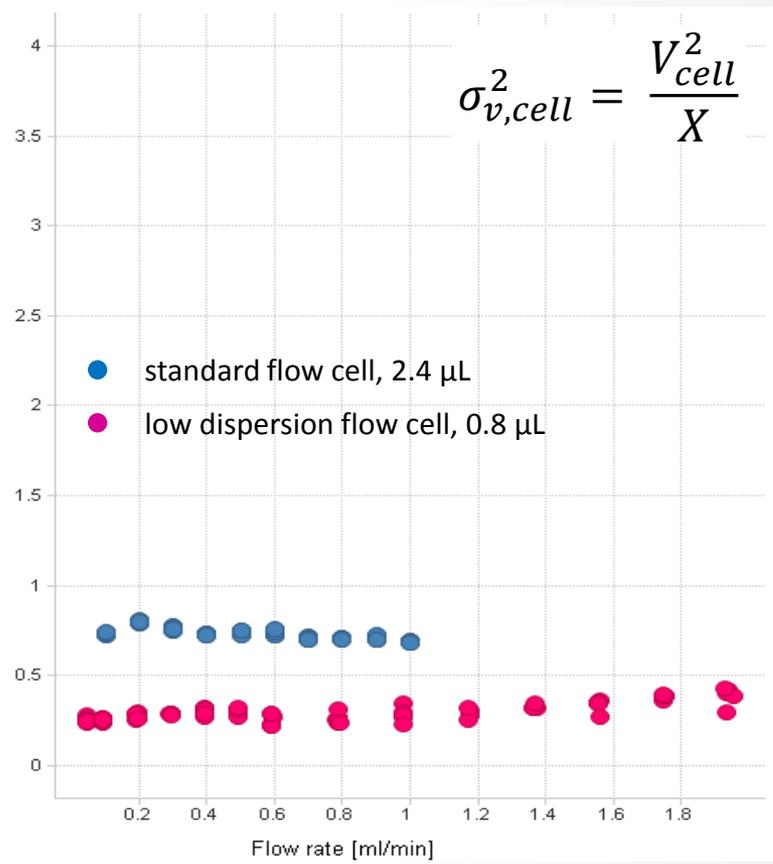
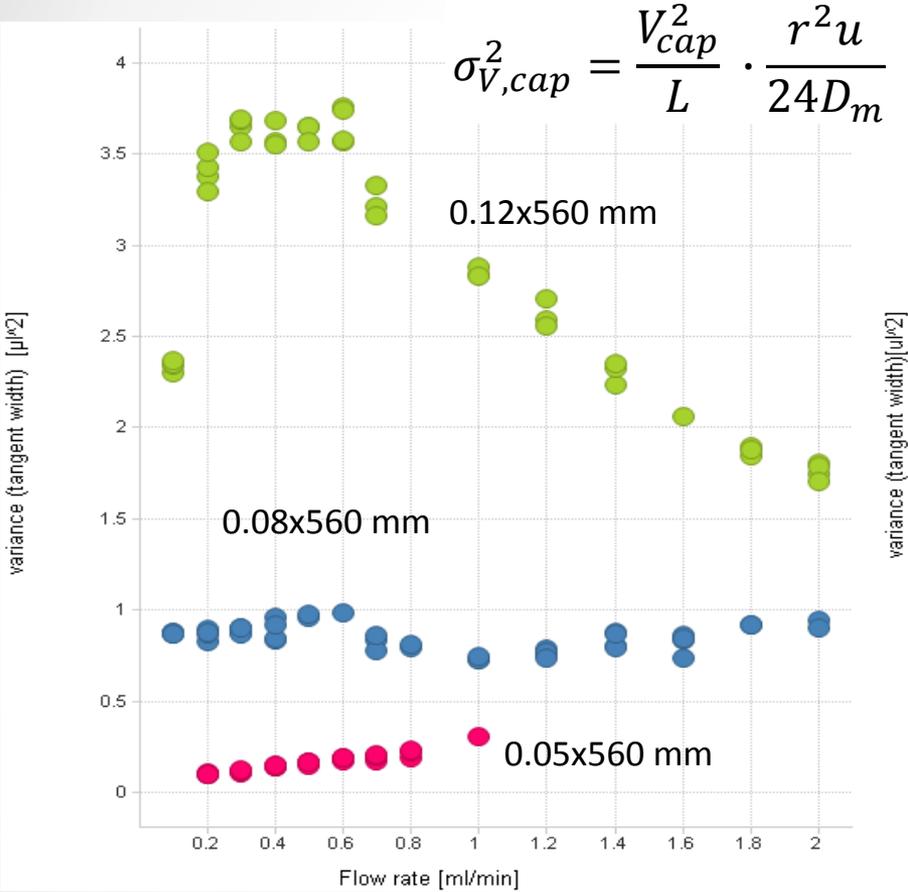
Experimental Determination of Ext. Band Broadening

