Top 10 HPLC Method Development Fails

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Trusted Answers

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Top 10 HPLC Method Development Fails

10. Not benchmarking a new column

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Benchmarking a New Column
And statistical quality control

Evaluation of column performance is critical for consistently reliable data.

- Quantitate and record the performance characteristics of a new column.
  - Subjective evaluations are extremely unreliable. For example, "I think I remember the last column having… [less pressure, sharper peaks, and so on] …when it was new [a year ago?]"
- Flush and equilibrate your new column
- Record the initial pressure on the included performance report.
- Inject an internal standard or control sample to:
  - Confirm retention time precision
  - Check peak shape (symmetry or tailing factor)
- Perform a system suitability check with the new column and look at RSDs of peak parameters
- Plot Area Under the Curve (AUC) on a Statistical Quality Control (SQC) chart
- Record peak symmetry or tailing factor, as well as final back pressure in a lab notebook or in performance maintenance records (an excel spreadsheet works well)

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10. Not benchmarking a new column

 9. Using the word "validated" incorrectly

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What Does 'Validated' Mean?

The FDA is the final authority on validations and has published a document called *Reviewer Guidance Validation of Chromatographic Methods.*

In this document, chapter IV is titled *Parameters for Validation of HPL Chromatographic Methods for Drug Substance and Drug Product*. It states that a validation includes:

A. Accuracy

B. Detection Limit and Quantitation Limit

C. Linearity

D. Precision
   1. Repeatability
      a) Injection Repeatability
      b) Analysis Repeatability
   2. Intermediate Precision (Ruggedness)
   3. Reproducibility

E. Range

F. Recovery

G. Robustness

H. Sample Solution Stability

I. Specificity/Selectivity

J. System Suitability Specifications and Tests (with six sections)

K. General Points to Consider

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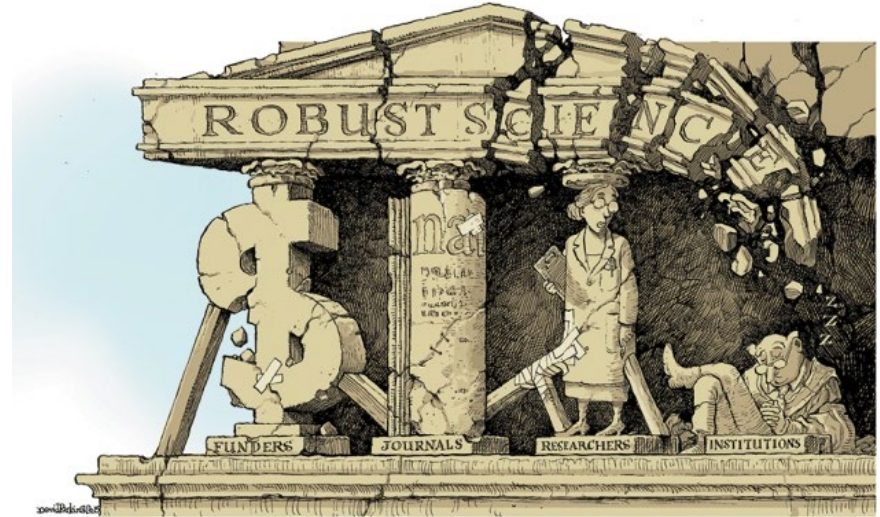
Studies estimate that only around 40% of published findings can be replicated reliably.[1]

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)

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Lack of Ruggedness
Or intermediate precision

"Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over.
Depending on time and resources, the method can be tested on multiple days, analysts, instruments, etc." - *Reviewer Guidance Validation of Chromatographic Methods*, p.15

Lack of ruggedness is one of the most common failings of what people (mistakenly) call a validated method. If your method does not give good results across multiple lots of the same column, the issue is not "a column problem". The issue is that the method is not rugged enough.

Methods I have validated included at least two instruments run by two analysts over three days, using three different lots of packing material. Typically, we collected 90–120 data points and looked at inter and intra variable results. The variables were different analysts, instruments, days, and lots of material.

Ruggedness is the component of our validation study that gives us confidence in the reliability of data generated by end users of this method, after it has been put into routine service.

