Improving HPLC Column Selection and System Performance

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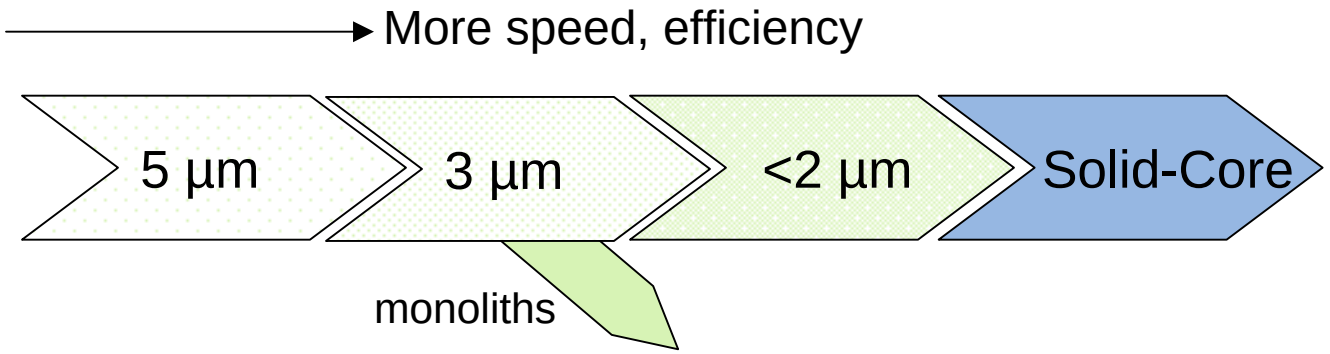
T410170

SIGMA-ALDRICH®

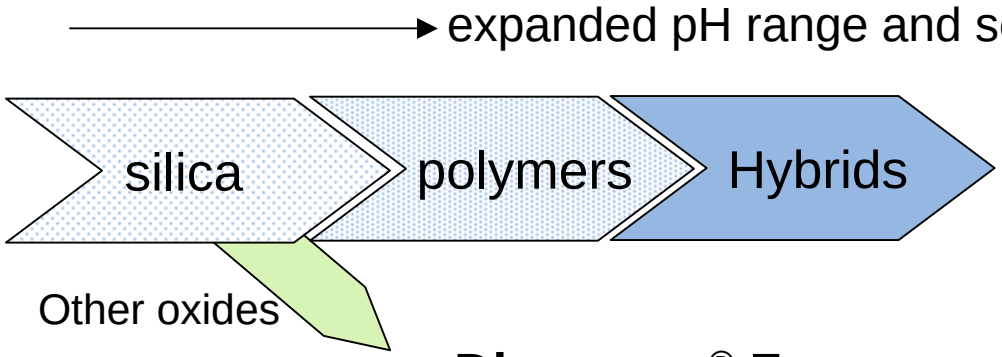
LC Particle Innovation Leads the Way*

Particle Size and Architecture (porosity)



Ascentis® and Ascentis® Express

Particle Composition (and stationary phases)



Kromasil® Eternity™

Discovery® Zr

* From Frank Michel

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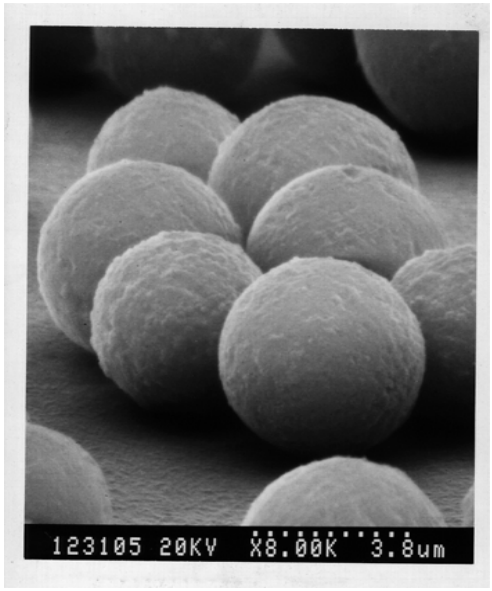
Evolution of HPLC Column Particle Shapes

Irregular particles

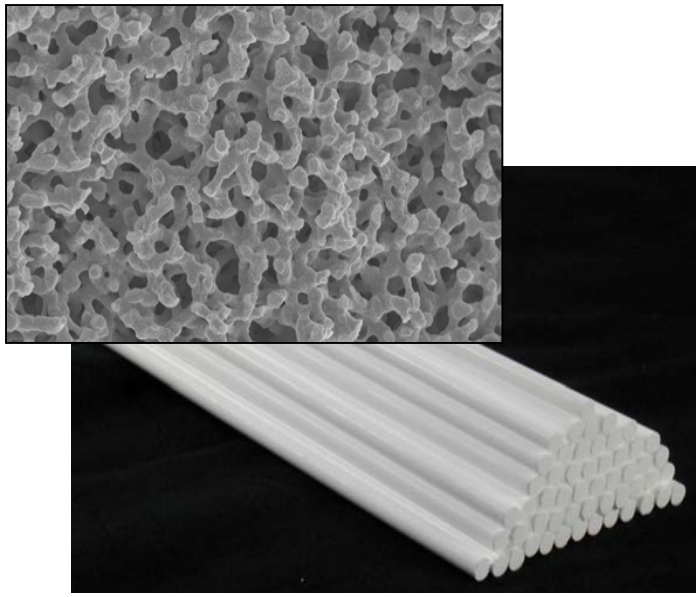
1970



Spherical particles

1980

Remain the workhorse



Monoliths

2000

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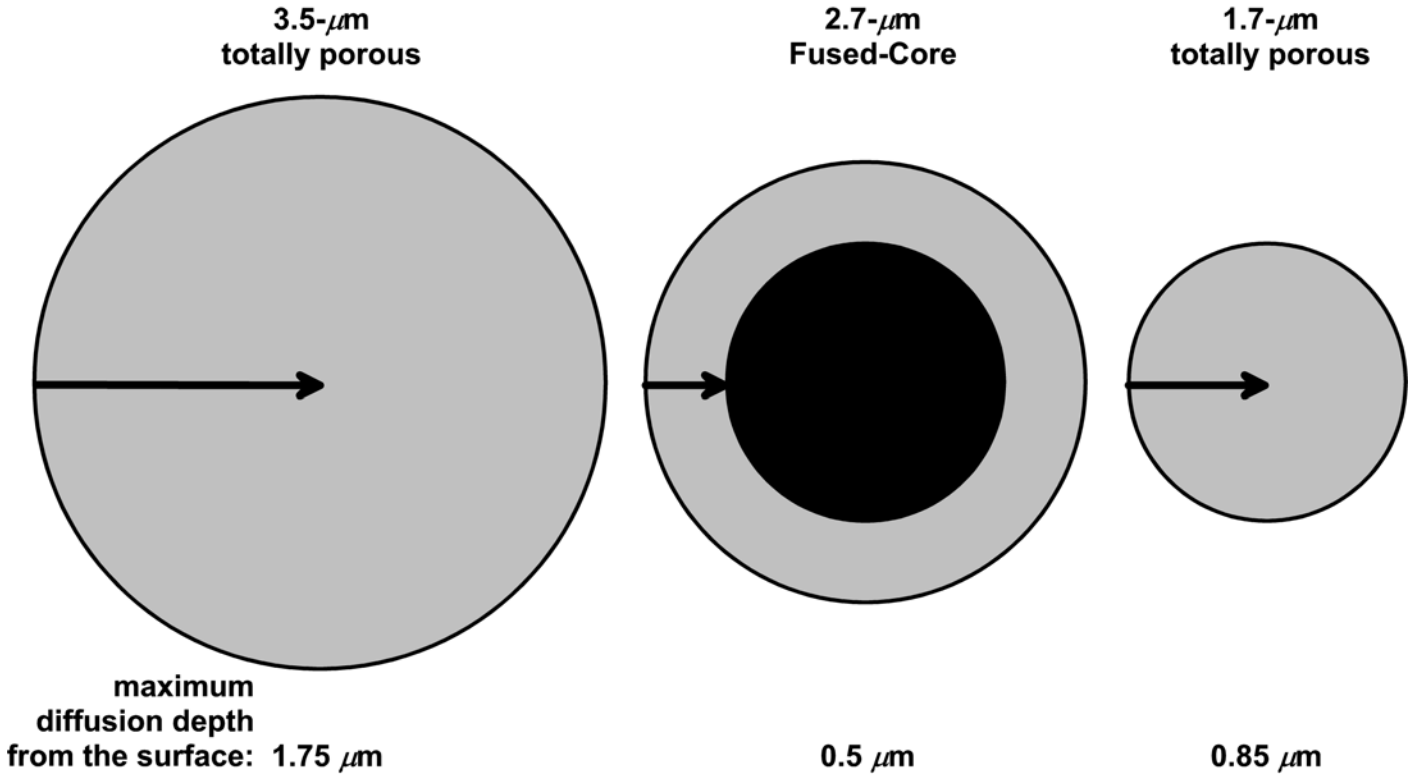
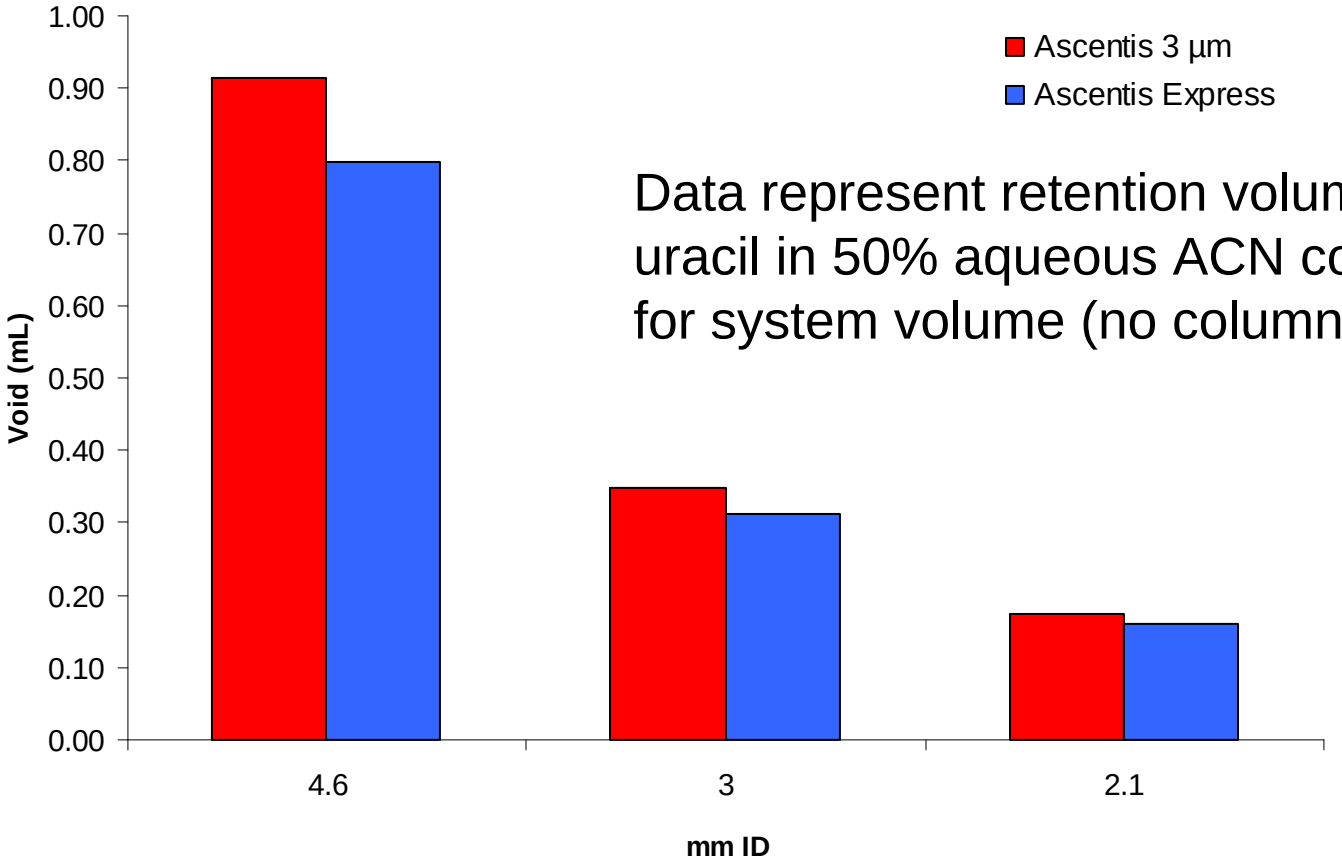
Particle Innovation has Reduced Column Dispersion*

...more efficiency, narrower peaks, higher peak capacity, more sensitivity.



* T. L. Chester, American Lab, Vol. 41 No. 4, pp 11-15, March 2009.

Measured Void Volumes: Solid-Core vs Porous



Data represent retention volume of uracil in 50% aqueous ACN corrected for system volume (no column).