Ultra low dispersion HPLC system



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

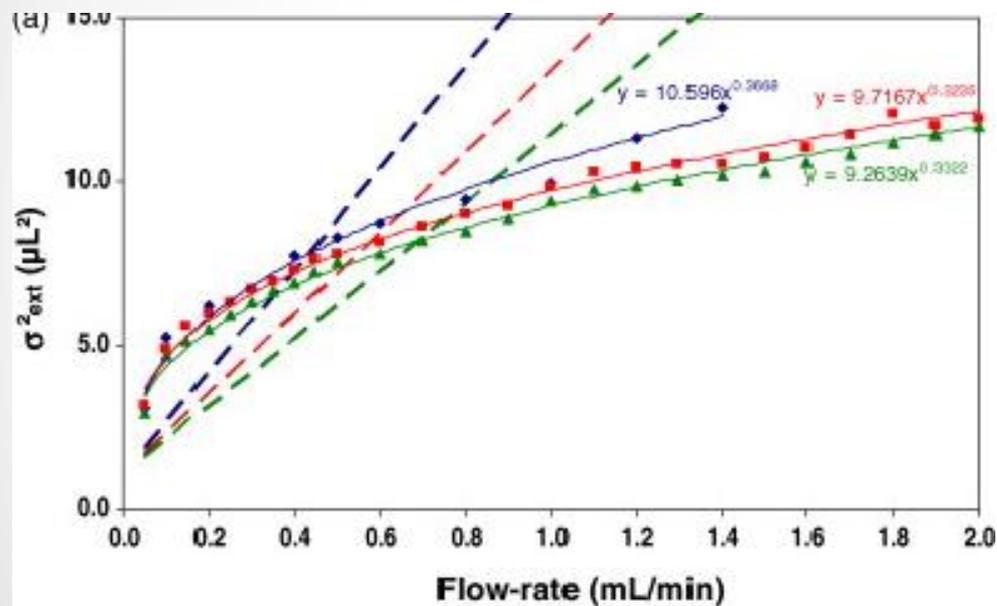
Flow Rate Dependence of Dispersion of UHPLC System Components