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Re-using an Old Column

On the far extremes of "lack of ruggedness" are issues relating to reusing old columns. Over the last four years I have received this call more times than you would believe.
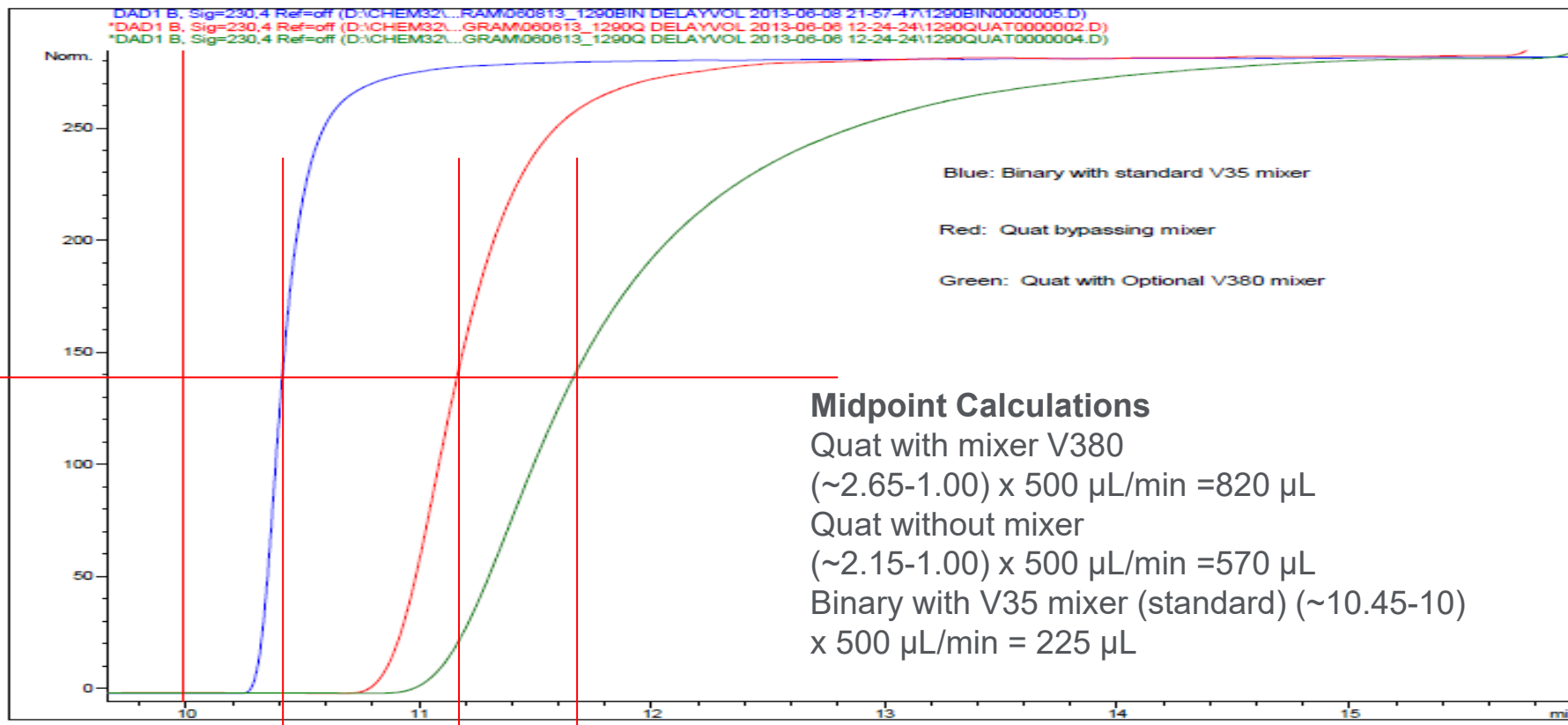
- How we use and store our columns can, potentially, change their surface chemistry. Bonded phase may be lost due to hydrolysis, over time. Contaminants can bond, irreversibly to the particles in our column, creating a new unique multimodal phase.

- After a method has been developed or validated, consider transferring those columns that were used in the validation to the labs that will be running this new method routinely. Transferring with the same exact columns is a good way to limit variables during transfer and minimize troubleshooting that might be required if the results differ.

- Transferring the original columns allows us to get the full lifetime of usage from these columns. Once the method and columns are transferred, replace them with a new set of columns for the next method development project. In this manner, we always have new columns for developing new methods but none of the old columns are wasted or disposed of prematurely.

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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

Blue: Binary with standard V35 mixer

Red: Quat bypassing mixer

Green: Quat with Optional V380 mixer

Midpoint Calculations
Quat with mixer V380
(~2.65-1.00) x 500 µL/min =820 µL
Quat without mixer
(~2.15-1.00) x 500 µL/min =570 µL
Binary with V35 mixer (standard) (~10.45-10)
x 500 µL/min = 225 µL

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Forgetting to Check Analyte Properties

- Physical-Chemical properties of our analytes significantly impact our choice of bonded phase

- Examples might include:
  - Log P – the octanol/water partitioning coefficient
  - pKa – the acid dissociation constant
  - Structure, for example Pi-electron density and dipole moment
  - Solubility (more on the next slide)

- These properties can be found at several different resources, such as PubChem, ChemSpider or others. Most can also be estimated through the use of commercially available software.

