Why Won’t My HPLC Method Transfer?

How to build a more robust method

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What Do We Mean By “a Well-Developed Method?”

The importance of ruggedness and robustness

Ruggedness

“Reproducibility of results when a method is performed as written under actual use conditions”[1]

- Different analysts
- Different instruments
- Different lots of reagents/columns
- Different days

Robustness

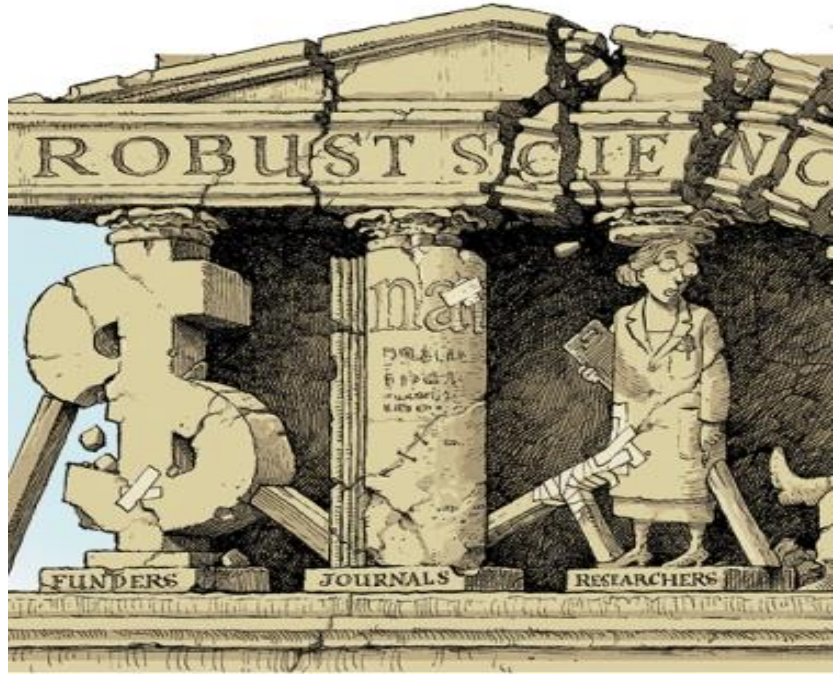
“Is a measure of [an analytical procedure] to remain unaffected by small, but deliberate variations in the method parameters”[2]

- Temperature
- Mobile phase pH
- Flow rates
- Composition of extraction solvents

1. According to The United States Pharmacopeia (USP)
2. International Conference on Harmonization (ICH) Guideline: Validation of Analytical Procedures: Text and Methodology, Q2 (R1), Nov. 2005.

When a Method is Not Rugged and Robust

- Method fails unexpectedly, halting production
- “Method creep”
- Results fail after maintenance
- Results fail after changing the column
- Risk of needing to redevelop method
- Compromise quality



Studies estimate that only around 40% of published findings can be replicated reliably.[1]

Cartoon Reference: Begley, CG, Buchan AM, Dirnagl. Robust research: Institutions must do their part for reproducibility. *Nature* ***525,*** 25-27 (03 September 2015)

1. Baker M, Penny D. Is there a reproducibility crisis? A Nature survey lifts the lid on how researchers view the ‘crisis’ rocking science and what they think will help. *Nature* ***533,*** 452-454 (26 May 2016)

Method Development and Method Transfer

The importance of ruggedness and robustness

Rugged methods do not depend upon a particular column, a senior chemist, or some other “secret sauce”.

If the analytical results change significantly when changing columns, users, instruments or just days, then the method is not rugged.

Robustness can be thought of as a measure of “flexibility” of our method. It addresses questions like “what happens if my buffer strength is a little off?” or “what happens when I change the tubing on my instrument (to a different length)?”

