Method Development 101: From Beginner to Expert Part 1

From column selection to the first injection

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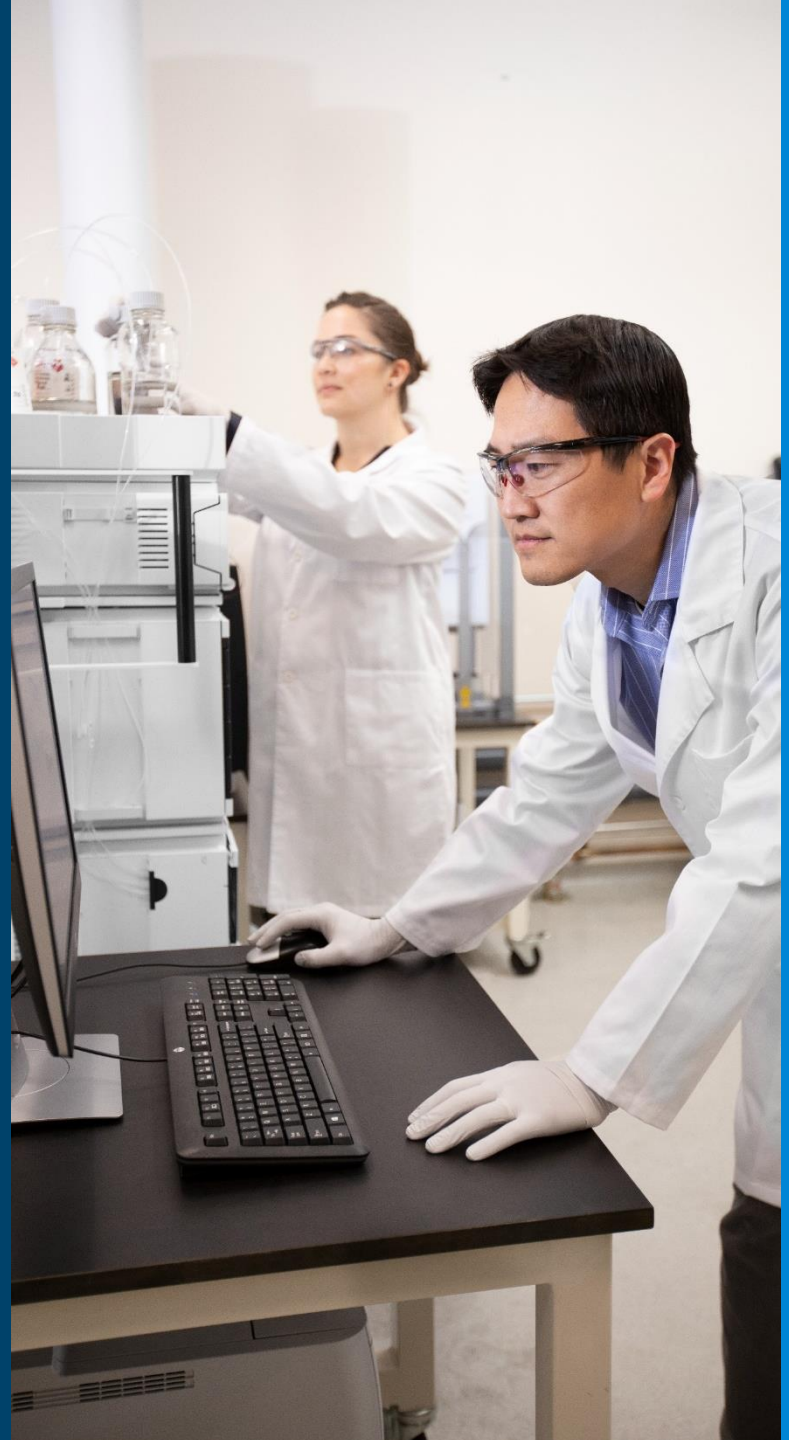

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Trusted Answers

Method Development 101

Agenda

- Key Terminology
- Method Goals
- Column Selection
- Mobile Phase Selection
- Flow Rates & Injection Volumes
- Scouting Gradient
- Q&A