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Selecting (or using) the wrong buffer or mobile phase

Buffer and mobile phase considerations:

- Sample solubility – ideally, sample should be solvated with initial mobile phase

- Buffer salt solubility – Remember, for example, that phosphate buffer is not very soluble in ACN

- Viscosity and back pressure – at what point in the gradient do we expect highest pressure

- Miscibility – use IPA to transition to and from solvents not miscible with water

- Detector specific issues such as UV cutoff, ionization potential and noise

Using the wrong mobile phase – tips for handling aqueous mobile phase:

- Aqueous reservoirs should be amber and not in direct sunlight

- Mobile phase should be replaced no less than once a week

- Always flush system with pure water for evening shutdown, following a buffered method

Note: The "right" mobile phase includes using a high organic hold at the end of a gradient or an organic flush after an isocratic sequence.

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3. Poor sample preparation

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Poor sample preparation

Sample cleanup:

- Options include – SPE, PPT, LLE, Quechers, filtration, etc.

- Cleaner samples should extend column life. SQC charts help track:
  - Peak shape
  - Retention time
  - Resolution of a critical pair

- Every additional step the sample is subjected to has the potential to impact accuracy and precision.
  - Consider recovery studies to confirm sample is not lost during cleanup
  - Use a second internal standard for cleanup

Sample presentation:

Sample solvent should match initial mobile phase. This not only prevents precipitation but also facilitates proper loading on the head of the column. When using a sample solvent that has a higher eluotropic strength than the mobile phase, we expect to see broader peaks due to poor sample loading. This can partly be compensated for by using a smaller injection volume.

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3. Poor sample preparation
2. Choosing the wrong column

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Choosing the Wrong Column

- "We had it in our lab already" is never the correct reason for choosing a column

- Bonded phase is best chosen based upon the physical-chemical properties of your analyte

- Column dimensions are often chosen based upon several different factors including, but not limited to:
  - The detector
  - Sample concentration
  - Throughput needs
  - Maximum system pressure
  - Delay volume

- We don't always want to focus all of our efforts on the first bonded phase that appears to work. If time permits, it's a good idea to run initial scouting gradients on several orthogonal phases

- If our intention is to transfer a method after development, then we need to remember that the correct column dimensions are those that best fit the instruments that will be performing the routine analysis.

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2. Choosing the wrong bonded phase
1. Using an HPLC system that needs maintenance

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Using an HPLC System that Needs Maintenance

- Tracking column performance with benchmarking should show variations which suggest the maintenance needed

- Most modern chromatography software includes options for tracking usage
  - Such as number of injections, number of valve switches, volume of solvent pumped, and others.

- Correlating this information with our SQC data allows us to develop a timely and efficient preventative maintenance plan

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Using an HPLC System that Needs Maintenance, Continued

- One of the most common issues we have is mistaking an instrument error for a problem with a column, especially if the column is new.

- Try putting a new column into service before the previous column is completely spent:

  – Tracking column performance (number of injections) should quickly establish an average and standard deviation for the number of injections the column in our method is expected to be capable of handling. At 50%-60% of that value, test the next column to ensure it behaves as expected. **You now still have a well-functioning column, the original one, to use as a reference if a new column gives unexpected results.**

  – If a new column appears to be problematic, reinstall the original column that you know is good, and re-evaluate the method's performance.

  – If the results are similar to the new column's results (not as expected), then the issue is most likely with the HPLC system and not the column.

System suitability, along with a strong program of preventative maintenance, are requirements for consistently generating strong, reliable data.

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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](#)

- Consumables Community: [Agilent Collection of Columns, Supplies, and Standards Resources - Consumables - Agilent Community](#)

- App finder: [Application Finder | Agilent](#)

- Agilent University: [Agilent University](#)

- YouTube: [Agilent Channel](#)

- Your local product specialists

- Agilent Peak Tales podcasts: [peaktales.libsyn.com](#)

- Webinars, upcoming and recorded: [LC & LC/MS Column Webinars | Agilent](#)

Contact Agilent Chemistries and Supplies Technical Support

Available in the U.S. and Canada, 8-5 all time zones

1-800-227-9770 option 3, option 3:

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for sample preparation, filtration, and QuEChERS

Option 4 for spectroscopy supplies

Option 5 for chemical standards

Option 6 for Prozyme products

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spp-support@agilent.com

spectro-supplies-support@agilent.com

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Questions?

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