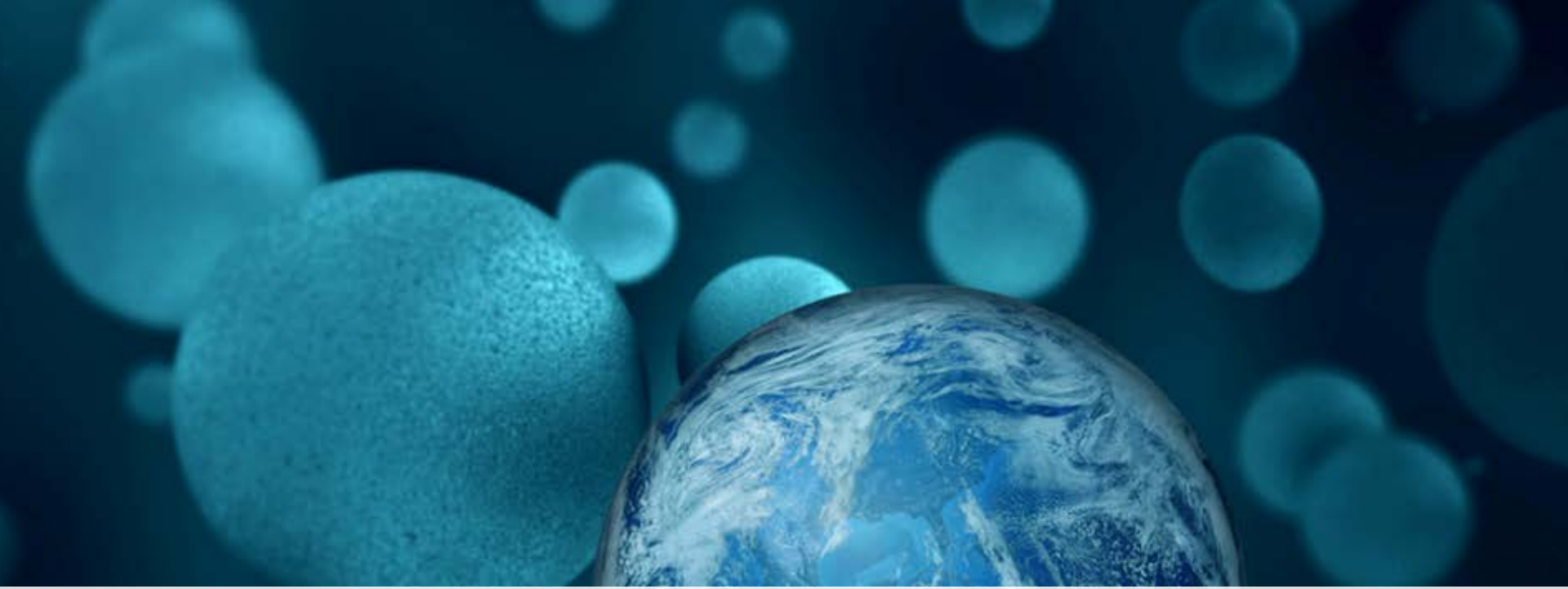

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From HPLC to UHPLC: What are the Instrumental Requirements and Pitfalls?

Dr. Markus M. Martin
Thermo Fisher Scientific, Germering/Germany

Strategy for Speed Optimization

Use small particles in short columns

Leads to same efficiency in shorter time

Enables faster separations with less peak broadening

Shorter analysis time
Less eluent consumption
Less sample needed

Same result!

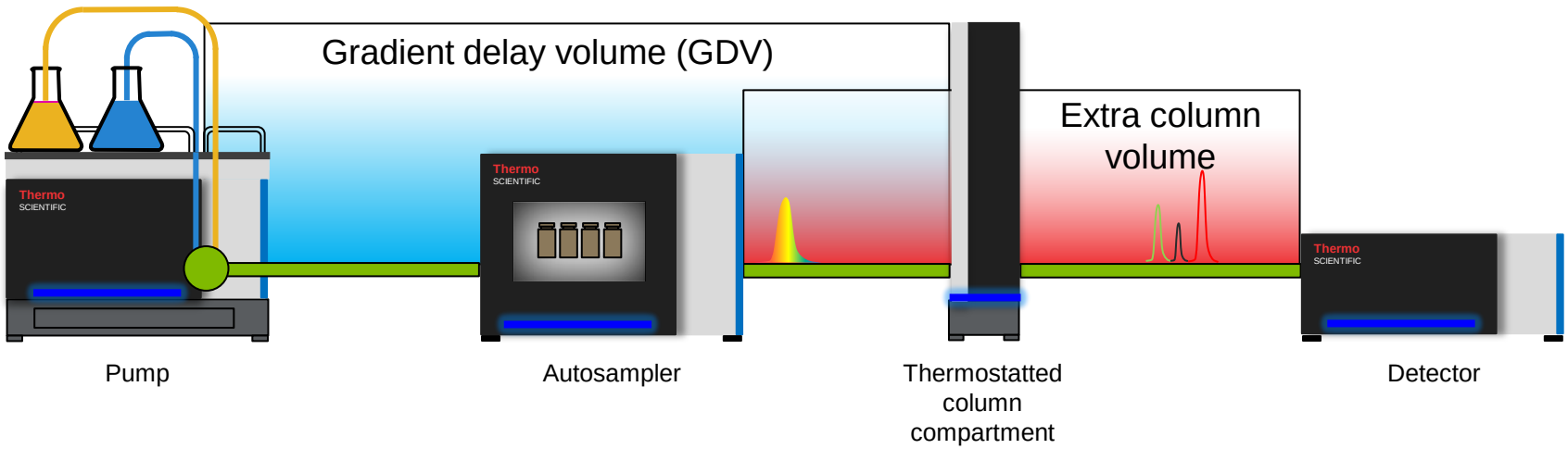
UHPLC Systems: What do I Need to Consider?

Detector:
- Flow cell
- Data rate (DCR)
- Filter parameters

Capillaries:
- Extra column volume (ECV)
- Diameter

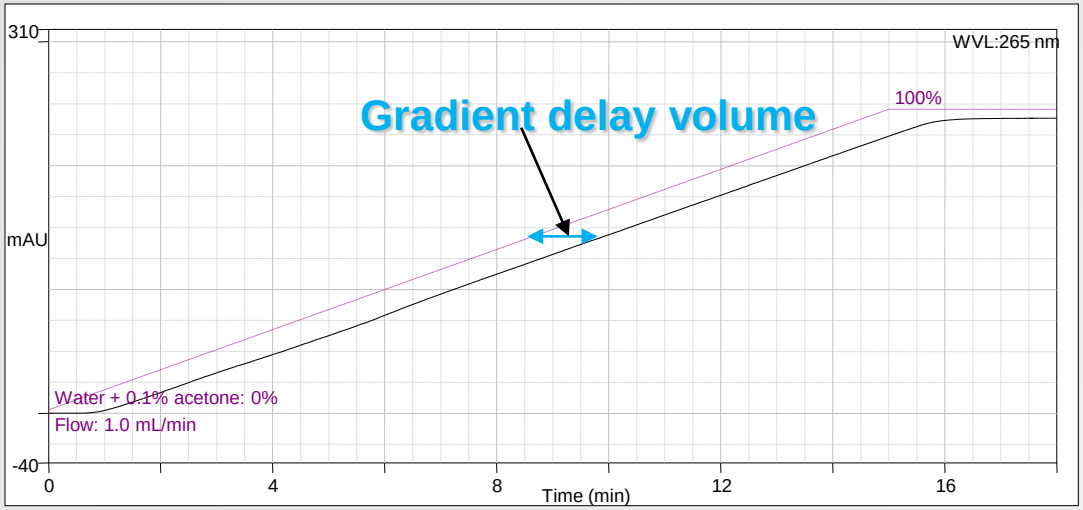
Pump:
- Operating pressure range
- Gradient delay volume (GDV)

Gradient Delay Volume and Extra Column Volume



- **Gradient delay volume (GDV):**
  Volume of fluid between mixing point of the gradient and column head

- **Extra column volume (ECV):**
  Volume of fluid between sample injection point and midpoint of the detector's flow cell.