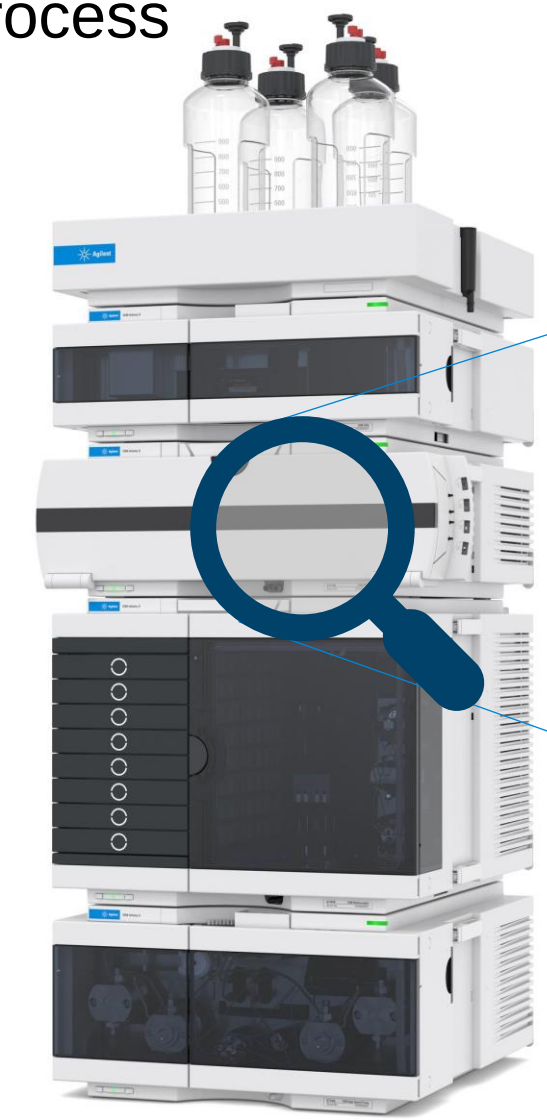
Method Development 101

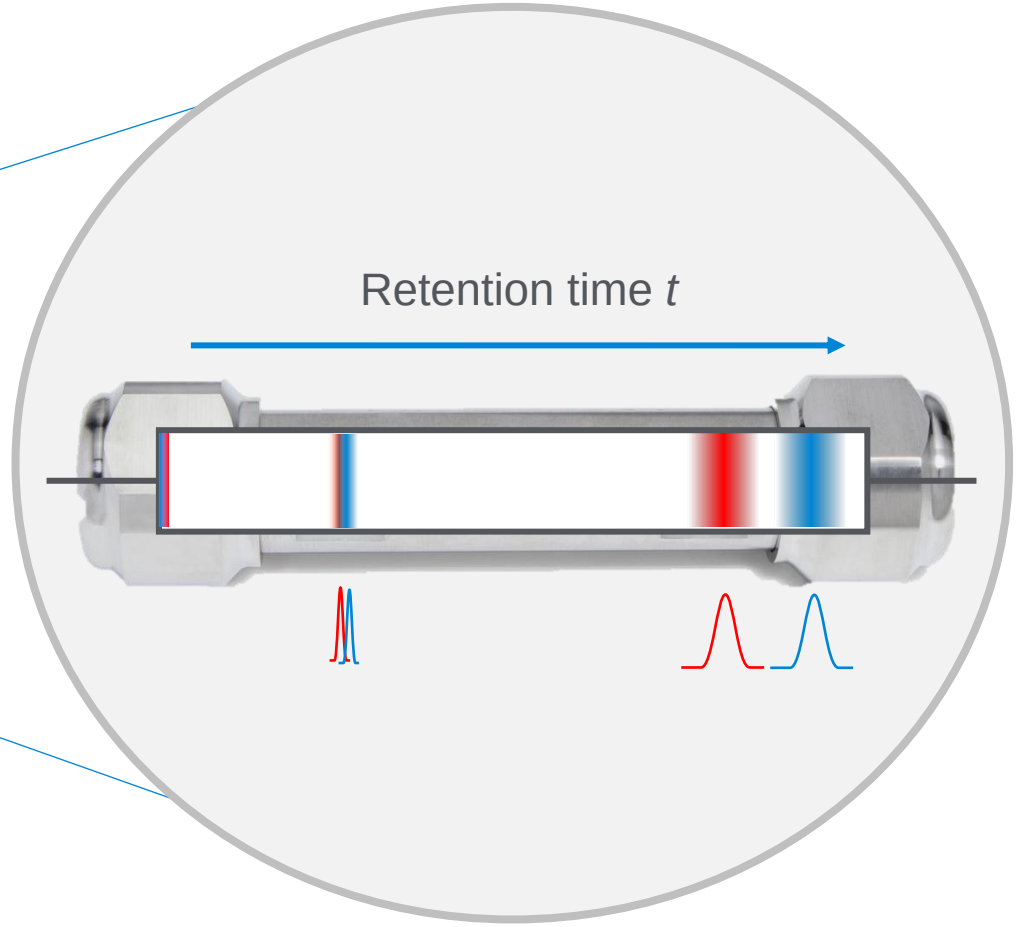
Key Terminology

Agilent

Key Terminology

Chromatographic process