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

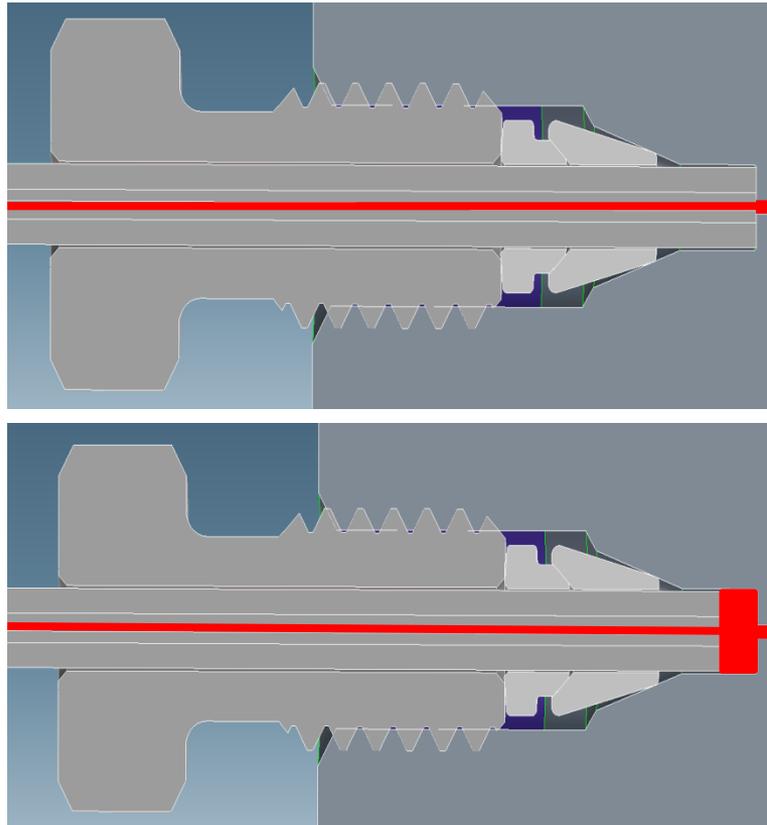
Extra-column variance versus mobile phase flow-rate

T= 30 °C (◆), 60 °C (■) and 90 °C (▲) with an UPLC System



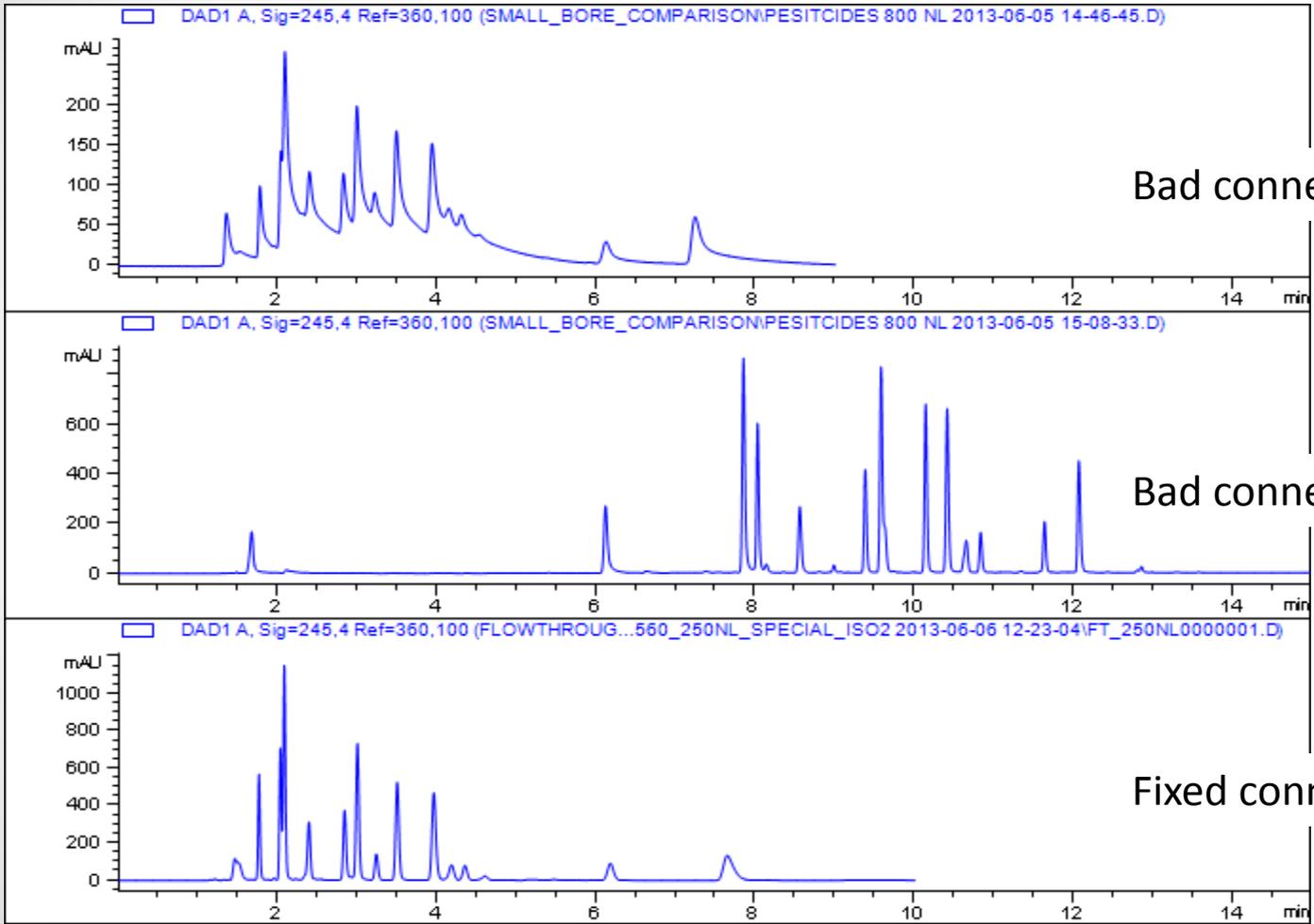
S. Heinisch et al., J. Chrom. A, 1203, 124 (2008)

