optimizing instruments for modern HPLC columns

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Analytical Products

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Abstract

Higher performance HPLC columns result in higher resolution chromatograms with very narrow peak widths that cannot be achieved by all HPLC instruments in the laboratory, independent of pressure limitations.

For example, porous-layer particles can deliver peak widths comparable to sub-2 µm particles with flow resistance comparable to 3 µm particles so that columns can easily be operated within the pressure range of traditional HPLC instruments.

However, UHPLC performance and narrow peak widths can only be observed with older, traditional instruments that have adequately low instrument bandwidth (i.e. dispersion). This paper will give an overview of the problem and describe some simple ways to qualify HPLC instruments, within their operating parameters, for use with modern, higher performance columns.

Band Broadening in Column (at two different times)

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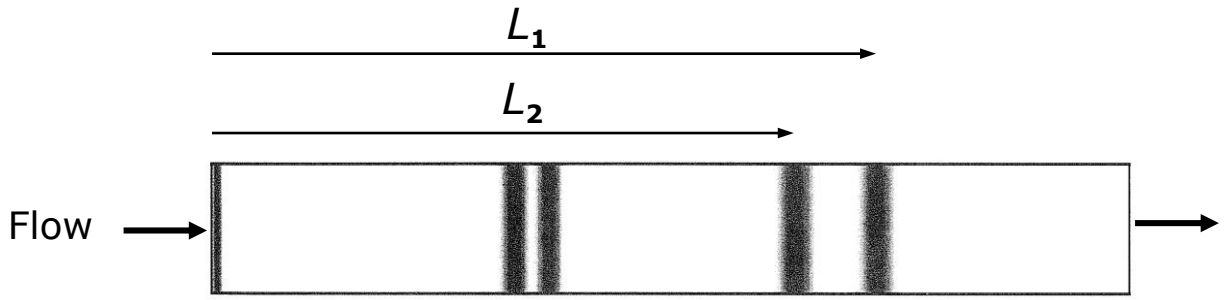

Peak width, w $\propto L^{1/2}$

$\Delta L \propto L$

Resolution, $R = \Delta L/w$

While peak width increases in proportion to the square root of the distance migrated, separation of peak centers increases in direct proportion to distance migrated; thus resolution increases as distance migrated increases. Longer columns produce higher resolution.

A chromatographic peak, modeled as a statistical distribution of molecules, has the property of standard deviation, σ. Therefore, total peak width can be closely estimated by $w = 4\sigma$, where w or σ is measured in units of length, time, or volume.

U. D. Neue, *HPLC Columns: Theory, Technology and Practice*, Wiley-VCH (1997).

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Chromatographic Peak Variance

$$\sigma^2_{obs} = \sigma^2_{col} + \sigma^2_{instr}$$

$$(\sigma^2_{instr} = \sigma^2_{extra\text{-}col})$$

$$\sigma^2_{instr} = \sigma^2_{inj} + \sigma^2_{det} + \sigma^2_{tubing}$$

- σ^2_{col} is a fixed parameter of a column (for a given k value)
- Σ^2_{instr} is something user can affect (though specific to a given flow rate)
- IBW = $4\sigma_{instr}$ (or 4σ)

Dispersion in Packed Column Bed

$$\sigma^2_{obs} = \sigma^2_{col} + \sigma^2_{instr}$$

$$\sigma^2_{col} = V_0^2(1+k)^2/N$$

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

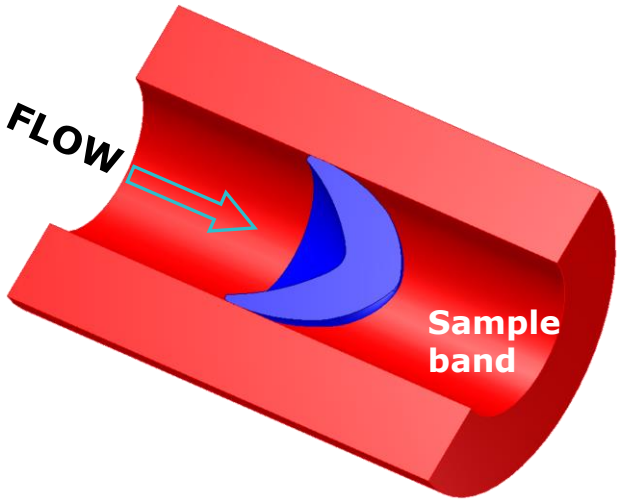
Dispersion within the column increases with retention factor, k.

Small geometry, short retention and high efficiency favor low dispersion (dilution) in columns.

When column volume (V_0) is small, excessive instrument bandwidth is harmful to efficiency and resolution, especially at low k values.