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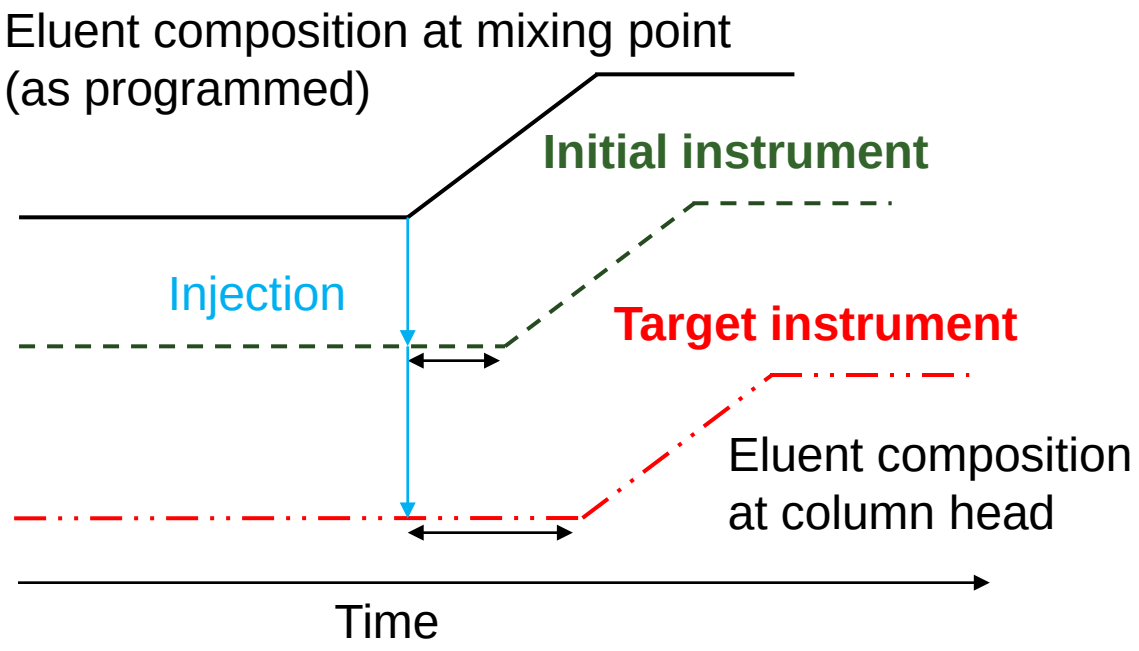
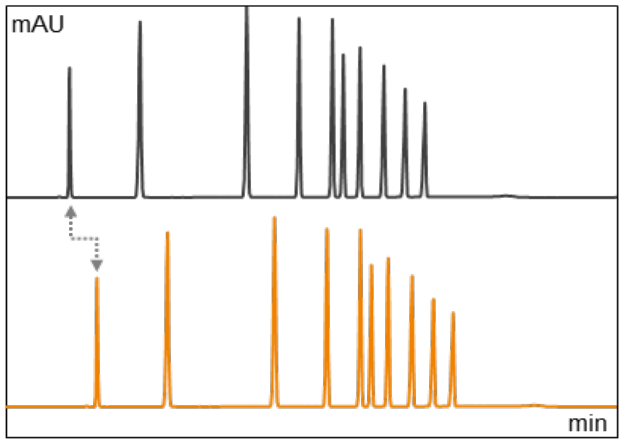
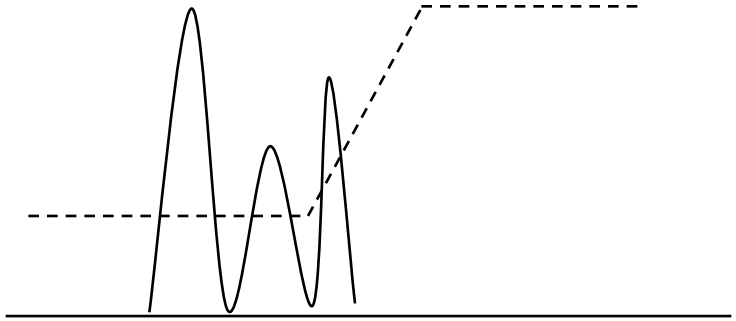
How Does the GDV Influence my Method Transfer?



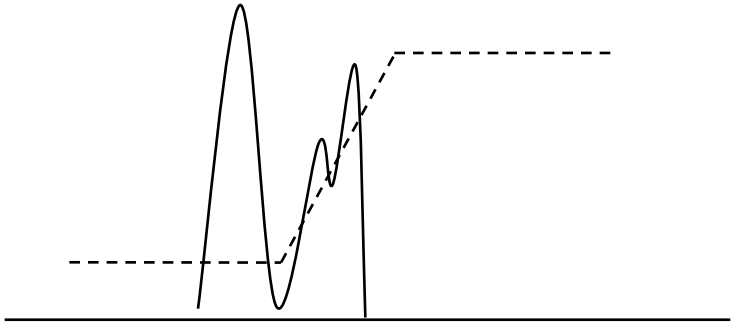


- Compare Gradient delay volumes
- If the target instrument has a smaller GDV:
  → Delay the start of the gradient program
- If the target instrument has a larger GDV:
  → Minimize the isocratic segment (Ex: Use micoflow kits, tubing with smaller ID, autosampler bypass)

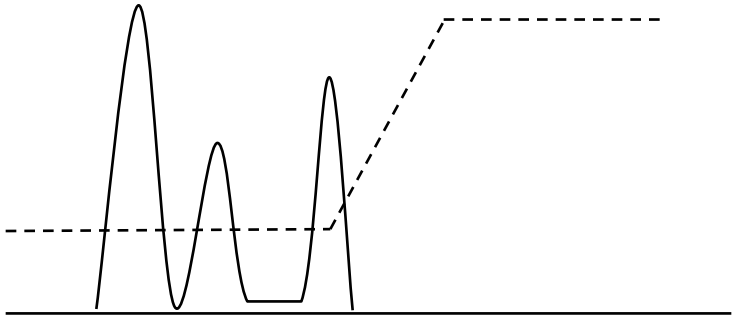
Various GDVs and their Influence on Peaks Eluting Early



Original system

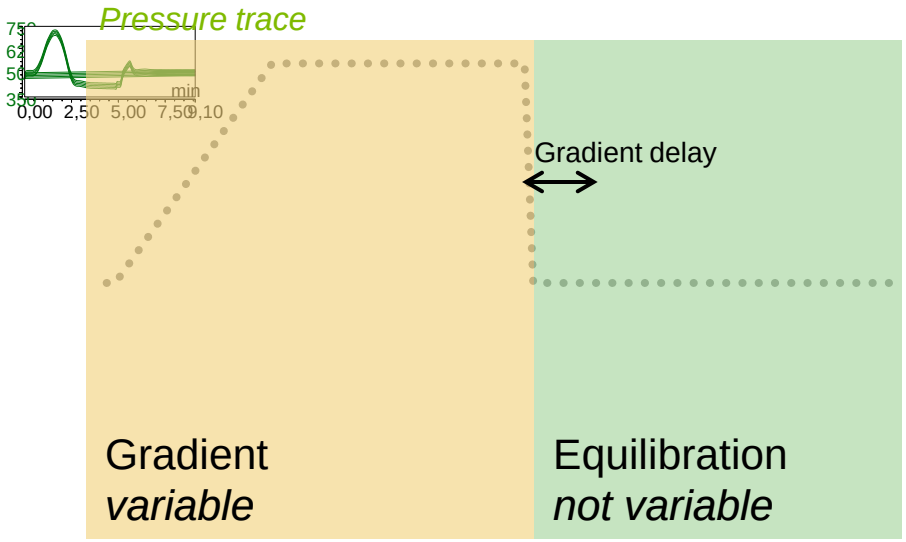


Target system with smaller GDV

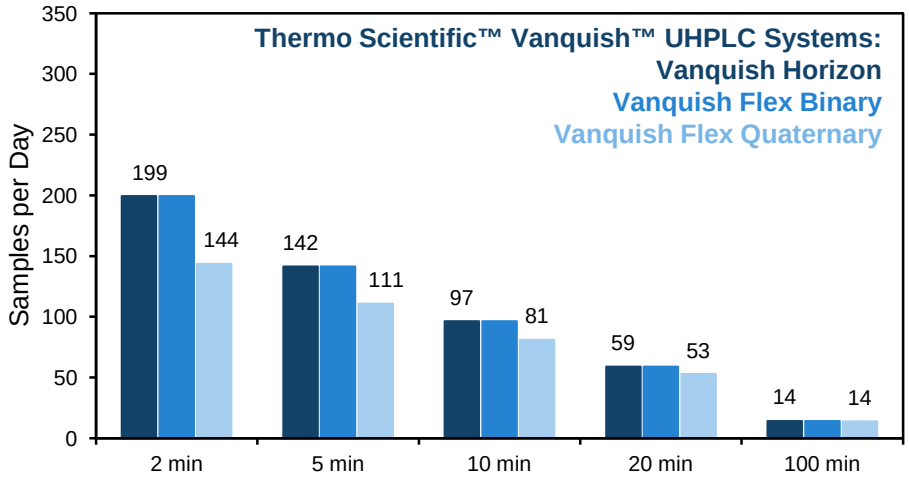


Target system with larger GDV

Influence of Gradient Delay Volume (GDV) on Throughput



Equilibration plays a larger role with shorter runs



Short methods and higher throughput

- The duration of column equilibration depends on GDV
- The smaller the GDV, the shorter the equilibration
- Column equilibration most impacts the method length of shorter methods.

