Multi-Stage Sample Injection: An Effective Way to Reduce Band Broadening in Liquid Chromatography

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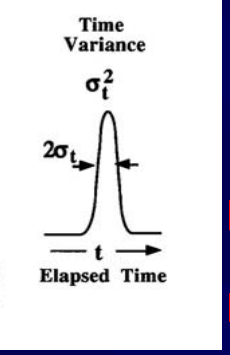
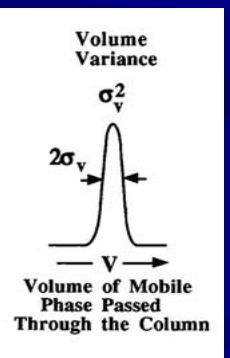
Summary: The customary automatic LC injection process using a full loop on a 6-port 2-position valve is a fundamental limit on the performance levels observed in reverse phase LC. Thus, we have explored ways to improve the injection process and found that automatic multi-staged injection implemented on a commonly used programmable autosampler provides measurable improvement.



Background – Variance, AKA peak width

- $\sigma^2_{observed} = \sigma^2_{injection\ process} + \sigma^2_{column} + \sigma^2_{extra\text{-}column}$
- $\sigma^2_{injection\ process}$ is as much as 80% of $\sigma^2_{observed}$ in an otherwise optimized LC system (assumes "good" column).*
- Guiochon et.al. says: "The contribution of the sampling device is particularly deleterious since, for a 2 μL injection, the maximum solute concentration in the peak that enters into the column is nearly ten-fold lower than that of the sample."
- $\sigma^2_{extra\text{-}column}$ can readily be made negligible.
- The ultimate speed and separation efficiency in LC is *not limited by mass transfer efficiency* in the column (i.e. not limited by d_p).*
- This presentation describes an approach for reducing the impact $\sigma^2_{injection\ process}$ has on $\sigma^2_{observed}$ during <u>fast</u> (high velocity) gradient operation using LEAP/CTC autosamplers along with otherwise ordinary HPLC instruments.



*F. Gritti, A. Felinger, G. Guiochon, *J. Chromatogr. A,* **2006**, 1136, 57.
*Henry, R.A., in Modern Practice of Liquid Chromatography,
J.J. Kirkland ed., Wiley-Interscience: New York, **1971**.

Background: Variance

AKA peak width

The limitation imposed by $\sigma^2_{injection\ process}$ on $\sigma^2_{observed}$ in an otherwise optimized LC system has long been known!

From:
L.R. Snyder & J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, 1974, Wiley, New York.

TABLE 4.7. MAJOR ADVANTAGES OF SAMPLING MODES

Direct on-column Syringe injection (see below)	Loop style Sampling Valve 6-port 2-position (ordinary autosampler)
Injected volume readily changed	Can be used at high pressures (5000 psi) without disturbing flow
Simple, convenient, low cost	More precise, less operator dependent
Permits very small sample volumes	Easy to automate
Produces very small band spreading	Accommodates large sample volumes

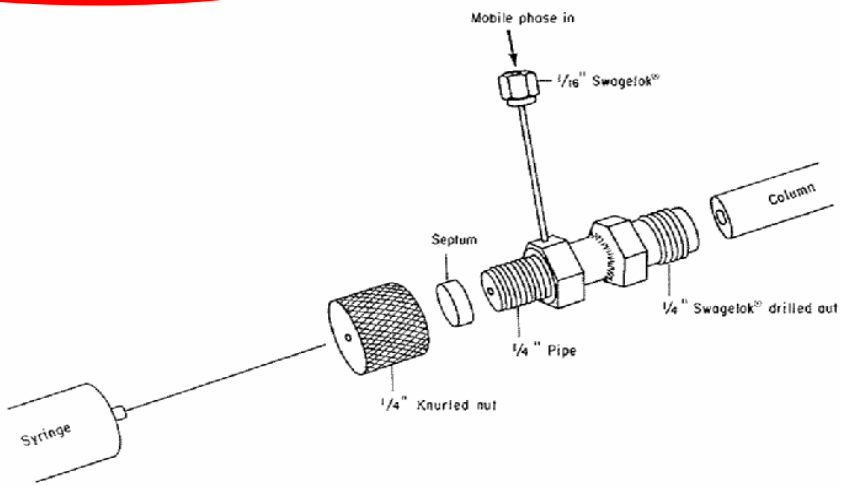
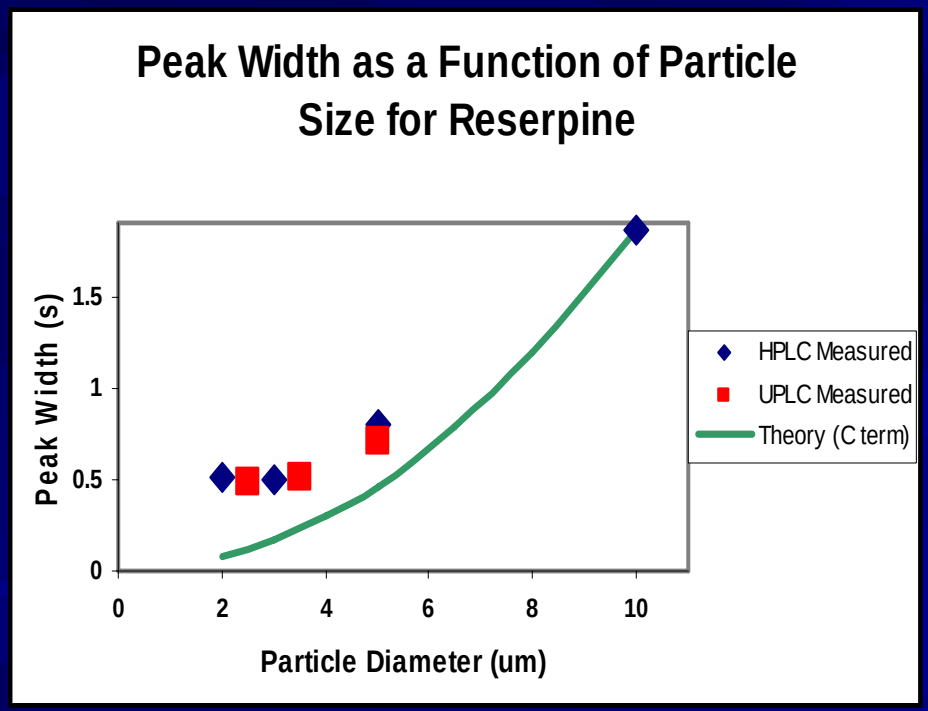


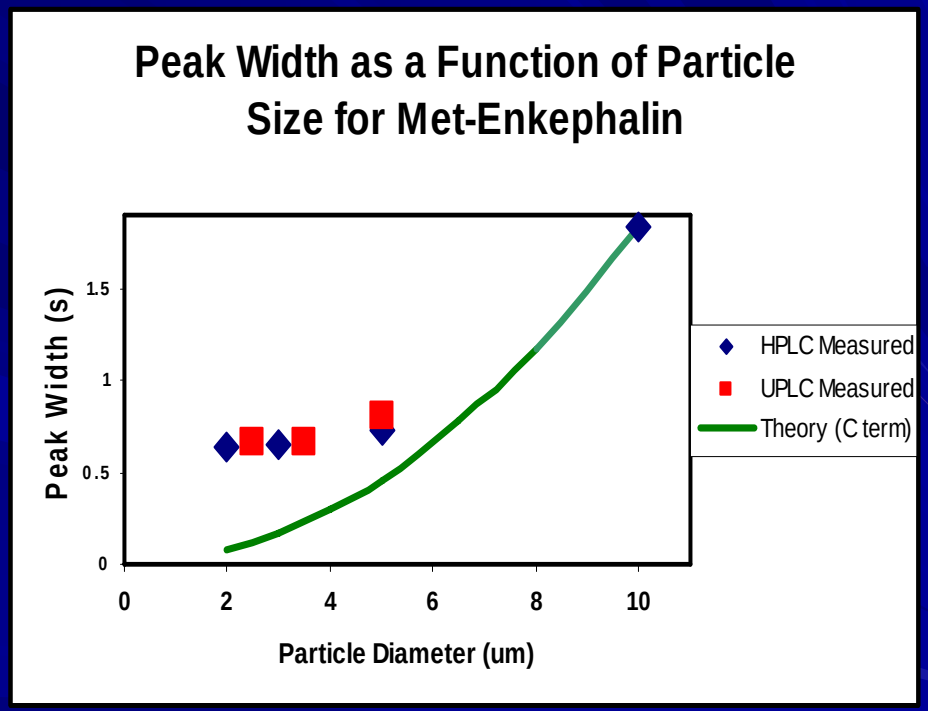
Figure 4.13 On-column injection port. R. A. Henry, in Modern Practice of Liquid Chromatography, J. J. Kirkland, ed., Wiley-Interscience, New York, 1971. Reprinted by permission of publisher.

Practical consequences of being injection process limited:

- Expected improvements with particle size reduction level off (can even slow separation).

*J. Kofman, Y. Zhao, T. Maloney, T. Baumgartner, R. Bujalski, *Am. Drug Discovery* **2006**, 1, 12.
T.L. Chester, S.O. Terami, *J. Chromatogr. A,* **2005, 1096, 16.





ACN soluble compounds: peak widths level out at 3 μm

3 μm looks like way to go.