Dispersion in Open Cylindrical Tube

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



d = cylinder diameter
L = cylinder length
F = flow rate
D = diffusion coefficient

Dispersion from volume elements is constant for a given flow rate, but bandwidth and dispersion increase as f(F).

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 [f(1/D)] in connectors.

Principle of Measuring IBW

Recall that for chromatographic peak,

$$\sigma^2_{obs} = \sigma^2_{col} + \sigma^2_{instr}$$

If therefore, σ^2_{col} is eliminated, the observed peak variance is due only to variance originating from IBW.

Two common methods for eliminating σ^2_{col}, each requiring different experimental setups:

- Extrapolation method
  - makes use of actual chromatographic data, simple data transformations, and graphical extrapolation to $V_0 = 0$.
  - This method was not used for this study because results vary with performance of column and relative size of IBW vs V_0.
- Direct method
  - directly connect injector to detector
  - make injection and record peak, retention time, N; calculate σ (and thus 4σ)
  - can also calculate 4σ by hand from print-out of peak

Direct Method for Measuring IBW

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Connect injector to detector

- ZDV union
- shunt

Inject small volume (µL or less) of chromophore

Record peak retention time and N

Calculate IBW:

$$\sigma = (r.t. * flow) / \sqrt{N}$$

$$IBW = 4\sigma$$

Devil is in the details

- data sampling rate
- detector response time
- flow rate
- calculation of N

These determinations of IBW are specific to the defined flow rate.

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Details of IBW Measurement

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Data Sampling Rate

- too low a sampling rate will result in truncated peak heights, thus yielding artificially low values of N; this leads to artificially higher calculated values of σ.

Detector Response Time

- too slow a detector response will result in artificially broad peaks and longer retention times.

Flow rate

- effects dispersion (and thus IBW) but also informs consideration of appropriate data sampling rate and detector response time.

Calculation of N

- To maximize data precision and accuracy, calculate N based on peak width-at-half-height rather than peak width-at-baseline; determination of peak width at baseline can be problematic and less certain.

Standard Protocol for Measuring IBW

mobile phase:	40:60, water:methanol
test probe:	1% (v/v) acetone in mobile phase
flow rate:	0.1 mL/min.
det.:	250 nm
injection:	0.5 µL
sampling rate:	≥ 10 Hz
detector response time:	≤ 0.1 sec

record peak retention time and N (N by peak width-half-height method)

calculate IBW:

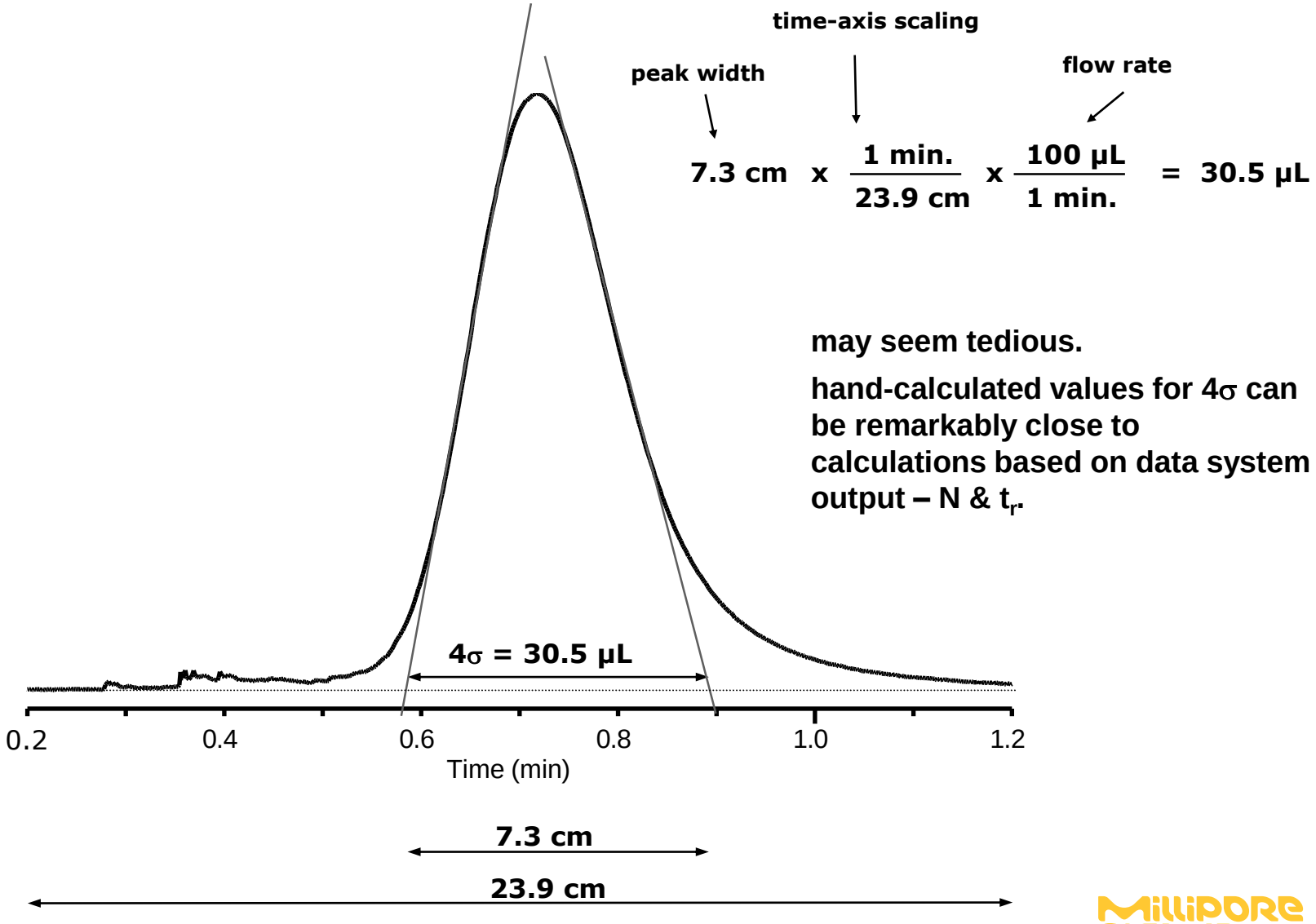
$\sigma = (t_r * \text{flow}) / \sqrt{N}$