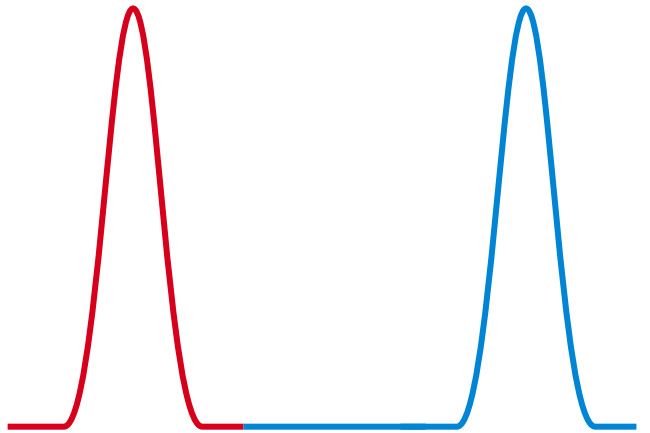




Key Terminology

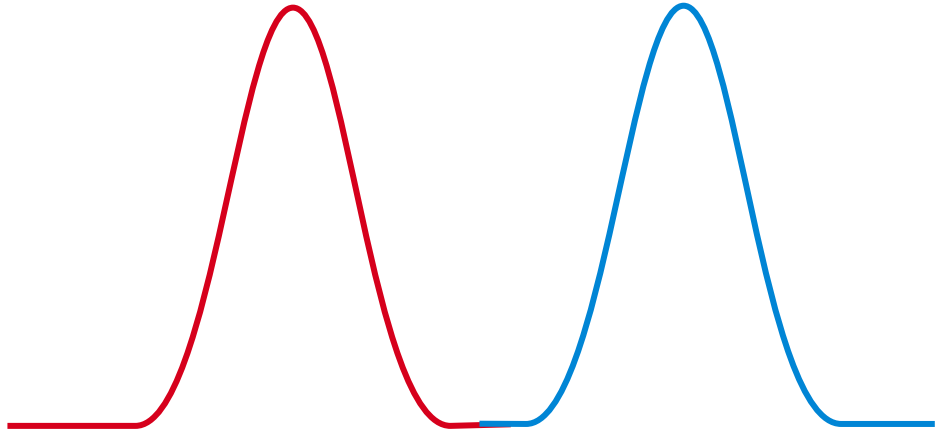
Chromatographic process

What makes one separation better than the other?



