Essentials for Good HPLC Method Development

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Outline

- The chromatographic process and resolution
- Band broadening in the column – The Van Deemter equation
- The power of selectivity – How to choose an orthogonal column
- How delay volume impacts method development and transfer
- Introduction to scouting gradients

Chromatographic Process

Partition between mobile phase and stationary phase ($K = C_s/C_m$)

Description of the separation:

- R_s – Resolution
- N – Column efficiency, plates
- *k, k'* – Retention factor, capacity factor
- *α* – Selectivity
- t_{ret} – Retention time

Definition of Resolution

Resolution is a measure of the ability to separate two components

$$R_s = \frac{t_{R-2} - t_{R-1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$$

Resolution

Determined by three key parameters:
Efficiency, selectivity, and retention

The fundamental resolution equation

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$

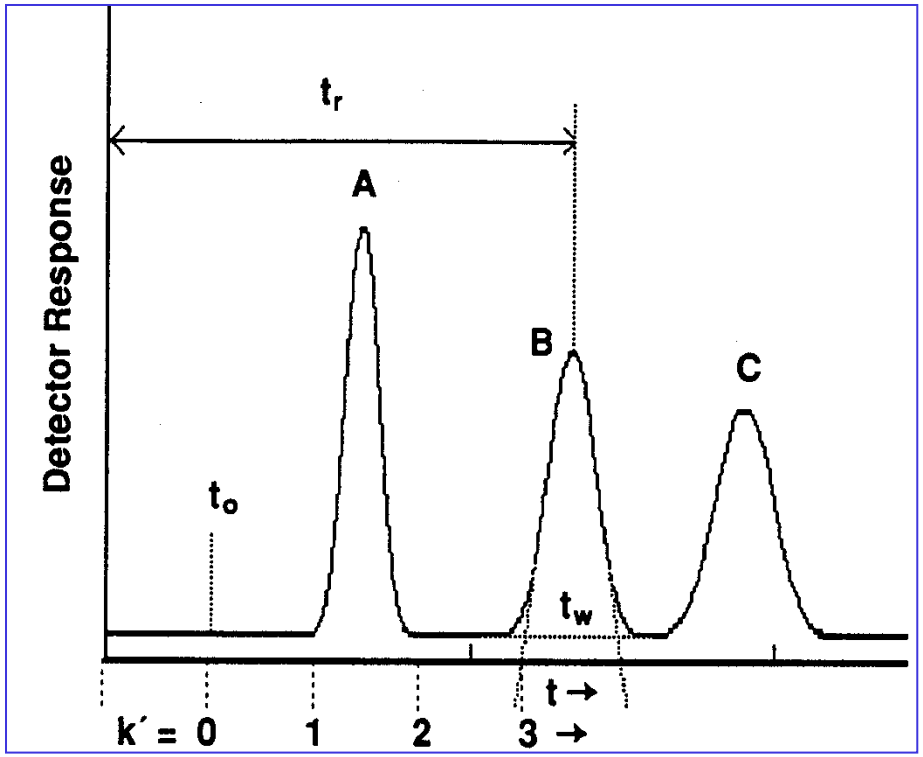
N = Column efficiency – Column length and particle size

a = Selectivity – Mobile phase and stationary phase

***k* = Retention factor** – Mobile phase strength

Chromatographic Profile

Equations describing factors controlling R_S



Retention factor

$$k = \frac{(t_R - t_0)}{t_0}$$

Selectivity

$$\alpha = k_2/k_1$$

Theoretical plates – efficiency

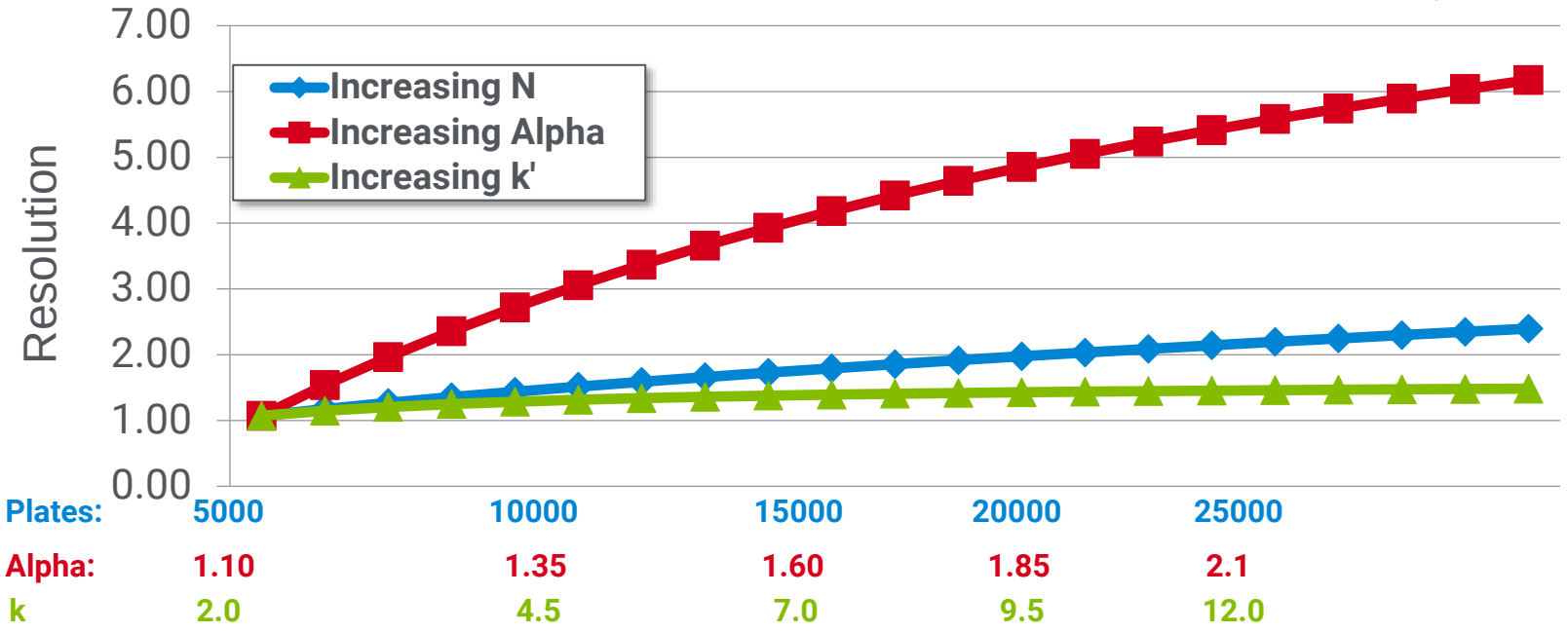
$$N = 16(t_R / t_{W\text{-base}})^2$$

$$N = 5.56(t_R / t_{W\text{-}1/2})^2$$

Factors That Affect Resolution

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution Efficiency Selectivity Retention