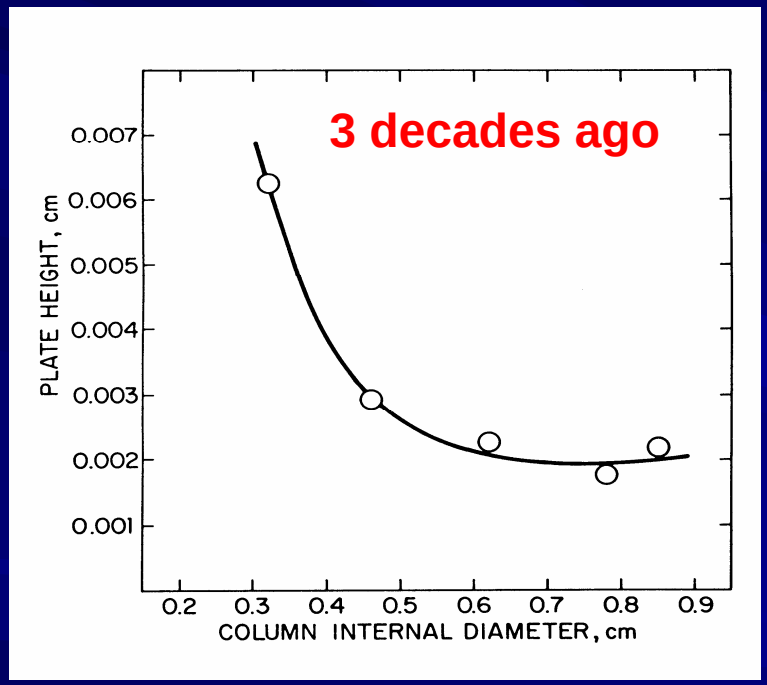
H_2O soluble compounds: peak widths level out at 3-5 μm

Minimum σ^2 exiting column slightly larger than σ^2 entering column (HPLC or UPLC, by connecting UV to inj valve).
Best half dozen columns all yield about the same performance (C_{18} Luna and Sunfire shown).
Velocity = 7 mm/s, T = 45°C, L = 50 mm, column diameter = 4.6 mm HPLC & 2.1 mm UPLC.

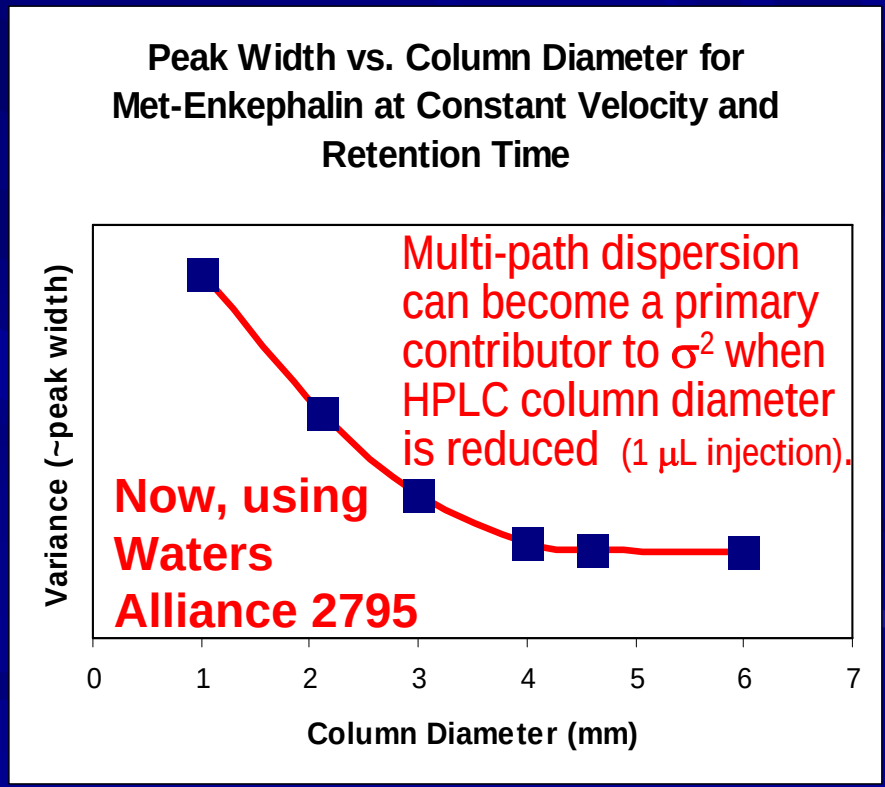
Practical consequences of being injection process limited:

- **Ideal column diameter – depends on performance of injector.**
  Well known in literature, see: L.R. Synder & J.J. Kirkland, "Introduction to Modern Liquid Chromatography," **1979**, 2nd Ed., John Wiley & Sons: New York

"Infinite Diameter Effect" or dispersion at column wall



4.6 mm ID looks like way to go (HPLC).



These curves can be flattened well below 1 mm diameter by using direct on-column syringe injection.

*Henry, R.A., in Modern Practice of Liquid Chromatography, J.J. Kirkland ed., Wiley-Interscience: New York, **1971**.

Practical consequences of being injection process limited:

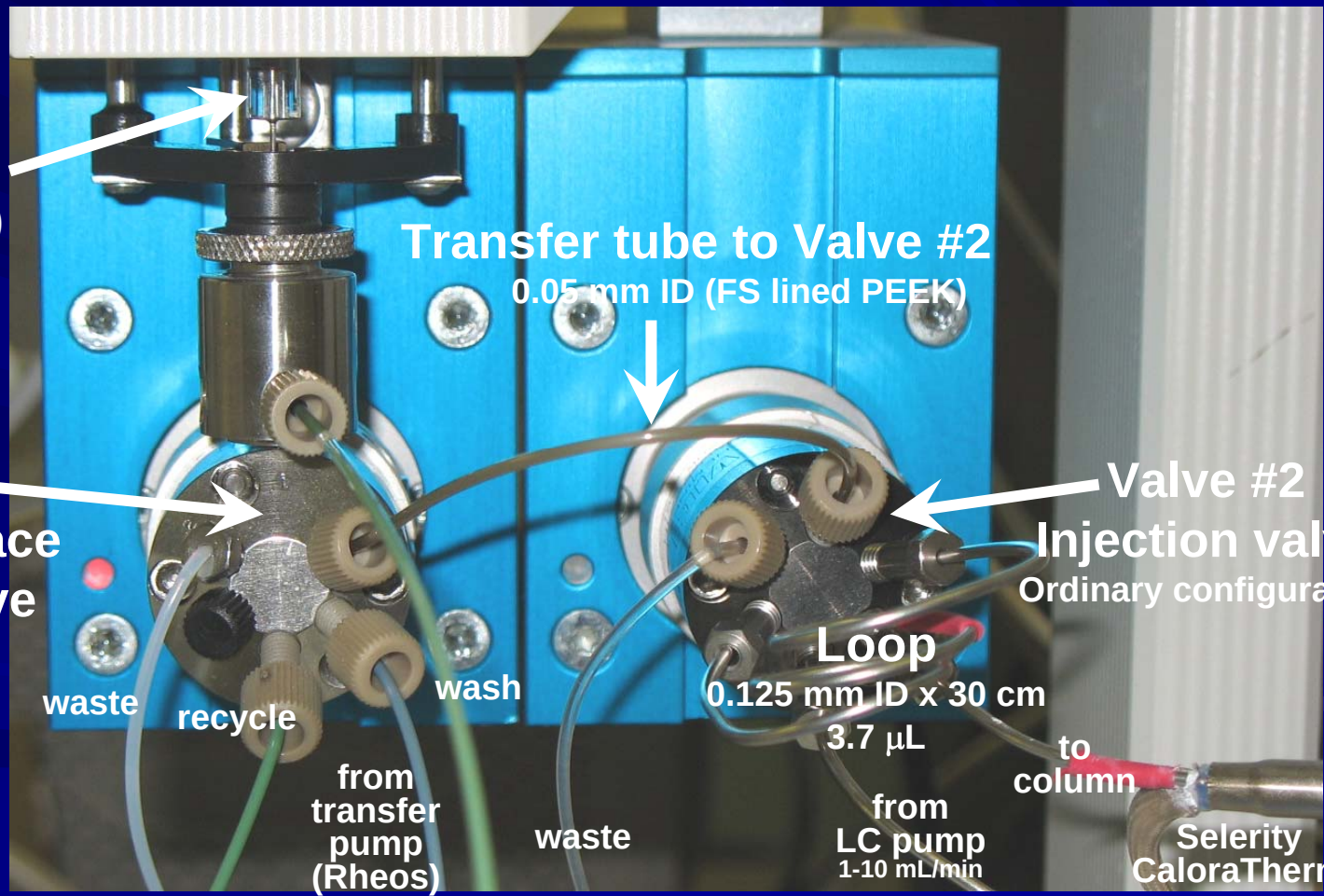
- **Operation under "infinite diameter" conditions gives best separation efficiency. Reducing diameter below that significantly sacrifices separation efficiency.**
- **Automation required: direct syringe on-column injection provides narrow peaks but is not sufficiently automated.**
- **Column diameter must be scaled to delivered injection volume to get best separation efficiency and speed.**
- **Delivered injection volume (2σ) can be measured by connecting UV detector directly to injection valve.**
- ***Instrument choice is one way to reduce column diameter for improved sensitivity without sacrificing separation efficiency.***
- **Key volumes / column diameters to maintain efficiency:**
  - **$2\sigma \approx 50\ \mu L$→ col. dia. 4 – 6 mm (ordinary HPLC)**
  - **$2\sigma \approx 10\ \mu L$→ col. dia. 1.5 – 2.1 mm (example: UPLC)**
  - **$2\sigma \approx 0.2\ \mu L$→ col. dia. 0.2 – 0.3 mm (example: Eksigent Express)**

Buying new instruments is nice!
Can anything be done with the instrument I've got?