Good separation

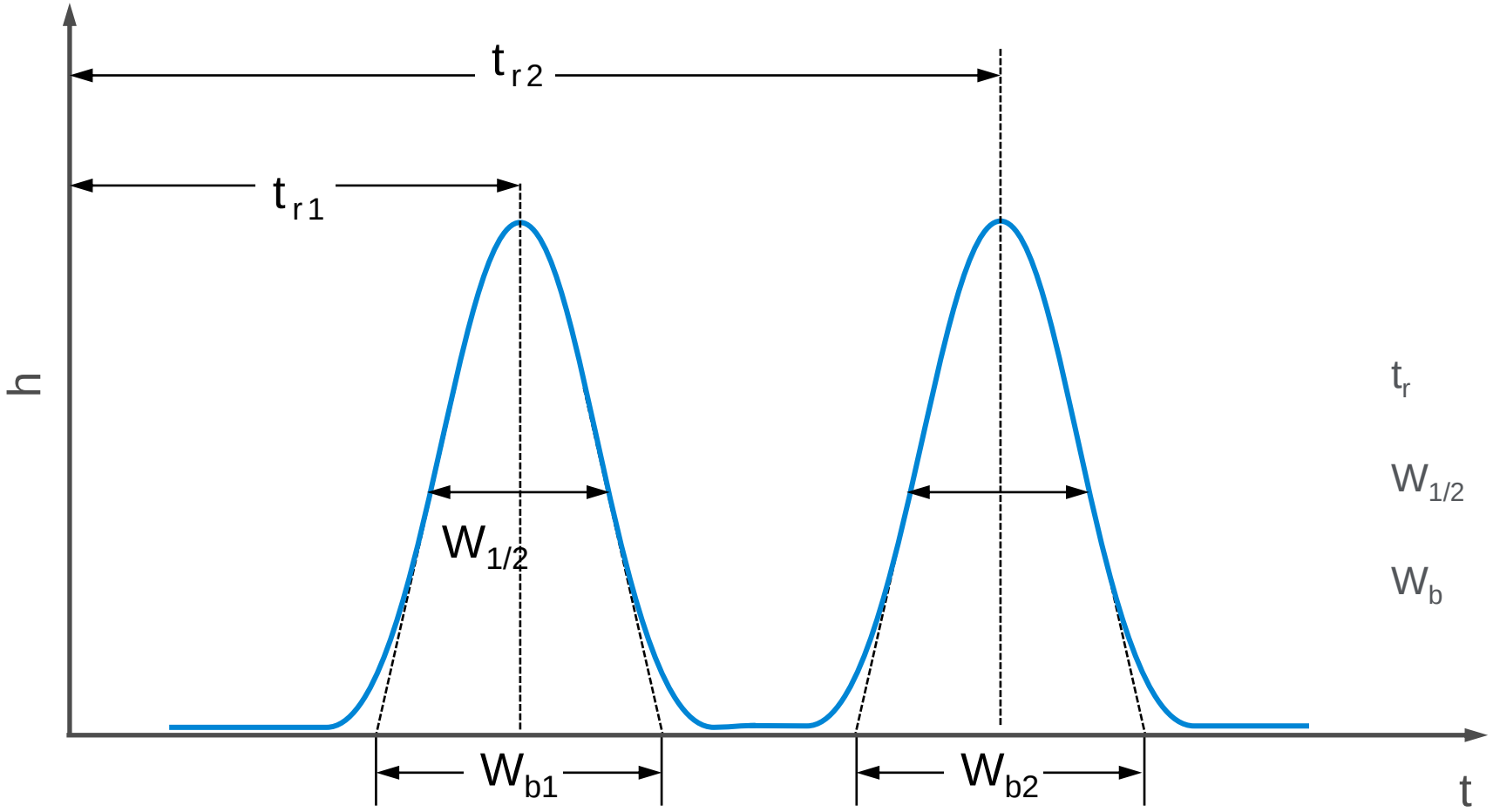
How do we design methods that result in consistent, good separations?