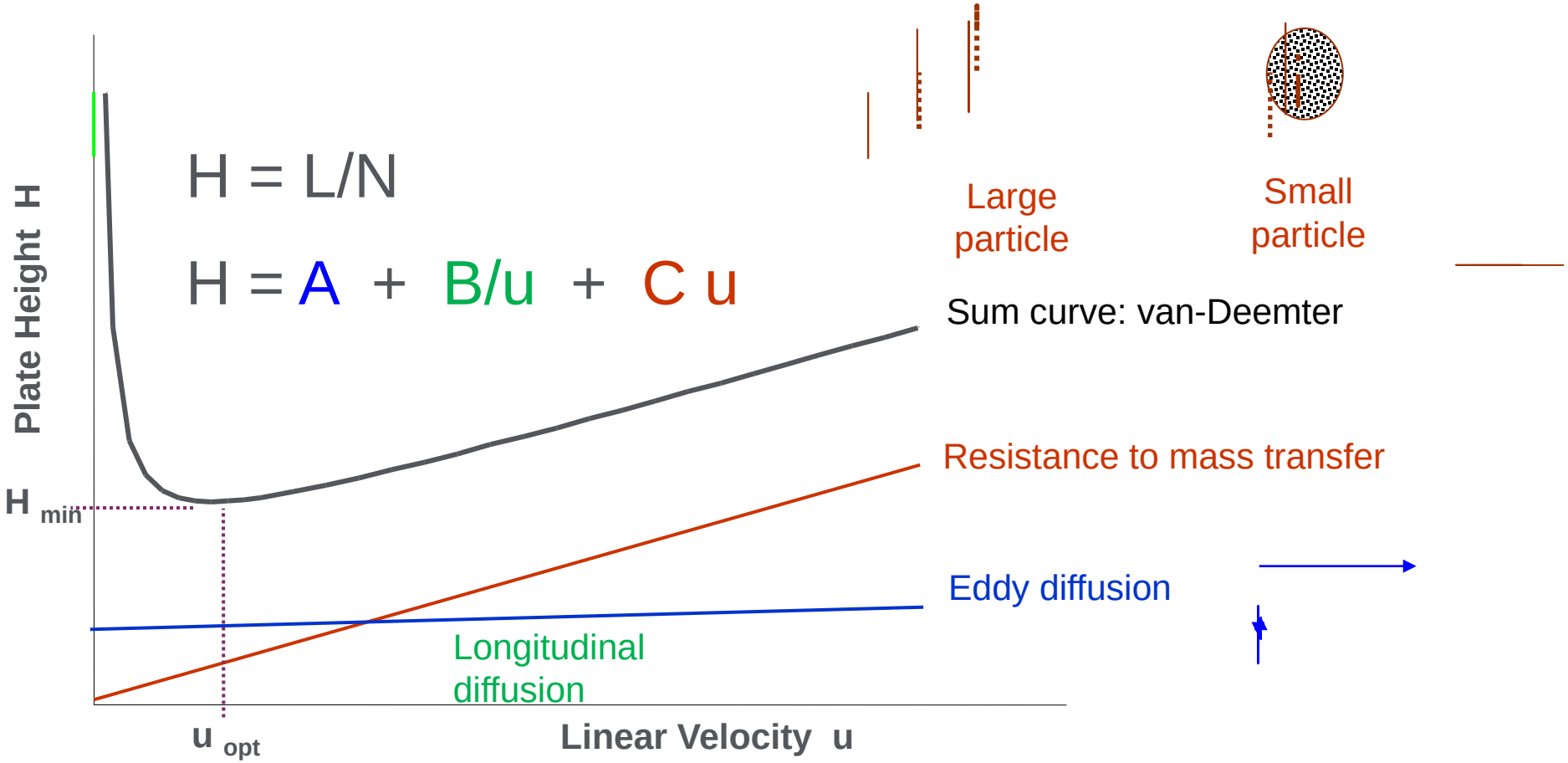
Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} Typical analytical method development parameters