Robustness

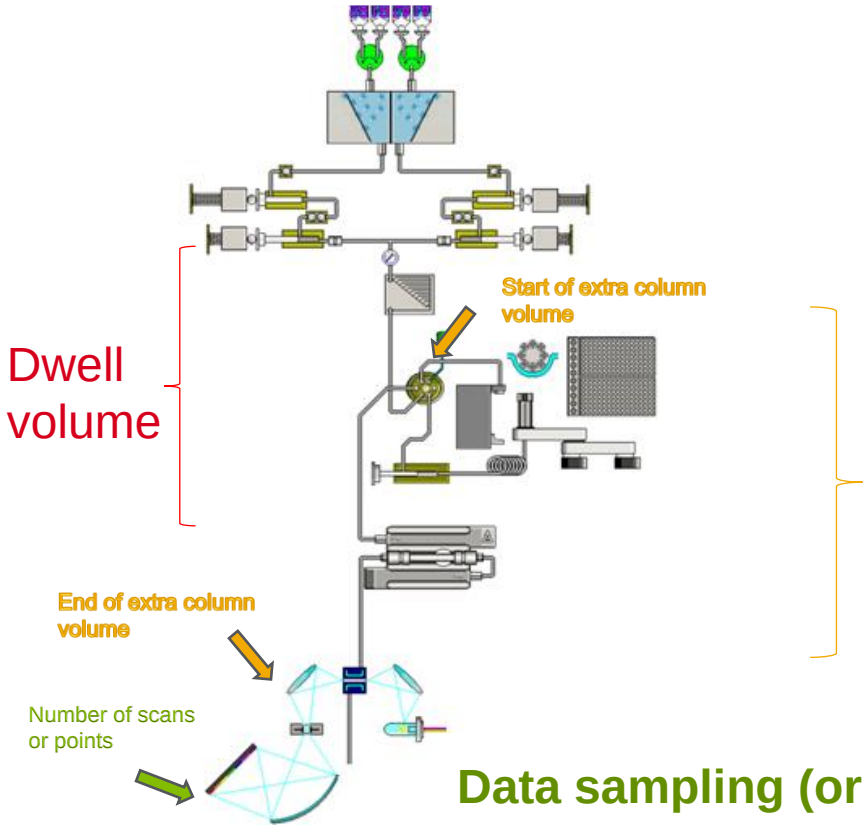
What do we want to test?

LC parameters:

- Gradient table variations
- Dwell volume
- Extracolumn volume
- Data sampling rates
- pH of mobile phase
- Buffer composition and concentration
- Temperature
- Flow rate
- Column choices, for example, different L1 columns

Gradient Separations

Instrument impact on column performance



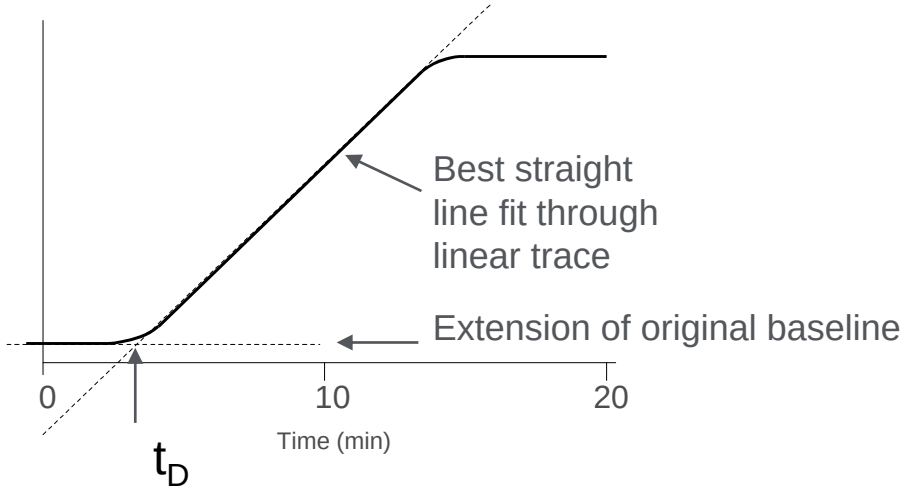
Data sampling (or acquisition) rate

Determining the Dwell Volume of Your System

- Look it up in the LC manual or follow the procedure below
- Replace column with short piece of HPLC stainless steel tubing
- Prepare mobile phase components
  A. Water – UV-transparent
  B. Water with 0.2% acetone – UV-absorbing
- Monitor at 265 nm
- Run gradient profile 0 to 100% B in 10 min at 1.0 mL/min
- Record
- Expected dwell volume in UHPLCs – µL range

Measuring Dwell Volume (V_D)