Less good separation

Key Terminology

Peak anatomy



t_r Retention time compound

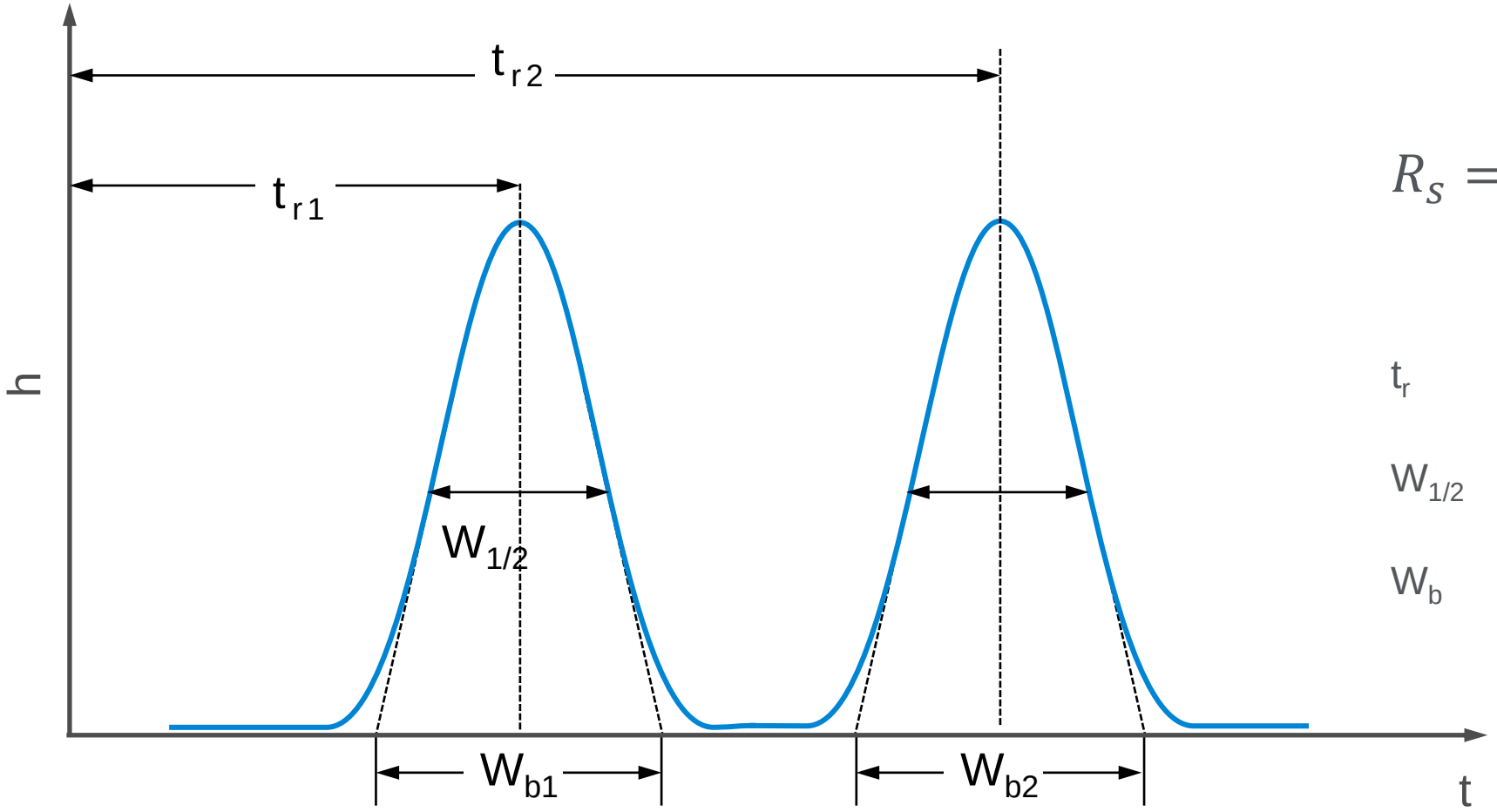
$W_{1/2}$ Peak width at half height

W_b Peak width at baseline

Key Terminology

Quantitative resolution

Resolution describes the ability of a column to separate the peaks of interest



$$R_s = \frac{t_{r2} - t_{r1}}{1/2 \cdot (W_{b2} + W_{b1})}$$

t_r Retention time compound

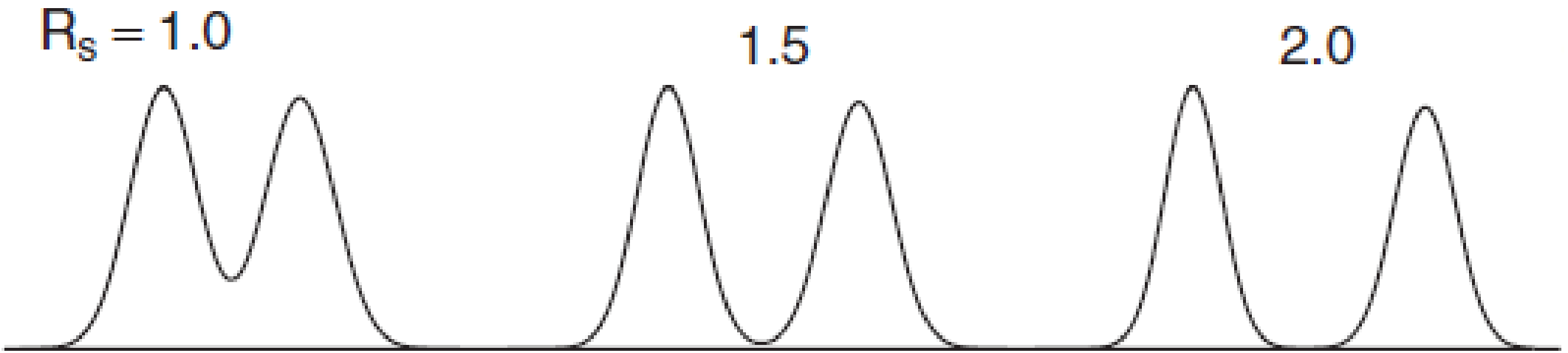
$W_{1/2}$ Peak width at half height