Void Volume Caused by Incorrect Fittings



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

Impact of Bad Connection on a 1 mm Column



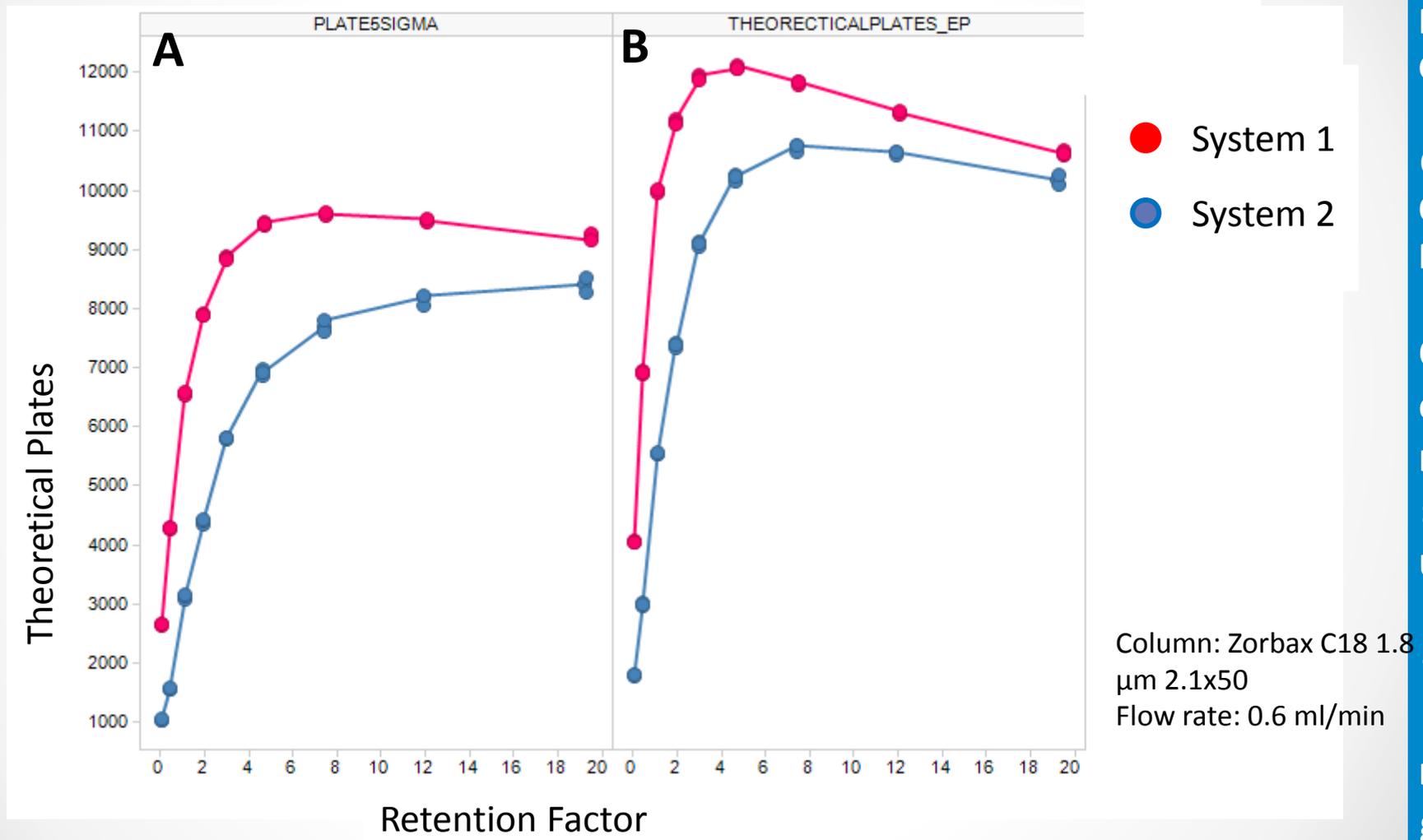
Bad connection isocratic