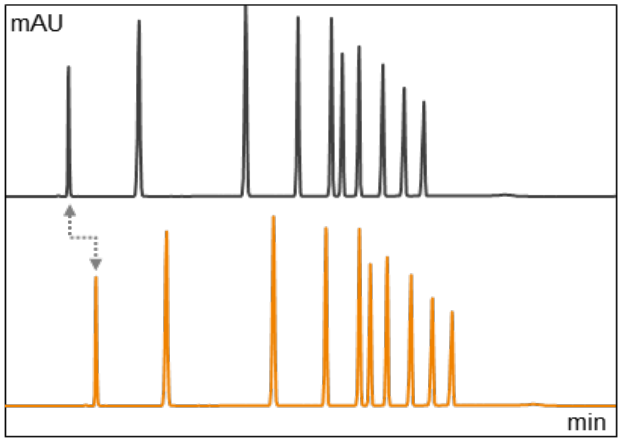
→ ***For throughput maximization: Binary pumps with smaller GDVs***

→ ***The GDV is negligible for longer runs.***

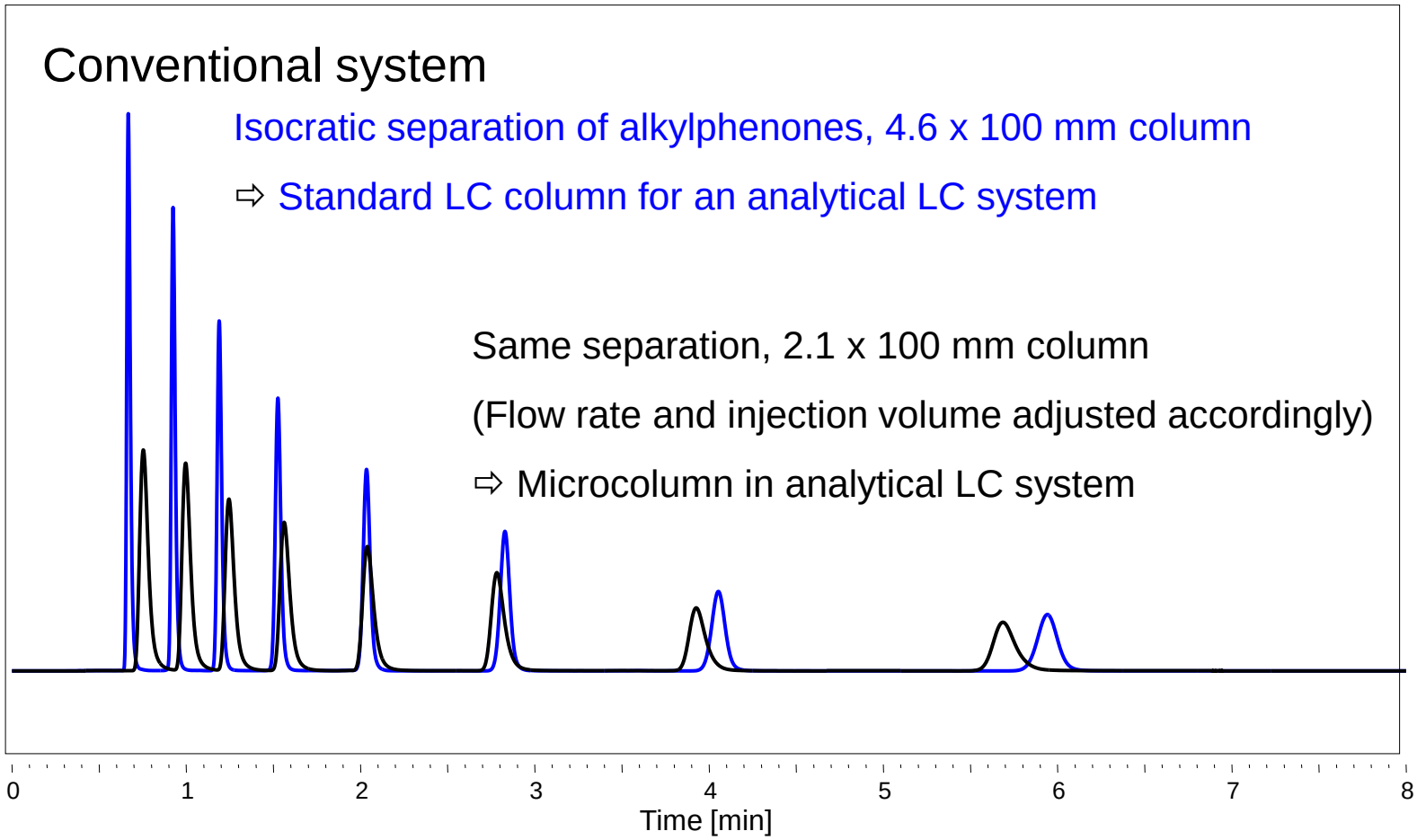
Effects of the Gradient Delay Volume (GDV)

- GDV influences/causes
  - An isocratic step at the beginning of every gradient separation
  - Accuracy of the gradient
  - Time required for column equilibration time and, therefore, the entire time required for the analysis
- Weakly retained analytes are generally more affected by the GDV than late-eluting analytes.

⇨ Special consideration in the case of steep gradients and low flow rates



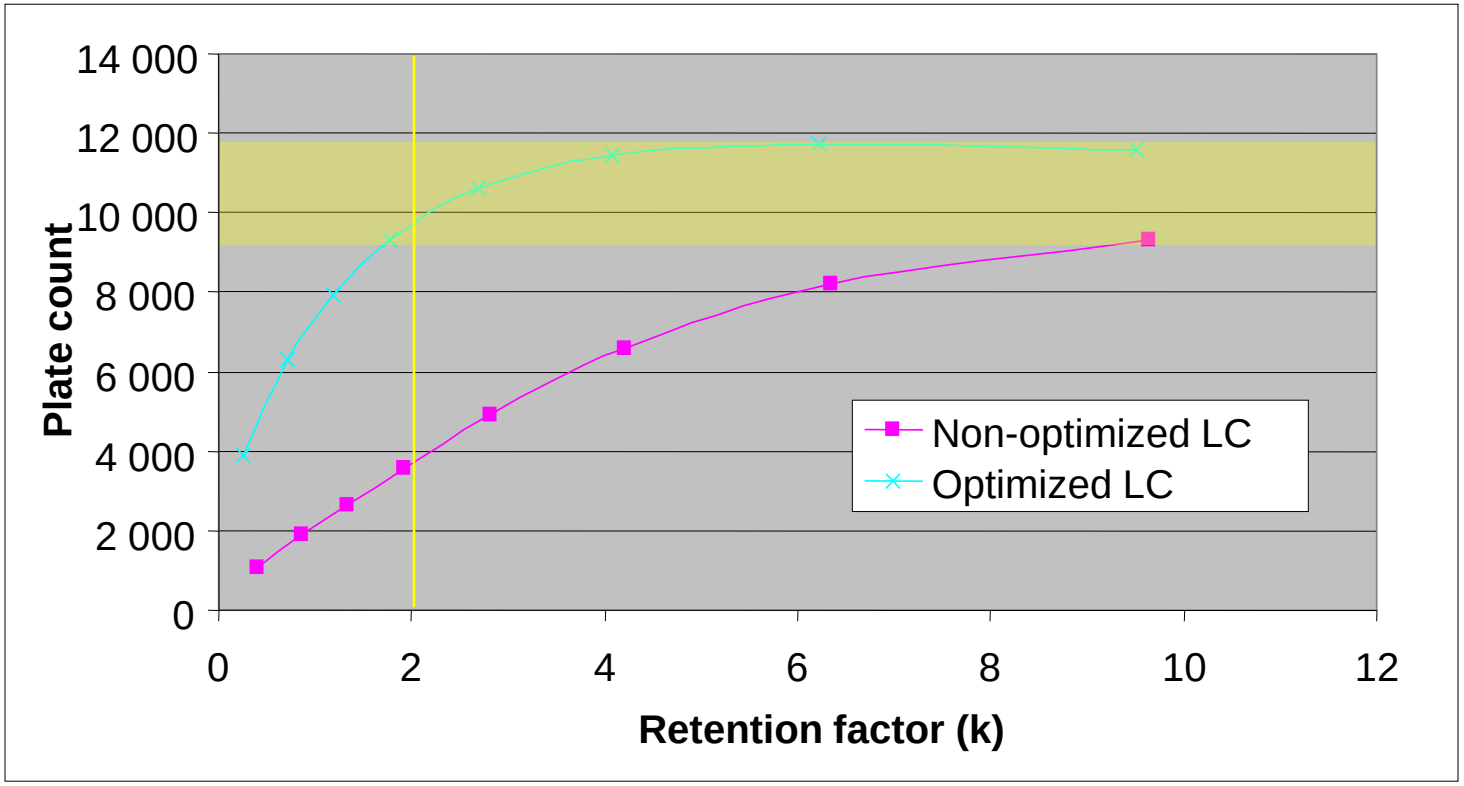
Effect of Extra Column Volume (ECV) on Efficiency and Resolution