Putting It Together

The van Deemter equation

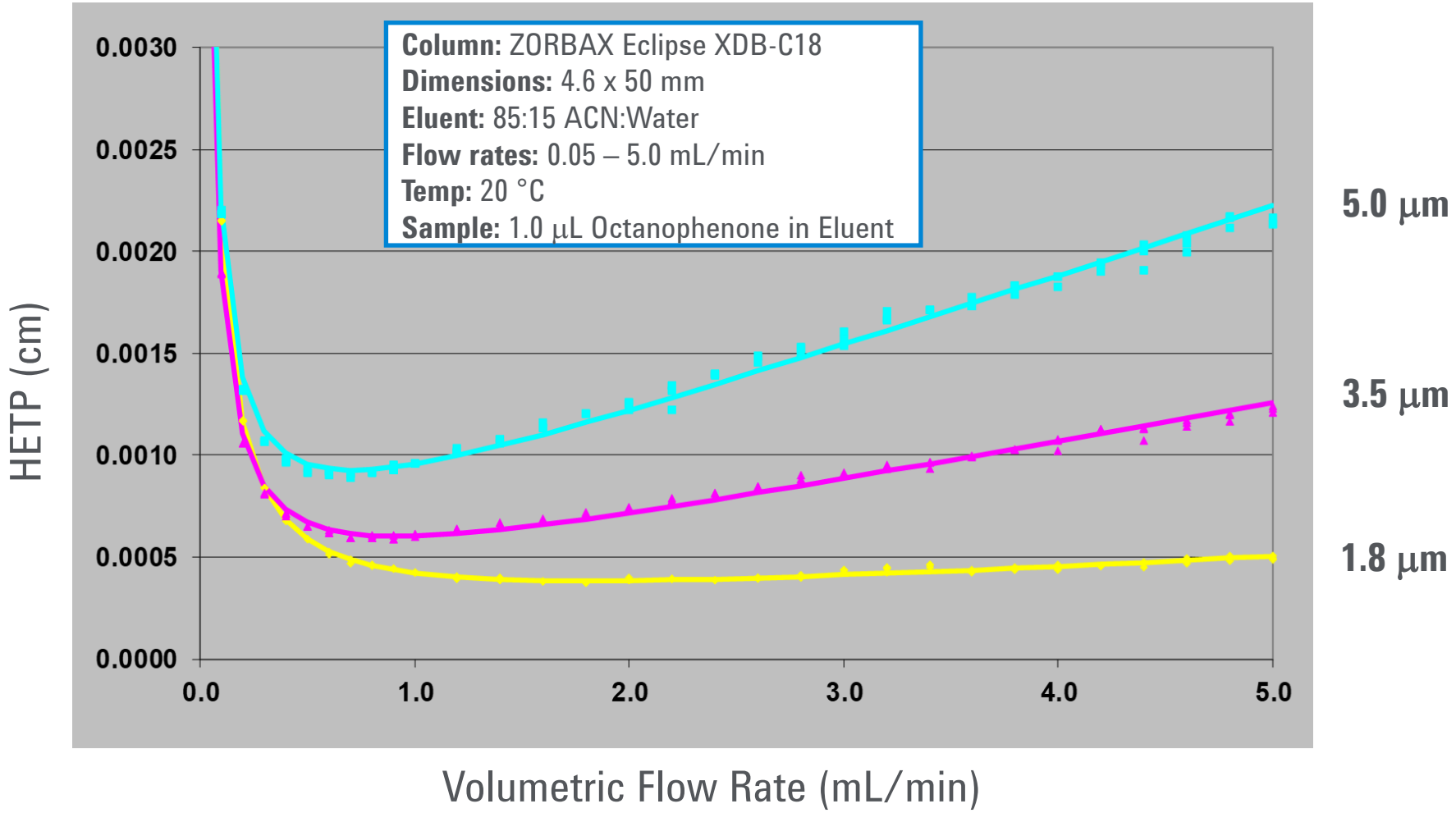


The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

Van Deemter Curve

Effect of particle size

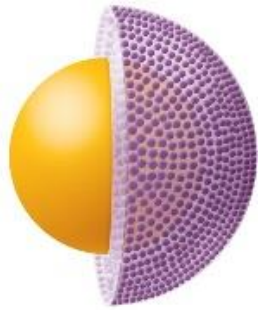
$$H = A + B/u + Cu$$



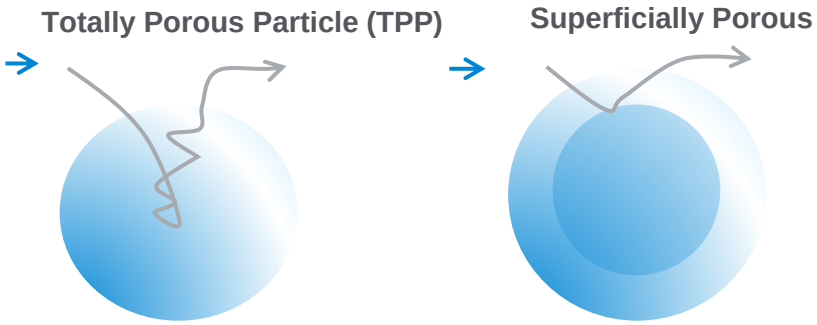
Smaller particle sizes yield flatter curves, minimal shift to higher flow rates

Poroshell Technology

What makes it better?



Poroshell is made of a solid core with a porous outer layer

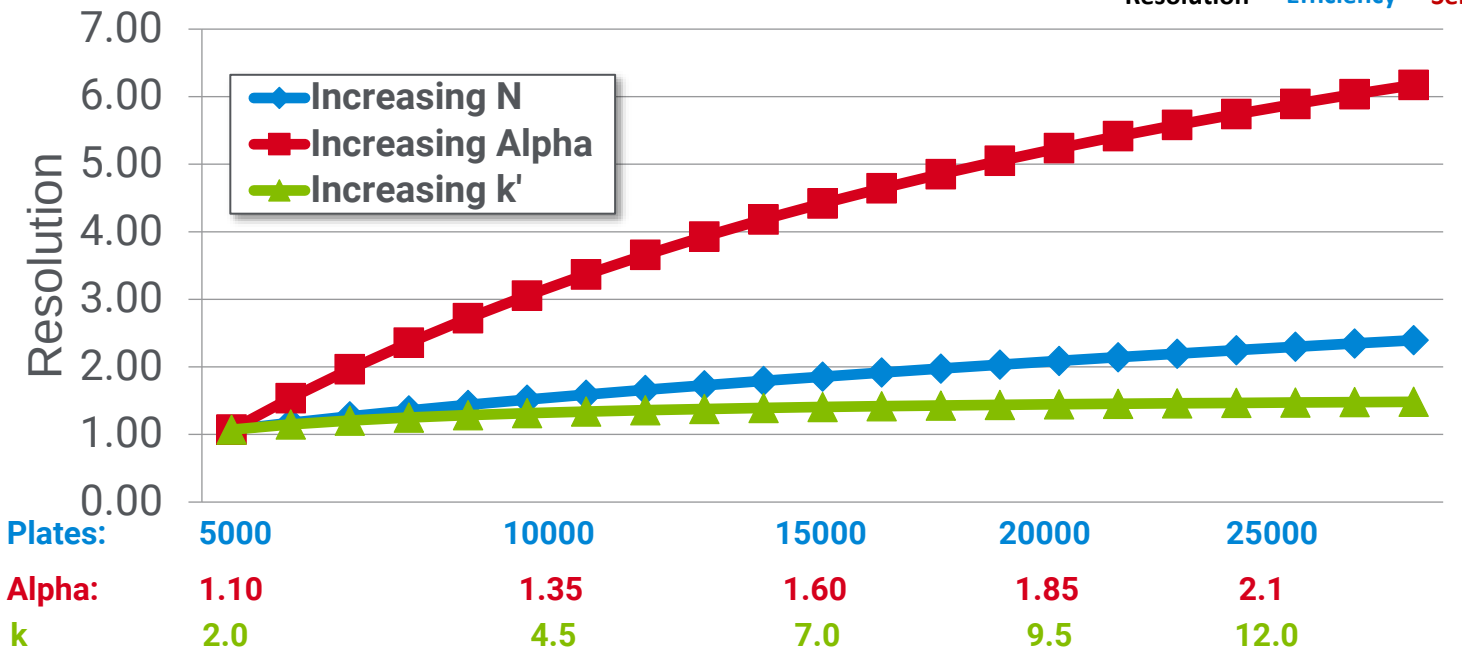


- Analytes travel though the particle more efficiently: improving peak shape and resulting in faster run-times
- High efficiency allows you to use a larger SPP (ie. 2.7 µm) for nearly equivalent performance to a smaller TPP column (ie. sub-2.7 µm)
- Using a larger particle allows for lower backpressure than comparable TPP columns, and flexible use on HPLC or UHPLC systems

Factors that Affect Resolution

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution Efficiency Selectivity Retention



Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} **Typical Analytical Method Development Parameters**