10cm x 4.6mm C18 Columns

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Resolution: Peaks Separate Faster Than They Spread

- Maximize: Differential migration (Δt_R) - selectivity
- Minimize: Band dispersion (w) - efficiency

$$R_S = \frac{\Delta t_R}{0.5\,(w_A + w_B)}$$

Thermodynamic (chemical)

$\Delta t_R \propto L$

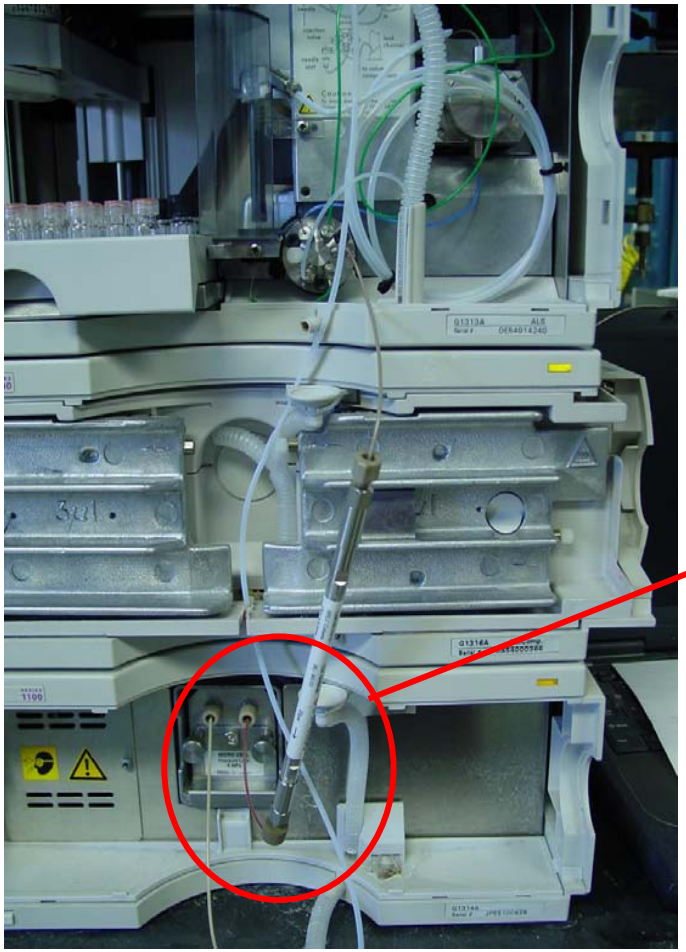
Kinetic (physical)

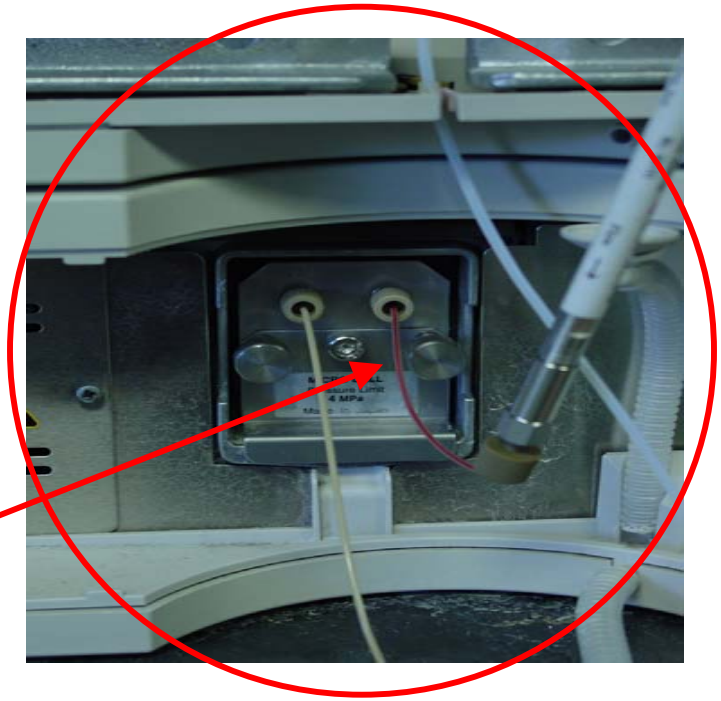
$w \propto L^{1/2}$

Improvements have been made in both areas

Measuring HPLC System Suitability

HPLC System Organization and Optimization





Injector and detector tubing for the Agilent 1100 were 0.007 inches ID. A 10 µL flow cell was employed. Experiments are underway to examine how tubing, flow cells and other HPLC system variables affect performance.

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Peak Band Spreading Equation

$$\sigma^2_{sys} = \sigma^2_{col} + \sigma^2_{instr} \qquad \sigma_{sys} = (\sigma^2_{col} + \sigma^2_{instr})^{1/2}$$

- **Band spreading (dispersion) can occur in both HPLC columns and instruments leading to a *system equation*; instrument dispersion is also referred to as instrument bandwidth (IBW)**
- **New developments in particles have greatly reduced column dispersion; instrument brand, model and configuration now matters; *system* performance can be dramatically different for column and instrument combinations; *can't use a column without an instrument!***
- **New instruments with higher pressure ratings and smaller volume tubing and components have been designed for modern, smaller particle columns; however, traditional instruments may also be suitable for modern columns, especially when optimized by the user; simple tests can qualify instruments for minimum dispersion.**

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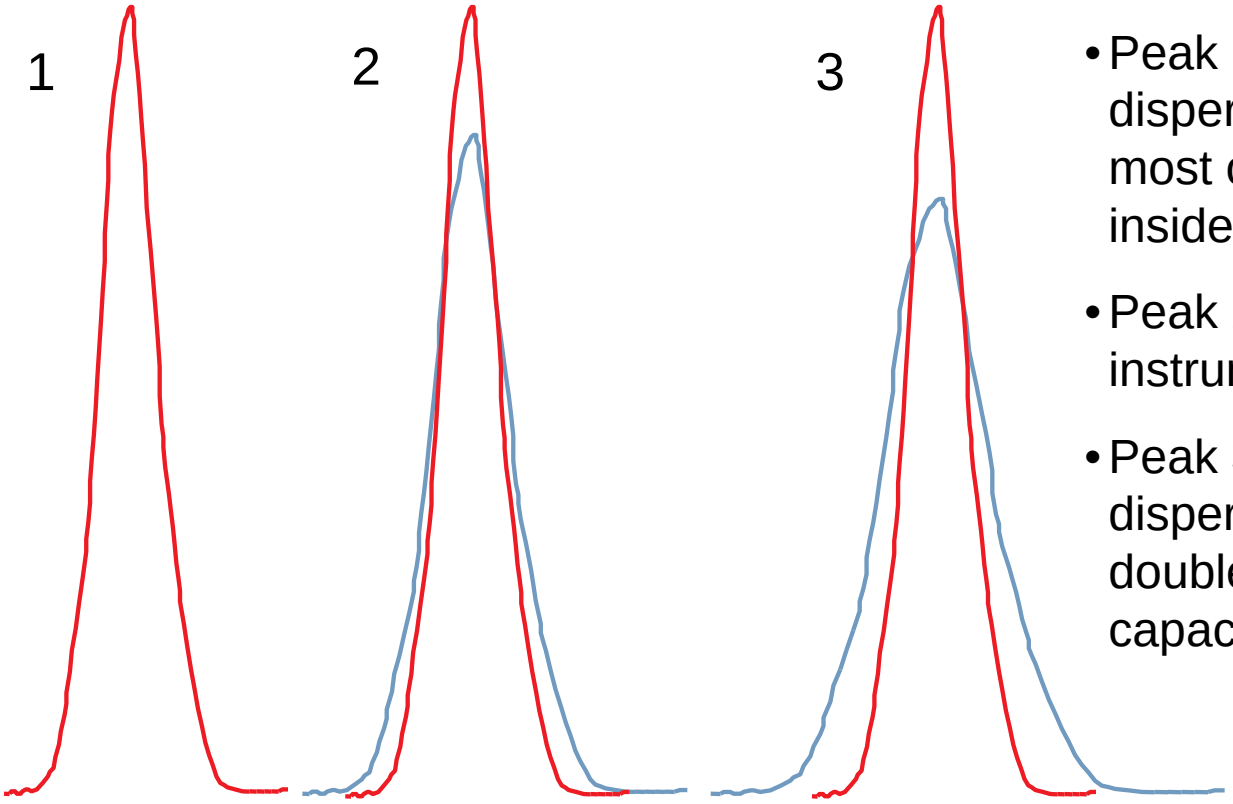
Illustration of Instrument Dispersion

A peak from the same column in three different instruments.



- Peak 1 shows a low dispersion instrument where most of the spreading occurs inside the column.
- Peak 2 shows moderate instrument dispersion in blue.
- Peak 3 shows high instrument dispersion; peak width has doubled and column peak capacity is halved.

Time spent outside the column destroys system efficiency

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Origins of Column Band Spreading* (van Deemter)

$$H = Ad_p + BD_m/u + Cd_p^2u/D_m$$

- A term- Multipath; bed uniformity; eddy diffusion
- B term- Longitudinal diffusion (axial in nature)
- C term- Rate of mass transfer from moving phase though stagnant mobile phase into stationary phase (radial in nature)

Instrument impact on H and N is often neglected

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Band Spreading Inside the Column Bed

$$\sigma_{col}^2 = V_o^2(1 + k)^2/N$$

V_o = mobile phase column volume (µL)
(unretained peak retention volume; void volume)
k = peak capacity factor
N = number of column theoretical plates

- **Small bed geometry, short retention and high efficiency favor low dispersion (dilution) within a packed column.**
- **Instrument bandwidth becomes more harmful to efficiency and resolution for short, small ID columns with low k values and high N.**

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Column vs System Band-Spreading

The effect of system band width can be calculated from the additive relationship of variances, where the total variance of the peak is equal to the sum of the true on-column peak variance plus the instrument variance.

$$\sigma^2_{system} = \sigma^2_{column} + \sigma^2_{instrument}$$

$$\sigma^2_{instrument} = \sigma^2_{injector} + \sigma^2_{detector} + \sigma^2_{connector\ tubing}$$

σ_{system} = Total peak dispersion in volume units (µL)

$$\sigma_{system} = W_b/4$$

$W_b = 4\sigma$ is an easy way to estimate dispersion

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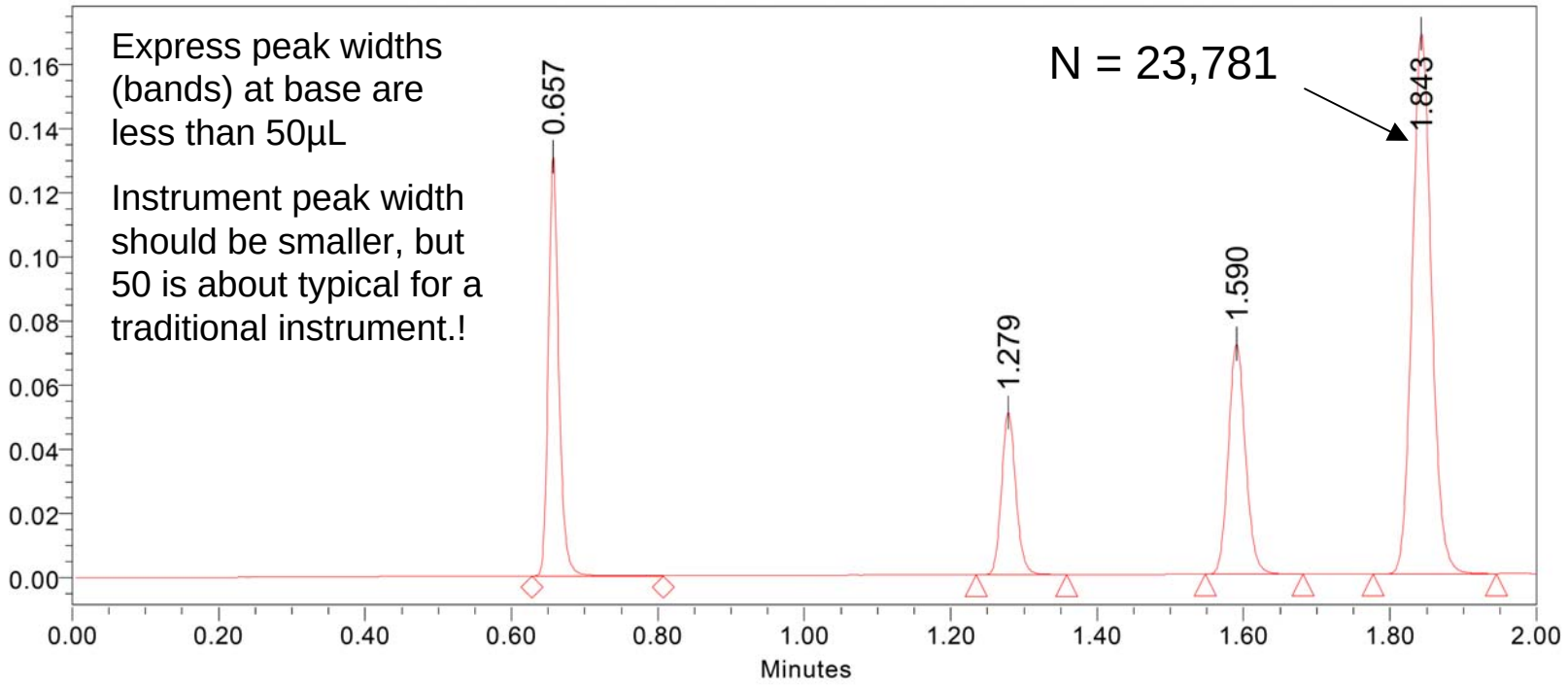
Test Mix Chromatogram on Ascentis Express Fused-Core C18*



Sample: Uracil, benzene, toluene and anthracene
Column: Ascentis Express C18, 100x4.6mm
Mobile phase: 30/70 water/ACN; Flow: 1.25 mL/min; T = 35°C
Instrument: Waters Acquity, 220 bar (3200 psi)

* T. L. Chester, American Lab, Vol. 41 No. 4, pp 11-15, March 2009.

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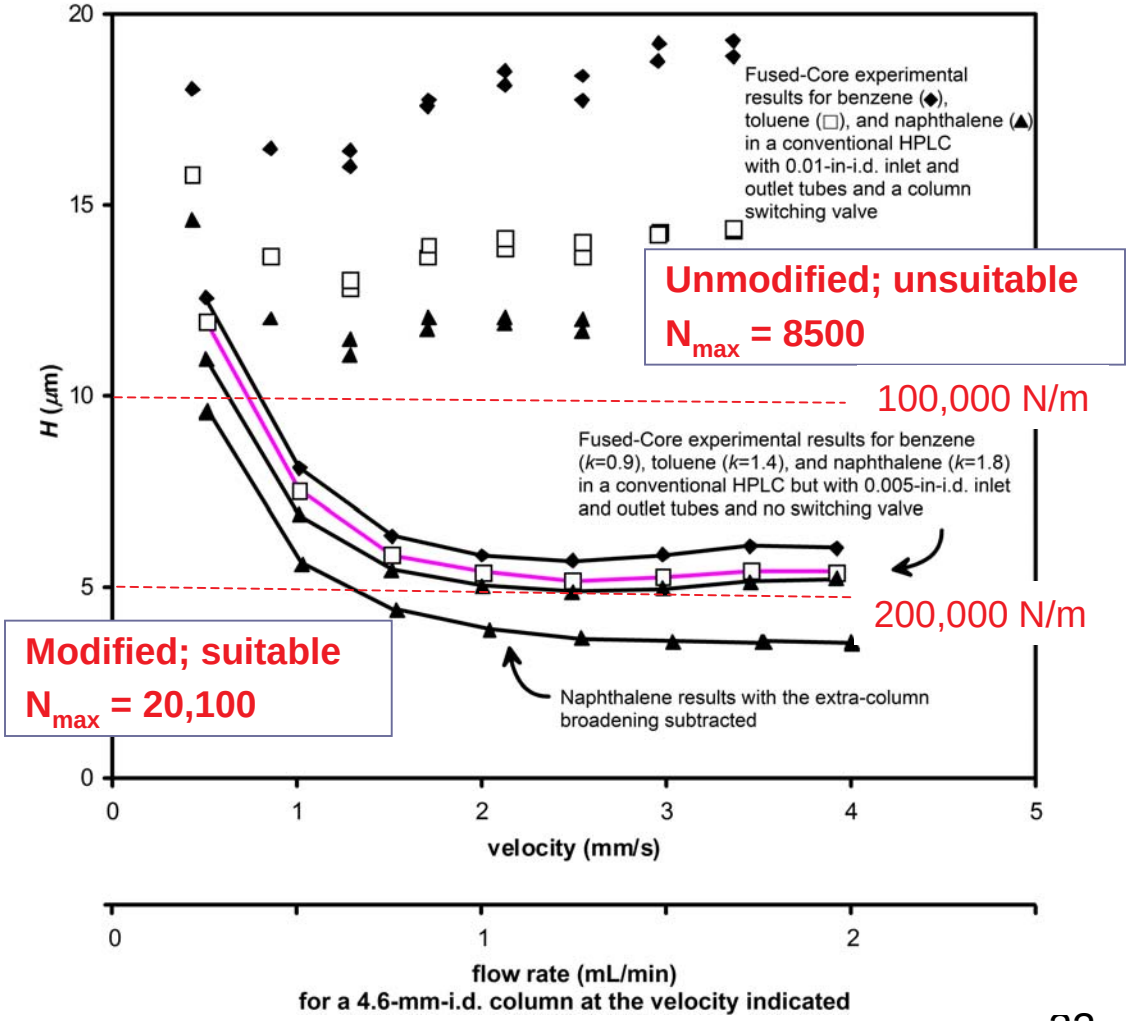
Fused-Core Measured Plate Height for Unmodified and Modified Instrument[3]