- Serious peak broadening outside the column impairs resolution and detection sensitivity

Effect of ECV on Experimental Chromatographic Efficiency

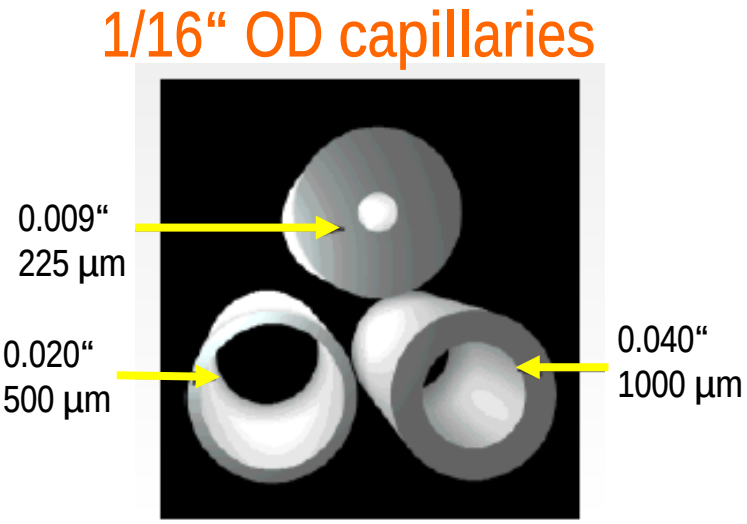
- Characterization of the efficiency of an ECV optimized LC system with a 2.1 mm ID column

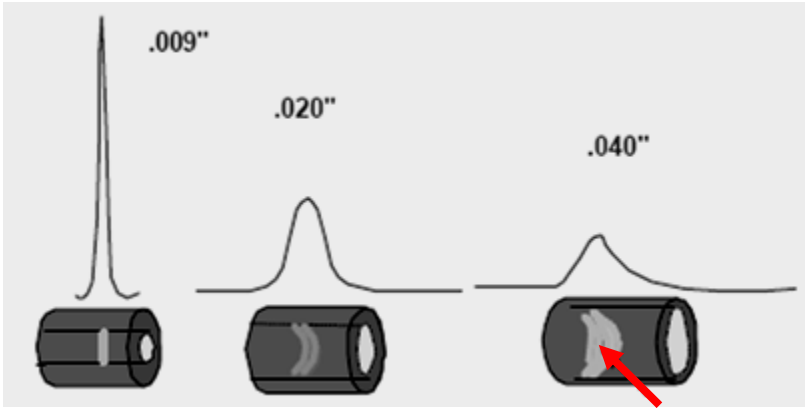


- The efficiency at k = 2 should be ≥80% of the typical column efficiency (plate number)!

Efficiency Loss Due to Incorrect Capillary Dimensions

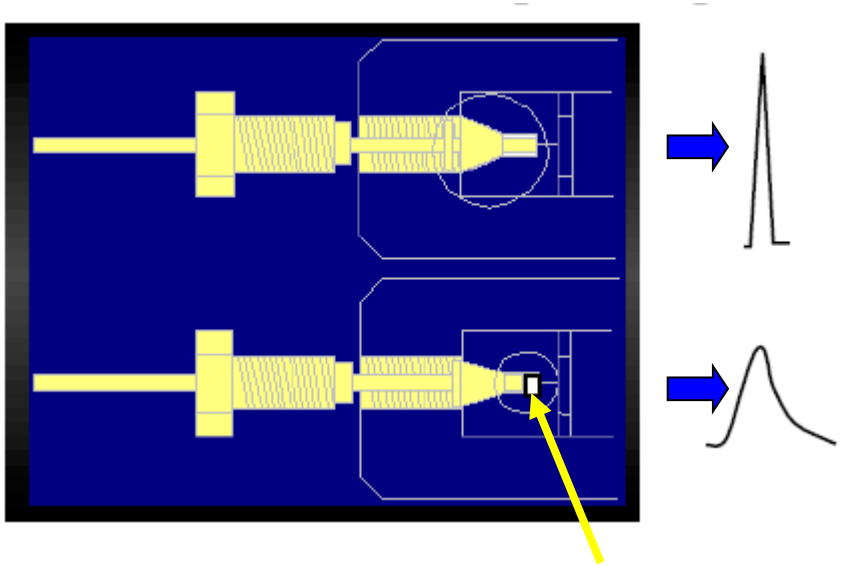
- The capillary volume must be reduced whenever...
  - …the volume of the column is reduced (applies to length and/or I.D.!).
  - …the efficiency of the column is increased.
- The capillary *inner diameter* has a significantly greater influence than the capillary *length.*





ECV – Quality of Threaded Connections and Capillary Construction

…a frequently neglected topic!



Dead volume if capillary not properly pushed into column during assembly

Fingertight

- Be sure to always use the correct type of ferrule and, upon tightening, to push the capillary into the head of the column.
- For capillaries in which steel ferrules have been used, never change the type of column hardware.
- UHPLC columns require special fitting systems in order to withstand higher system pressures.

System Contribution to Minimal ECV – Viper Fitting System

Cutaway of an assembled Viper fitting :

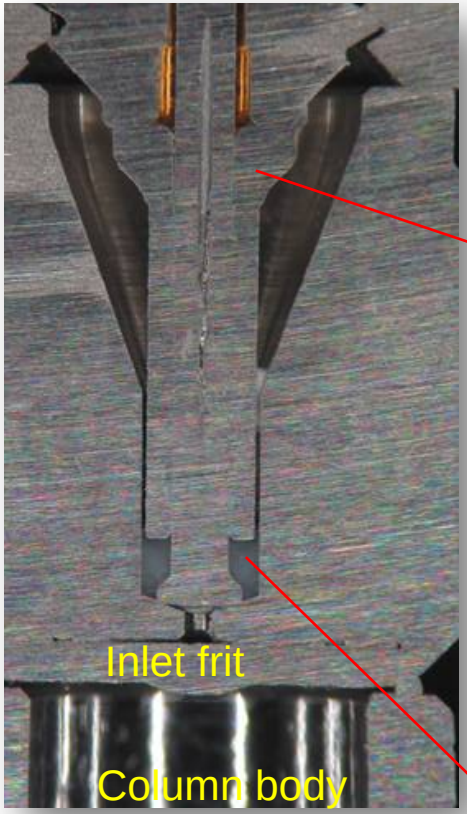
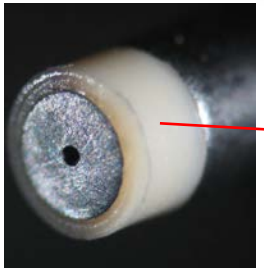
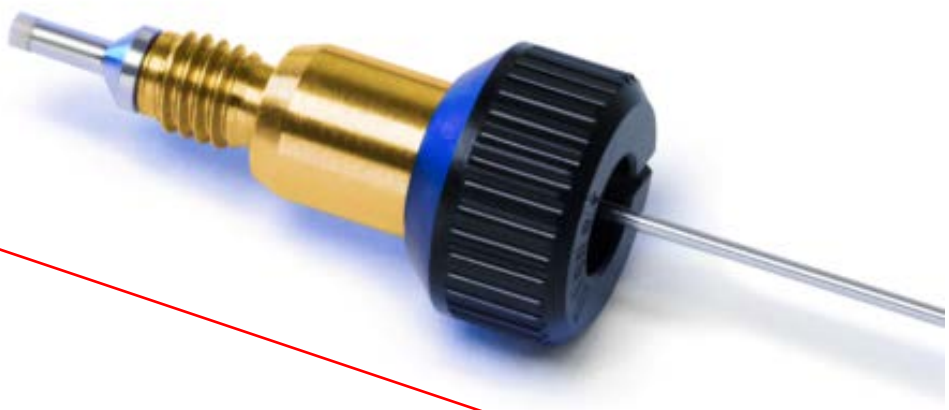