W_b Peak width at baseline

Key Terminology

Resolution

Resolution describes if baseline separation was achieved



- R_s = 1 separation of two peaks
- $R_s \geq 1.5$ Baseline separation and exact quantitation is possible
- $R_s \geq 1.7$ desirable for robust method

Key Terminology

Resolution: Influencing factors

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

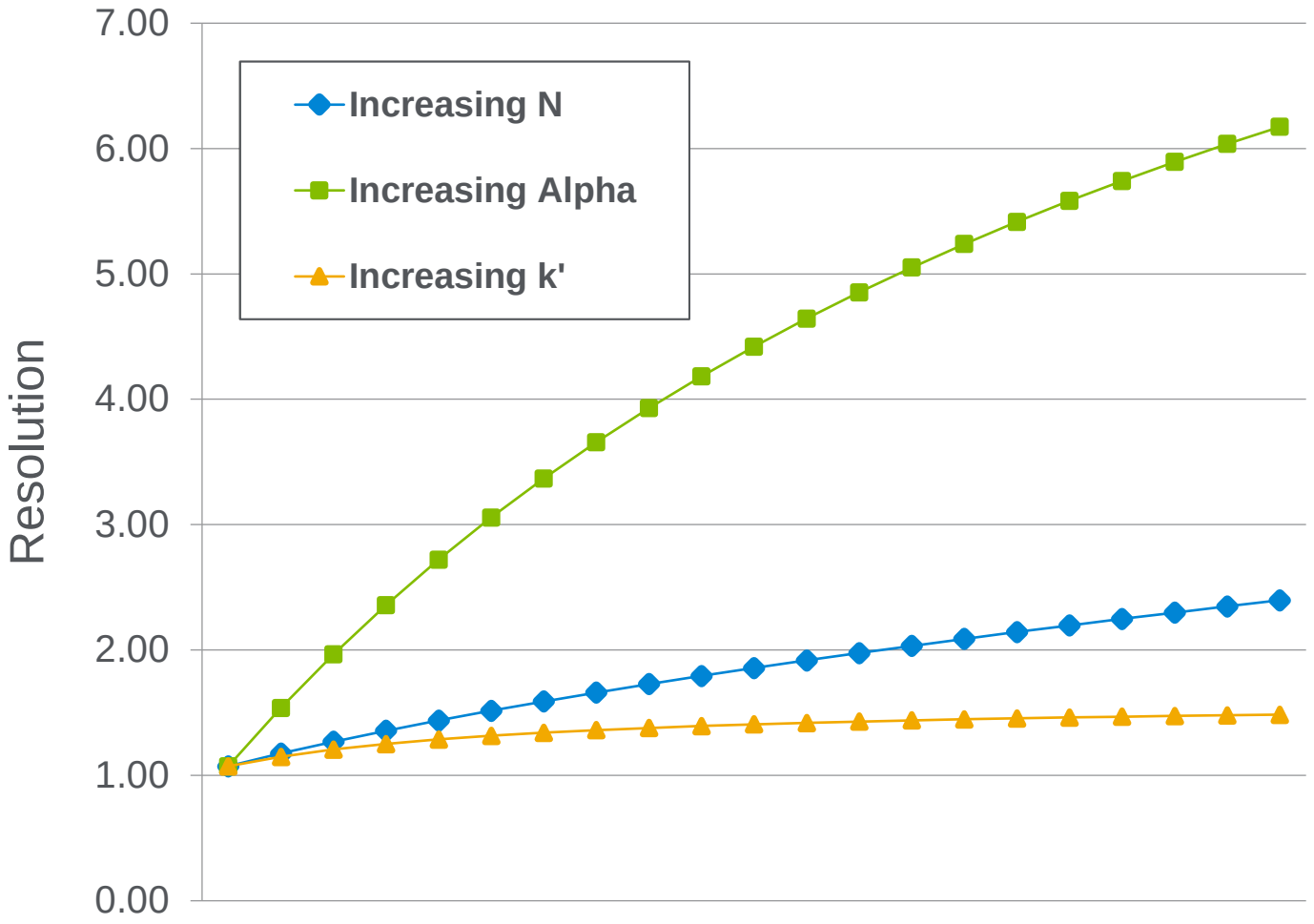
Efficiency **Selectivity** **Retention**