% of Total Dispersion

	Unmodified	Modified
Inj	<1	1
Col	30	72
Conn Tubes	66	8
Det*	4	19

*** detector cell had a welded heat exchanger that could not be conveniently replaced (Waters Alliance).**



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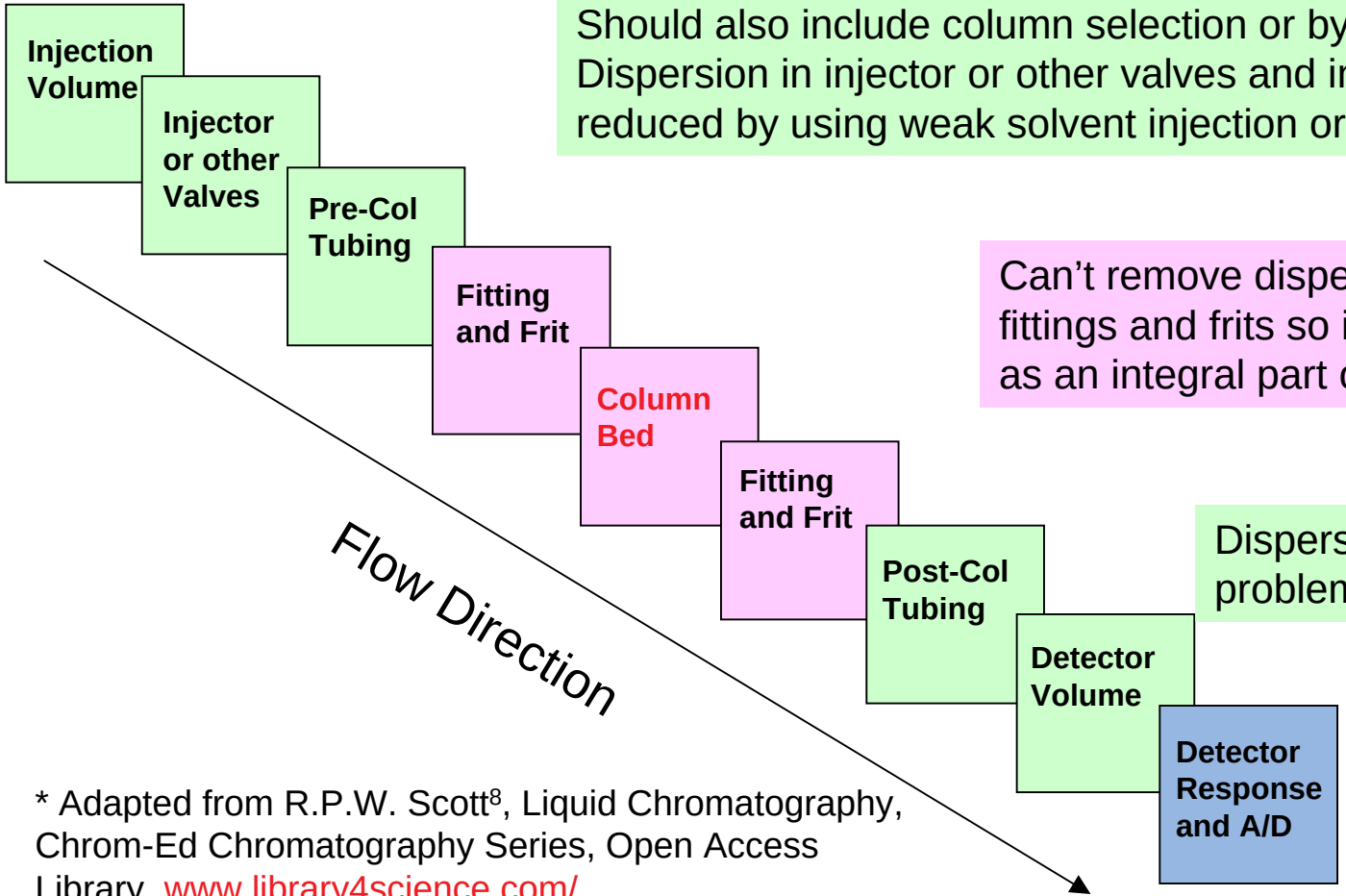
What Causes HPLC Instrument Dispersion?

.... Time sample spends outside the column bed.

HPLC System Components that Affect Measured Peak Bandwidth*



Should also include column selection or bypass valves. Dispersion in injector or other valves and inlet tubing can be reduced by using weak solvent injection or gradients.

Can't remove dispersion in column fittings and frits so it must be treated as an integral part of the column bed.

Dispersion is always a problem after the column.

Electronic and often neglected

* Adapted from R.P.W. Scott[8], Liquid Chromatography, Chrom-Ed Chromatography Series, Open Access Library, www.library4science.com/

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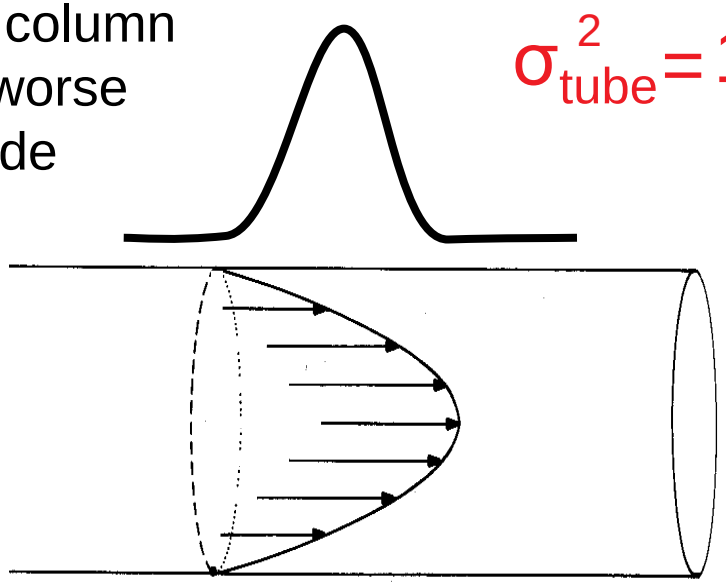
Dispersion in Open Cylindrical Flow Path

Time outside column bed is much worse than time inside



$$\sigma^2_{tube} = 1.36 \times 10^{-3} d_t^4 L_t F/D$$

d = cylinder diameter

L = cylinder length

F = flow rate

D = diffusion coefficient

- **Dispersion from volume elements is constant for any given flow rate and analyte, but note that dispersion (bandwidth) increases with flow.**
- **Velocity at the wall is essentially zero under laminar flow conditions. Small inside diameter, short length, low flow and fast solute diffusion favor low dispersion in connection tubes and accessories. Larger molecules show greater dispersion (as 1/D) in connectors.**

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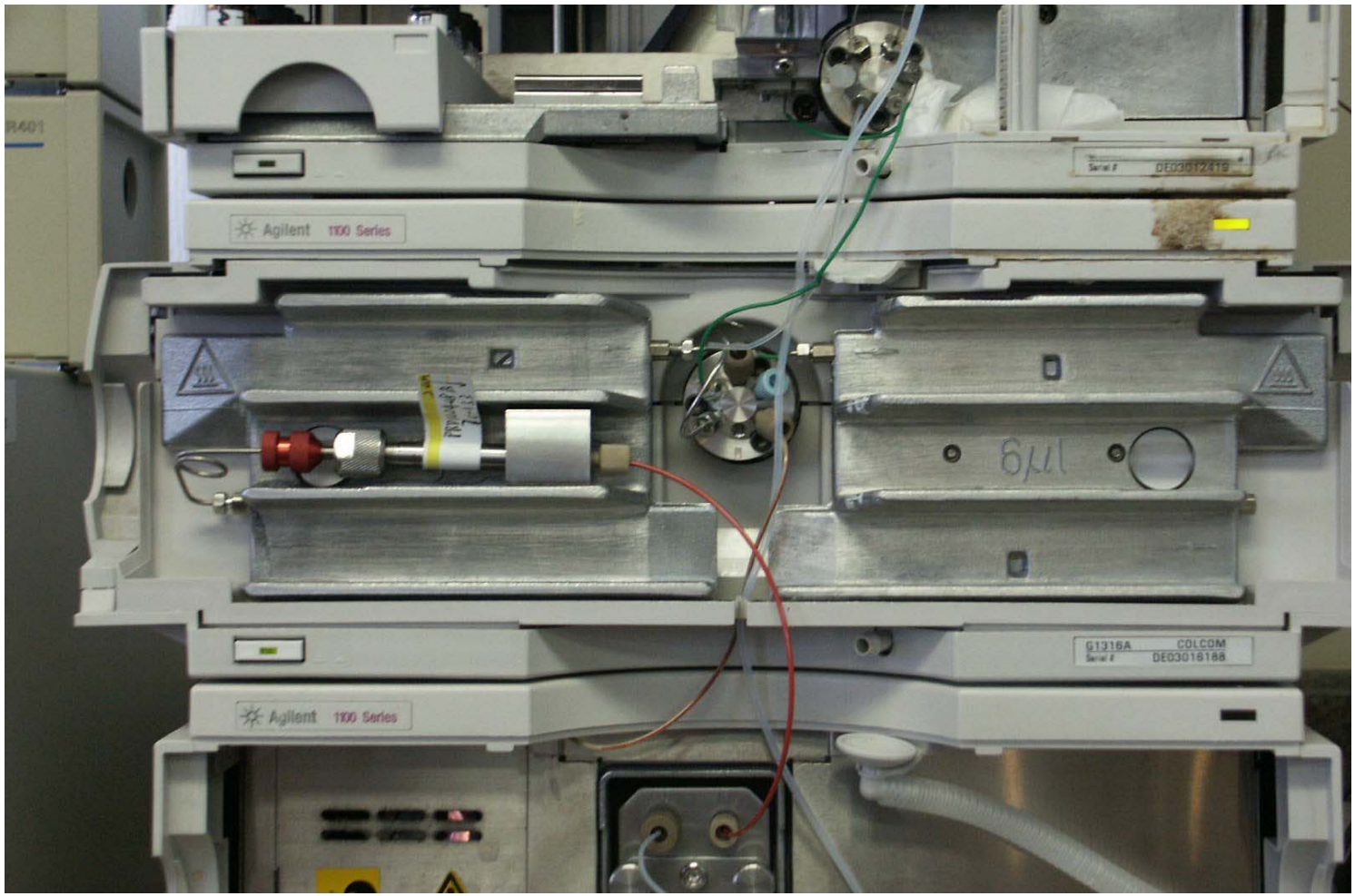
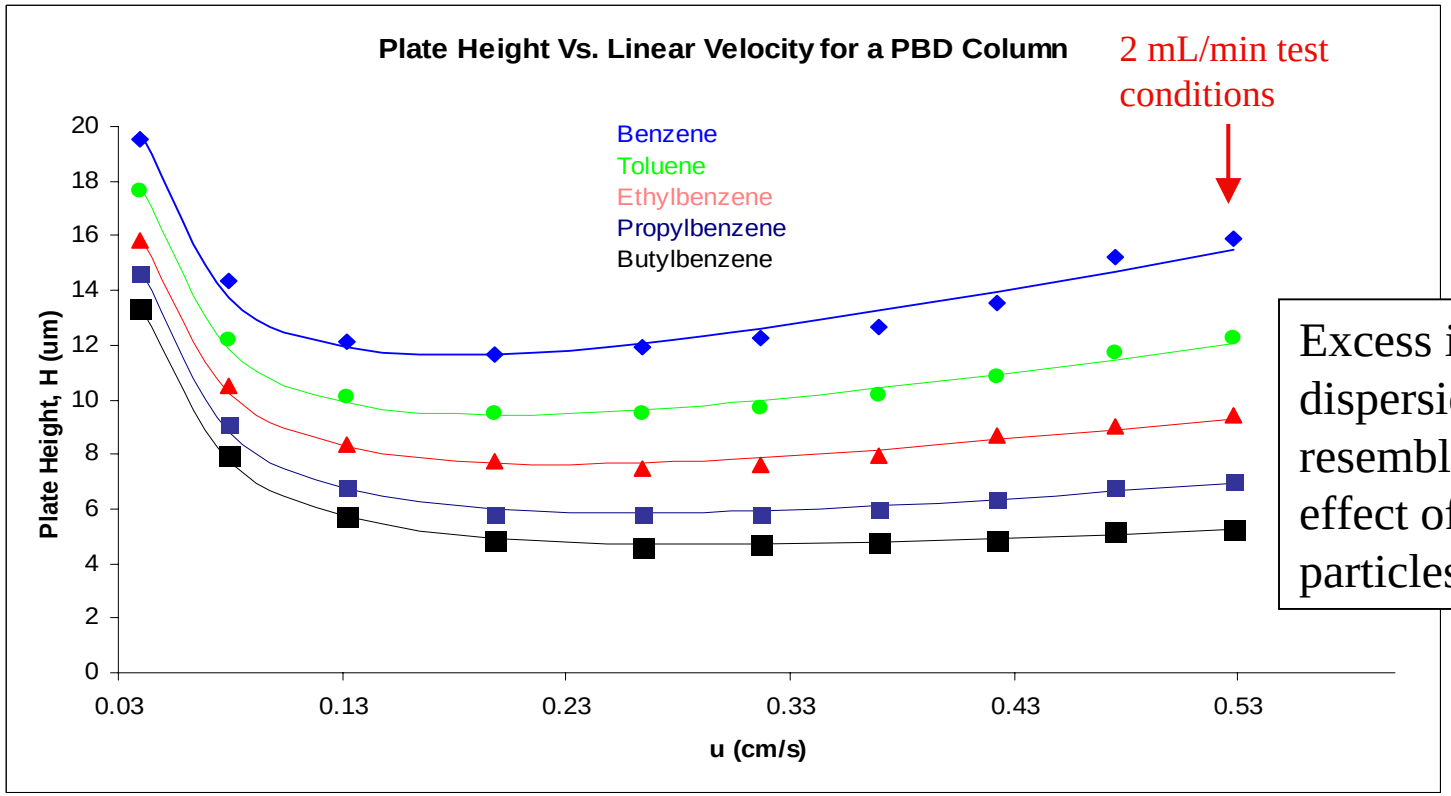
Optimizing Instrument Configuration for Elevated Temperature Operation*



R. A. Henry 2010 *Agilent 1100 photo compliments of Dan Nowlan at ZirChrom

Performance of a Factory Agilent 1100 Instrument with Sub-2µm Zirconia

A family of curves with H_{min} ranging from <5µm to >12µm indicates strong instrument impact on H_{system}



Excess instrument dispersion resembles the effect of larger particles (C-term).