Column Choice

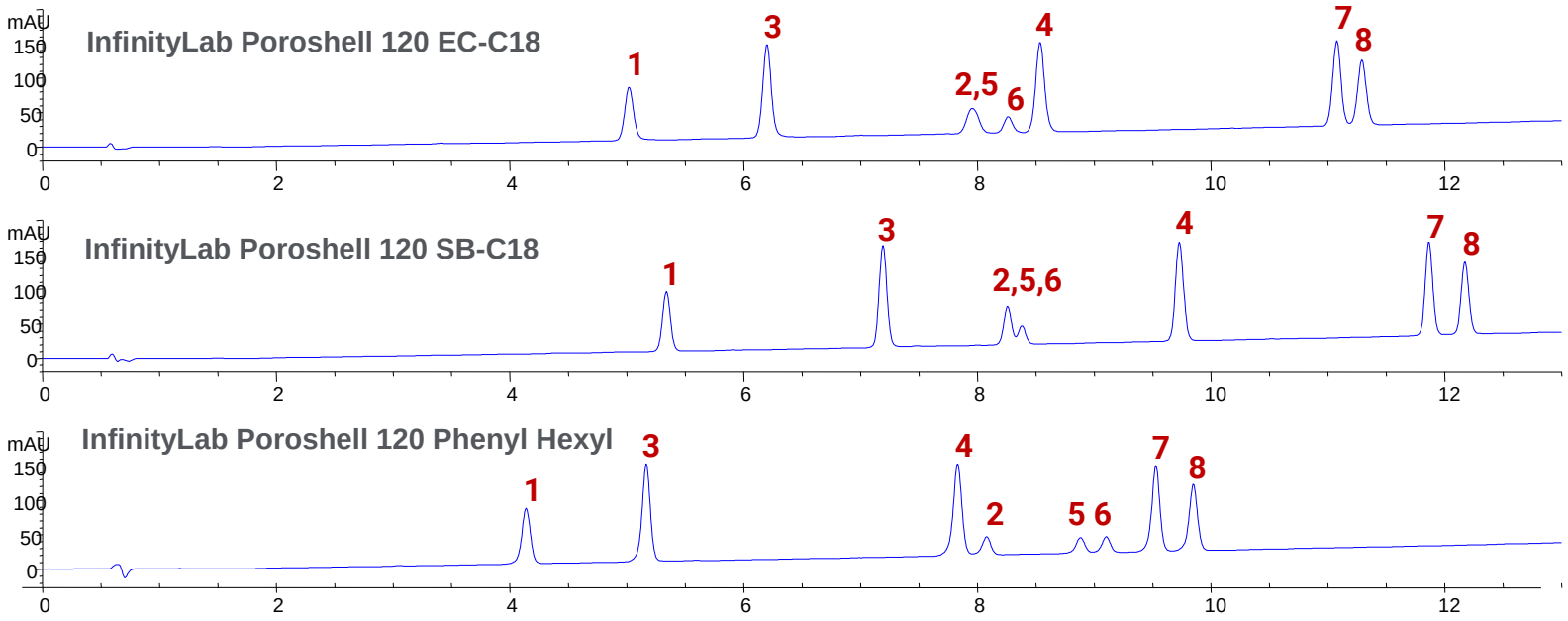
Evaluate different bonded phases

- Bonded phase affects selectivity (alpha)
- Different interactions for polar and non-polar compounds.
- Exploit other interactions with bonded phase (e.g., pi-pi)
- Changing the bonded phase can improve selectivity/resolution, reduce analysis time
- Using superficially porous particles (SPP) decreases Van Deemter band broadening

Evaluating different bonded phase chemistries early can save time in optimization and generate a more robust method

Selectivity Differences

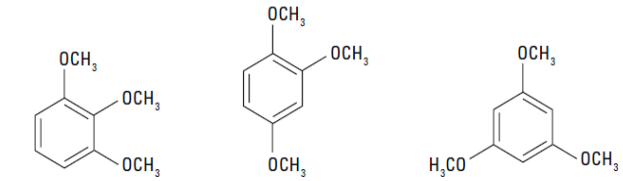
Across InfinityLab Poroshell bonded phases



1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone
5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

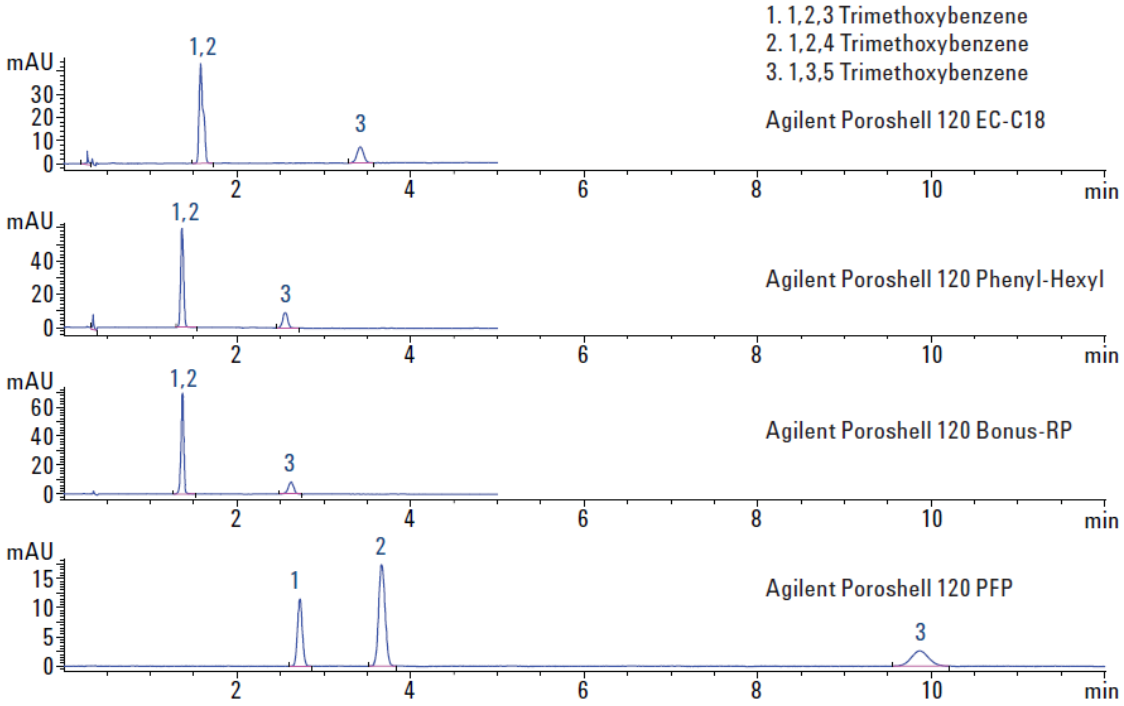
40-80 % Methanol in 14 min, DAD 260, 80 nm 0.4 ml/min, 2.1 x 100 mm column, 40 C, 0.1% Formic Acid in Water and Methanol, Agilent 1260 Method Development Solution

Importance of Alternate Selectivity Chemistries



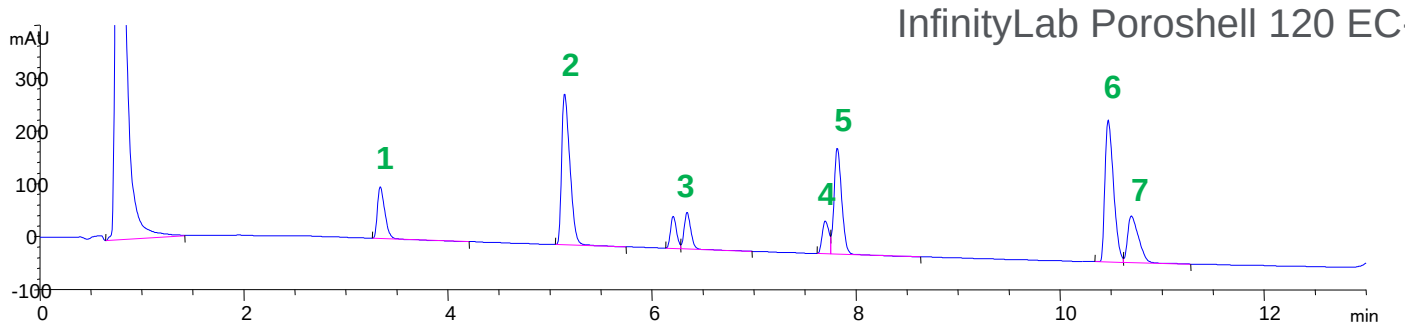
Three compounds

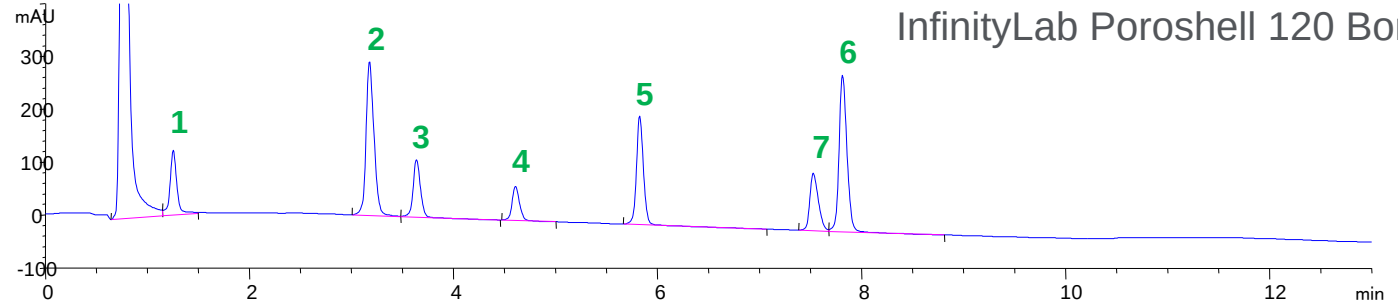
- Same molecular weight
- Only differ by positional location of the functionality