$\text{IBW} = 4\sigma$

Alternate Method for Hand-Calculating IBW

Identical experimental protocol to calculated method:

1. Expand chromatogram region of interest.
2. Make hardcopy.
3. Draw tangent lines and baseline with ruler.
4. Measure distances.
   - peak width measured where tangent lines intersect baseline.
   - time axis scaling.
5. Calculate peak width as shown in example.



may seem tedious.

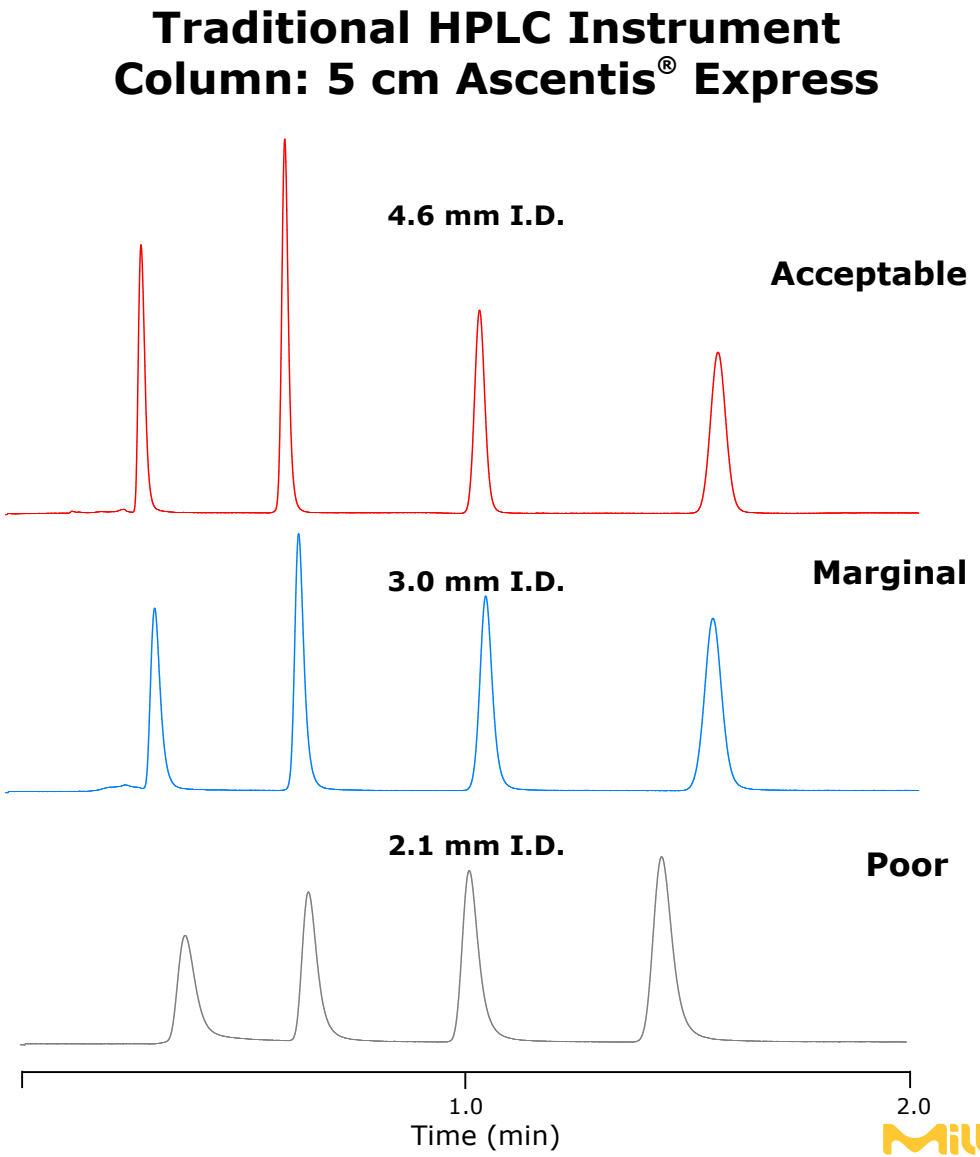
hand-calculated values for 4σ can be remarkably close to calculations based on data system output – N & t_r.