Plate height vs linear velocity, Temperature 30 ºC, Mobile phase: 50/50 ACN/water, Column: 50 x 4.6mm, Agilent 1100/UV with Standard Cell and 0.007'' i.d. tubing.

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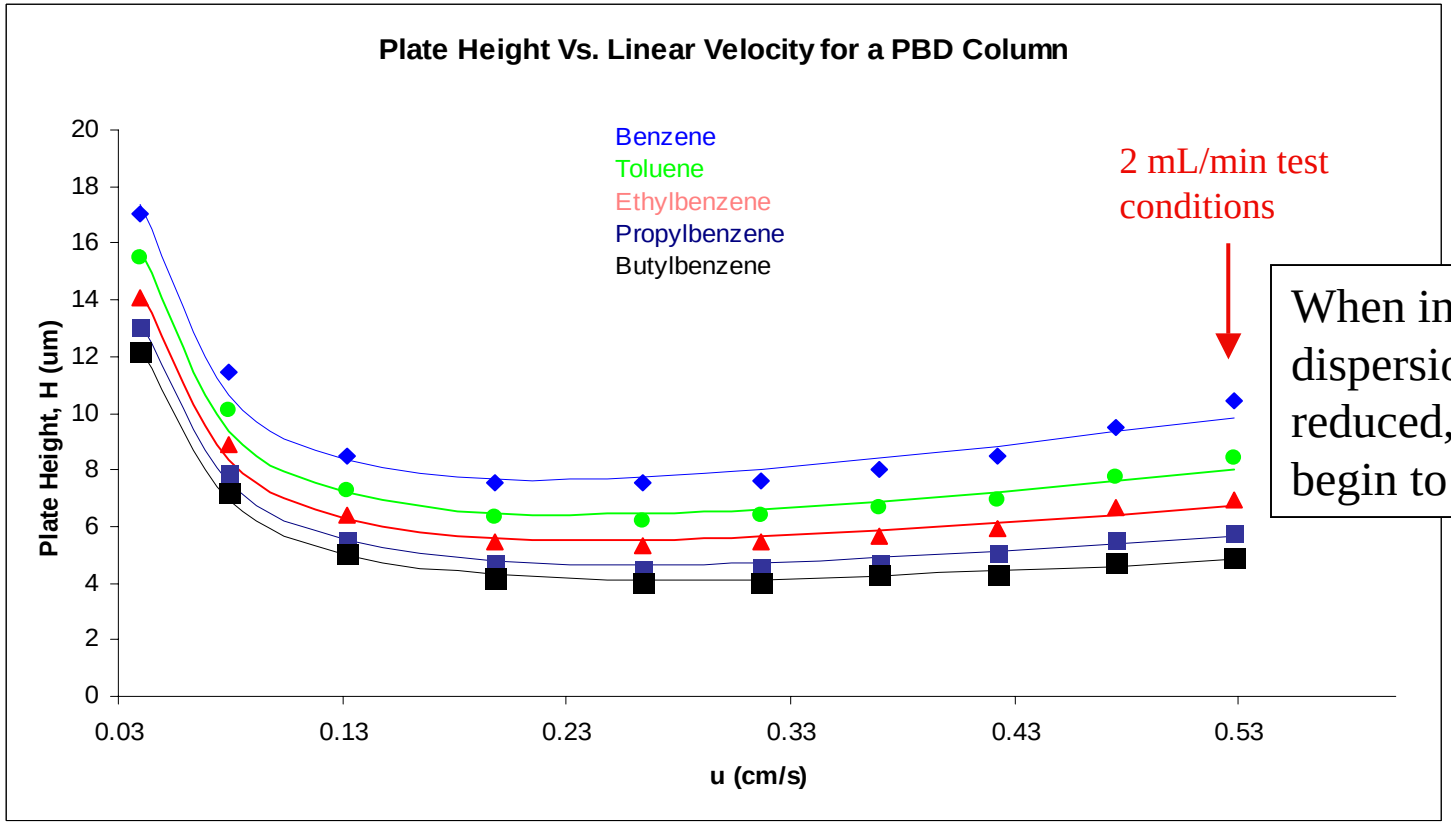
Optimizing Factory Agilent 1100 by Adding Micro Flow Cell

New family of curves with H_{min} ranging from <5µm to 8µm indicates lower instrument impact on H_{system}



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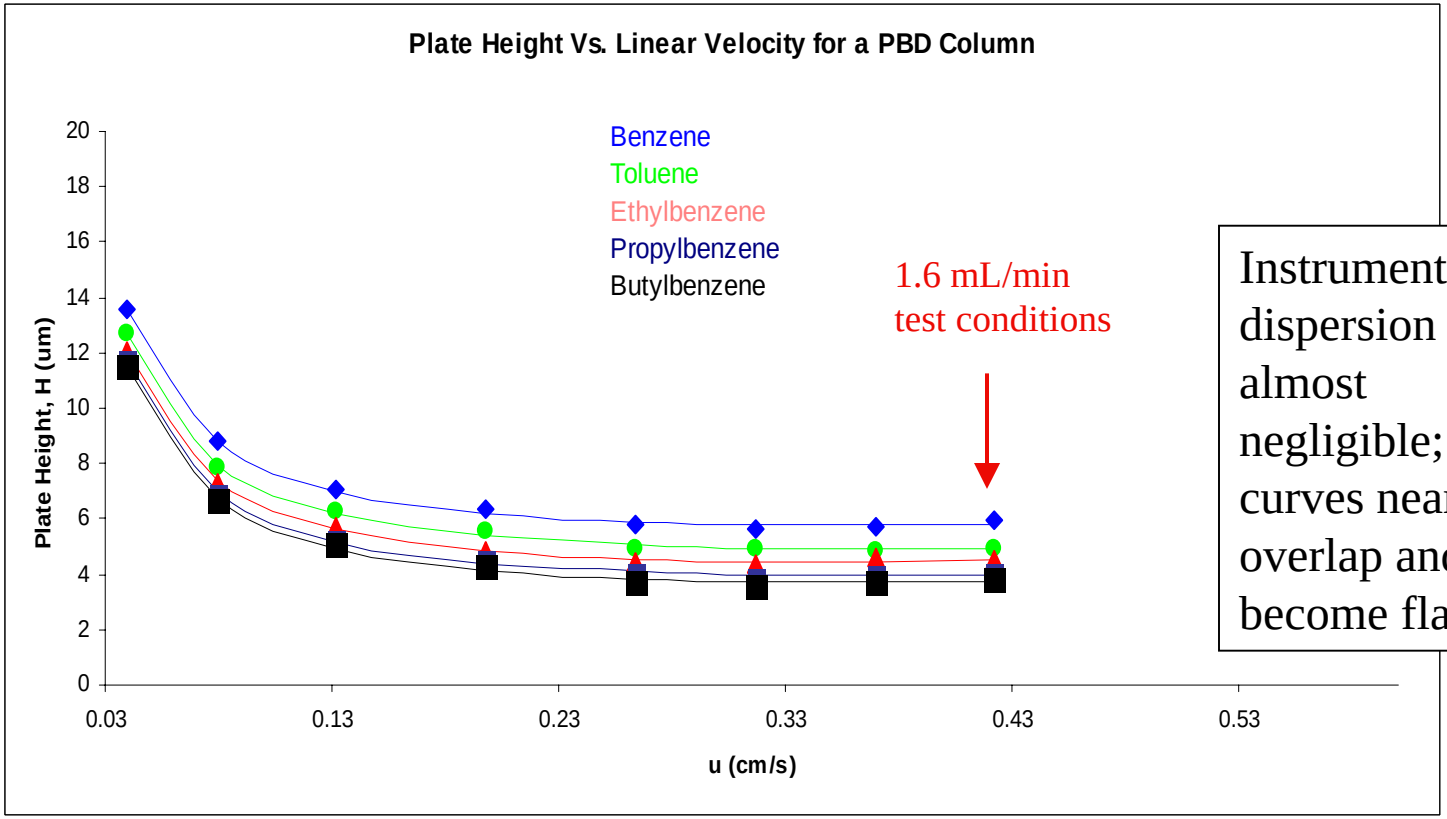
Optimized Factory Instrument with Micro Cell + 0.005” ID Tubing

New family of curves with H_{min} ranging from 4µm to 6µm indicates very low instrument impact on H_{system}



Instrument dispersion is almost negligible; curves nearly overlap and become flat.

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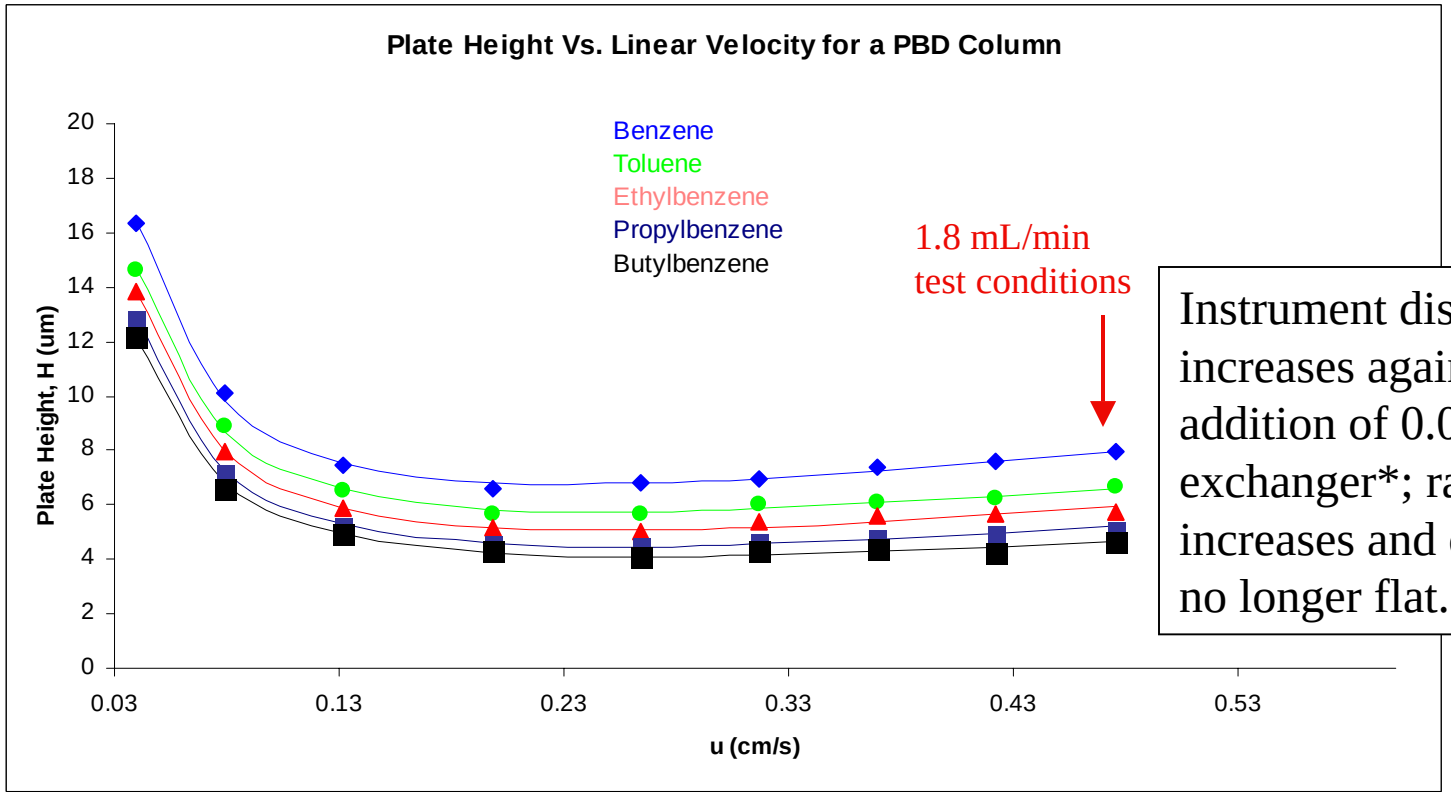
Factory Instrument with Micro Cell + 0.005” ID Tubing + Heat Exchanger

New family of curves with H_{min} ranging from <5µm to >7µm indicates moderate instrument impact on H_{system}



Instrument dispersion increases again due to addition of 0.007” heat exchanger*; range of H increases and curves are no longer flat.

* An Agilent Model 1200 heat exchanger may be surface mounted to the heater block to maintain low dispersion for heated applications.