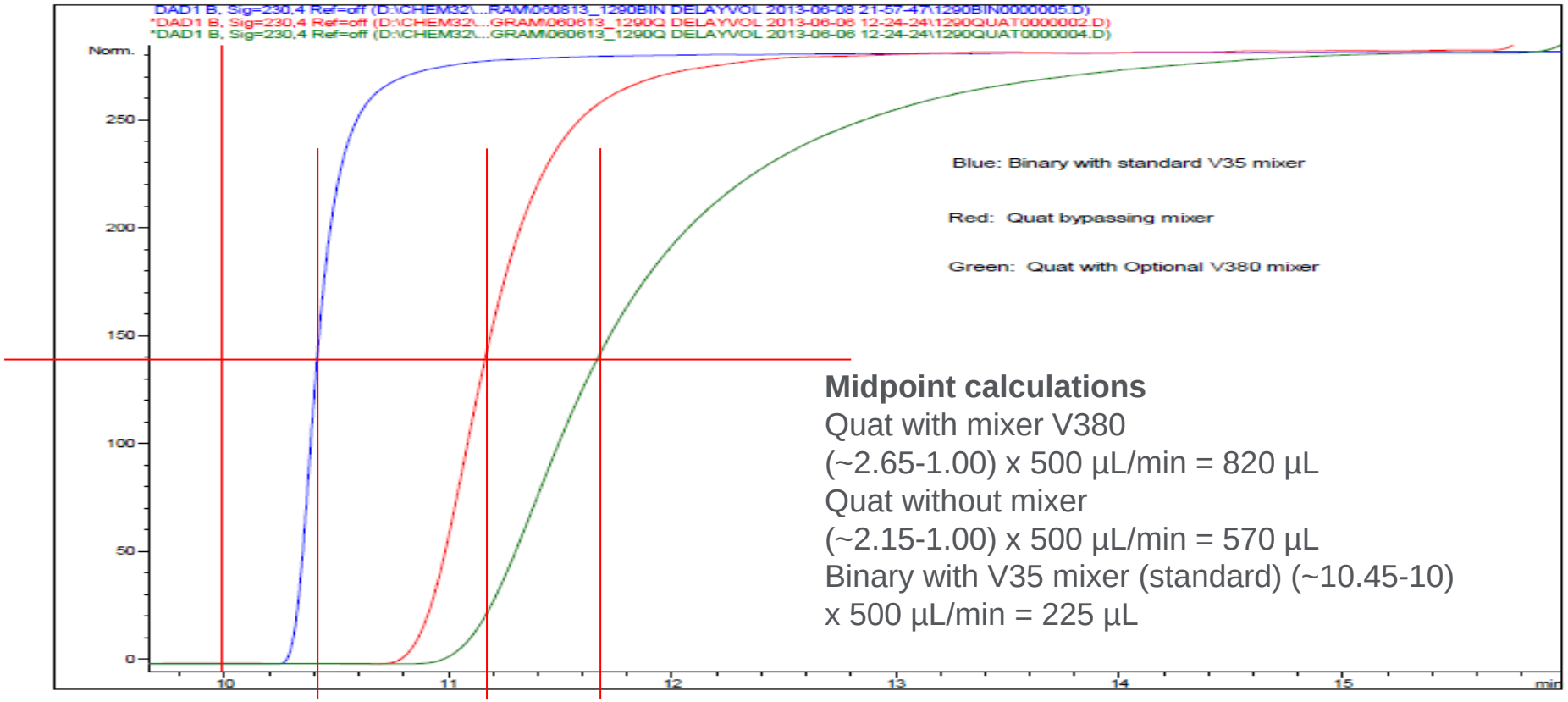
$V_D = t_D \times F$



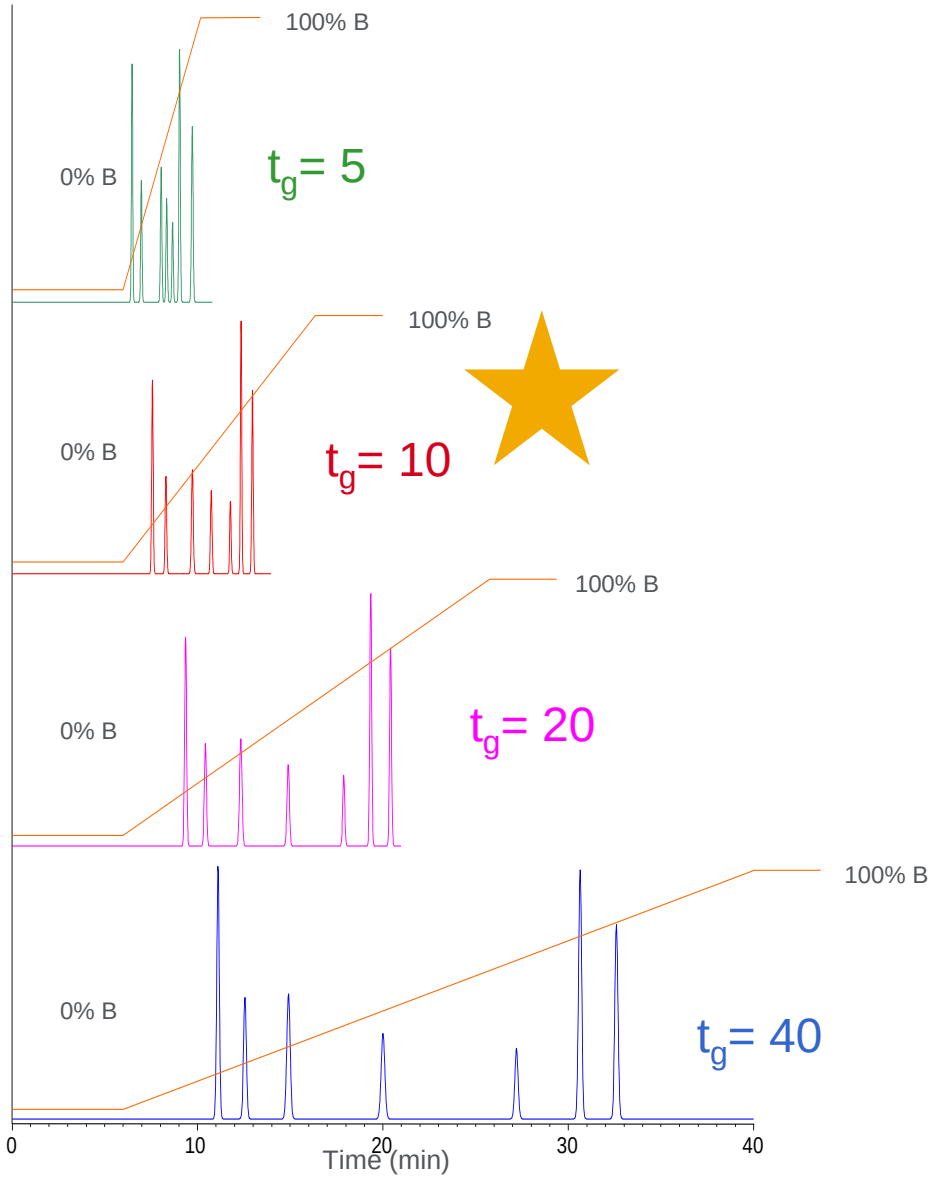
- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time

Disregarding Delay Volume

- Measure instrument delay (dwell) volume; V_D
- Simulate larger V_D with initial isocratic hold. Simulate smaller V_D with injection delay.
- Model delay volume changes with simulation software, such as iSET
- Compare performance on different instruments



Changing Gradient Time to Affect Retention (k*) and Resolution



$$k^* = \frac{t_g F}{S \, \Delta\%B \, V_m}$$

1/k* = gradient steepness = b

$\Delta\Phi$ = Change in volume fraction of B solvent
S = Constant
F = Flow rate (mL/min)
t_g = Gradient time (min)
V_m = Column void volume (mL)

- S ≈ 4–5 for small molecules
- 10 < S < 1000 for peptides and proteins

Scenarios and Strategies

First scenario: Developing a method that will be transferred to instruments that match the method development HPLC system.

- Measure dwell volume on method development instrument
- Measure extracolumn volume on method development instrument
- Confirm the range of the above two volumes for the set of instruments that will routinely run this method
- Include the measured ranges of dwell and postcolumn volumes in the robustness testing
- Restrict the range allowed to the range used during robustness testing

Scenarios and Strategies

Second scenario: Developing a method where the dwell volumes of the method development systems and routine analysis systems differ significantly.

- Software compensation: iSET
  - An HPLC or UHPLC with very low dwell volume can be programed to behave like an older system with a larger dwell volume.