IBW with Various Column I.D.s

Traditional LC plumbed with 0.007” I.D. tubing except the column outlet which is 0.005” I.D.

column:	5 cm
mobile phase:	50% acetonitrile
flow rate:	0.30 (2.1 mm I.D.), 0.60 (3 mm I.D.), 1.44 (4.6 mm I.D.)
det.:	250 nm
inj.:	1 (2.1 mm I.D.), 2 (3 mm I.D.), 5 (4.6 mm I.D.)
sample solvent:	25% acetonitrile
analytes:	acetophenone, benzene, toluene

Same y-axis scale



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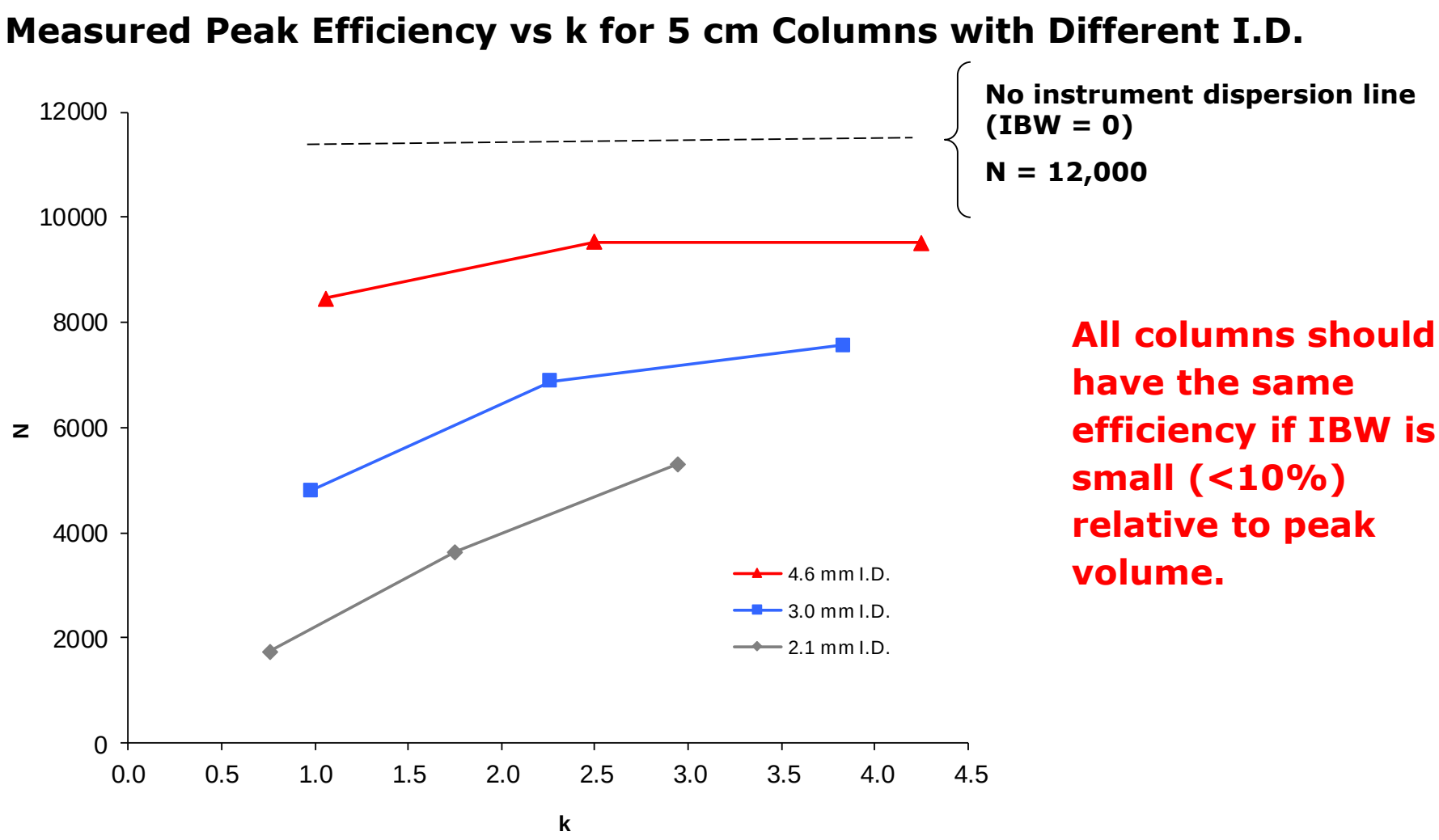
IBW Effects *vs* Column I.D.