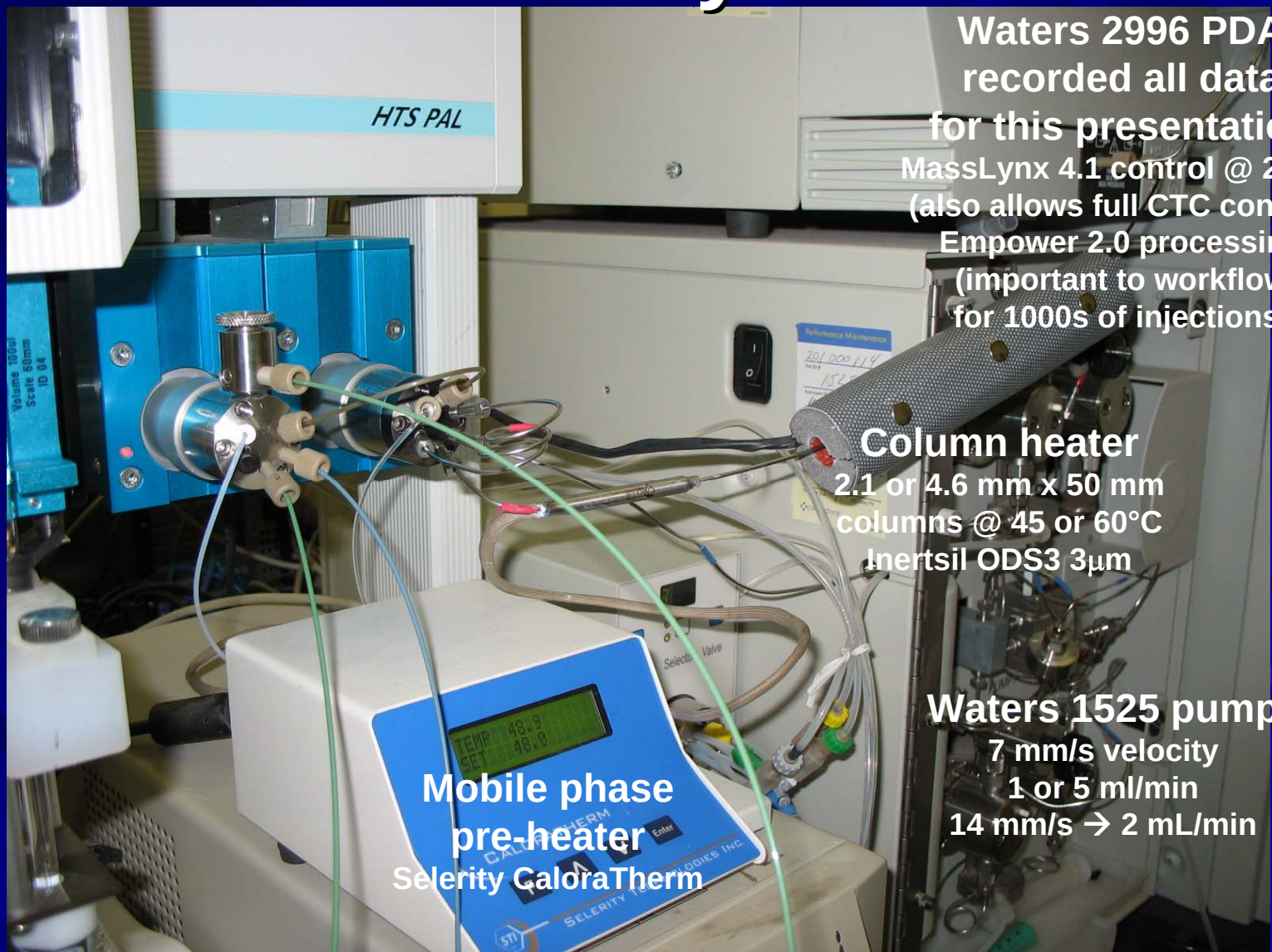
- The greater the flexibility of your existing autosampler, the greater the possibilities.
- We have explored the possibilities using a LEAP/CTC HTS PAL (we already had 7 of these).
- The HTS PAL has been fitted with:
  - 2 Valco valves (6-port 2-position) on CTC MV-3 drives
    - Valve 1-"heart cut" - C2V-0006D-CTC, 0.15 mm ID ports, 6k psi.
    - Valve 2-"normal injection" - C72VX-6696D-CTC, 0.15 mm ID ports, 15k psi.
  - Fast wash (for syringe) & Self wash 2 (for valves)
  - 46 Line macro (written at LEAP, Raleigh, NC for our specifications)
- Allows "heart cutting" or metered injection.
- Allows partial loop injection with plug positioning control.

Our LEAP/CTC HTS PAL valve configuration

(reverse flow through loop)



A step back
Our LC System



Waters 2996 PDA recorded all data for this presentation
MassLynx 4.1 control @ 20 Hz
(also allows full CTC control)
Empower 2.0 processing
(important to workflow for 1000s of injections)

Column heater
2.1 or 4.6 mm x 50 mm columns @ 45 or 60°C
Inertsil ODS3 3μm

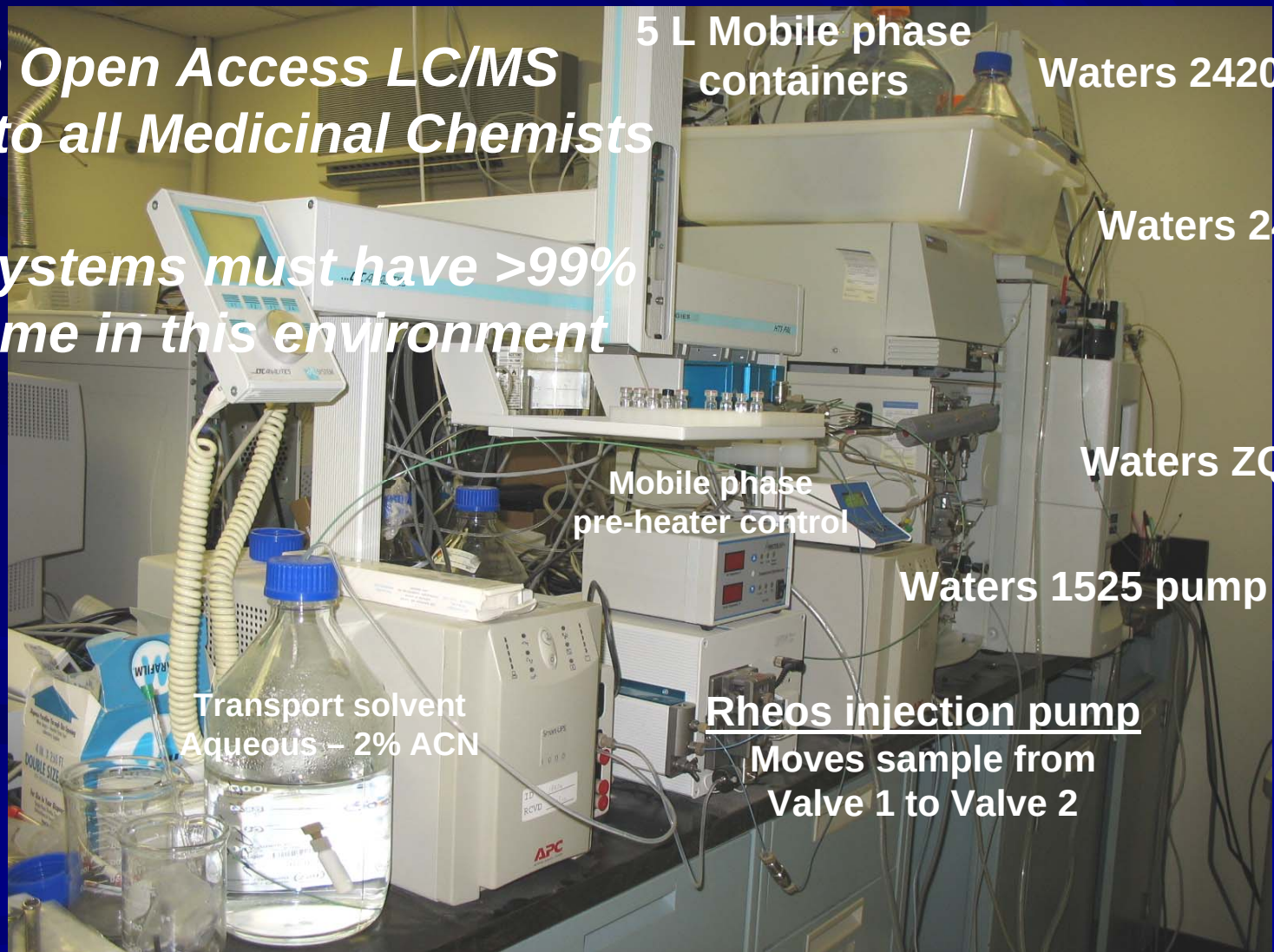
Waters 1525 pump
7 mm/s velocity
1 or 5 ml/min
14 mm/s → 2 mL/min

Mobile phase pre-heater
Selerity CaloraTherm

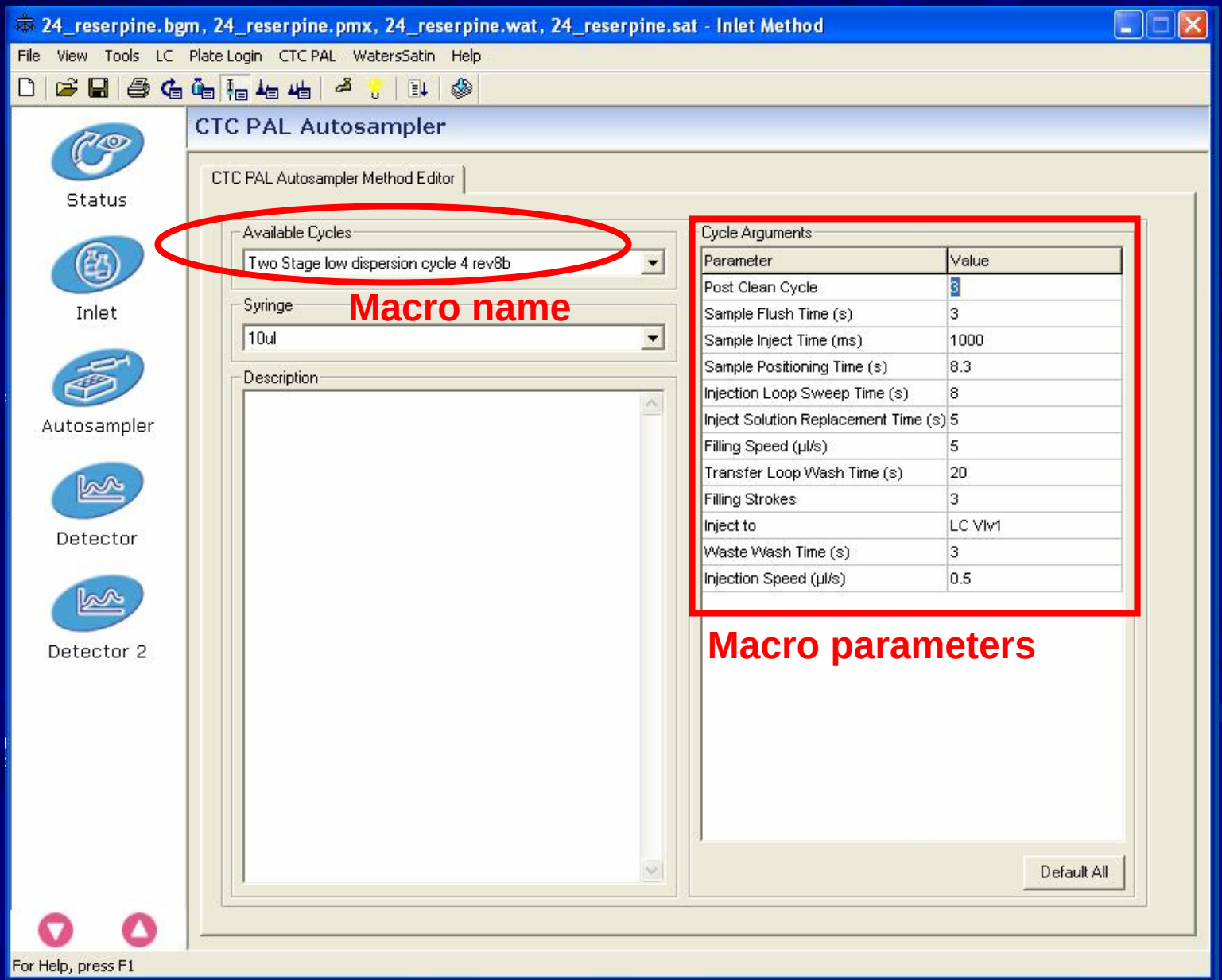
Another step back
Our Open Access LC/MS System