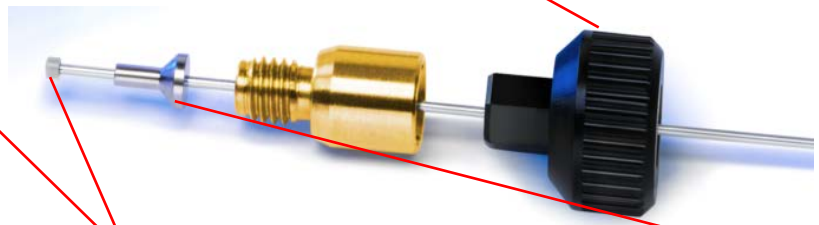
Photo of the front end:



Thermo Scientific™ Viper™ Fitting System



Removable knurl



PEEK™ (Victrex PLC) seal

Adapter (No ferrule!)

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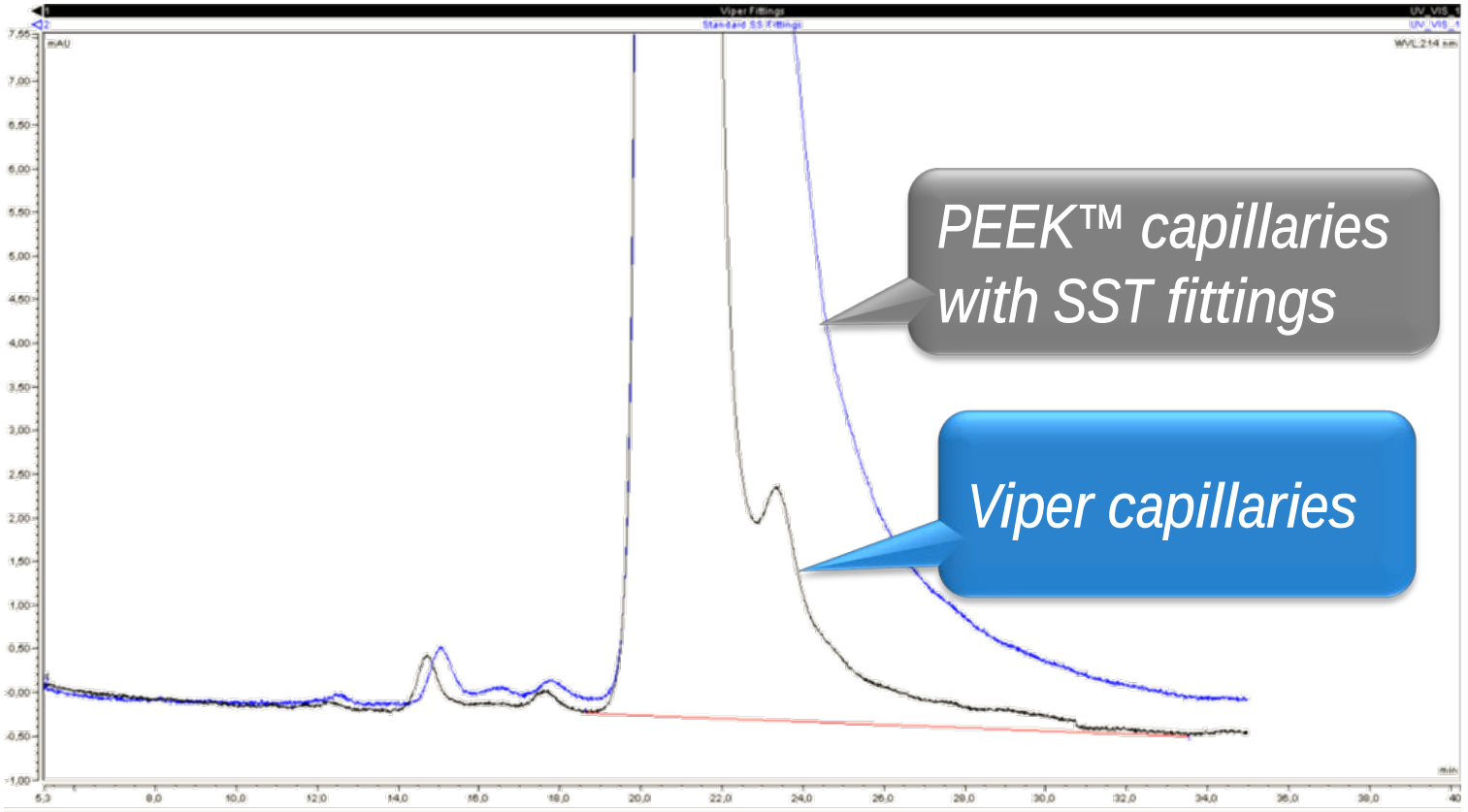
Example of Improved Separation Thanks to Viper Fittings

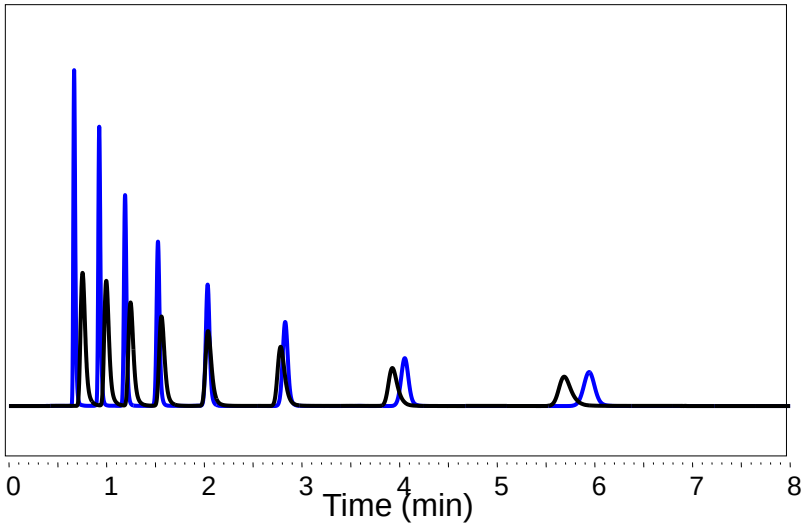


Both capillary sets with 180 µm i.d.