InfinityLab Poroshell 120 columns 4.6 x 50mm, 2.7um
70:30 – MeOH/H2O, 1.5 ml/min, 40º, 254nm

Polar Embedded Phase for Alternate Selectivity





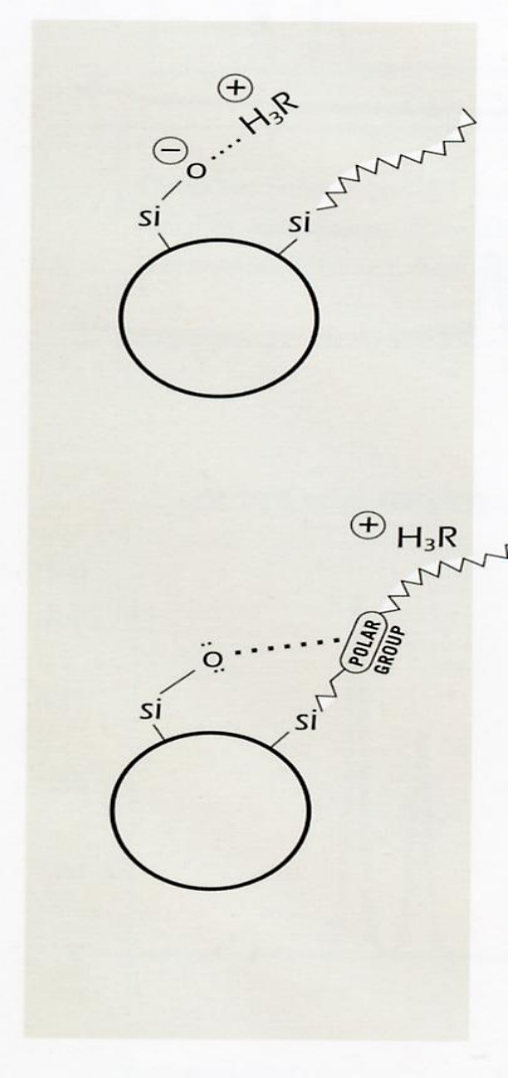
Beta Blockers

1. Atenolol
2. Pindolol
3. Naldolol
4. Metoprolol
5. Acebutolol
6. Propranolol
7. Alprenolol

Polar embedded group provides unique selectivity

10-70 % Methanol/12 min, DAD 260 nm 0.35 ml/min, 2.1 x 100 mm 40 C 10 mM pH 3.8 Ammonium Formate Buffer and Methanol

Improved Resolution and Peak Symmetry

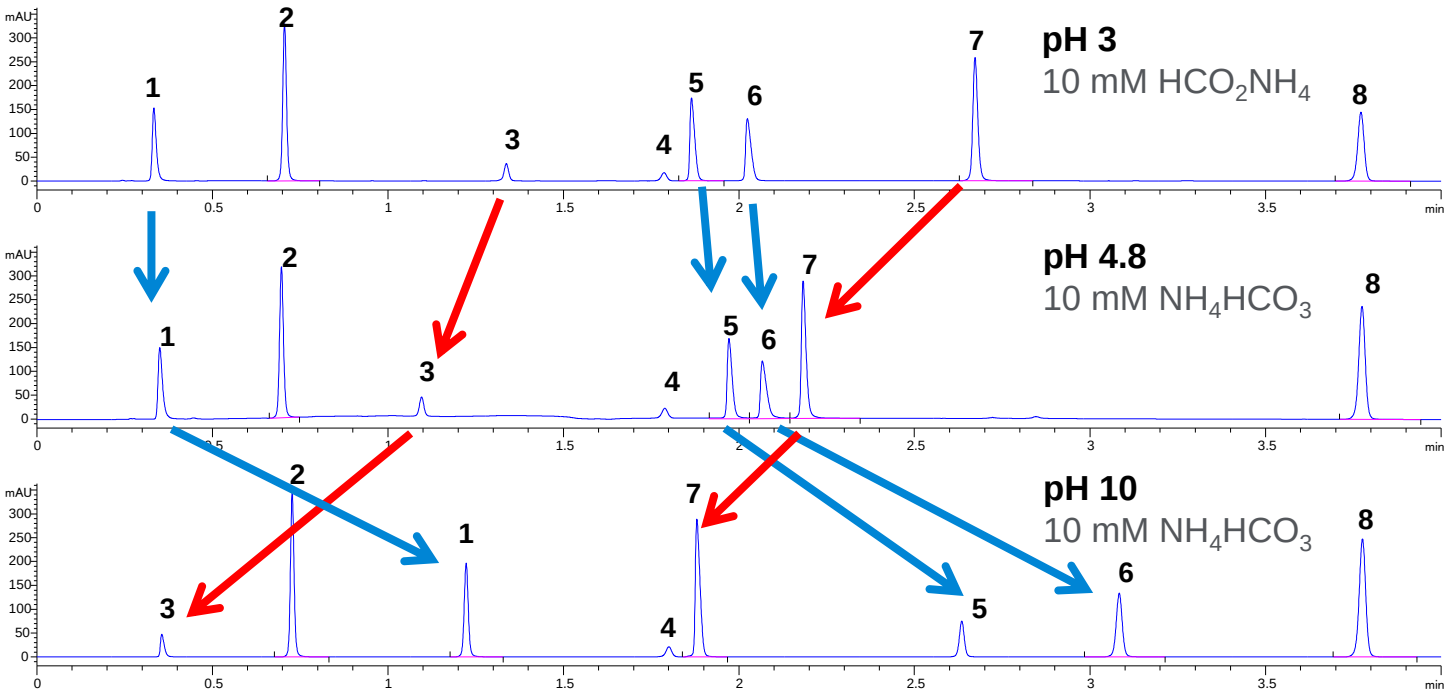


On conventional media, exposed silanols can cause mixed mode effects and poor peak shape.

Polaris embedded phases provide additional charge density to adjacent silanols through electron delocalization, thereby removing mixed-mode interactions and improving peak symmetry.