- Physically changing the dwell volume
  - Additional tubing can be added between the pump and the autosampler to increase the delay volume of the method development instrument until it is reasonably close to the dwell volume of the instruments that will be routinely running the method.
- Changing the gradient table
  - After measuring the slope of the gradient on both the method development and routine assay systems, we can change the gradient table of the routine assay systems to approximate the slope on the method development system.

Scenarios and Strategies

Third scenario: Recreating a method from literature. Transferring from a system with an unknown delay volume.

- Look at the delta retention time of the first peak
  - If the retention time shifts lower on the new instrument, this suggests that the delay volume of the system in the literature was larger.
    - Try to compensate by adding tubing

- If the delay time on the new system is later, this would suggest that the delay volume of the system from the literature was smaller.
  - Try reducing the extracolumn volume
  - Try to compensate by making the gradient steeper. This is now method development, not a simple transfer.

LC Columns and Supplies Resources

- InfinityLab Poroshell Columns catalog: InfinityLab Poroshell 5991-8750EN
- Agilent BioHPLC Columns catalog: BioHPLC columns 5994-0974EN
- InfinityLab Supplies catalog: InfinityLab LC Supplies (agilent.com)
- LC Handbook: LC-Handbook-Complete-2.pdf (Agilent.com)
- LC troubleshooting poster: LC Troubleshooting Guide (Agilent.com)
- Agilent Community: Agilent Community
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