Improve resolution by improving any of these parameters:

- **Efficiency** describes the separation power of the column
- **Selectivity** has the highest influence on the resolution. Small changes in selectivity can lead to big changes in resolution
- **Retention** has only a significant influence at small k values

Key Terminology

Resolution: Influencing factors



Note:

Selectivity has highest impact on resolution

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

Efficiency | Selectivity | Retention

LC-Handbook-Complete-2.pdf (agilent.com)

Key Terminology

Resolution: Retention factor

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

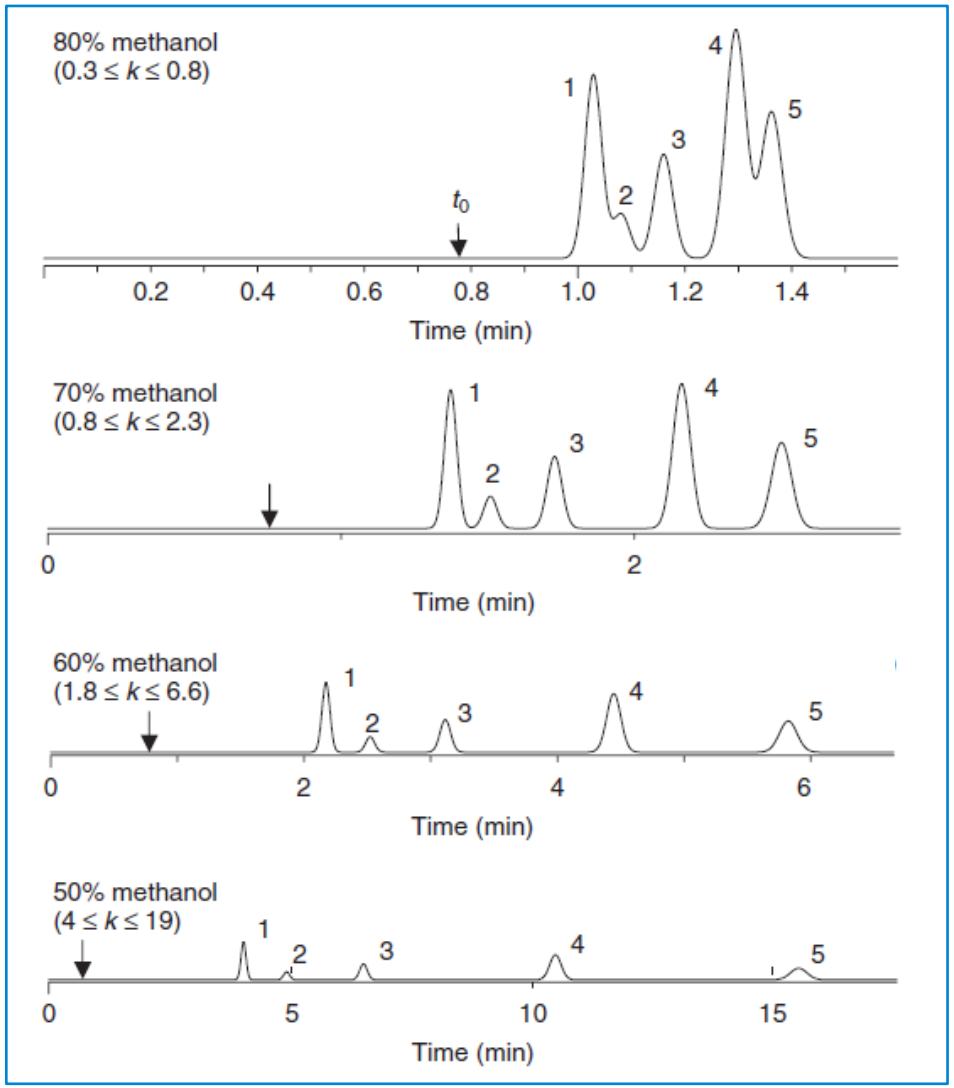
t_R = retention time for sample peak

t_0 = retention time for unretained peak

The **retention factor** measures the period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase.