An Open Access LC/MS
Open to all Medicinal Chemists

Our systems must have >99% up time in this environment



Our software control of the HTS PAL within MassLynx 4.1



What's known about injection?

- **Very little literature is available (almost all from 1970s).**
  - *Larger injection = higher sensitivity, Karger, Guiochon, Rozing, & their coworkers.*
  - *Syringe approach also adapted to split flow with 6 port, 2 position valve and it works the same, but still requires custom column, Coq, Kelsey & their coworkers.*
  - *Theory, Guiochon et.al.*
- **Smaller tubing should help (Taylor equation) and smaller injection volumes often yield narrower peaks.**
- **Fixed full loop injection has best precision and widest peaks.**
- **Partially filled loop injection (smaller volumes) produces narrower peaks (but not as precise).**
- **Lower flow rates should result in less dispersion upon injection (less change in momentum).**
- **Faster operation times should result in less dispersion by allowing less time for sample diffusion.**
- **Heart cut (metered) injection successfully delivers volumes <1 μL for column IDs <1 mm (@ low flow, example Eksigent).**
- **There seems to be a preference for backfilling the loop.**
- **Some newer autosamplers position small sample plug within a larger sample loop (example Waters).**
- **<u>OUR GOAL</u>: learn about and improve our HPLC injections with a focus on narrowest peaks for <u>fast</u> analysis speed** *(for ordinary analyses, neither sensitivity nor sample quantity limited)***.**

Characterization of the Multi-Stage Injection System

- Tubing sizes
- Loop loading flow rate selection
- Injection volume and syringe speed
- Loop loading flow direction
  - Effect of sample position within loop
- Example data
- Summary & conclusions

Unless otherwise specified, all trends are reserpine peak width / area eluting from a 4.6 x 50mm (3μm C_{18}) column at 7 mm/s (5 mL/min) and 45°C.

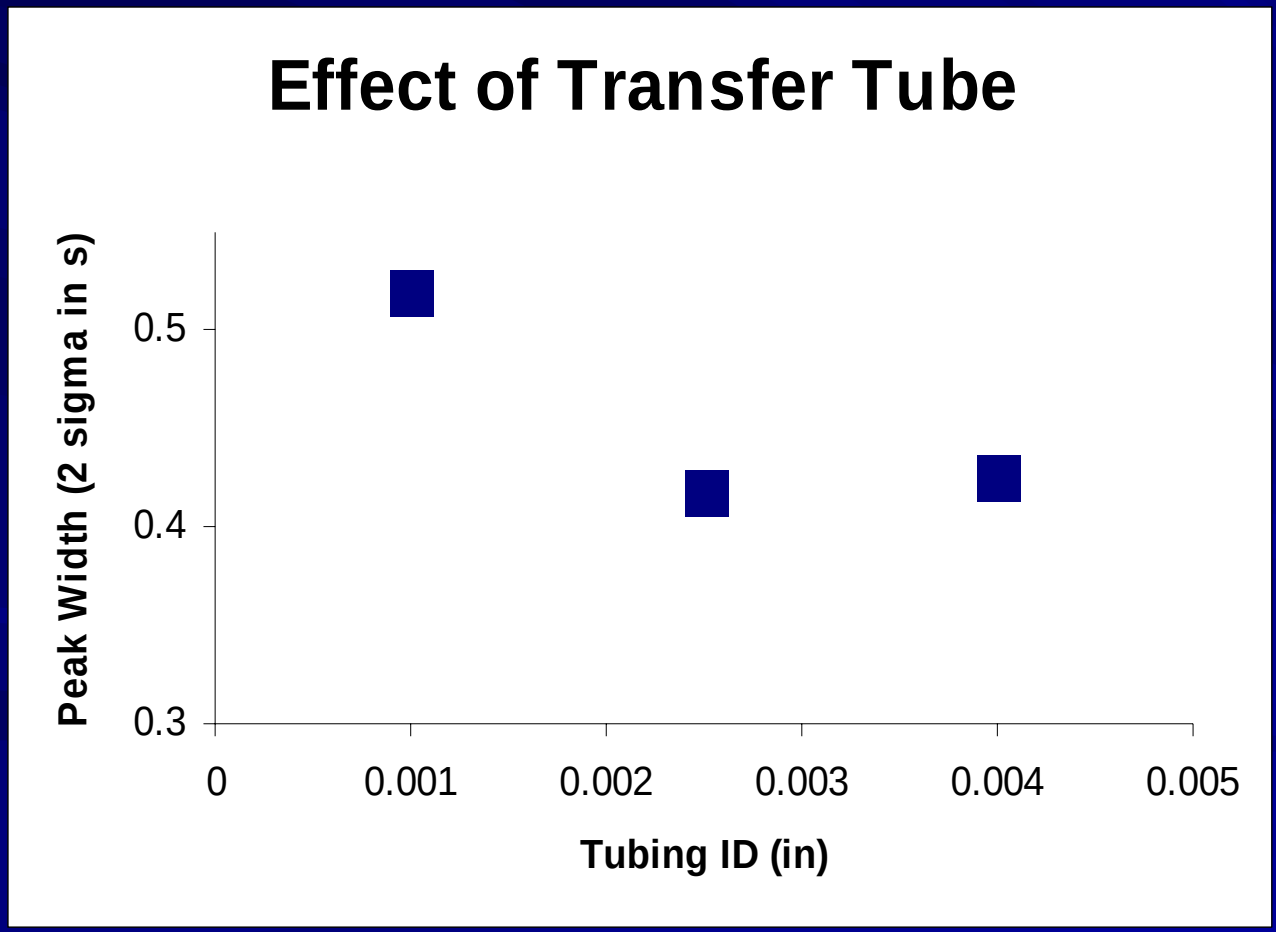
Also, transfer flow is 100 μL/min through 0.002" or 0.0025" ID tube, injection volume is 0.5 μL

Tubing sizes

- For ordinary HPLC at 7 mm/s (5 mL/min through 4.6 mm ID column):
  - At 1 m length post column, going from 0.25 to 0.175 mm ID tubing reduces 2σ peak width less than 0.05 s.
  - $\sigma^2_{extra\text{-}column}$ agrees with Taylor equation which describes post column behavior well.
  - In contrast, $\sigma^2_{injection\ process}$ does not agree with Taylor equation.
  - Regardless of length pre-column, going from 0.175 to 0.125 mm ID tubing (loop and column connection tube) reduces 2σ peak width $\approx$ 0.1 s (also applies to UPLC conditions and all injection process types).
- Minimizing cross section in loop and prior to column generally visibly improves performance.
- 0.10 to 0.125 mm ID is currently the lower limit for widely available SS components (we use 0.125 mm, 1/16" OD).
- Depending on the configuration, use of these small cross sections can add significantly to pressure (100+ bar).

Tubing size prior to loop

- No impact on peak width for areas that have no sample flow.
- Key aspect of sample flow involves doing things quickly so that opportunity for diffusion is minimized.



Small improvement with tubing ID reduction.