- By-product is hidden by extensive extra-column band broadening caused by standard tubing

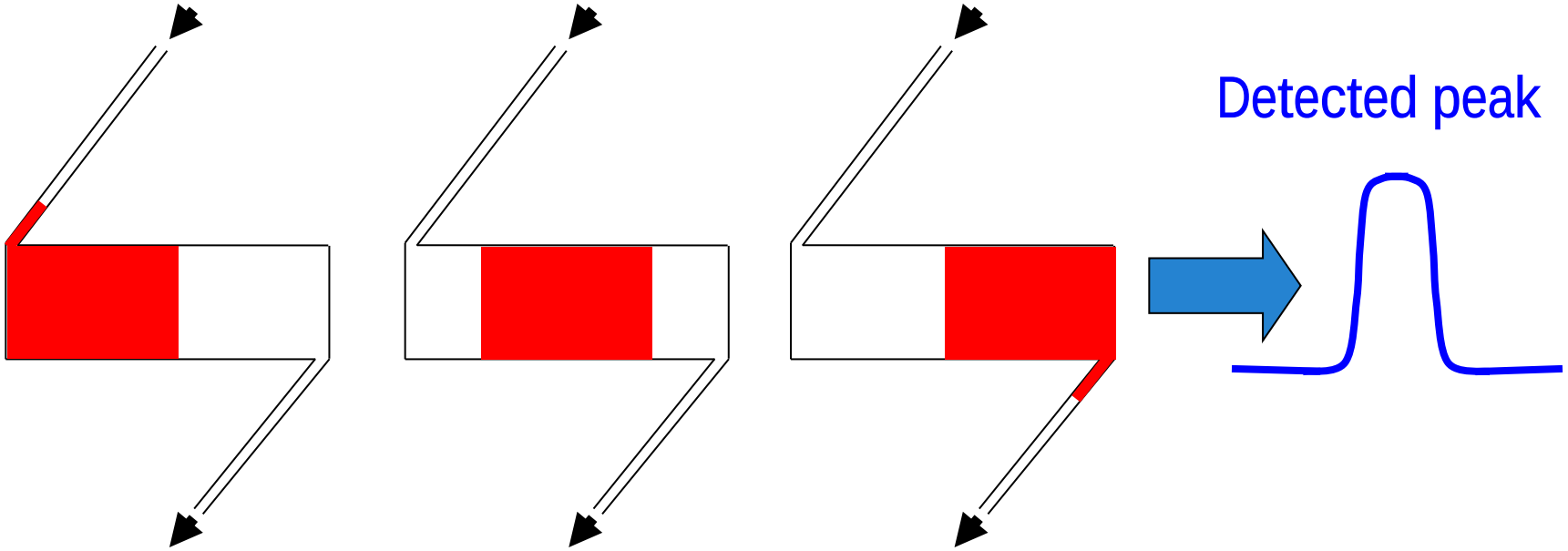
Effects of Extra Column Volume (ECV)

- ECV becomes more relevant for smaller columns (Shorter, reduced inner diameter)
- An ECV that is too large causes:
  - Peak broadening
  - Loss of separation efficiency
- ECV can be reduced by the use of:
  - Capillaries that have small diameters
  - Viper capillaries

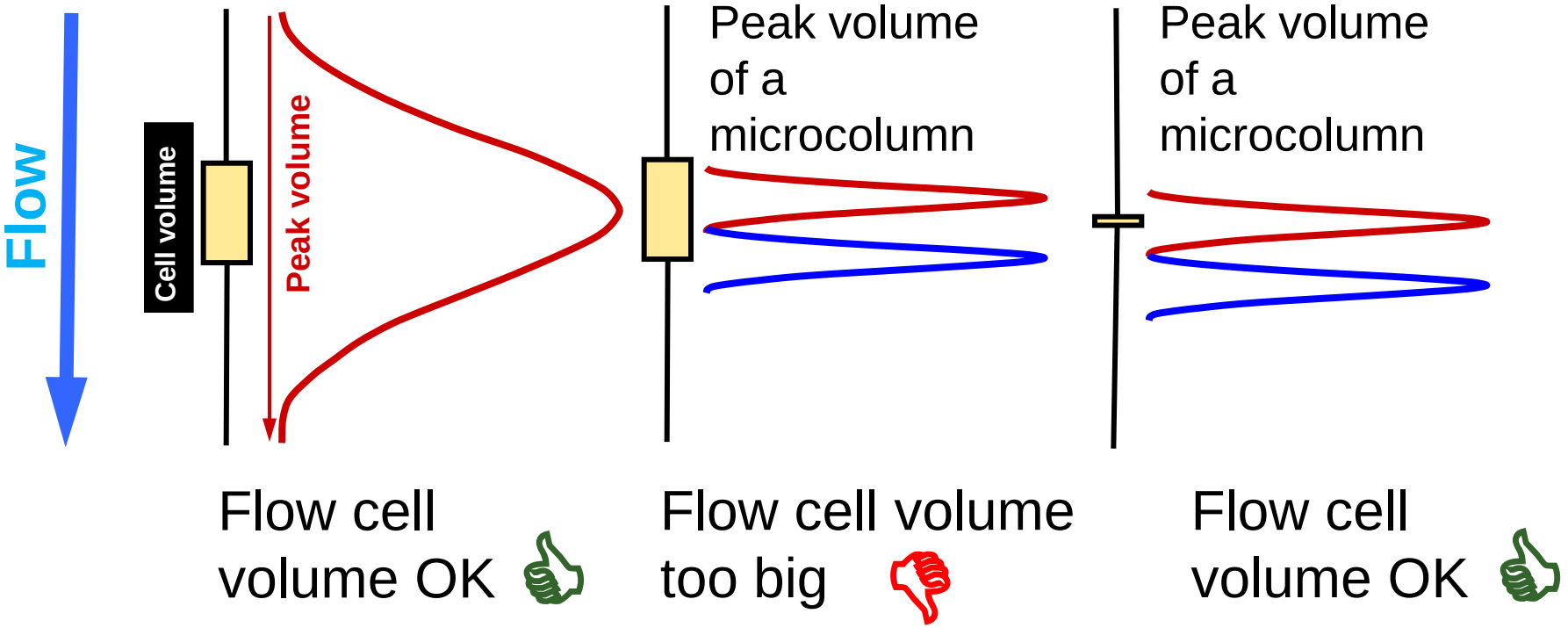


Effect of Detector Cell Volume

- What happens when a peak that has a significantly smaller volume than the detector cell enters the detector?



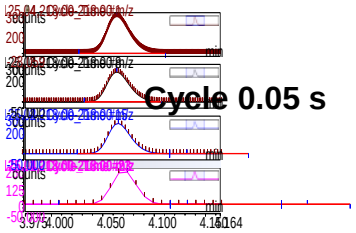
How Large Can a Suitable Detector Cell Be?



- If the cell volume is significantly greater than the peak volume, the detector will record separated peaks as one, not separately.
- The volume of the detector cell should be no more than 1/10 the volume of the smallest peak.

Data Rates/Cycle Times and Retention Time Precision

Data rates/cycle times can negatively affect retention time precision.