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Efficiency Plot for Agilent 1100 with Micro-Cell and Factory Tubing

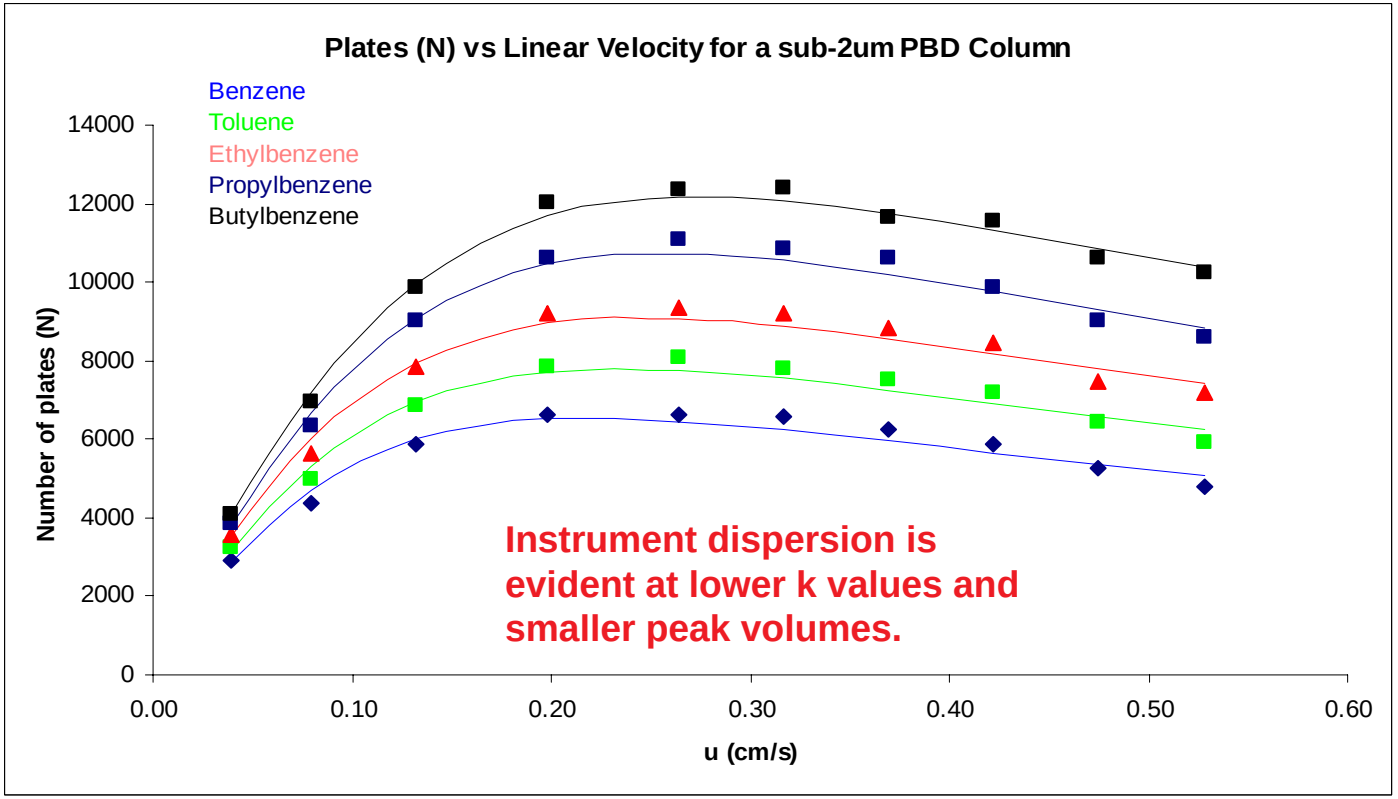

Plate height vs linear velocity for retained solutes: Alkylbenzenes, Temperature 30 ºC, Mobile phase: 50/50 ACN/water; Column: 50 x 4.6mm, Agilent 1100/UV with Micro Cell and 0.007'' i.d. tubing.

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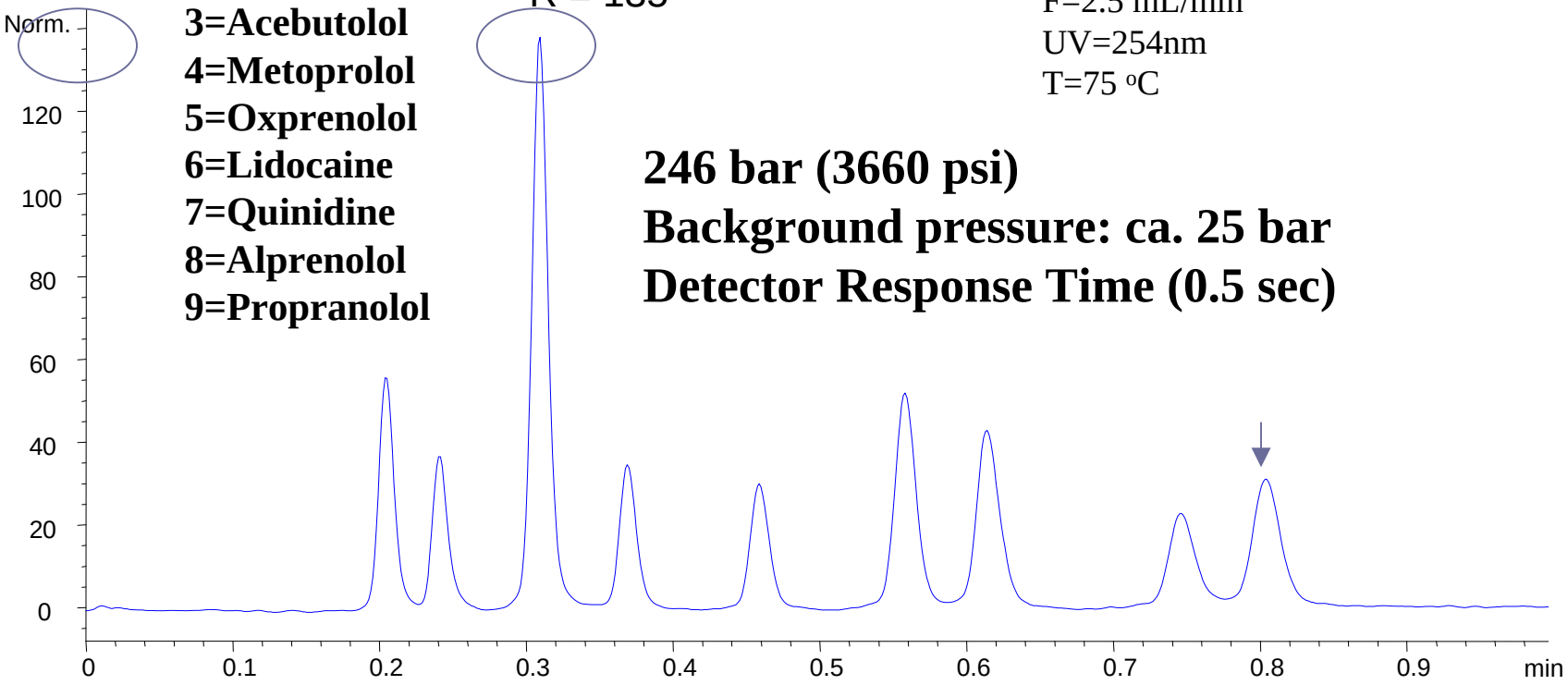
Beta-Blockers on Zr-PBD Sub-2µm at 75 ºC

Analytes
1=Labetalol
2=Atenolol
3=Acebutolol
4=Metoprolol
5=Oxprenolol
6=Lidocaine
7=Quinidine
8=Alprenolol
9=Propranolol

Zr-PBD
50mm x 4.6mm, sub-2µm
22/78 ACN/20mM K_3PO_4 at pH=12
F=2.5 mL/min
UV=254nm
T=75 ºC



246 bar (3660 psi)
Background pressure: ca. 25 bar
Detector Response Time (0.5 sec)

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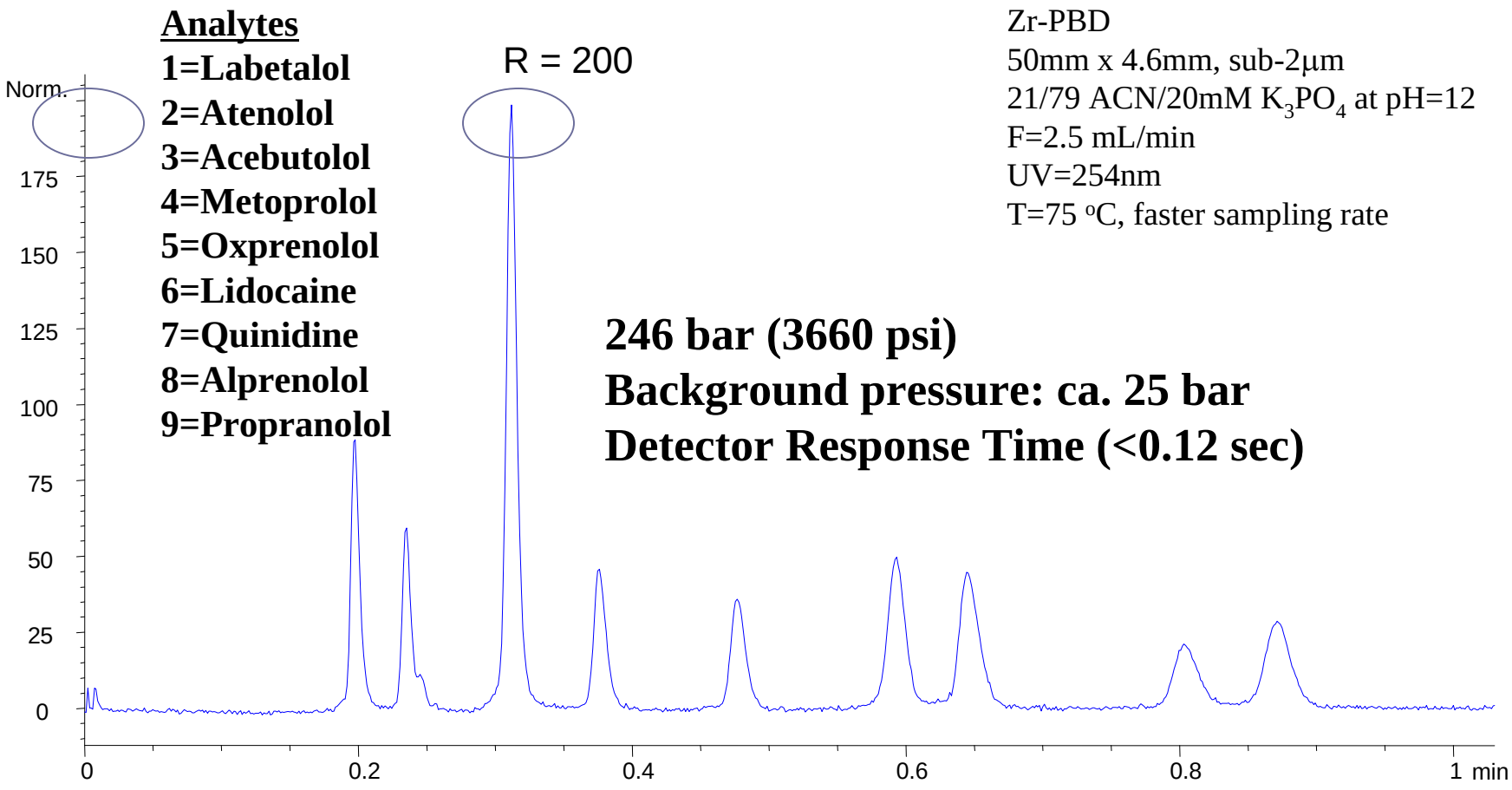
Beta-Blockers Optimized with Faster Detector Response



Zr-PBD
50mm x 4.6mm, sub-2µm
21/79 ACN/20mM K_3PO_4 at pH=12
F=2.5 mL/min
UV=254nm
T=75 °C, faster sampling rate

246 bar (3660 psi)
Background pressure: ca. 25 bar
Detector Response Time (<0.12 sec)

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Importance of Injection Volume and Gradient Focusing

Performance of Some Factory HPLC Instruments

Column Plates Determined from Toluene (k = 2) Half-Height Width Isocratic Elution with 60% ACN 10 µL Injected in Mobile Phase (All instruments in standard configuration with analytical flowcells)				
Column	Agilent 1100	Agilent 1200	Waters 2695	Column Supplier Test Data
Ascentis C18 150x4.6mm 5µm	16911 (6.7% loss)	18034 (0% loss)	16874 (6.9% loss)	18119
Ascentis Express (Fused-Core) C18 100x4.6mm 2.7µm	18649 (34% loss)	22001 (22% loss)	18666 (34% loss)	28164

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Effect of Variables Using Agilent 1200*

Summary of Toluene Plates from 2.7-µm Supelco Ascentis® Express FCP Column Isocratic Elution with 60% ACN on Agilent 1200 10 µL Injected in Mobile Phase*	
Non-Optimized (10 mm / 13 µL flowcell)	22001
Change to 6 mm / 5 µL flowcell	21912
Minimize tubing lengths (don't bother)	22104
*10x sample dilution with 10x volume increase	25738

S. Bannister studies related to ref. 7 (Xcelience Labs, Tampa, FL).

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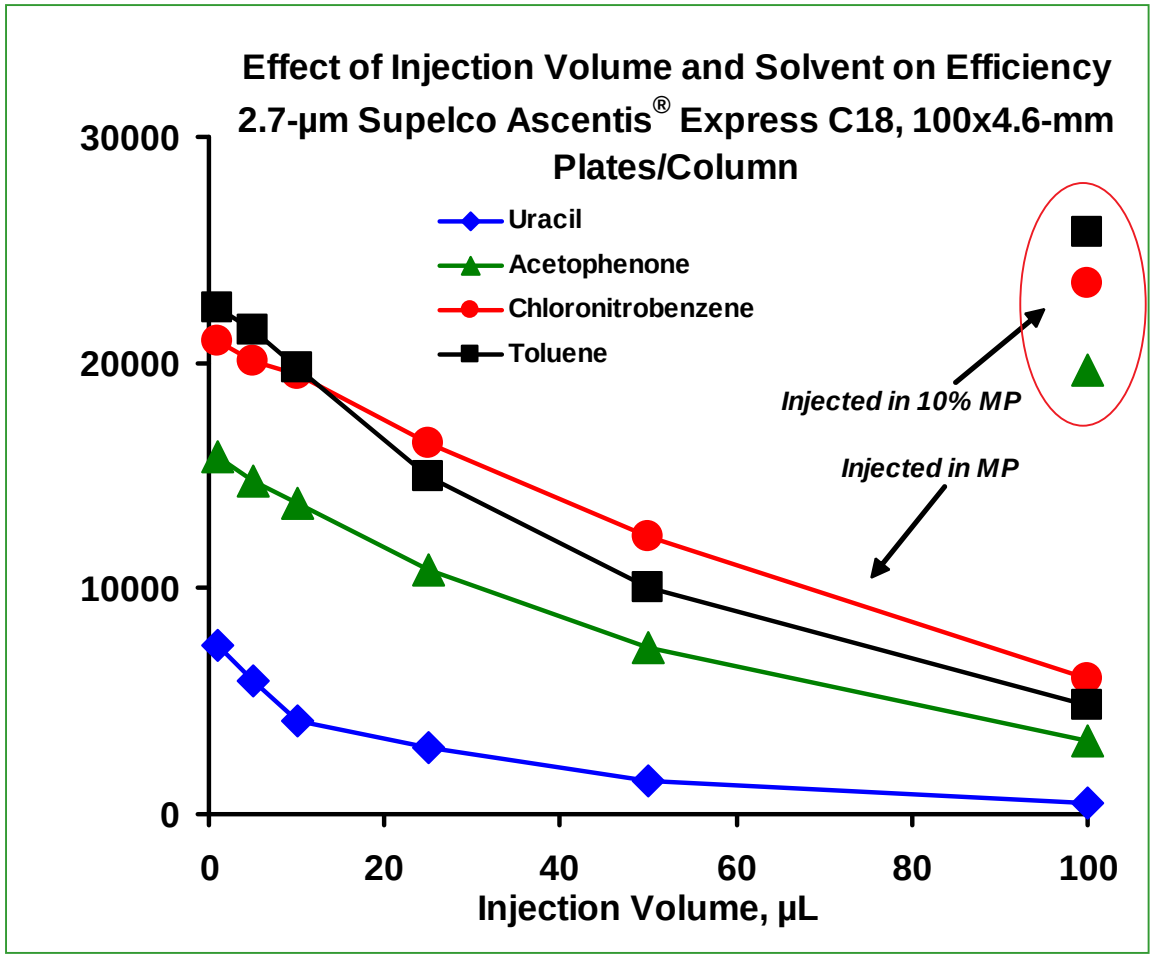
Dispersion Caused by Injections in Mobile Phase



- Sample was diluted 1:10 with water for weak solvent injection.
- Note that efficiency performance was greater with a 100µL injection that has been diluted with water than the original 1µL injection in mobile phase.