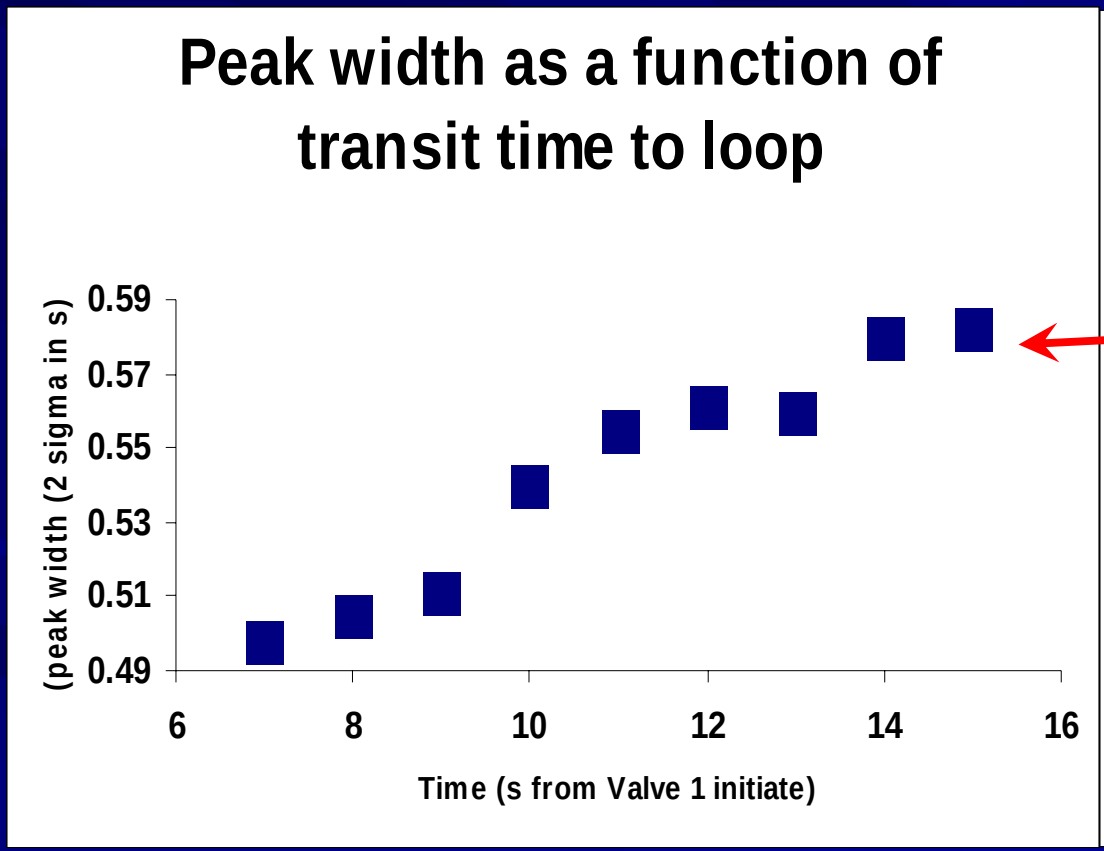
At 0.001” ID (25 μm) flow must be reduced (pressure) and additional diffusion time seems to outweigh other effects.

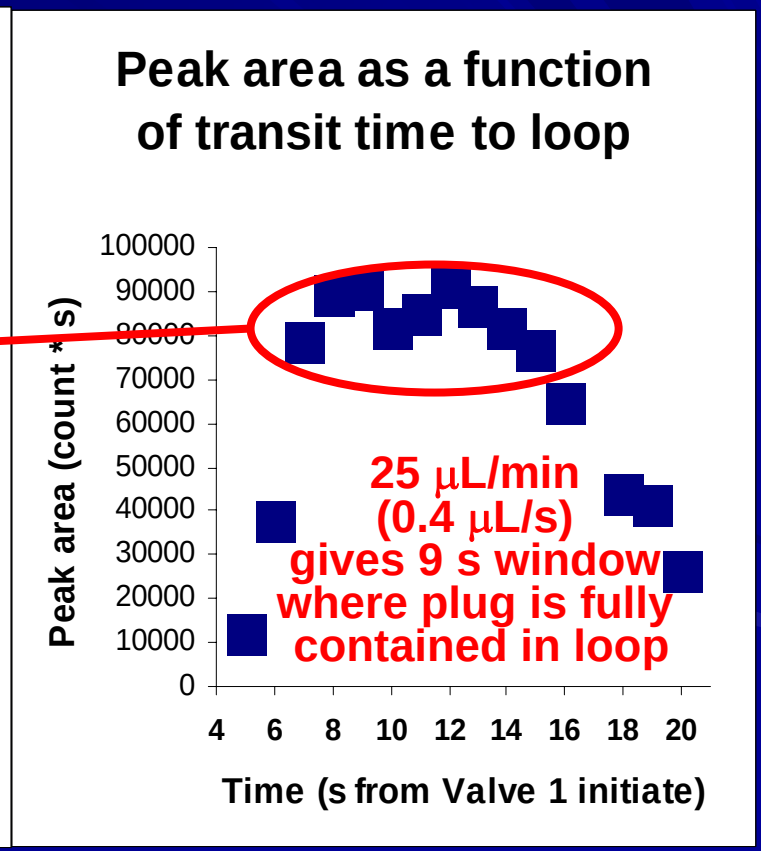
0.0025” PEEK (or 0.002” FS lined PEEK) works nicely in an Analytical lab.

0.004” PEEK provides ruggedness for Open Access with Med Chemists.

Transfer flow rate (25 μL/min shown)

- Transfer tube + loop + valve ports ≈ 4 μL
- Transport flow rate of 100 μL/min (1.7 μL/s) gives 2 s transport time before peak is being pushed out other end of loop.
- In a trade-off between more mixing from more velocity and diffusion time, time appears to be the more important element.

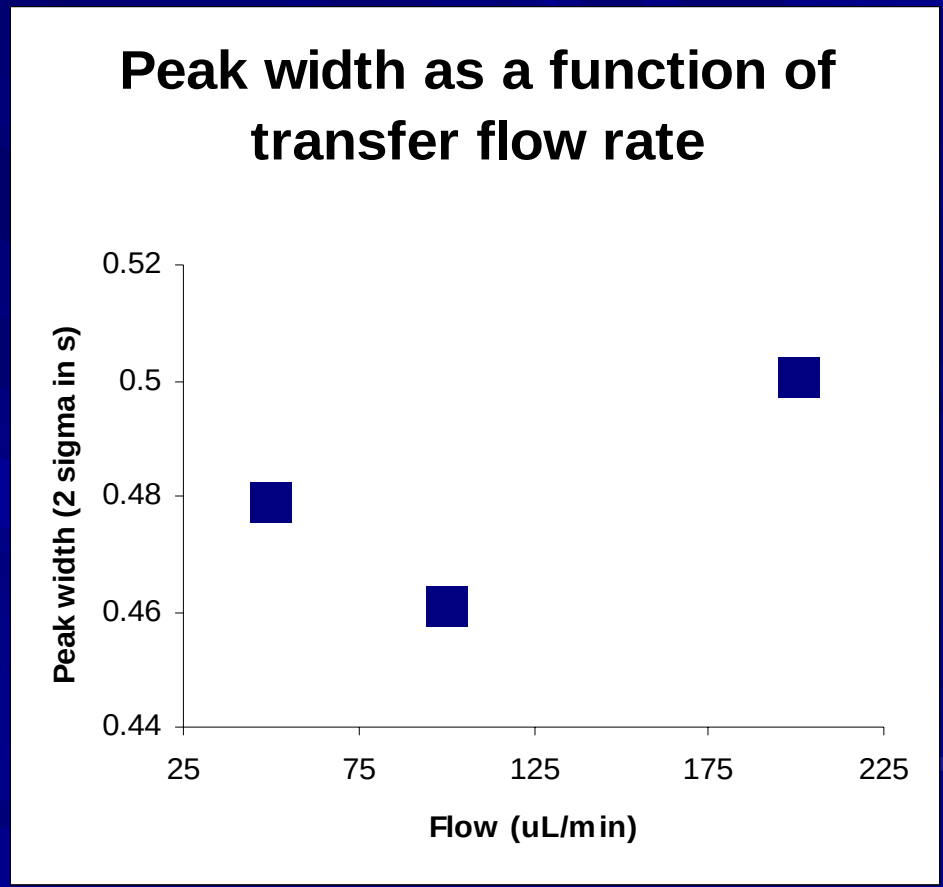




0.5 μL injection volume, 5 mL/min, 4.6 x 50 mm column (7 mm/s)