Selectivity Can be Controlled by Changing pH

Agilent InfinityLab Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm



1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

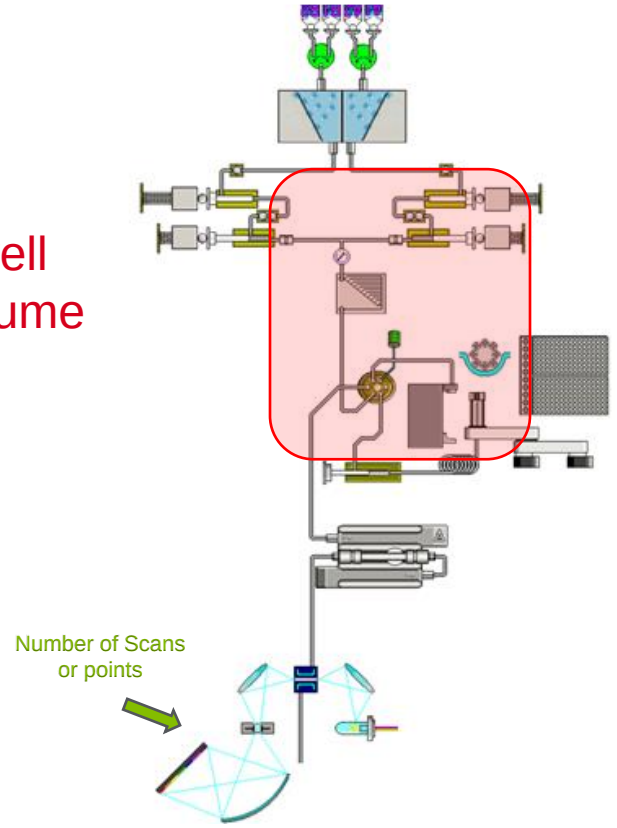
Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 ml/min		254 mn

Acids
Bases

Instrument Impact on Column Performance

Dwell volume

Dwell volume



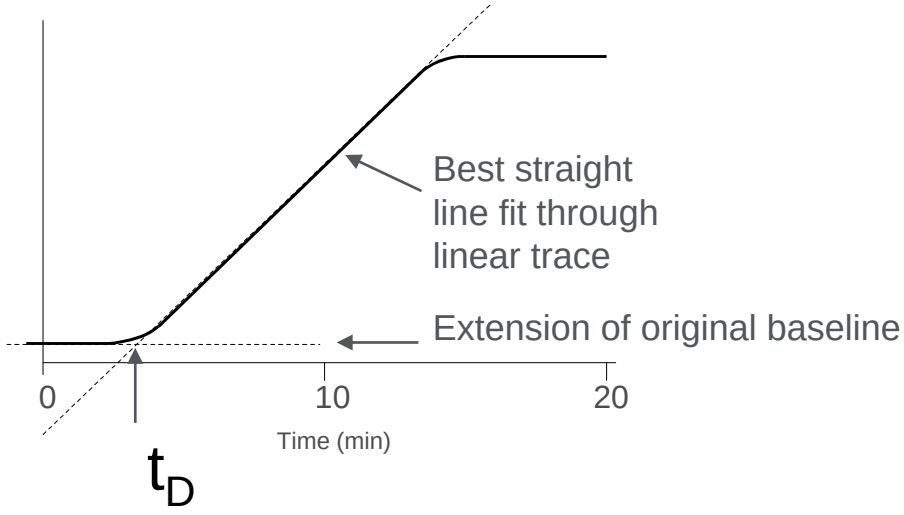
Dwell volume = volume from formation of gradient to the column

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 0–100% B in10 min at 1.0 mL/min
- Record
- Expected dwell volume in UHPLCs – µL range!

Measuring Dwell Volume (V_D)