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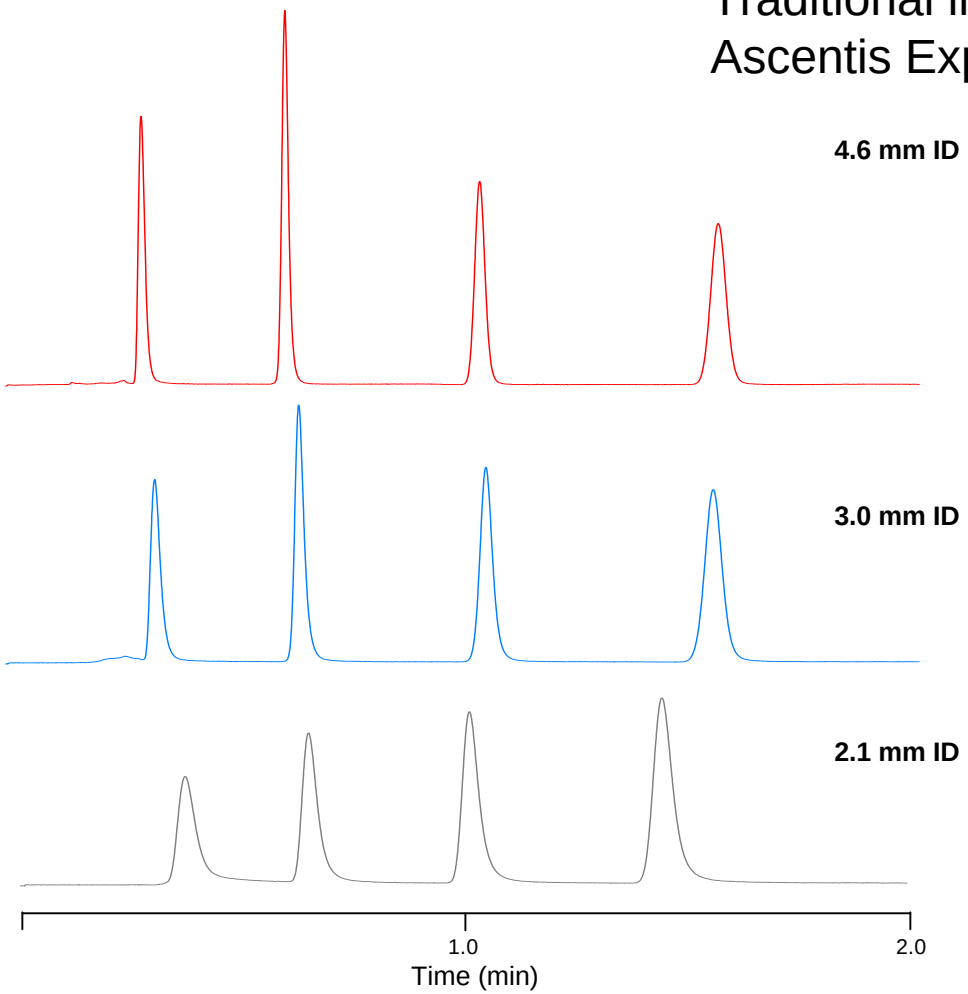
Estimating Instrument Bandwidth

Simple System Suitability (Bandwidth) Test

...one instrument with three columns (three *systems*)

Traditional instrument with 5 cm Ascentis Express columns



Choose test columns that can show >200,000 N/m at k = 2-3.

4.6mm ID columns usually look pretty good due to large void volume and peak volumes.

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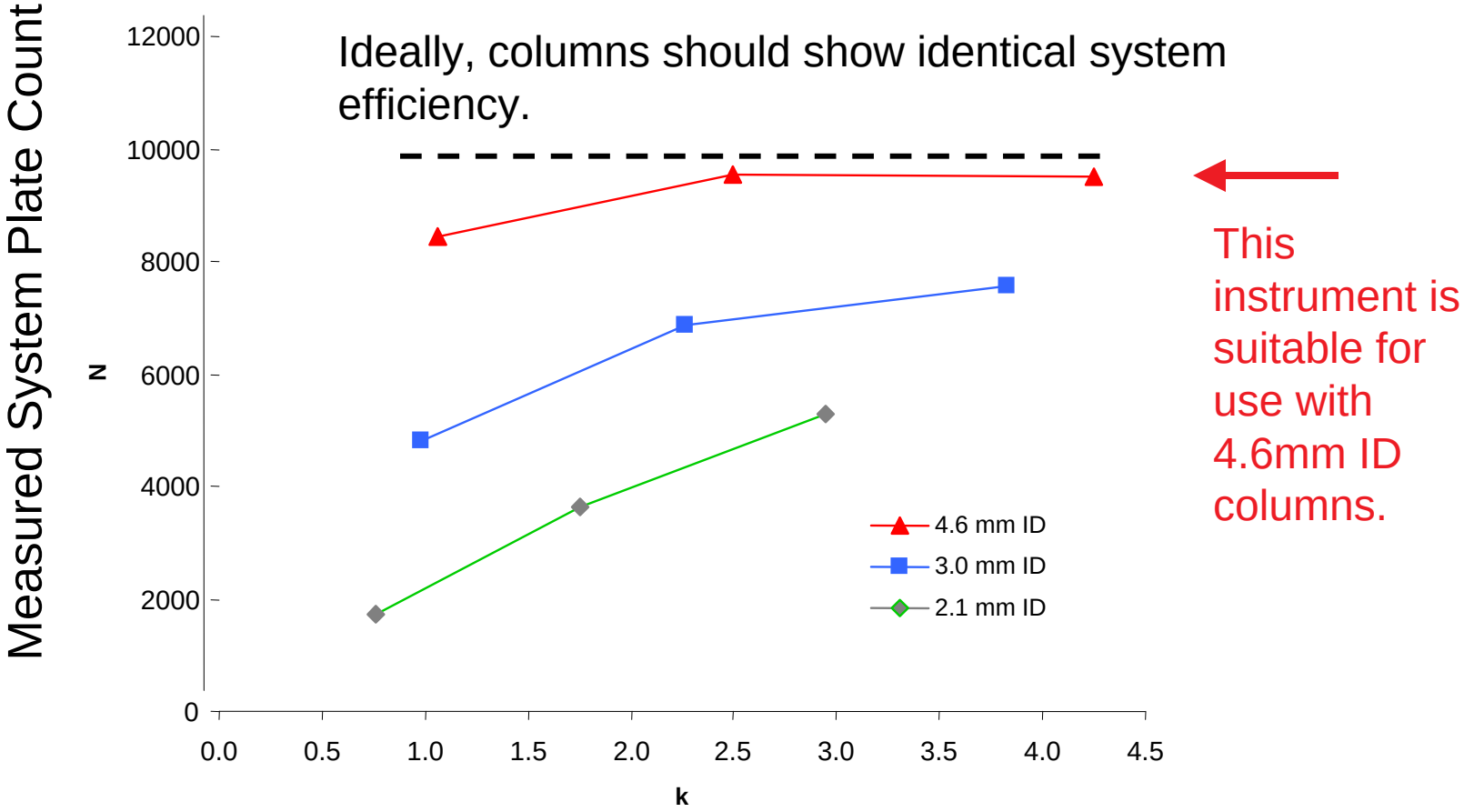
Ranking System Performance with Three Columns



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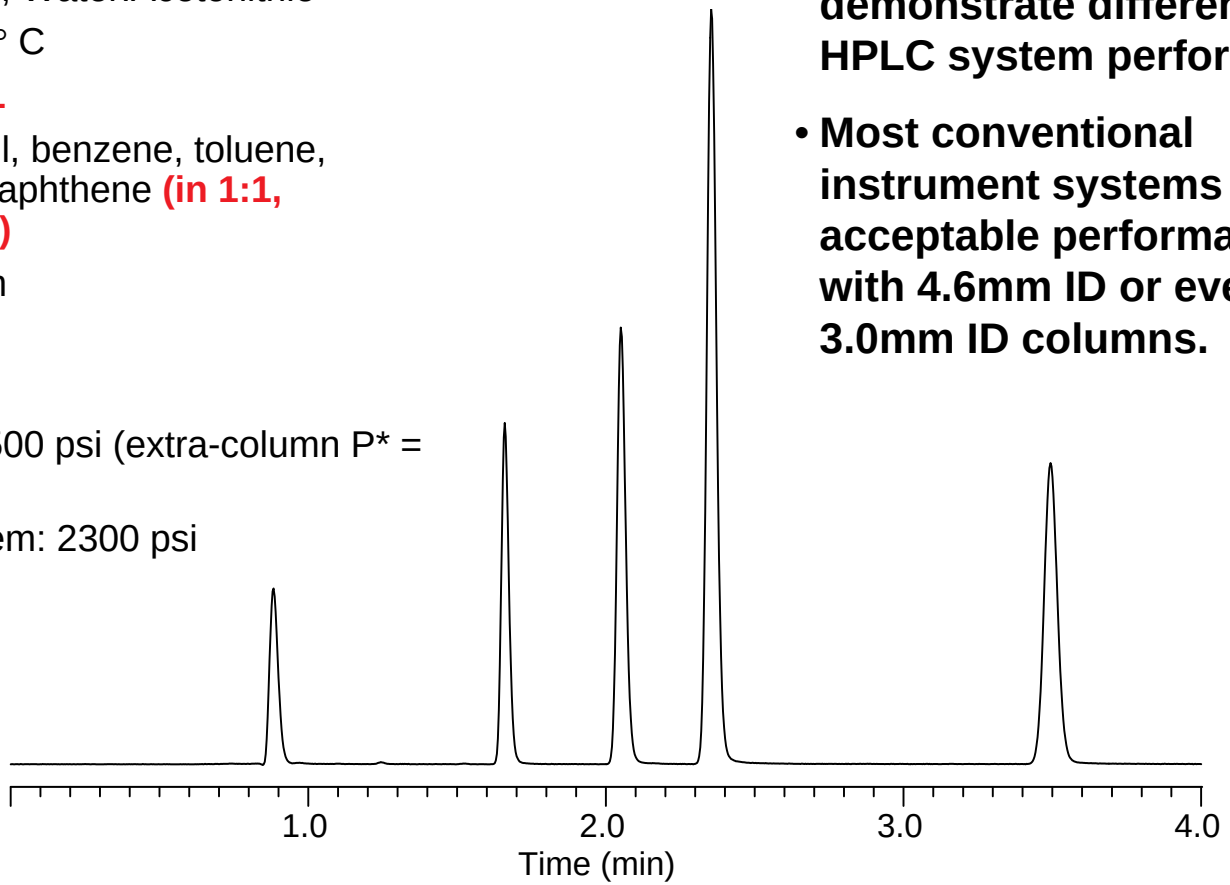
Another System Suitability (Bandwidth) Test

...one column with three instruments (three *systems*)

- **Column:** **Ascentis Express C18, 2.1 x 150 mm**
- **Mobile phase:** 3:7, Water:Acetonitrile
- **Column temp:** 35° C
- **Injection vol:** **1 µL**
- **Sample mix:** uracil, benzene, toluene, naphthalene, acenaphthene **(in 1:1, water:acetonitrile)**
- **Detection:** 250 nm
- **Flow:** 0.3 mL/min
- **Pressure:**

  Ultra System: 3500 psi (extra-column P* = 1200 psi)

  Traditional System: 2300 psi



- **2.1mm ID columns were used in the study to demonstrate differences in HPLC system performance.**
- **Most conventional instrument systems show acceptable performance with 4.6mm ID or even 3.0mm ID columns.**