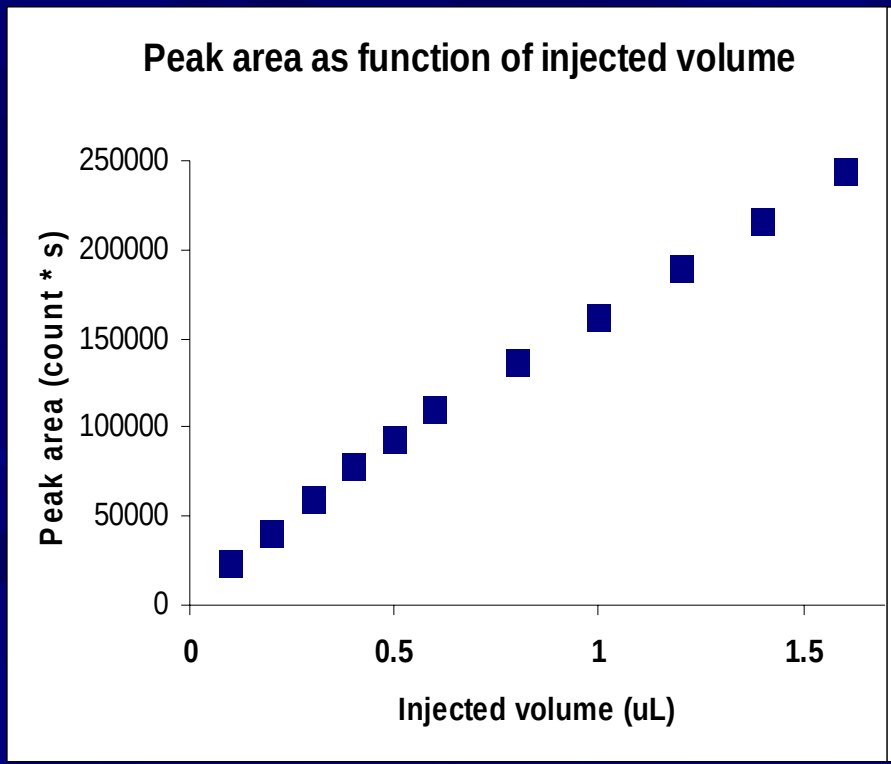
Transfer flow rate

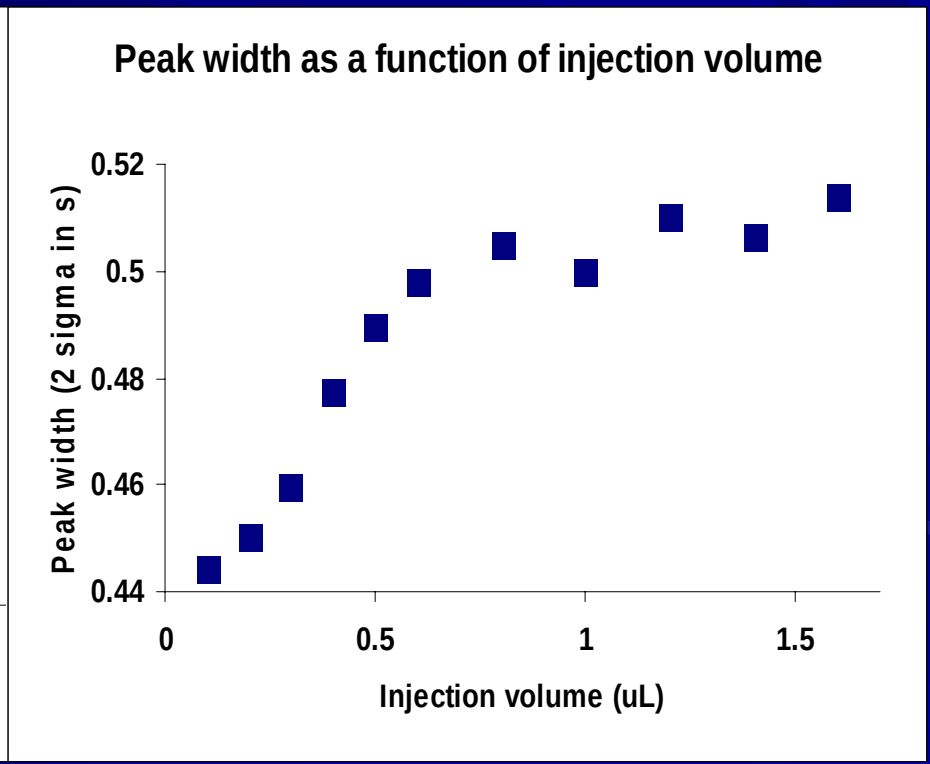
- Speed (less diffusion time) seems to be crucial.
- Timing control at 0.1 s intervals.
- Transport flow rate of 100 μL/min (1.7 μL/s) gives 2 s transport time before peak is being pushed out other end of loop. Reproducible operation can be readily achieved at this speed.
- Peaks areas for ≥200 μL/min show that part of plug is cut by Valve 2.



Injection volume

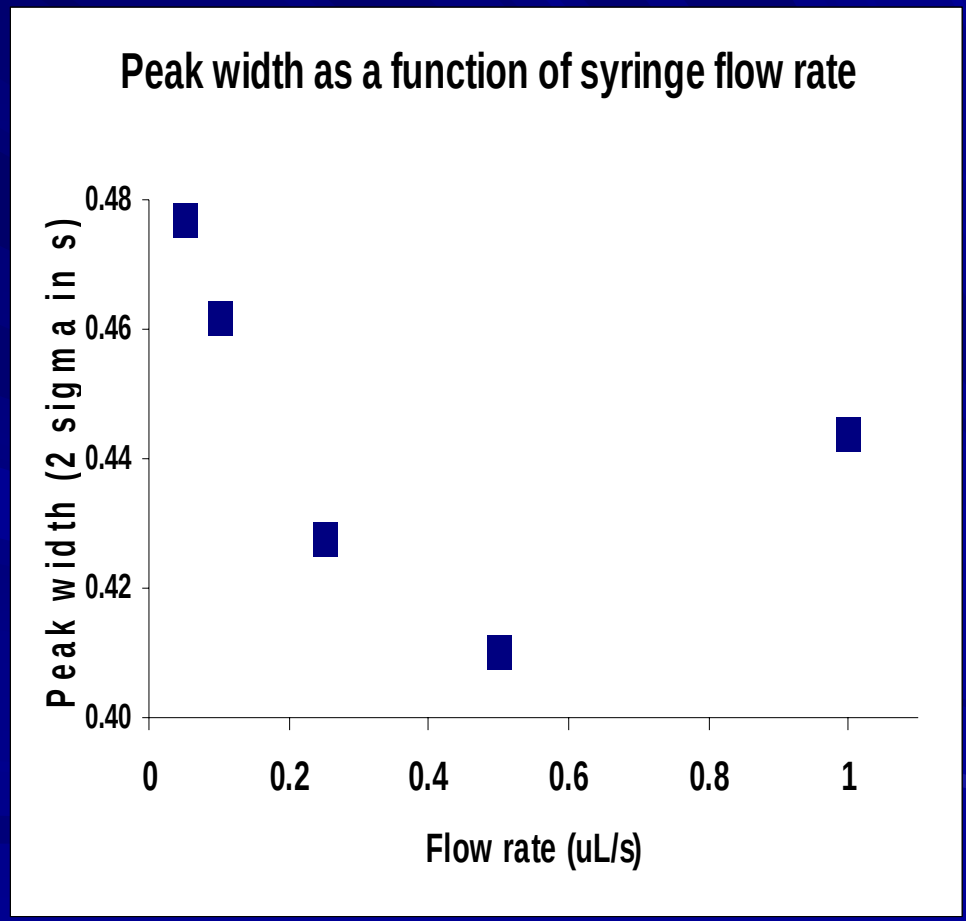
- Controlled with combination of syringe speed (flow rate) and Valve 1 timing.
- Linear response verified 0.1 to 1.6 μL with 2 distinct response ratio ranges (tail is cut off by Valve 2 at higher volumes, > 0.6 μL). Precision better than 2% RSD at 0.5 μL.
- Smaller volumes lead to narrower peaks!





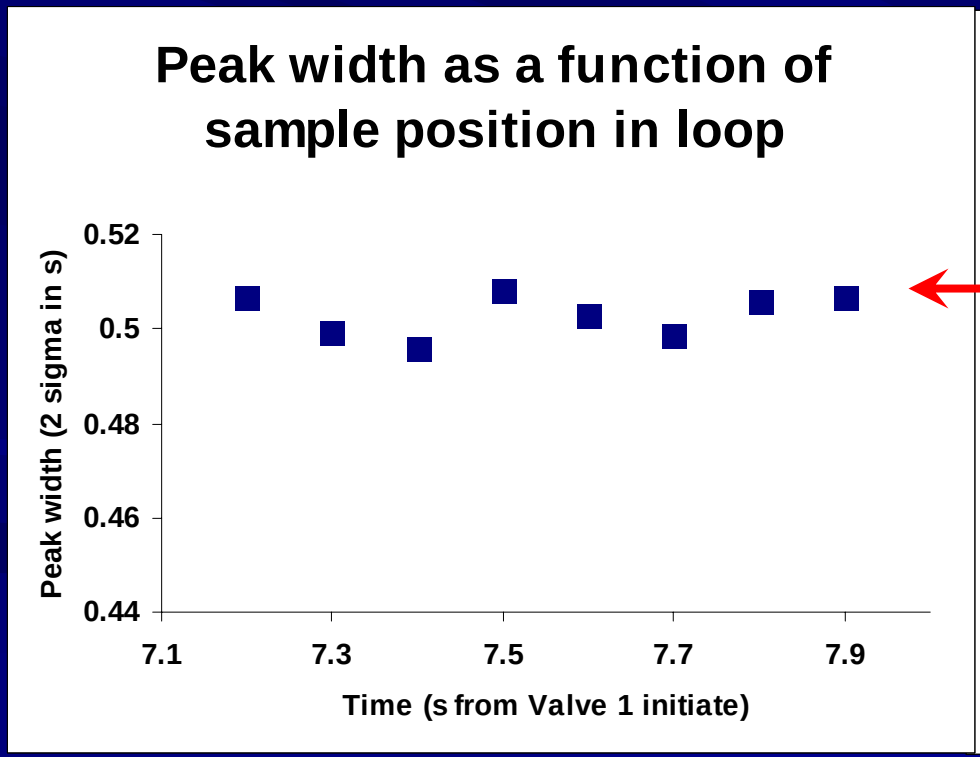
Syringe speed (flow rate)

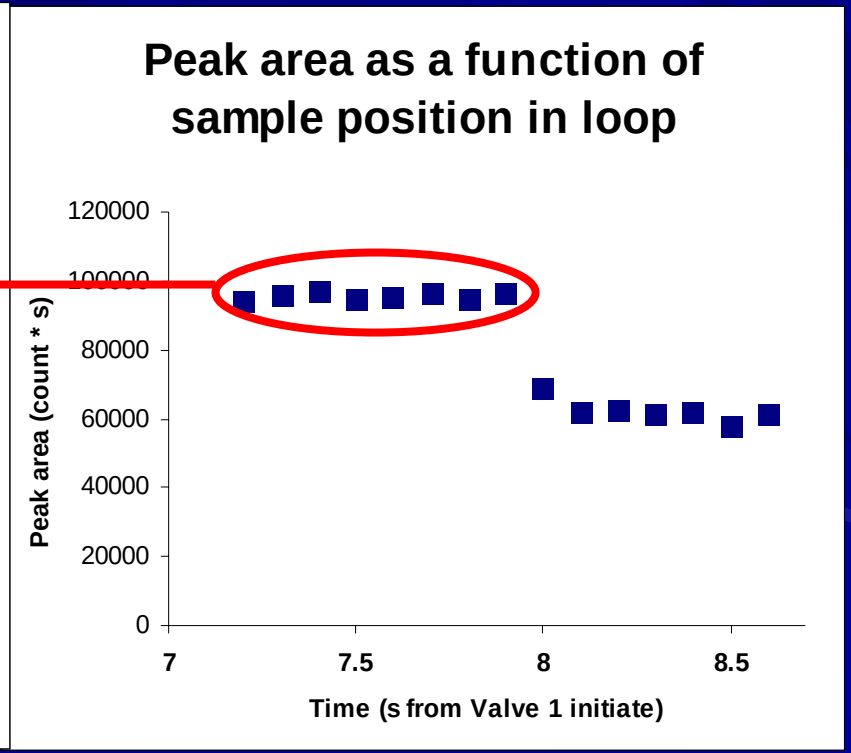
- Syringe flow into transfer tube also seems to favor avoiding excess time (excess diffusion).
- High flow drives up injection volume thus moderation is required to keep injection volume below 0.6 μL.