Bad connection gradient

Fixed connection isocratic

*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

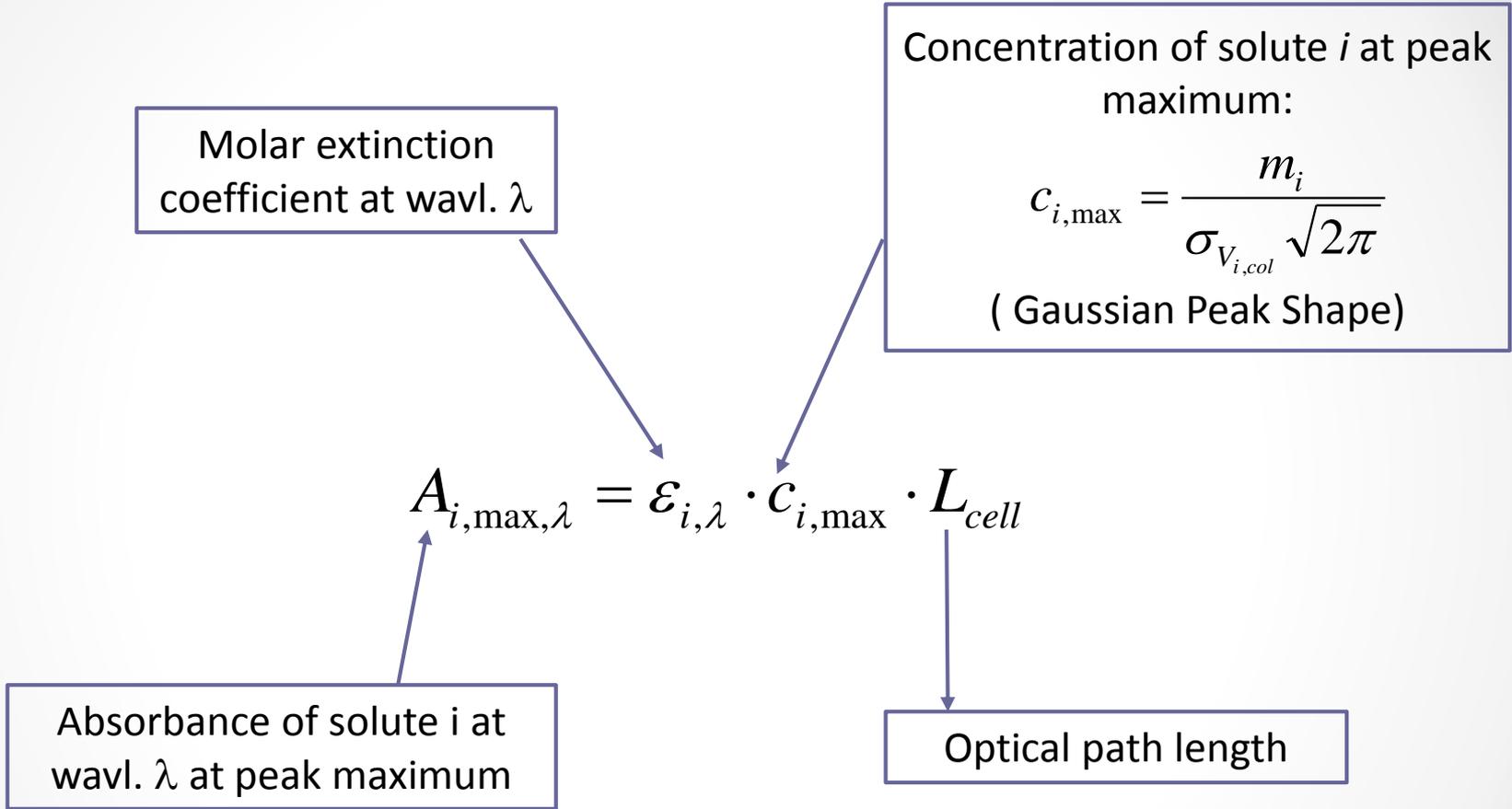
Impact of System Dispersion on Isocratic Separation I