All columns should have the same efficiency if IBW is small (<10%) relative to peak volume.

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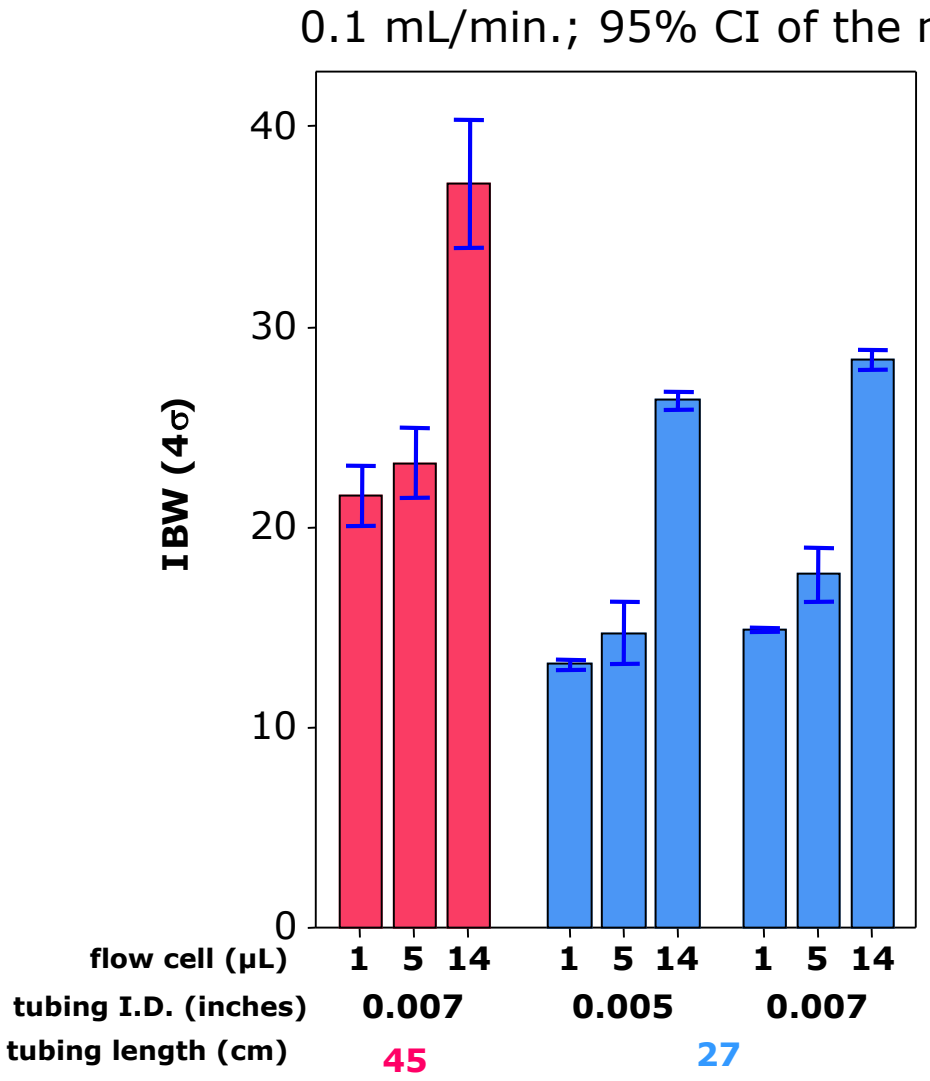
Affecting IBW

IBW impacts column performance more as column internal volume becomes smaller; instrument internal volume should always be kept negligible with respect to column internal volume for high system efficiency.