Loop loading in same direction as flow to column

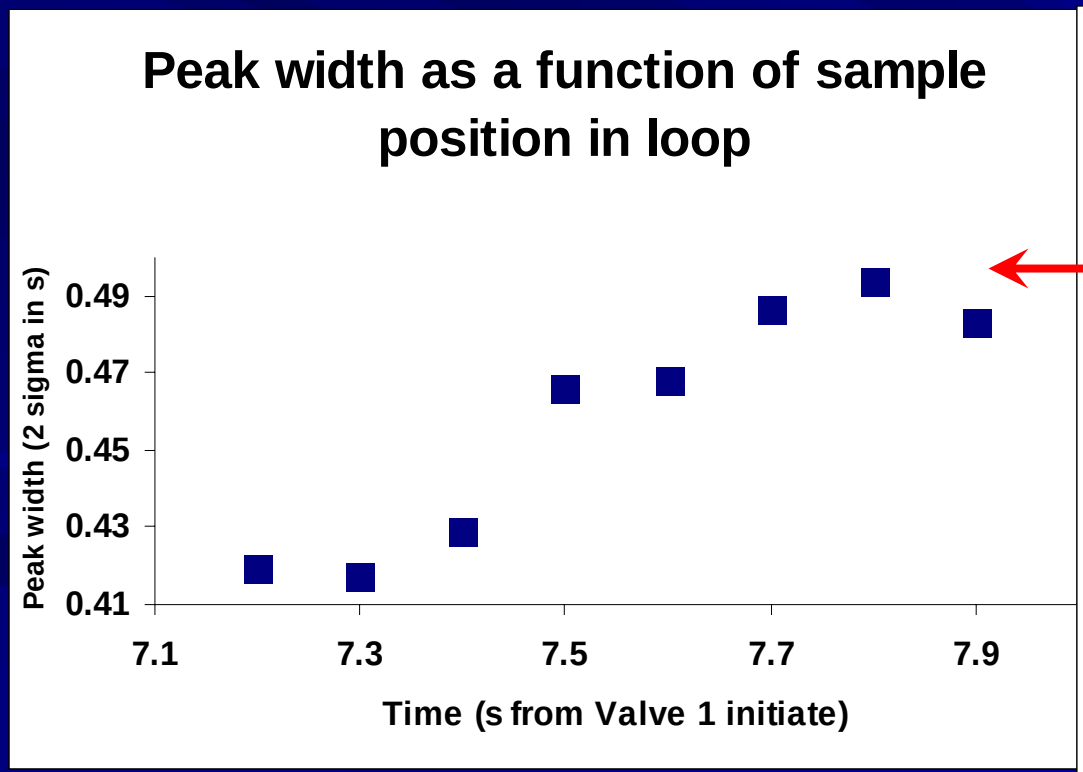
- No clear trend based on position or time.
- Performance is good and fairly consistent.

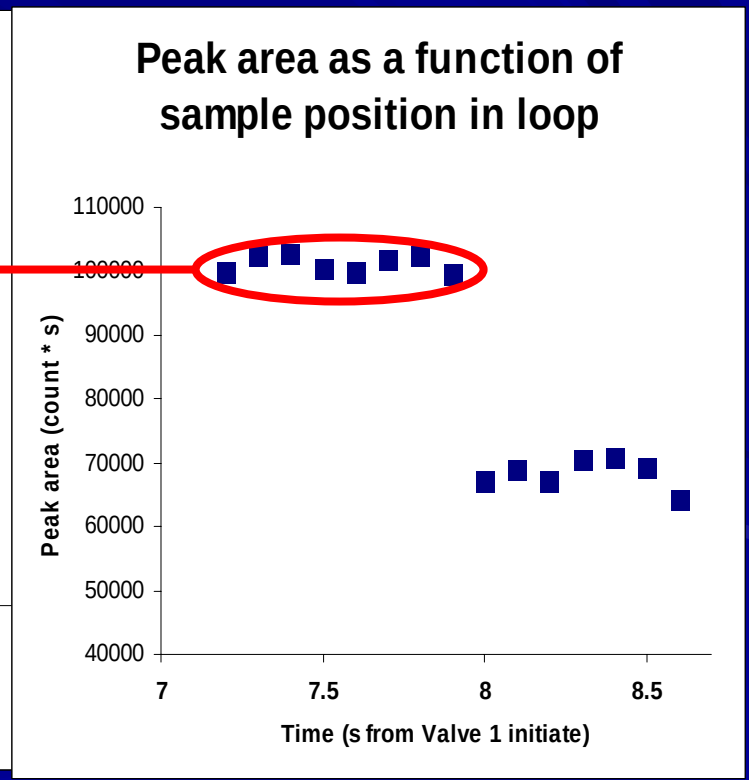




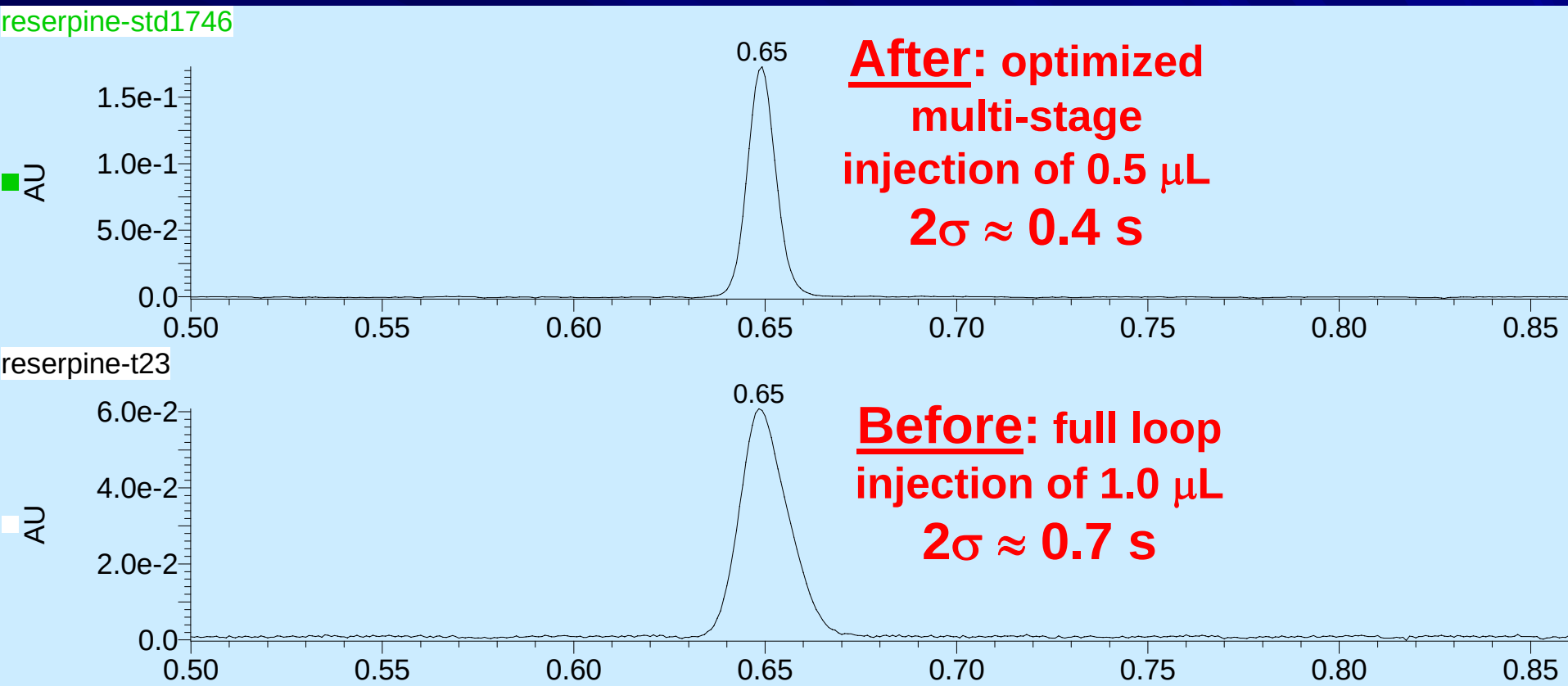
Loop loading in reverse direction

- Clear trend toward minimized time.
- Clearly better performance for reverse flow.
- Reversing direction appears to re-focus wall dispersion occurring during loop loading