*Slide courtesy of Dr. Monika Dittman, Agilent Technologies

Signal-to-Noise Ratio in Spectrophotometric Detectors

Lambert Beer's law



How does the Flow Cell Design in Spectrophotometric Detectors Affects the (U)HPLC Separation?

In practice however:

$$\sigma_{v_{i,tot}}^2 = \sigma_{v_{inj}}^2 + \sigma_{v_{i,col}}^2 + \sigma_{v_{cap}}^2 + \sigma_{v_{det}}^2$$

minimize



$$\sigma_{v_{i,tot}}^2 \cong \sigma_{v_{i,col}}^2 + \sigma_{v_{det}}^2$$

Equation describes well designed HPLC system, where column and flow cell are the dominant sources for dispersion.

$$\sigma_{v_{i,tot}}^2 \cong \sigma_{v_{i,col}}^2 + \frac{V_{cell}^2}{X}$$



$$C_{i,max} \cong \frac{m_{inj,i}}{\sqrt{2\pi} \cdot \sqrt{\sigma_{v_{i,col}}^2 + \frac{V_{cell}^2}{X}}}$$

Signal-to-Noise Ratio in Spectrophotometric Detectors

Extending Baumann's Principle^{*,**}

Lambert Beer's law

$$A_{i,\lambda} = \varepsilon_{i,\lambda} \cdot c_{i,\max} \cdot L_{cell}$$

Photon flux converted into electrical signal

$$m_{0,\lambda} = M_{\lambda} \cdot \Delta\lambda \cdot G \cdot T_{optic,\lambda} \cdot \eta_{\lambda}$$

Optical Signal ~ chromatographic Signal

$$S = (m_{0,\lambda} - m_{\lambda}) \cdot \tau$$

Standard deviation of photon flux = rms Noise

$$N_{rms} = \sqrt{m_{0,\lambda} \cdot \tau}$$

The rms-value of the Signal-to-Noise Ratio

$$SNR_{rms} = \frac{(m_{0,\lambda} - m_{\lambda}) \cdot \tau}{\sqrt{m_{0,\lambda} \cdot \tau}}$$