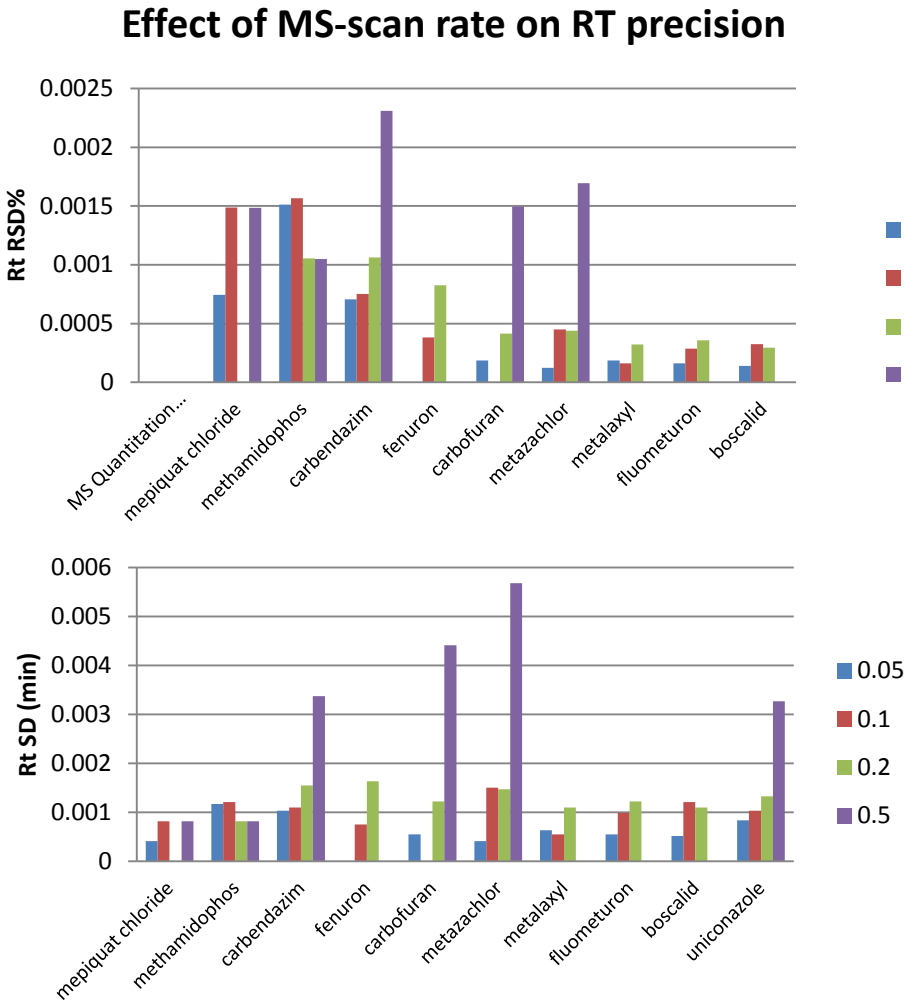
Cycle 0.05 s **20 Hz**

Cycle 0.1 s **10 Hz**

Cycle 0.2 s **5 Hz**

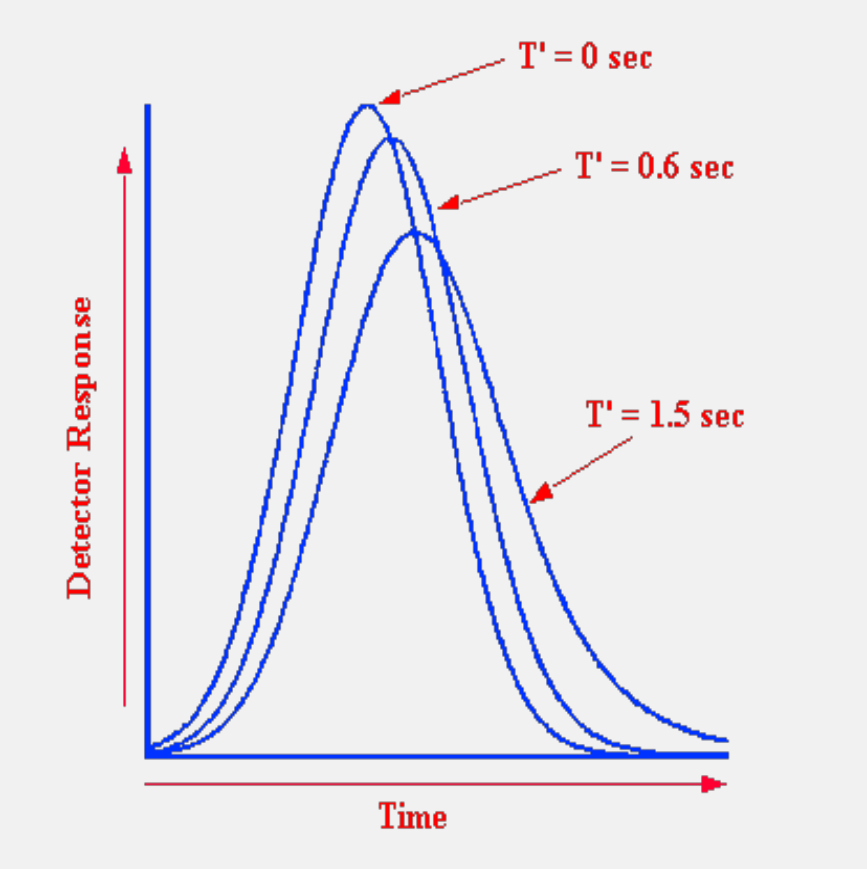
Cycle 0.5 s **2 Hz**

Averaged results of 6 replicates, 10 ppb pesticide mix, Thermo Scientific™ Vanquish™ Horizon and Thermo Scientific™ TSQ™ Quantiva™ LC-MS



Data rates of at least 10 Hz are necessary to achieve a retention time precision of SD=0.001 min (RSD% < 0.1%) for peaks with a half-height width of 0.025 min (1.5 s).

How Does a “Time Constant” or “Response Time” Change a Peak?



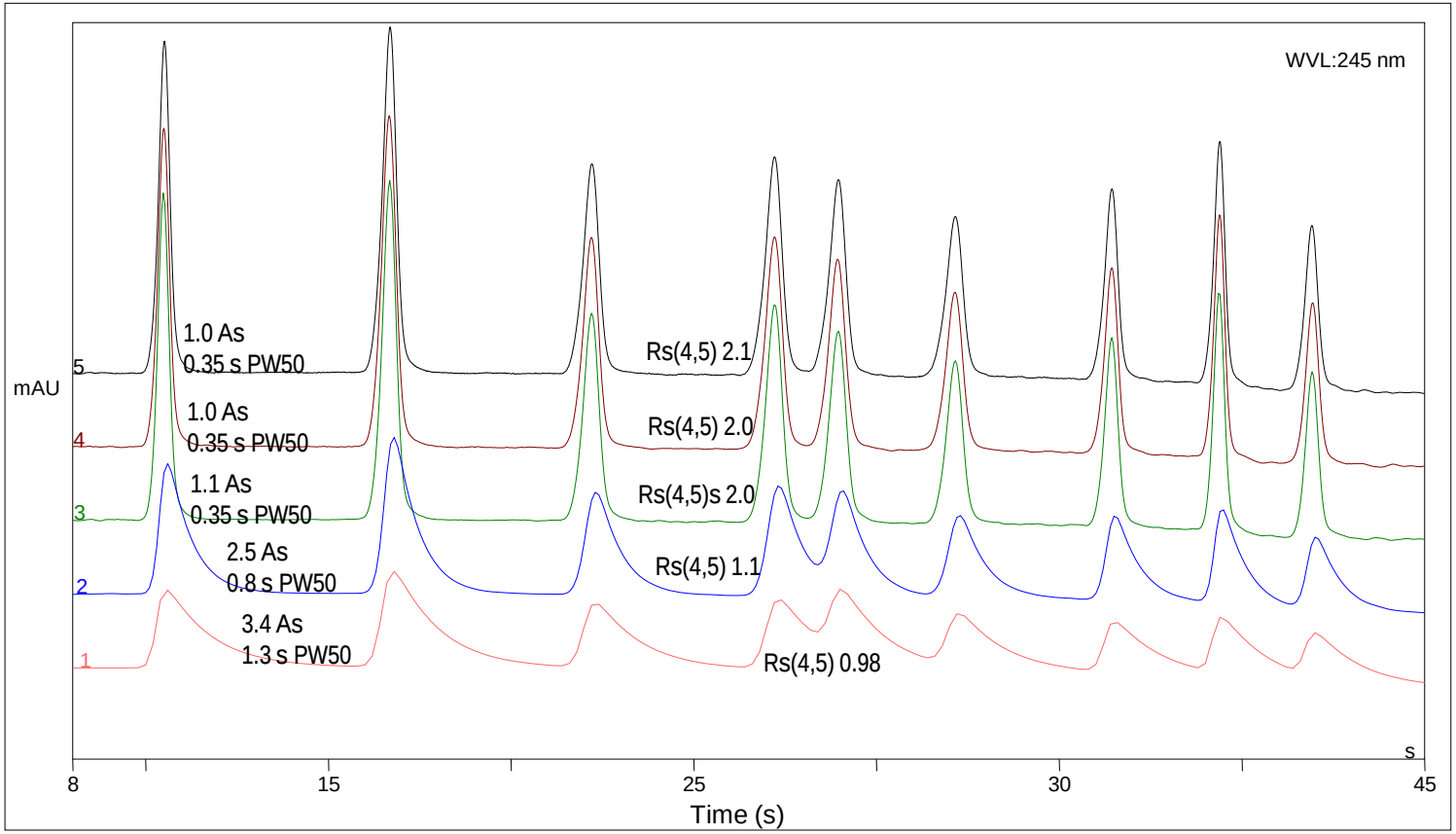
<u>As the time constant/response time increases:</u>

- Peak height decreases
- Peak width increases
- Retention time increases
- Peak symmetry becomes worse

<u>But also:</u>

- Noise decreases
- The optimal S/N relationship depends on maximized values for both data rate **and** response time.

What happens when I Vary the Detector Settings?