IBW can be minimized by the following steps:

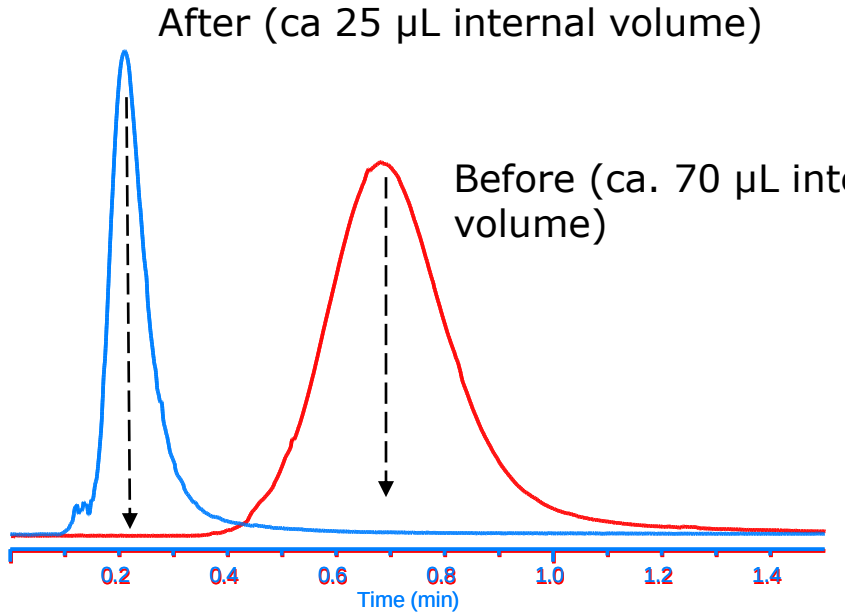
- reduce tubing I.D. (and volume)
- reduce tubing length (and volume)
- reduce detector flow cell volume

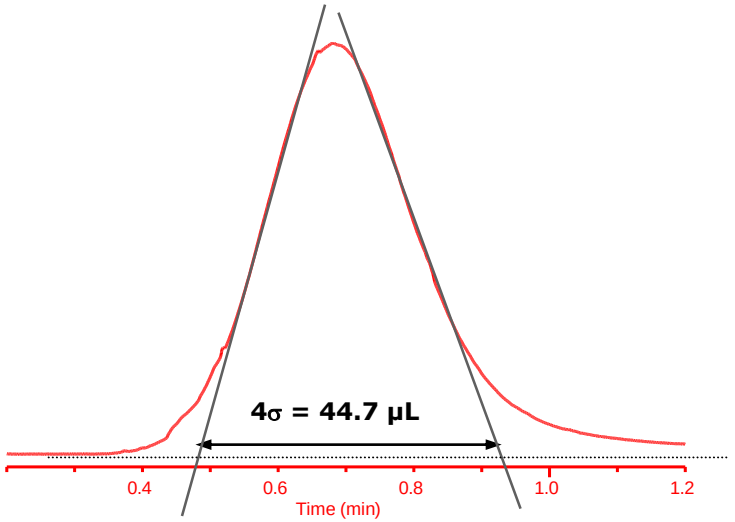
IBW of Agilent 1100 vs Instrument Configuration

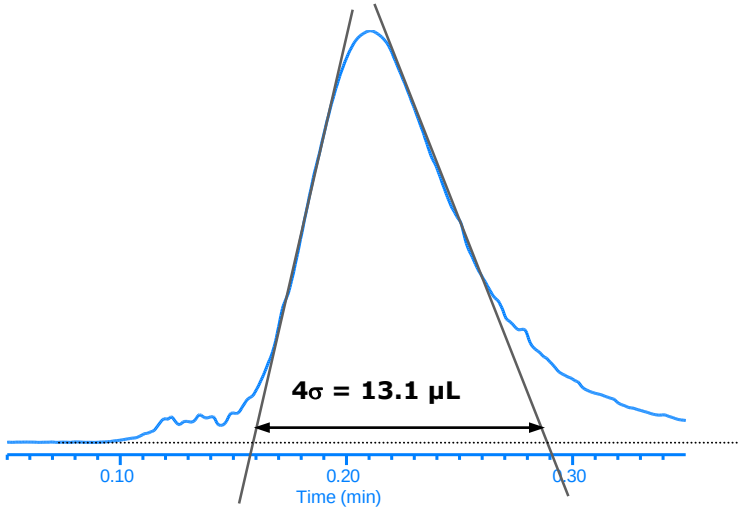


- tubing length affects IBW
- tubing I.D. affects IBW
- flow cell volume affects IBW, but likely more so flow cell design and engineering
- *flow affects IBW*

Example of Graphical Comparison of IBW Before and After Instrument Optimization







Clear contrast between peak profiles before and after optimization.

Graphically demonstrates band-broadening (dispersion) that increases as a function of IBW.

Note that t_R measurement also provides an estimate of instrument internal volume.

Agilent 1100 Optimized for Minimum IBW (IBW = 12.4 µL)

Tubing I.D.

- small I.D. tubing
  - 0.005” I.D. (instead of 0.007” or 0.010” I.D.)