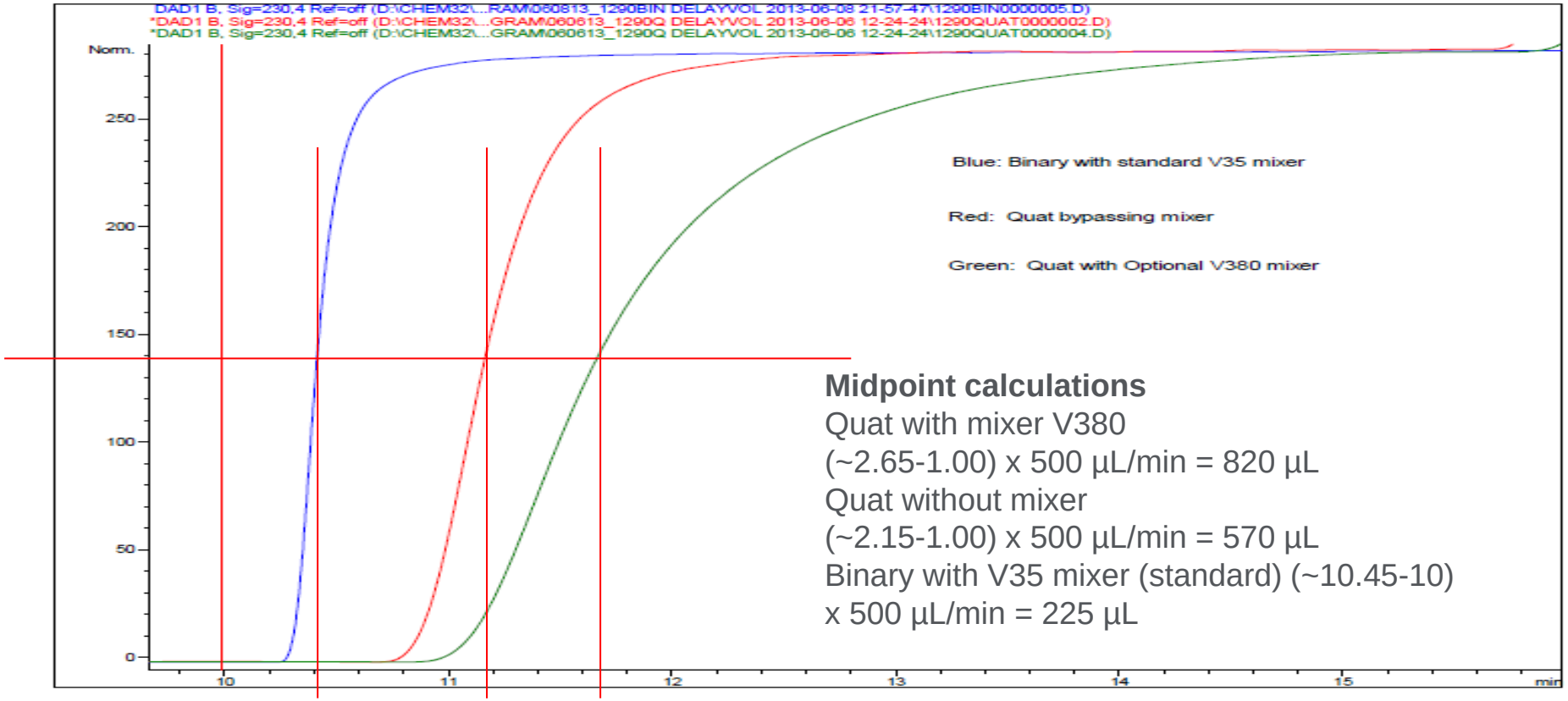
$V_D = t_D \times F$



- 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



Starting Point
Scouting gradient

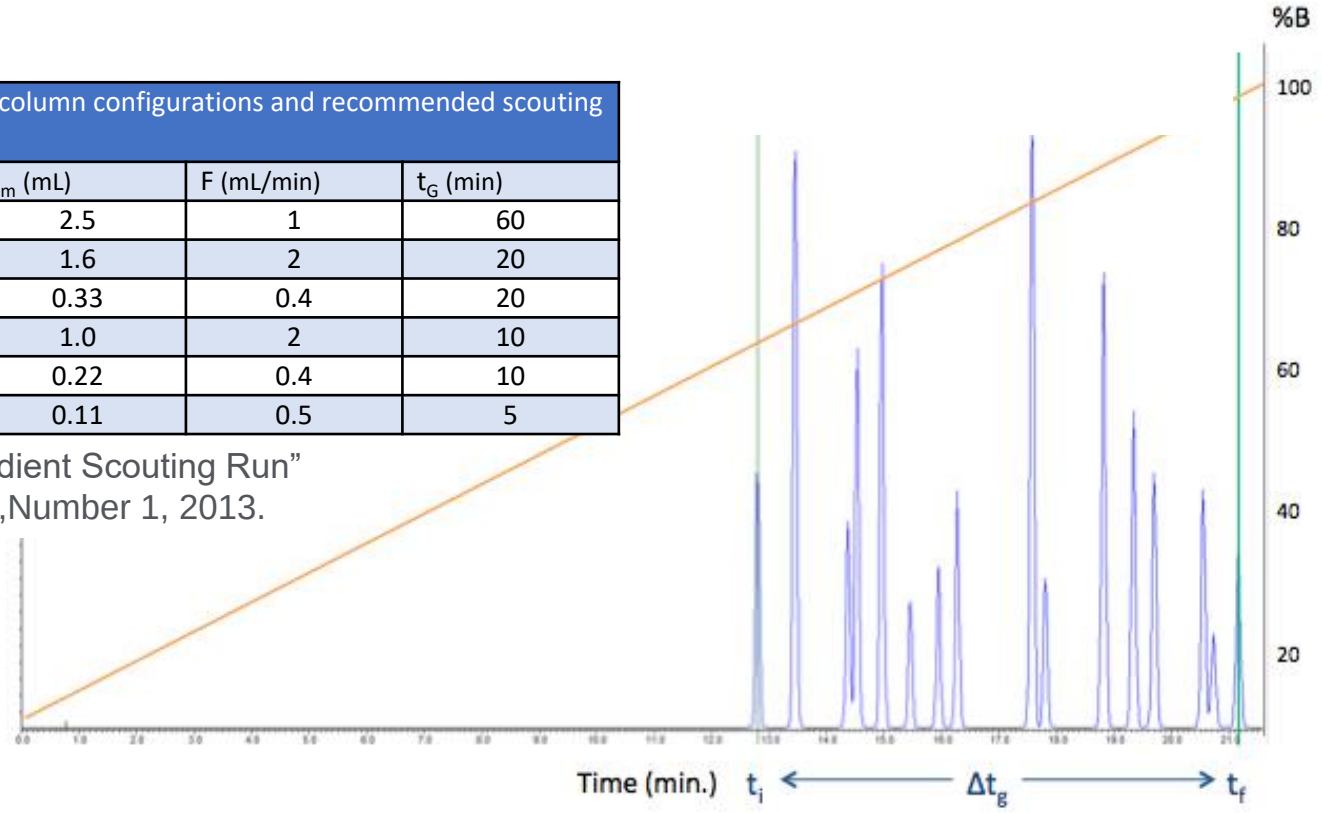
A good starting point when developing a method is a scouting gradient. The conditions recommended by John Dolan are 5–95% acetonitrile, low pH, and are dependent on the column length.

Where 10 cm columns are chosen, use a 10 minute gradient. This example shows a 150 mm column.

Table 1: Column volumes for various column configurations and recommended scouting gradient times

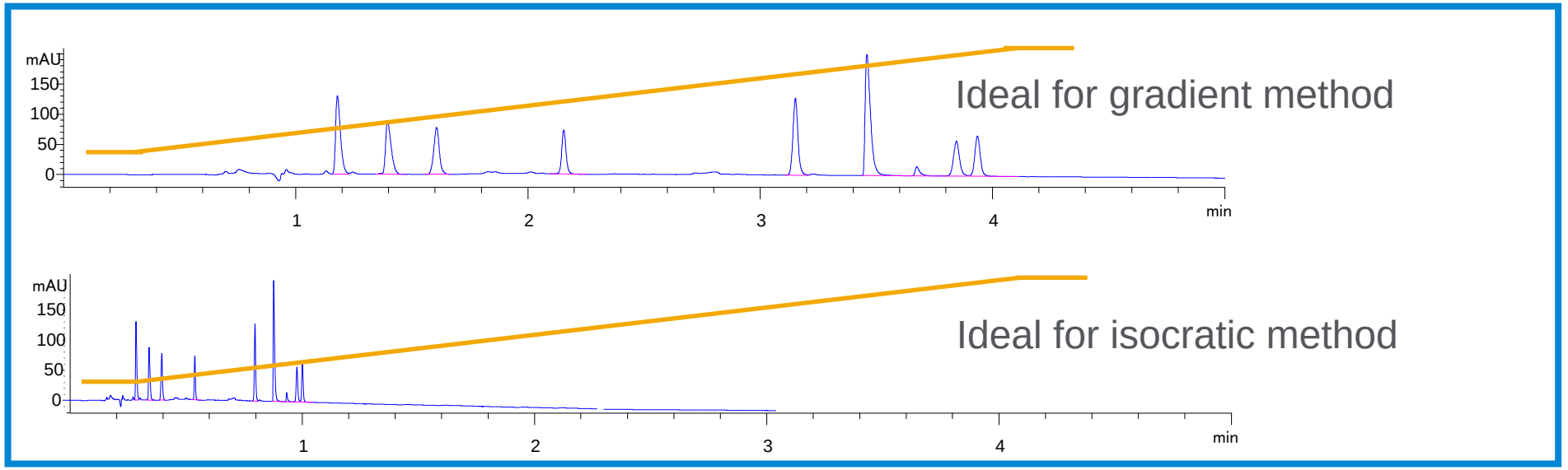
L (mm)	d_c (mm)	V_m (mL)	F (mL/min)	t_G (min)
250	4.6	2.5	1	60
150	4.6	1.6	2	20
150	2.1	0.33	0.4	20
100	4.6	1.0	2	10
100	2.1	0.22	0.4	10
50	2.1	0.11	0.5	5

"Making to the most of a Gradient Scouting Run"
LCGC North America Vol. 31,Number 1, 2013.