100 Hz, time const 0.01s

50 Hz, time const 0.02s

25 Hz, time const 0.025s

10 Hz, time const 0.5s

5 Hz, time const 1s

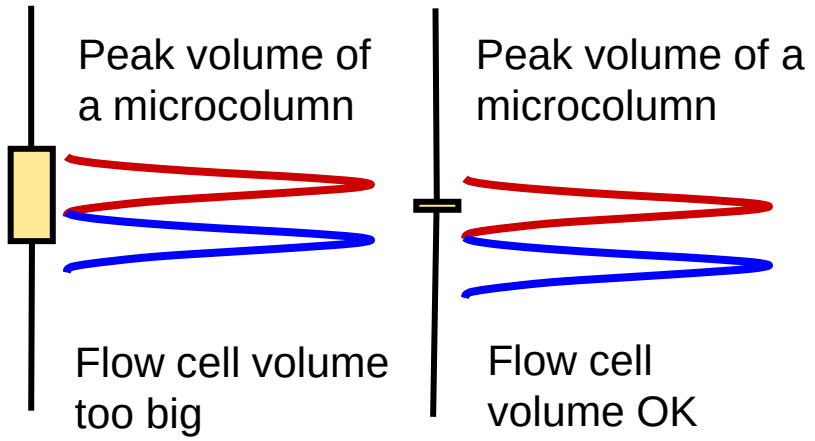
As: Peak asymmetry; Rs: Resolution between two peaks

- Smaller peaks
- Modified retention times
- Peaks distorted beyond recognition

Effects of the Flow Cell and the Detector Settings

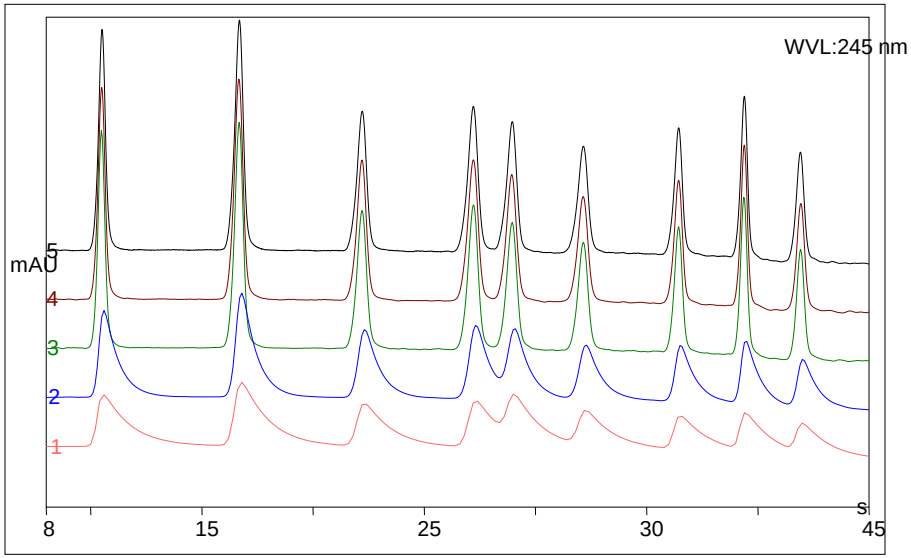
- The optimal flow cell should be
  - As big as possible
  - No more than 1/10 the volume of the smallest peak

- The optimal data collection rate should be
  - As small as possible, to avoid unnecessary large files
  - As large as needed to represent the peaks well (30-40 data points per peak)
  - Chosen in combination with a suitable response time

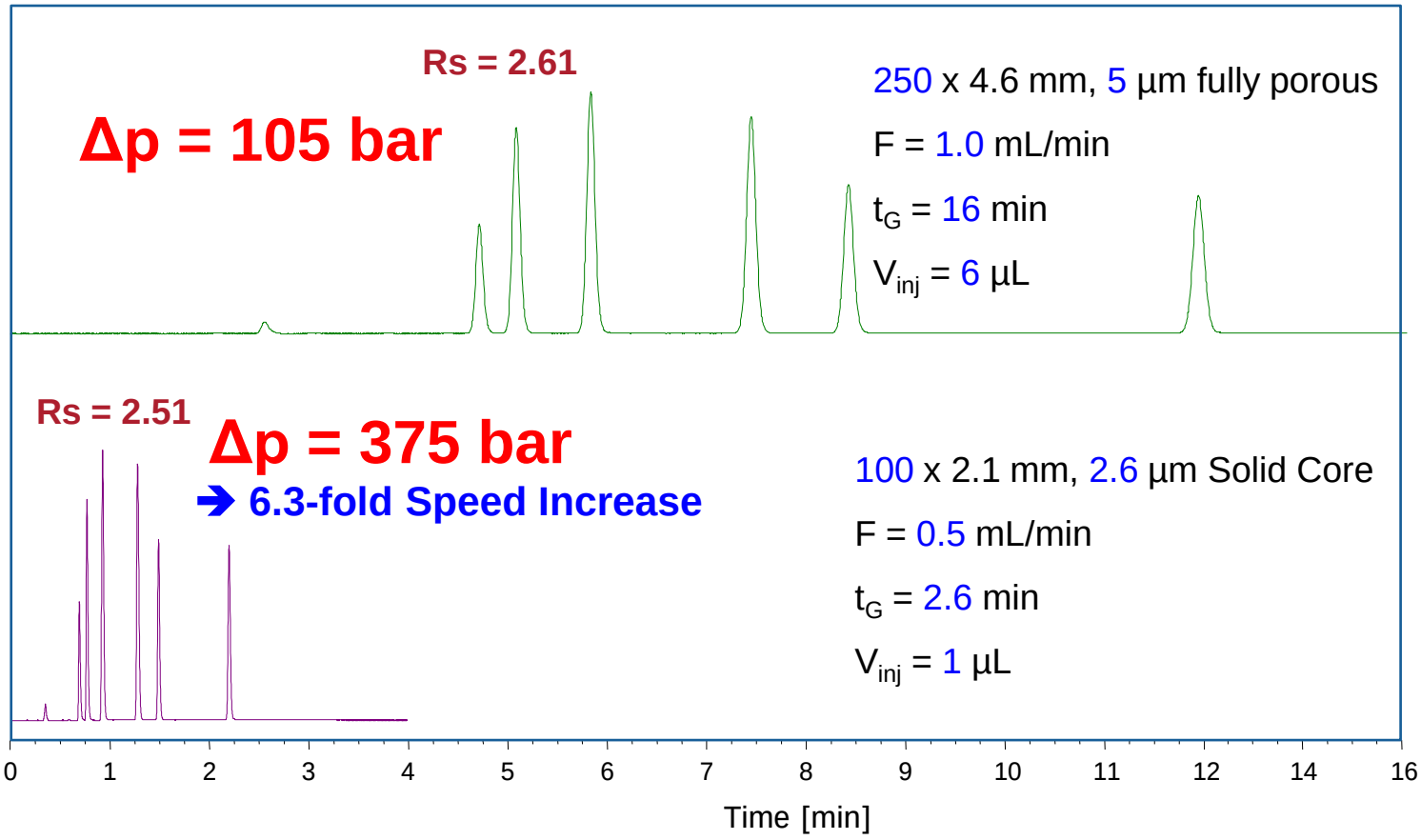


Rules of Thumb for Detector Settings

- Ideally collect 30-40 data points for each peak between the limits of integration.
- The response time should be the reciprocal of the optimal data rate times five ((1/DCR)·5 = Response Time= 2.2·time constant).
- Poor peak symmetry (efficiency) can be improved by reducing the response time.
- High baseline noise can be improved by increasing the response time.



UHPLC Method on HPLC System Thanks to Core-Shell Technology



- Despite appropriate method scaling, resolution decreases due to peak broadening in the fluidics of the analysis system (And different selectivities of the two columns).

From HPLC to UHPLC, What Do I Need to Consider?

- Minimize gradient delay volume (GDV). High pressure gradient instruments are at an inherent advantage due to their design.
- The GDV is more than just the pump's mixer. The entire volume from the point of gradient formation to the head of the column contributes to GDV.
- The band-broadening effect of the extra-column volume *before* the column can generally be disregarded in gradient mode thanks to band refocusing.
- After the column: The shorter the fluidic path, the better. Be aware of the back pressure associated with very narrow capillaries, especially when a pressure-sensitive UV flow cell is used before a mass spectrometer.
- Reduce as best you can any contribution to extra column volume (ECV) due to imprecise capillary connections.
- The volume of the detector flow cell should be no more than 1/10th the volume of the smallest peak.
- The data rate and response time/time constant should be determined based on the narrowest peak.

Thank You for Your Attention!

Any questions?

Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an E-Mail to analyze.eu@thermofisher.com and we will get back to you.