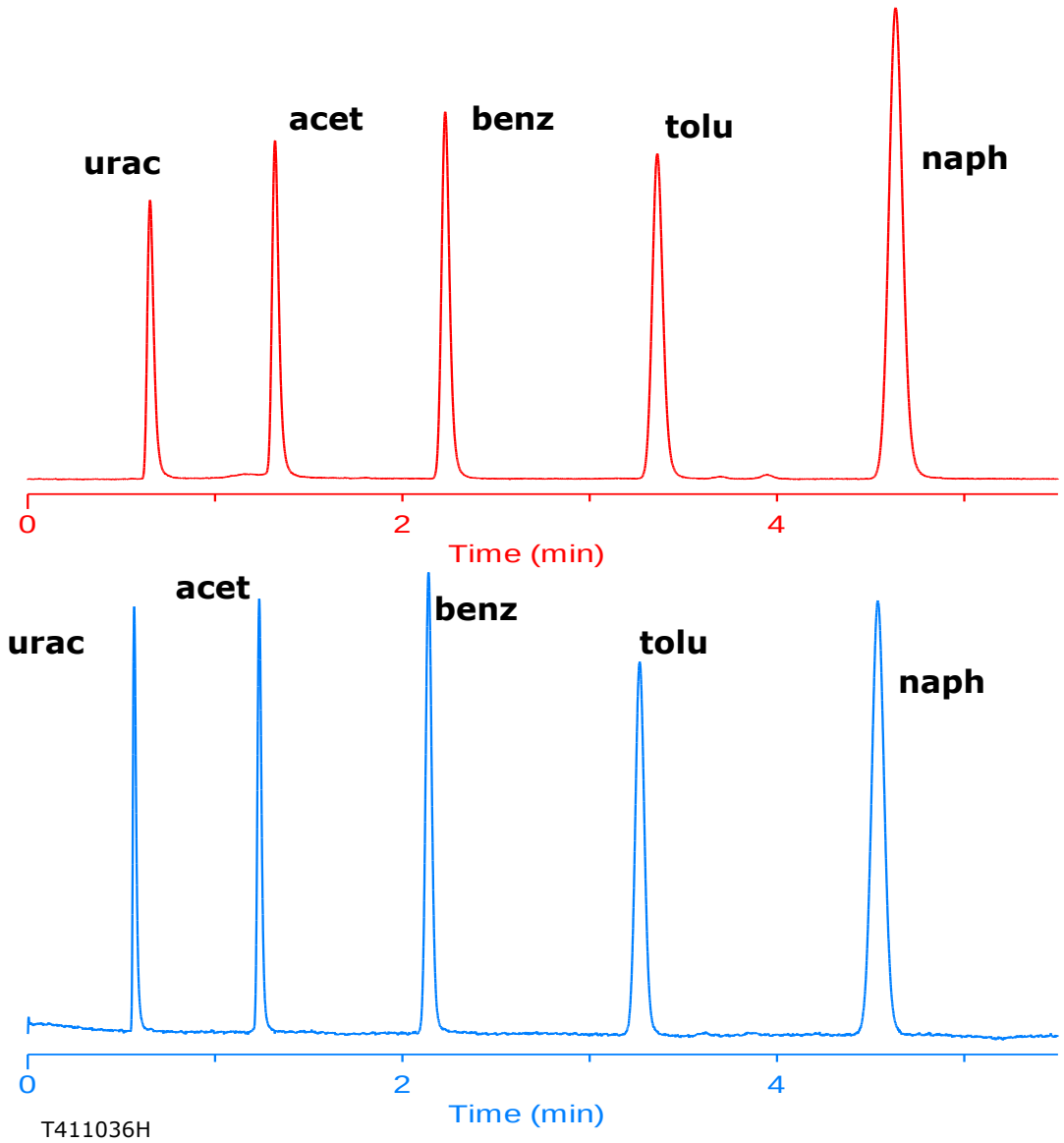
Tubing Lengths

- minimized column inlet and outlet lengths
- low-volume tubing
  - needle seat 10 cm (std is 10 cm as well, but 0.007” I.D.)
  - inlet 7 cm (std is 7 cm as well, but 0.007” I.D.)
  - outlet 15 cm (instead of 38 cm)

Flow Cell (VWD detector)