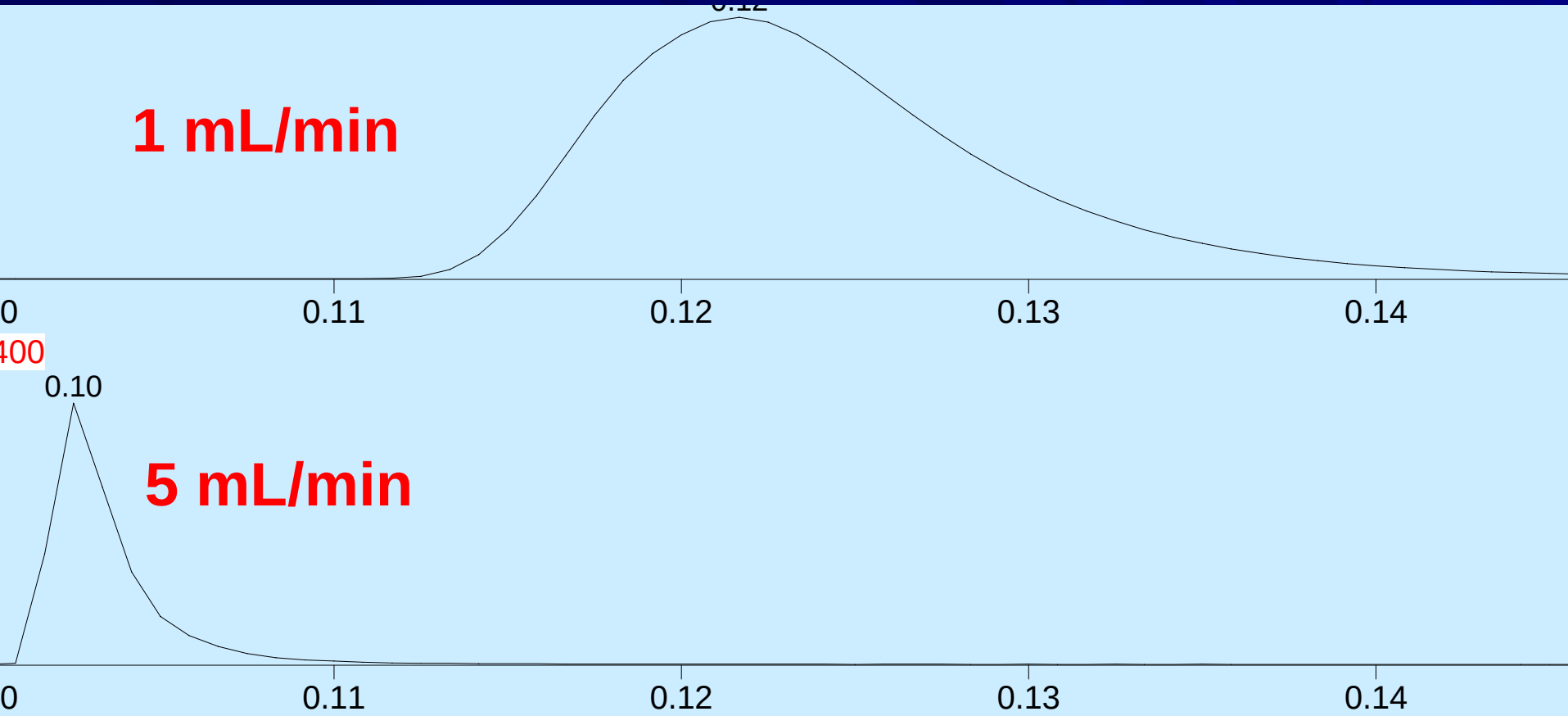


Results: overall effect on peak width
40% reduction



Impact ≈ 1/3 from tubing + 1/3 from heart cutting + 1/3 from reverse loading / position

Results: measuring 2σ for injection process in the multi stage injector



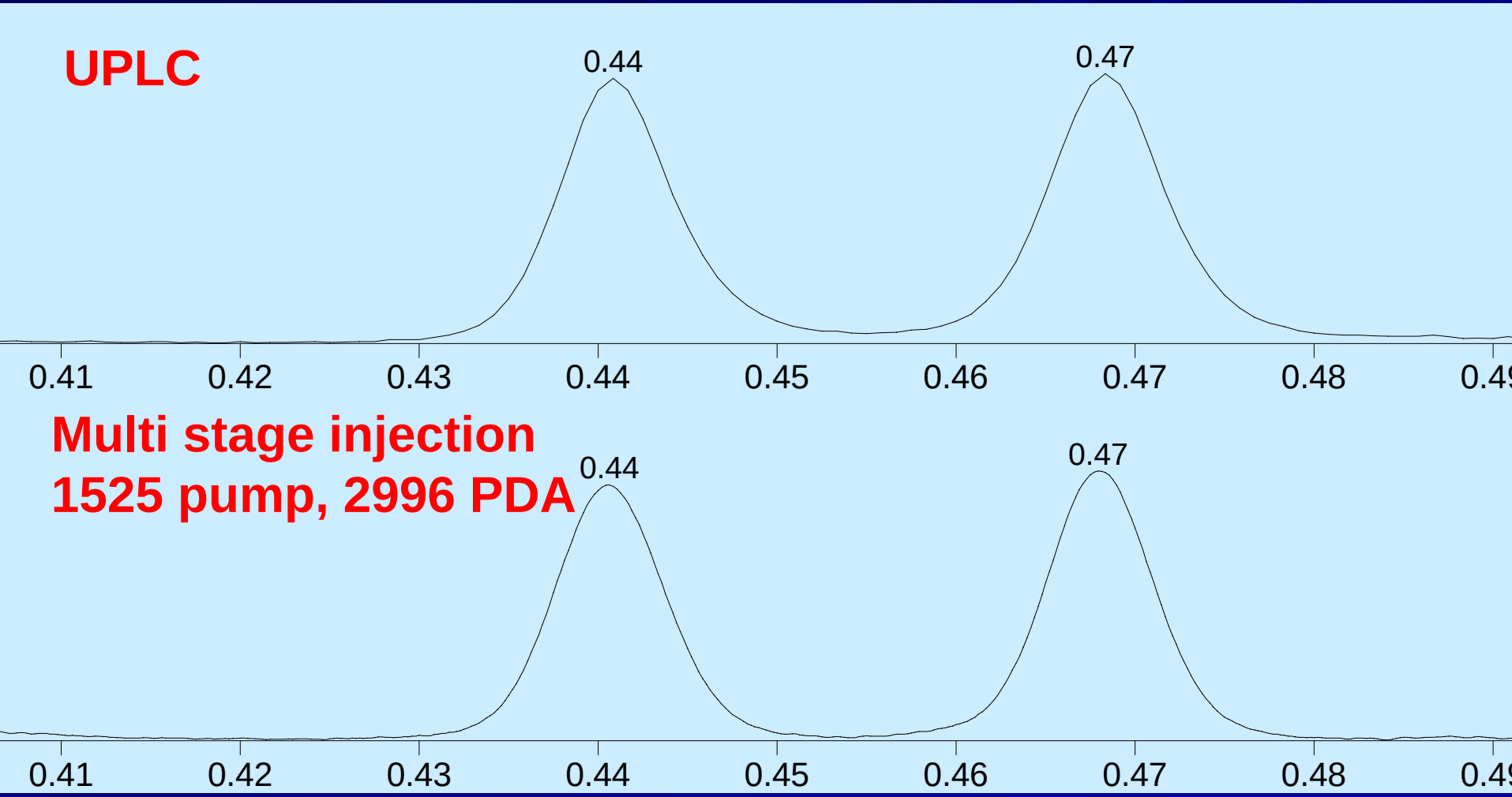
Injection volumes observed by direct connection to PDA:

9 – 10 μL, compound dependent, 0.5 μL sample injected,

Increasing flow from 1 to 5 mL/min increases volume ≈ 5%.

Should be comparable to UPLC.

Comparison with UPLC
Leu & Met Enkephalin at 14 mm/s



Both cases: 2 mL/min through 2.1 x 50 mm (3 μm) at 60°C & ≈400 bar max

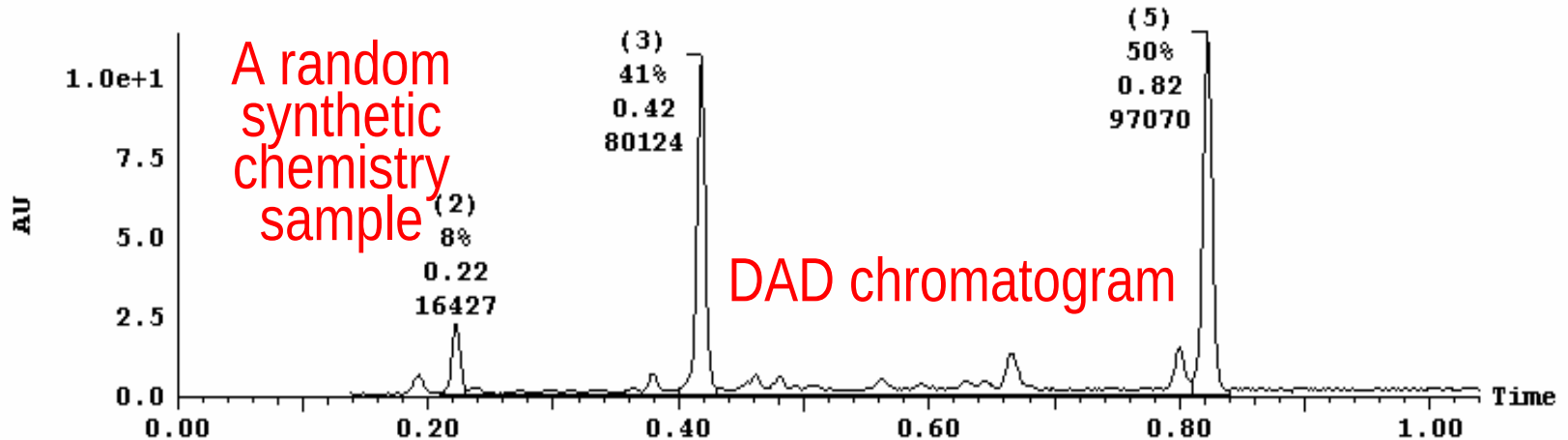
$2\sigma \approx 0.36$ s (or 12 μL, volume increased ≈ 20% in column)

Examples: Open Access LC/MS
under "ultra fast" conditions (14 mm/s)

Openlynx Report LC/MS Page 1

Lab journal #: ____-000-000 Report file name:SPH20 Raw data file name (Batch ID):- ____000-000K
Submitter:SPH Project..... Method:C:\MassLynx\9B_Fast_LCMS--C8_Neutral_pH.olp
Vial:1:55 Date:22-Feb ____ Time:18:30:10

3: UV Detector: 240_400 1.14e+1
Range: 1.137e+1



Peak Number	Compound	Time	Area (Abs)	Area % Total	Mass Found
2		0.22	1.64 e+004	8.48	
3		0.42	8.01 e+004	41.38	
5		0.82	9.71 e+004	50.13	

14 mm/s HLPC reaches k' = 17 in 1 min.

(achieved with ordinary low cost LC/MS components via recalibration of HPLC pressure limits for 400 bar operating pressure, *using a 6 year old system*)

Conclusions

- The injection process is a fundamental limit on performance in reverse phase LC as shown in literature by direct on-column syringe injection and by measuring bands at injection valve with UV detector.
- This limit has been reconfirmed by showing that improvement in the valve based injection process alone dramatically improves the observed chromatograms *in an otherwise ordinary LC system*.
- Relative to the standard full loop injection, several peak narrowing (speed enhancing due to increased peak capacity) improvements have been made using:
  - Smaller ID tubing
  - Small injection volumes (0.1 to 0.5 μL)
  - Heart cut sample plug creation
  - Partial loop injection with position control & loop loading in reverse direction.
- 2σ injection volumes have been reduced to 10 μL (comparable to UPLC).
- UPLC performance levels are achieved using otherwise ordinary LC instrumentation.
- These results are delivered routinely and reliably in a demanding Open Access environment at a rate of 1.5k samples / month.