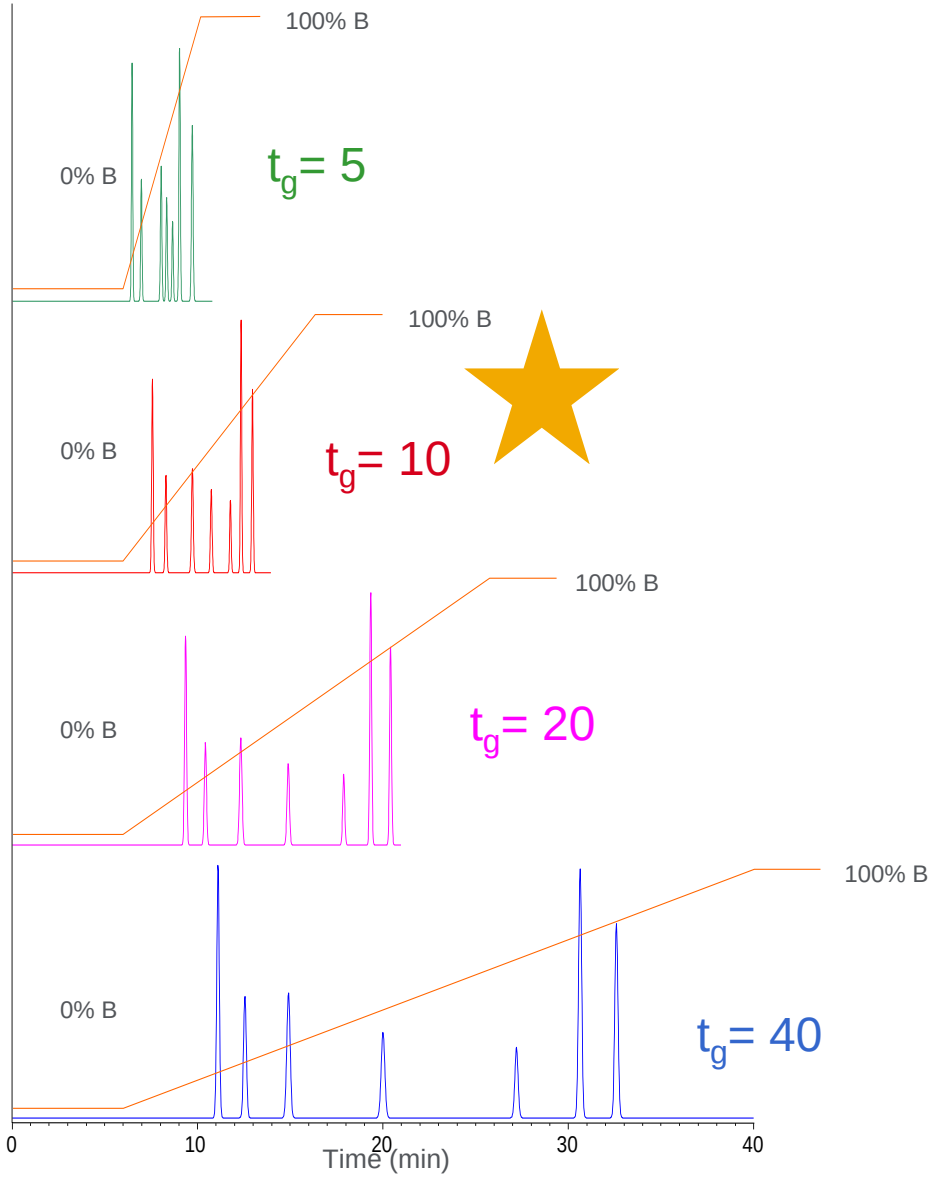


Gradients are Critical Tools for Faster Methods

- Run a scouting method 5% to 95% organic (reversed phase)
- Quick evaluation: how much of the gradient is occupied
  - $\frac{\Delta t_G}{t_G} \leq 25\%$ isocratic is recommended
  - $\frac{\Delta t_G}{t_G} \geq 40\%$ gradient is recommended



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



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

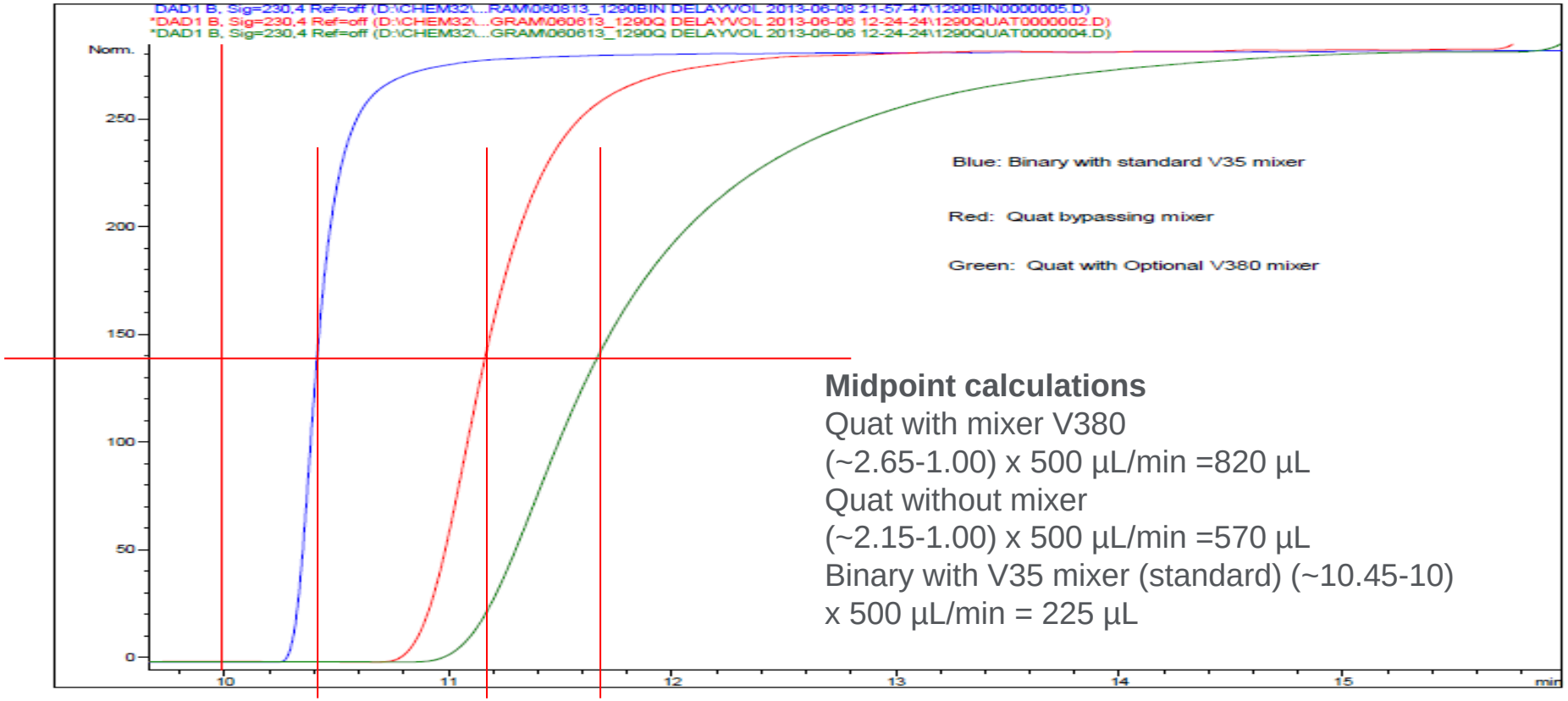
1/k* = gradient steepness = b

$\Delta\Phi$ = 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

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



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)
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Questions?