- M_{λ} = spectral output of light source
- $\Delta\lambda$ = spectral bandwidth
- G = (lowest) light conductivity of optical system
- $T_{optic,\lambda}$ = overall transmission of optic
- η_{λ} = quantum efficiency of photo detector at λ
- τ = detectors time constant
- $m_{0,\lambda}$ = fraction of photon flux converted into electrons
- m_{λ} = reduced photon flux caused by sample absorption
- $\varepsilon_{i,\lambda}$ = molar extinction coefficient of solute i
- c_i = concentration of the solute i
- L_{cell} = optical, sample path length of detector flow cell



1st order of Taylor expansion

Lambert-Beer's law

$$\frac{m_{0,\lambda} - m_{\lambda}}{m_{0,\lambda}} = 2.3 \cdot \varepsilon_{i,\lambda} \cdot c_i \cdot L_{cell}$$

*W. Baumann, Z. Anal. Chem. 284, 31-38 (1977)

**K. Kraiczek et al., Anal. Chem., 2013, 85 (10), pp 4829–4835

Slide courtesy of Karsten Kraiczek, Agilent Technologies

The Dilemma in Spectrophotometric HPLC detection^{*,*}

^{*}in HPLC detection the ptp value of SNR is relevant

Signal Parameters
Solute and Chromatography

Noise Parameters
Optics and Detector Settings

$$SNR_{ptp}(L_{cell}, V_{cell}) = \frac{2.3}{6} \cdot \epsilon_{i,\lambda} \cdot c_{\max,i} \cdot L_{cell} \cdot \sqrt{M_{\lambda} \cdot \Delta\lambda \cdot G \cdot T_{optic,\lambda} \cdot \eta_{\lambda} \cdot \tau}$$

Flow Cell Geometry and Flow Cell Dispersion Behavior

$$c_{\max,i} \approx \frac{Q_{inj,i}}{\sqrt{2\pi} \cdot \sqrt{\frac{V_{R,i}^2}{N} + \frac{V_{cell}^2}{X}}}$$

- ◆ $c_{i,max}$ highest if dispersion is absent i.e. cell volume $\rightarrow 0$:
 \rightarrow then pathlength is 0; signal can be measured
- ◆ Absorbance is highest with high L_{cell} and maximum light throughput (diameter, maximum light intensity optics, spectral bandwidth) \rightarrow increase cell volume
 \rightarrow then dispersion increases, $c_{i,max}$ decreases, signal and resolution is lost
- ◆ Hence there must be a compromise in flow cell dimensions for best signal to noise ratio
 \rightarrow Total Internal Reflection (TIR) flow cell is the best compromise

^{*,*}K. Kraiczek et al., Anal. Chem., 2013, 85 (10), pp 4829–4835

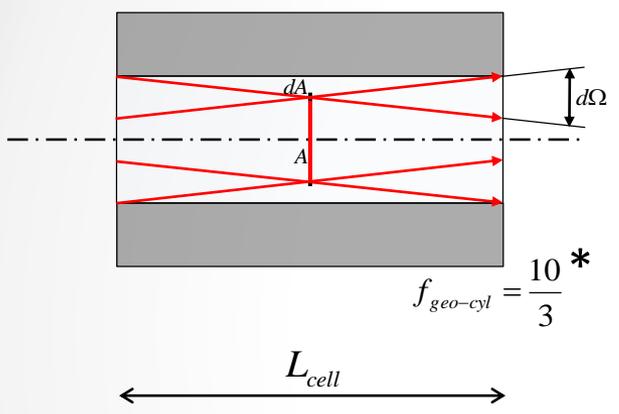
^{*}Slide courtesy of Karsten Kraiczek, Agilent Technologies