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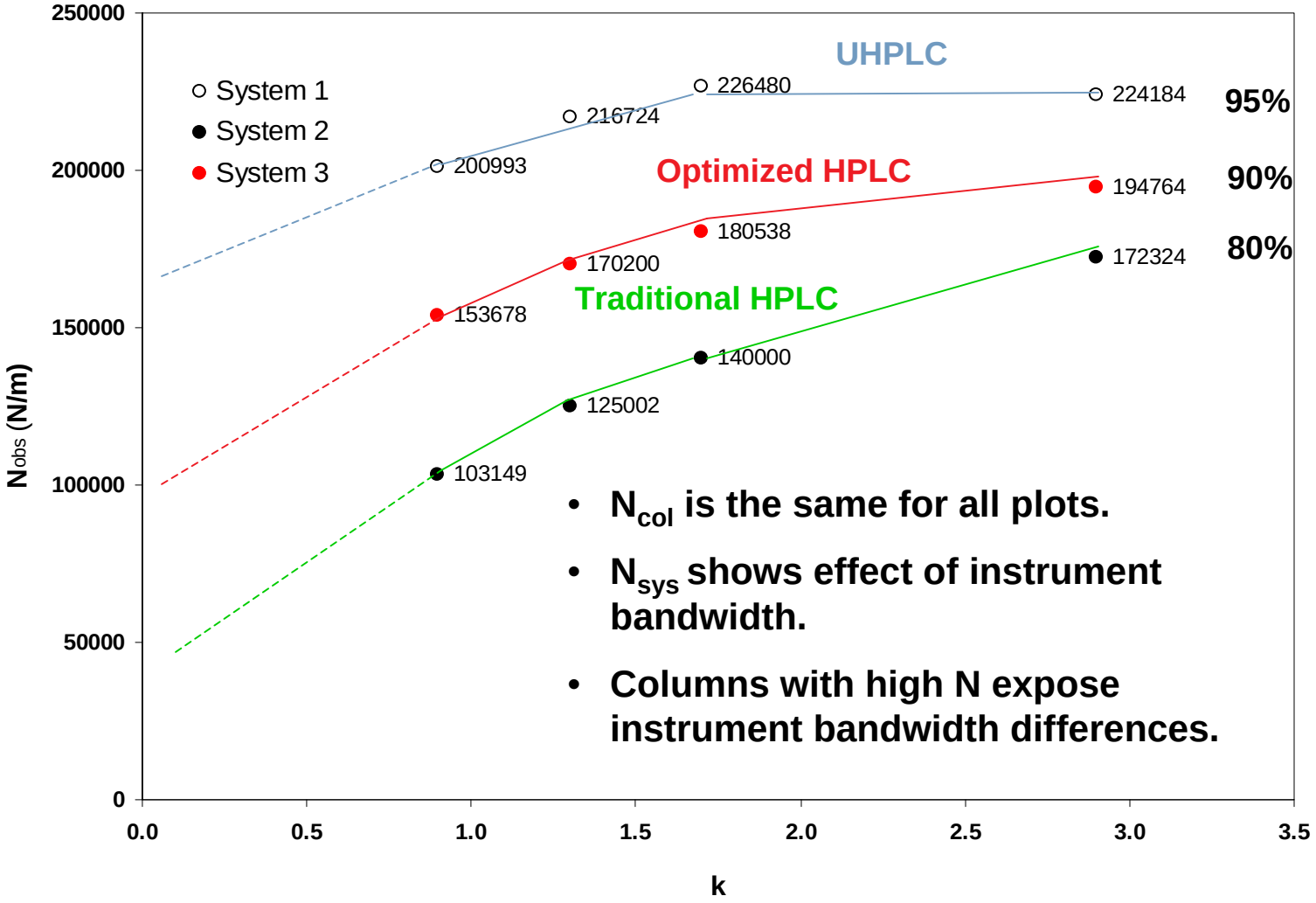
Ranking System Suitability Performance (Bandwidth)

The blue instrument is suitable (maybe the red one too)



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Direct Method for Measuring IBW

- Connect injector to detector
  - ZDV union
  - shunt
- Inject small volume (µL or less) of chromophore
- Record peak (or retention time and N)
- Calculate IBW (flow in µL) or measure directly from peak retention

  $\sigma = (t_r \times \text{flow}) / \sqrt{N}$

  $\text{IBW} = 4\sigma$
- Common mistakes
  - data sampling rate too slow
  - detector response time too slow
  - flow rate too fast (or variable)
  - calculation of N

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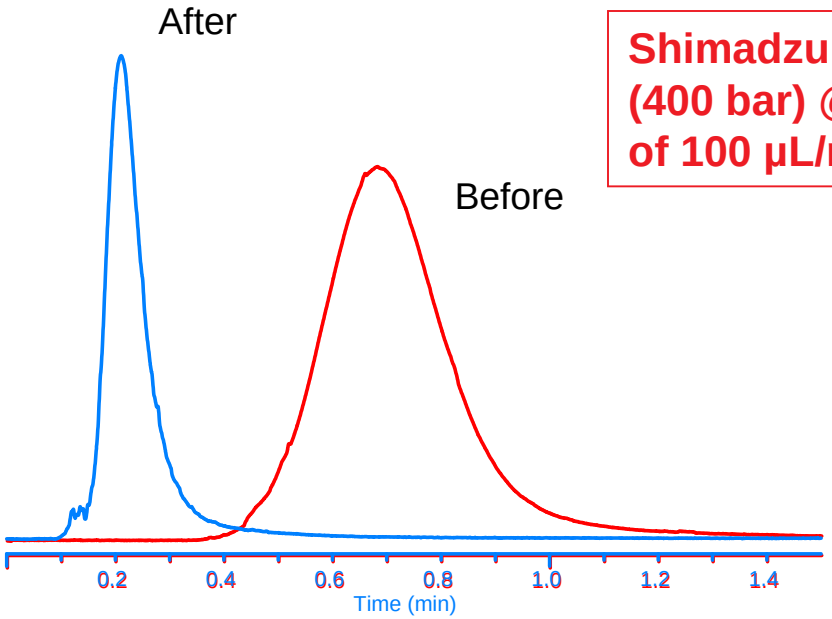
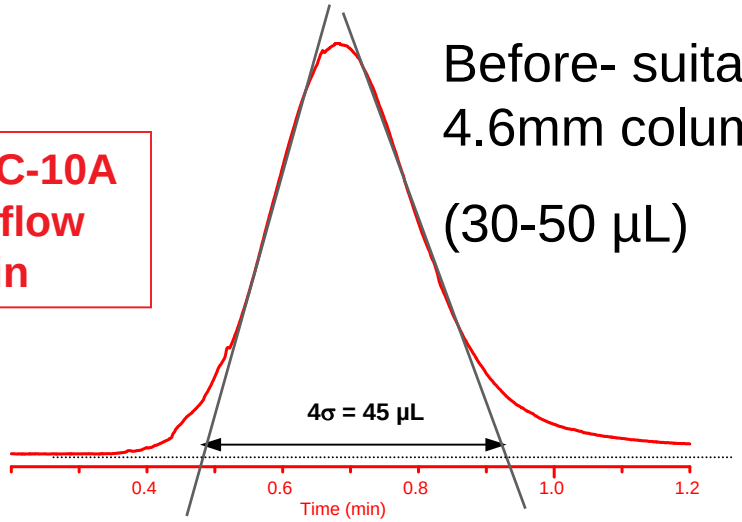
Comparison of IBW Before and After Instrument Optimization

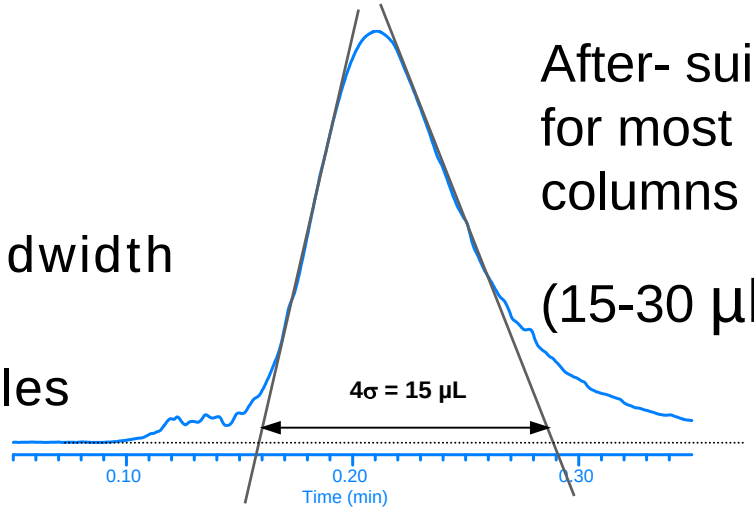


Shimadzu LC-10A (400 bar) @ flow of 100 µL/min



Before- suitable for 4.6mm columns

(30-50 µL)



After- suitable for most columns

(15-30 µL)

- Remove column and measure bandwidth at base for small volume injection
- Clear contrast between peak profiles before and after optimization

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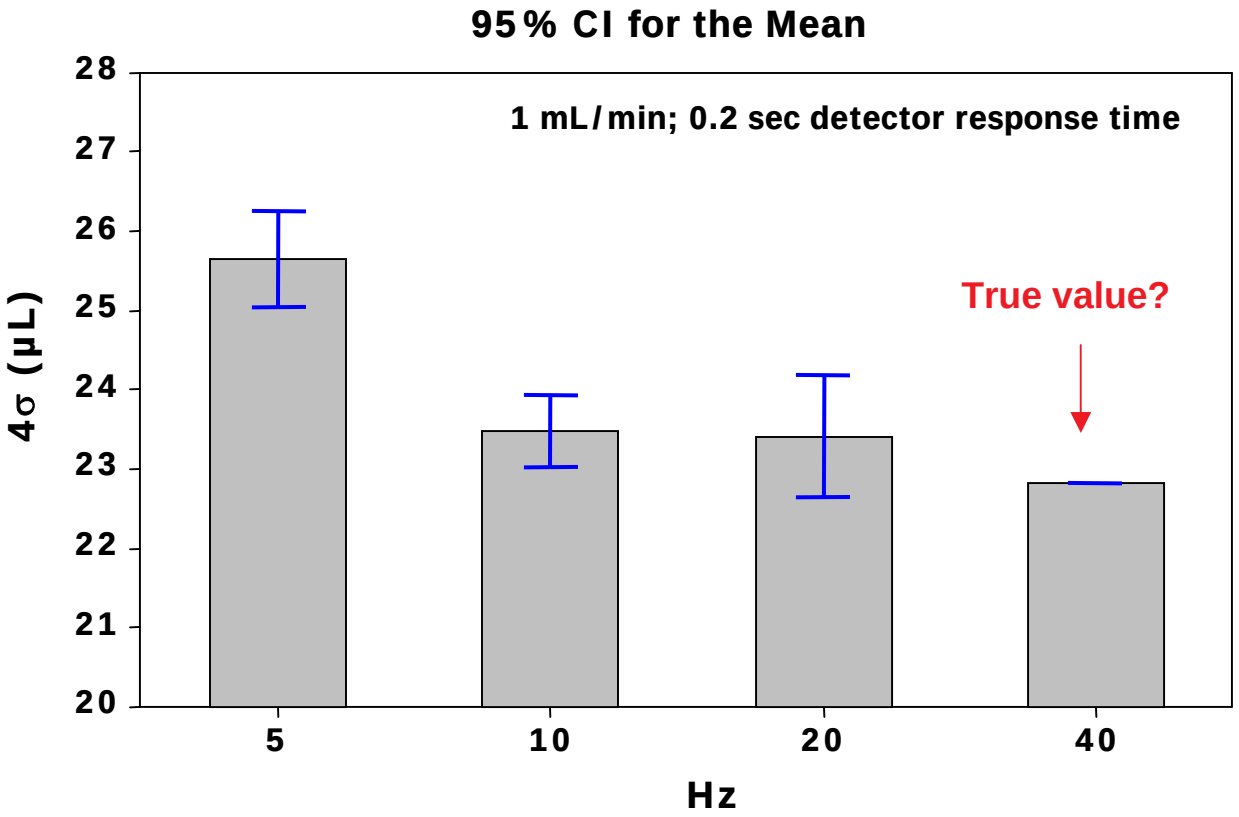
Effect of Data Sampling Rate on Measured IBW



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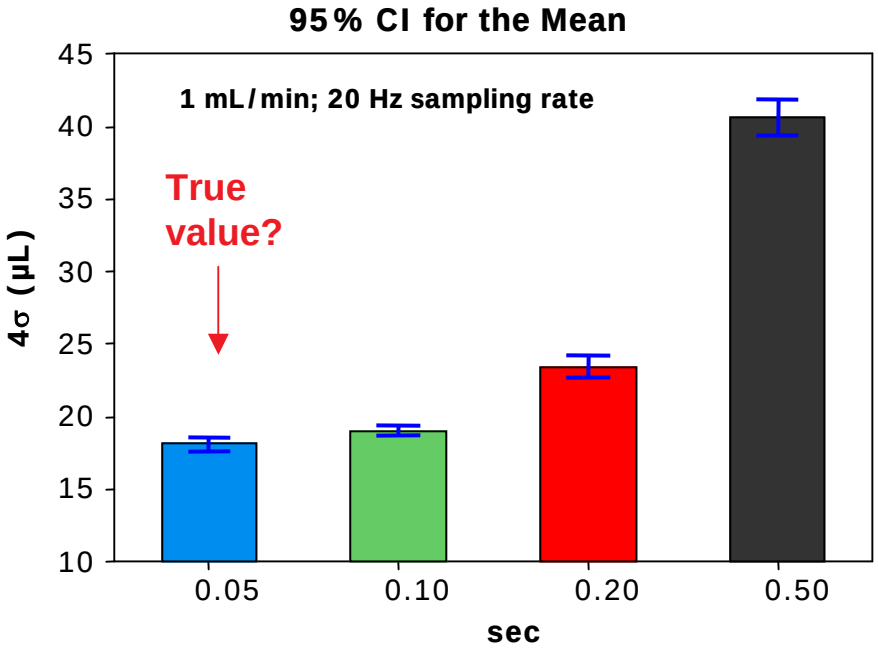
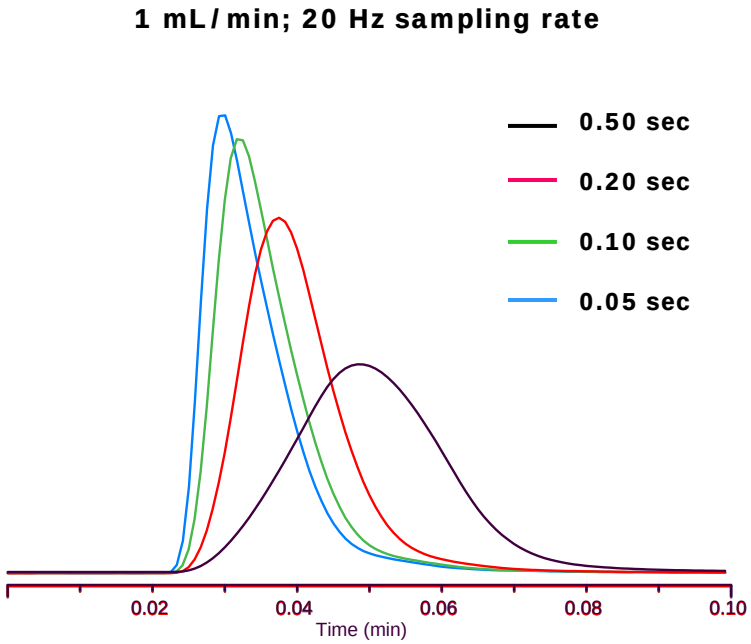
Effect of Detector Response Time on Measured IBW





- Slow filter response time adversely effects accurate capture of peak dimensions by data system