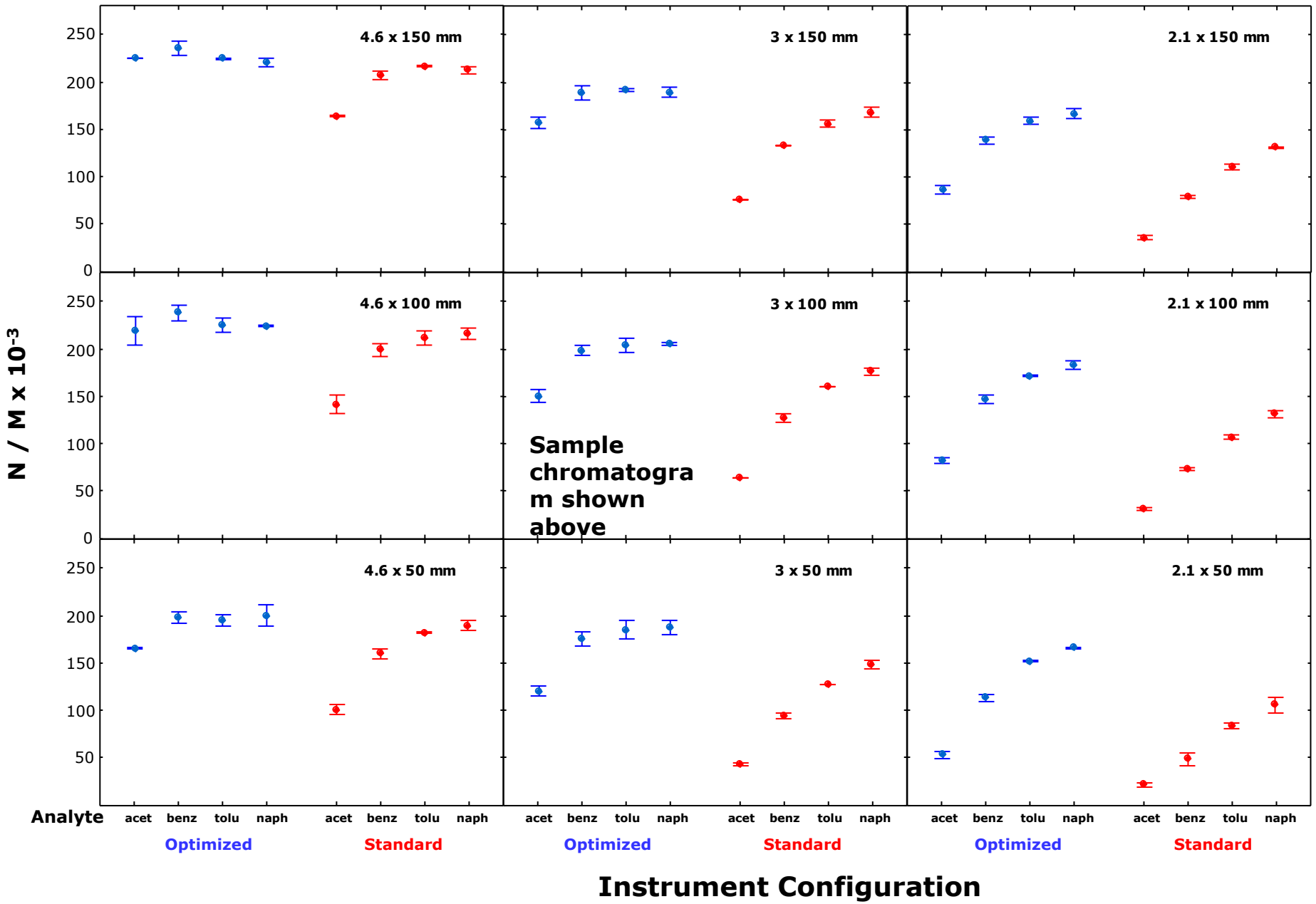
- 1 µL, 5 mm path length (instead of 5 µL, 6 mm or 14 µL, 10 mm)

Column Performance Example: Before and After Optimization



3 x 100 mm Ascentis® Express

	Peak Efficiency			
	$N_{(acet)}$	$N_{(benz)}$	$N_{(tolu)}$	$N_{(naph)}$
standard	6410	12720	16030	17640
optimized	15040	19790	20430	20470
percent improvement	135	56	27	16



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Conclusions

The following performance can be expected from Ascentis® Express columns, for k ≥ 2, as a percentage of specified peak efficiency:

		IBW (4σ)	
I.D. (mm)	L (cm)	≤ 15 µL	≥ 25 µL
2.1	5	70-100	40-80
2.1	10	90-100	60-90
2.1	15	90-100	60-90
3.0	5	80-100	50-80
3.0	10	90-100	70-100
3.0	15	100	70-100
4.6	5	90-100	80-90
4.6	10	100	90-100
4.6	15	100	100

Conclusions (contd.)

- IBW directly affects the performance that can be realized from high-resolution (small particle) HPLC columns.
- Assessment of IBW can be made in a straightforward manner if details of the experimental protocol are followed.
- Due to differences in system design and engineering, all contributions to system volume must be considered in minimizing system IBW.
- This includes tubing I.D., tubing length, and flow cell volume.