Light Conductivity (Light Throughput)

Conventional and Liquid Core Waveguide Flow Cells

$$G = \int \int_{A \Omega} dA \cdot d\Omega$$

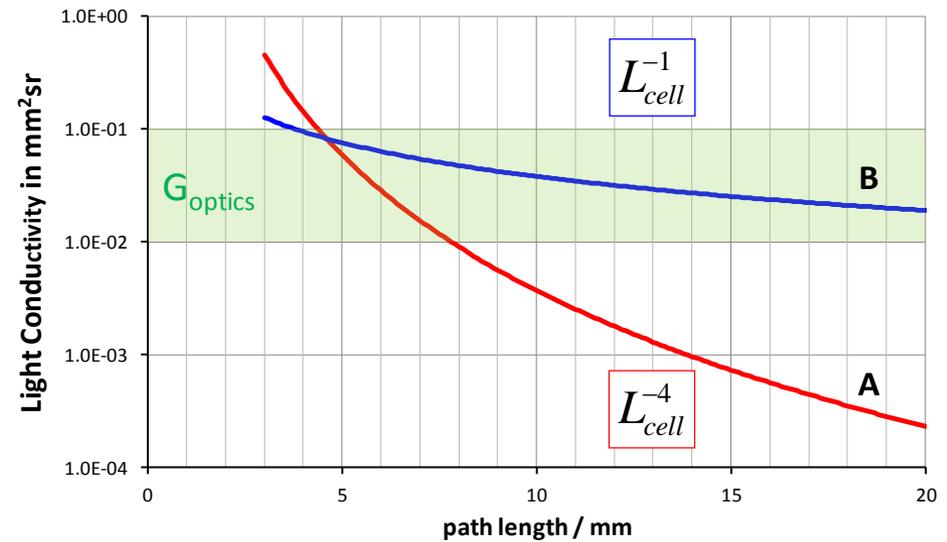
e.g. Conventional Cylindrical Flow Cell



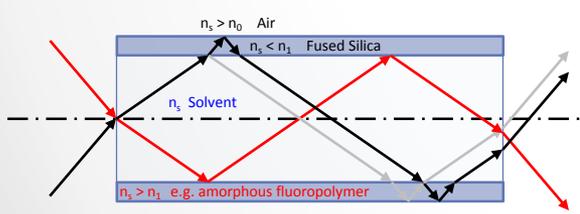
$$G_{conv-cell} \cong f_{geo} \cdot n_{solv}^2 \cdot \frac{V_{cell}^2}{L_{cell}^4} \cdot \Omega_0$$

Typically large in volume and short in path length

e.g. flow cell volume 2.5 μL



LCW Flow Cell



$$G_{LCW-cell} = \pi \cdot NA_{cell}^2 \cdot A_{cell} \cdot \Omega_0$$

$$NA_{cell} = \sqrt{n_{solv}^2 - n_{clad}^2}$$

$$G_{LCW-cell} = \pi \cdot NA_{cell}^2 \cdot \frac{V_{cell}}{L_{cell}} \cdot \Omega_0$$

Allow longer path length even at lower volume

*W. Baumann, Z. Anal. Chem. 284, 31-38 (1977)

Lessons Learned so far

- Ext. band broadening will significantly affect column efficiency of early eluting peaks in isocratic mode
- Chromatographic determination of ext. band broadening is easy
- Direct determination of dispersion behavior mandates a sophisticated system
- In UHPLC usage of very narrow i.d. capillaries is required
- Flow cell volume needs to be as low as possible

Outlook on UHPLC

Further increase of ΔP_{max} will:

- Result in moderate increase of N_{max}
 - Over proportional effort in engineering to provide equally robust and reliable equipment
 - Compliance with regulatory authorities
- Mandate usage of very narrow i.d. columns to counteract the frictional heat problems
 - Zero dead volume systems; detection?

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Thank You for Your Attention

谢谢

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