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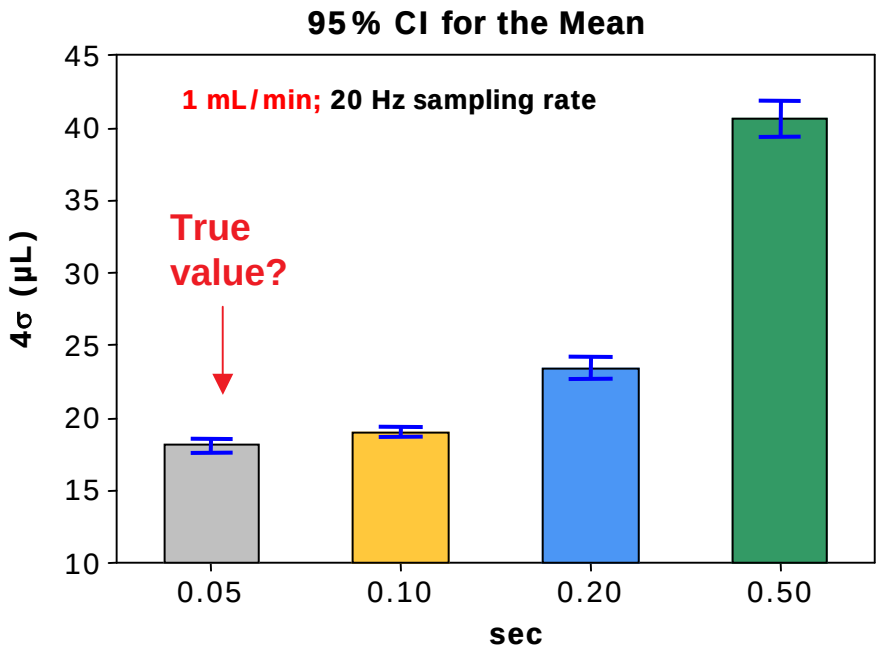
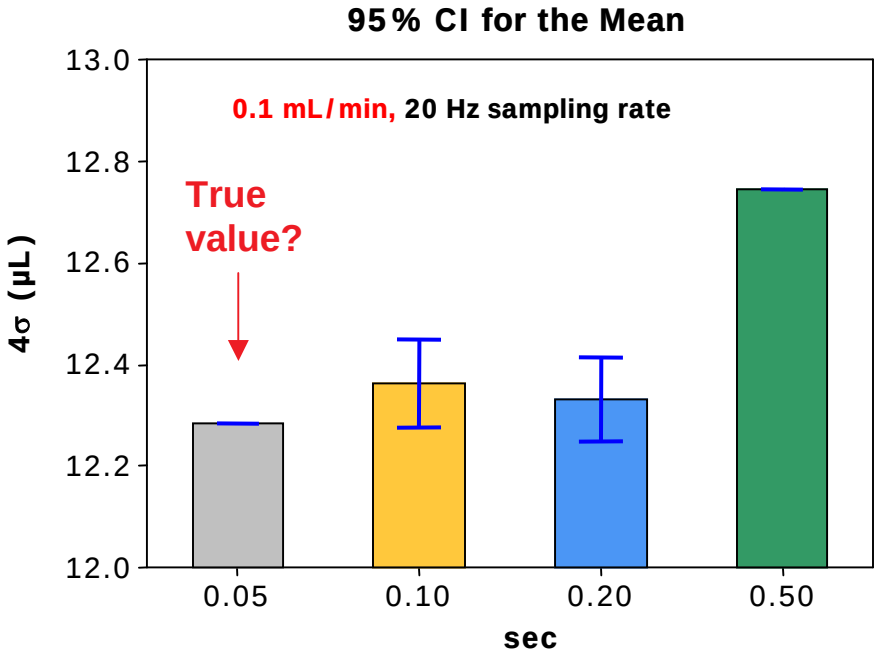
Effect of Flow rate on Measured IBW





- Flow rate of 0.1 mL/min will inherently yield less dispersion, but also permits accurate capture of peak dimensions well within capabilities of data system

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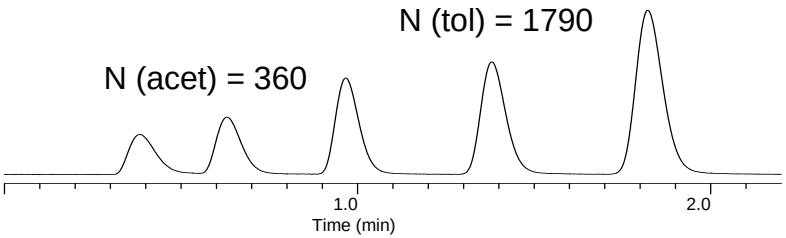
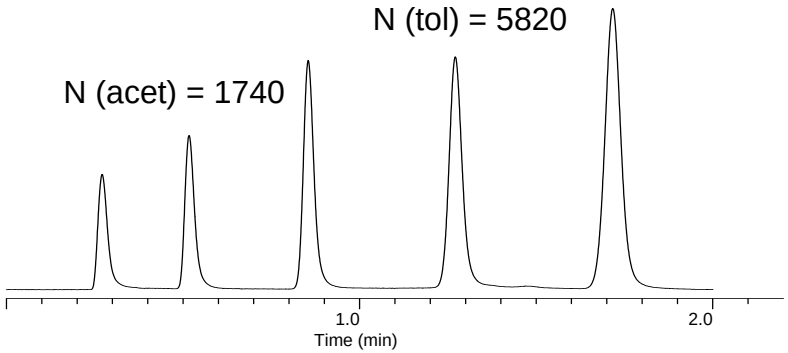
2.1 mm ID x 5 cm, 0.4 mL/min

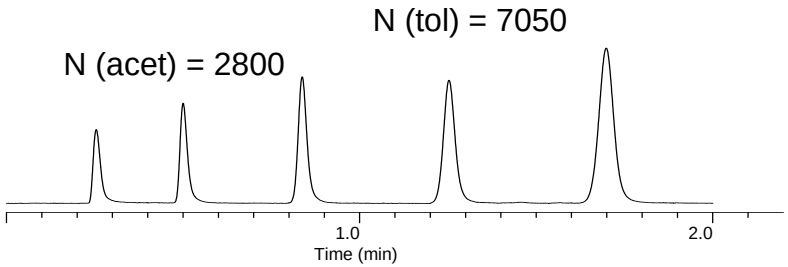


IBW: 42 µL
Tubing ID: 0.010”
Flow cell: 16 µL, 10 mm path
Pressure: 1560 psi

True N = >10,000



IBW: 17 µL
Tubing ID: 0.005”
Flow cell: 16 µL, 10 mm path
Pressure: 1680 psi



IBW: 12 µL
Tubing ID: 0.005”
Flow cell: 2.5 µL, 5 mm path
Pressure: 1680 psi

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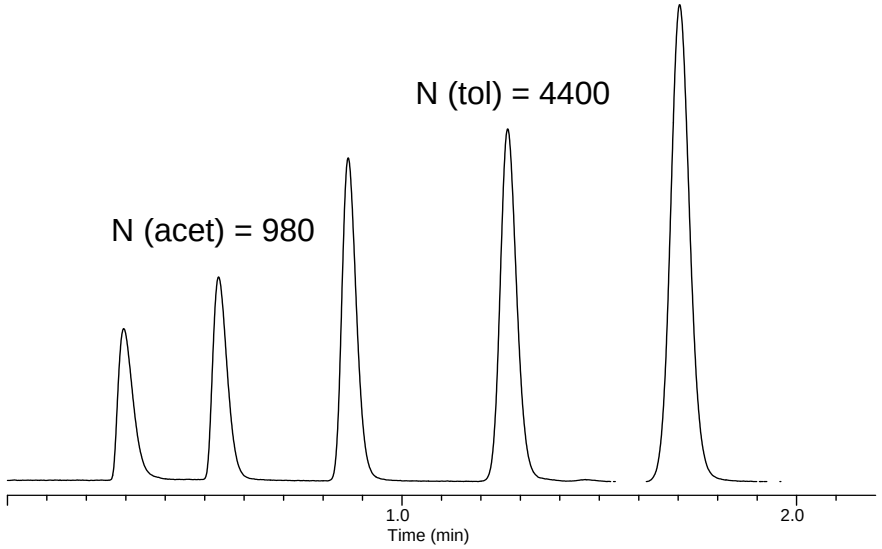
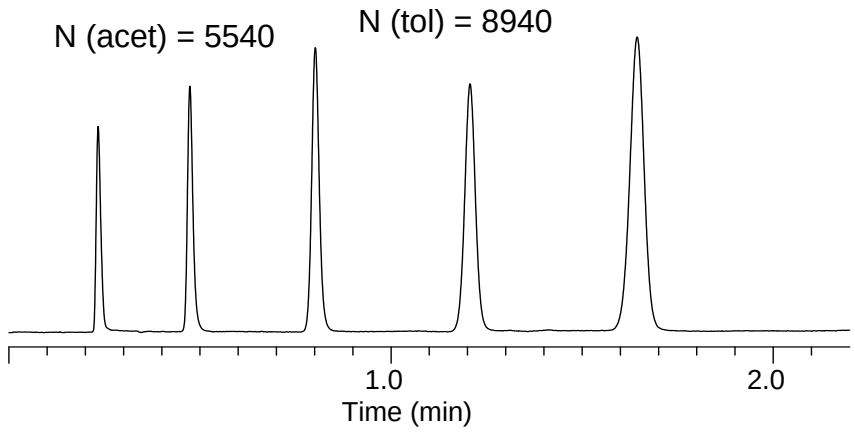
3 mm ID x 5 cm, 0.8 mL/min



IBW: 42 µL
Tubing ID: 0.010”
Flow cell: 16 µL, 10 mm path
Pressure: 1600 psi

True N = >10,000



IBW: 12 µL
Tubing ID: 0.005”
Flow cell: 2.5 µL, 5 mm path
Pressure: 1640 psi

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Fekete Direct Measurements of IBW[4]

0.04 min x 0.5 mL/min = 0.02 mL

IBW ≈ 20 µL

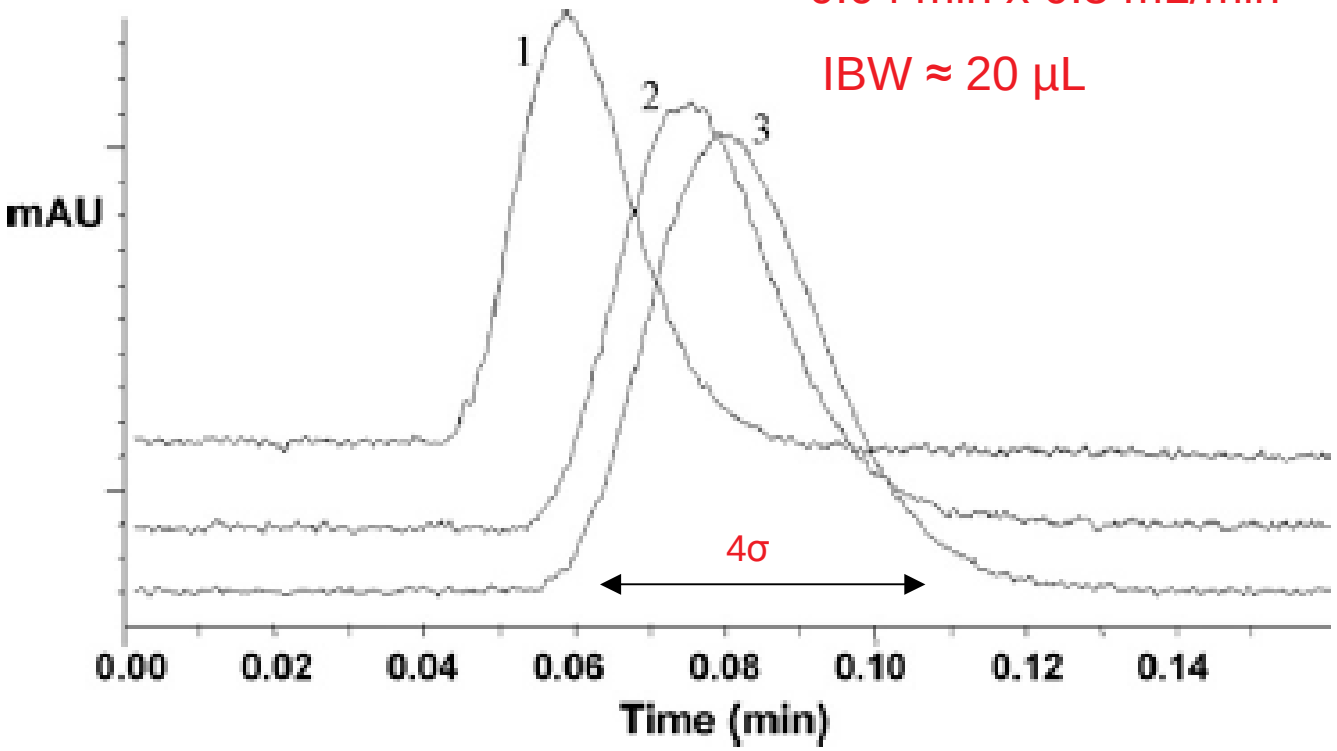

Fig. 9. Extra-column band profiles of the different liquid chromatographic systems. 1: Waters Acquity UPLC; 2: Agilent 1200 RRLC; 3: Shimadzu Prominence UFLC.

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Summary of Variables that Affect Instrument Bandwidth and System Suitability

Instrument volume should be small with respect to column internal volume which determines peak volume at base.

- Reduce tubing ID and volume
- Reduce tubing length and volume
- Reduce detector flow cell volume
- Improve detector response time
- Match data collection rate to peak width in time
  - At least 20-30 points across the peak

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Dispersion References

1. R. Majors, *Are you Getting the Most Out of Your HPLC Column?,* LCGC NA, Vol. 21, No. 12, 1124-1133 (December 2003).
2. R. A. Henry and D. S. Bell, *Important Guidelines for Optimizing Speed and Sensitivity in Small Molecule LC-UV and LC-MS,* LCGC NA, Vol. 23, No. 5, 2-7 (May 2005).
3. T. Chester, *Sub-2µm Performance with a Conventional Instrument Using 2.7µm Fused-CoreTM Particles,* American Lab, Volume 41, No. 4, 11-15 (2009).
4. S. Fekete, et. al., *Shell and Small Particles: Evaluation of New Technology,* J. of Pharmaceutical and Biomedical Analysis, 49, 64-71 (2009).
5. F. Gritti, G. Guiochon, et. al., *Achieving Full Performance of Columns by Optimizing HPLC Instruments,* J. of Chromatogr. A, 1217, 3000-3012 (2010)
6. H. Brandes, unpublished data, Sigma-Supelco Applications Lab Reports and Notebooks (2008-2009).
7. R. A. Henry and D. Nowlan, *Use of Sub-2µm Zirconia-PBD at Elevated pH and Temperature,* Oral Paper, EAS 2009, Somerset, NJ.

Thanks for your attention!

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