Main parameter affecting retention:
Mobile phase

Influence of Mobile Phase:



Key Terminology

Resolution: Selectivity

$$\alpha = \frac{k_2}{k_1}$$

α = selectivity

k_1 = retention factor of 1st peak

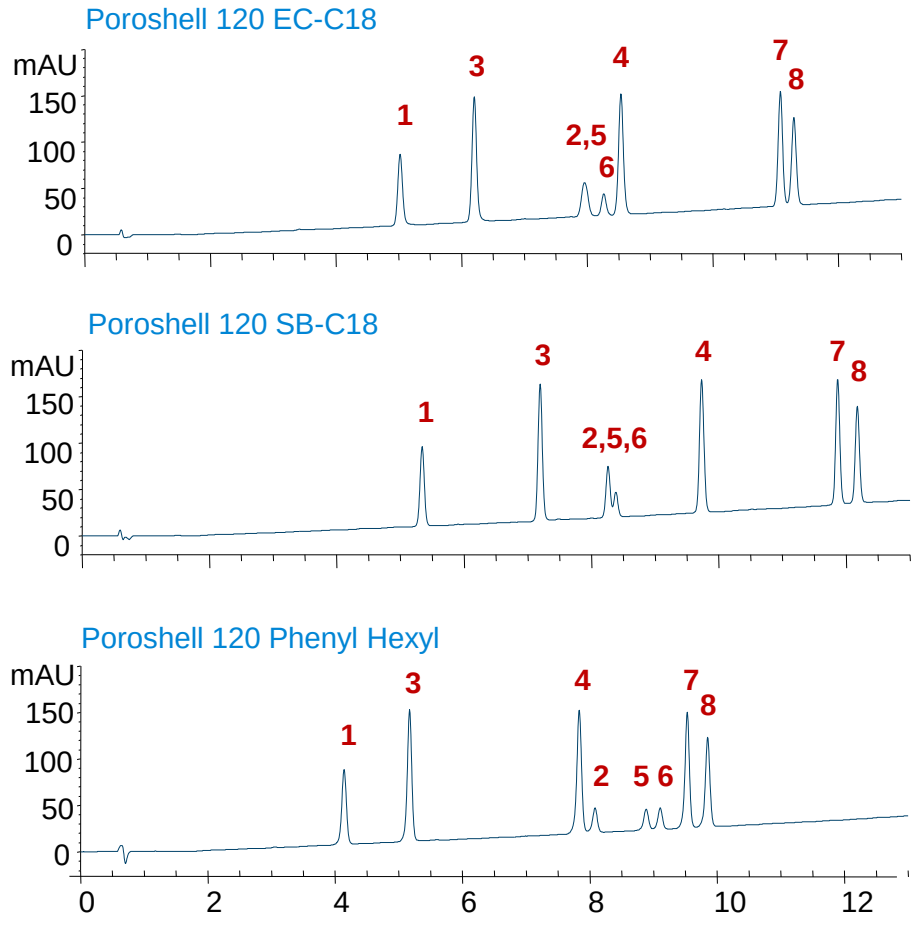
k_2 = retention factor of 2nd peak

Selectivity is a measure of the time or distance between the maxima of two peaks. If α = 1, the two peaks have the same retention time and co-elute.

Main parameters influencing selectivity:

- Stationary phase
- Mobile phase

Influence of Stationary Phase:



1. Hydrocortisone 2. β-Estradiol 3. Androstatriene-3,17-dione 4. Testosterone
5. Ethinyl estradiol 6. Estrone 7. Norethindrone acetate 8. Progesterone

Key Terminology

Resolution: Efficiency

$$N = 16(t_R/\mathrm{w}_b)^2$$

N = efficiency

t_R = retention time

w_b = peak width at base

Columns with high plate numbers are more efficient. A column with a high N will have a narrower peak at a given retention than a column with a lower N number.

$$N \propto \frac{L}{\mathrm{d}_p}$$

N = efficiency

L= column length

d_p = particle size

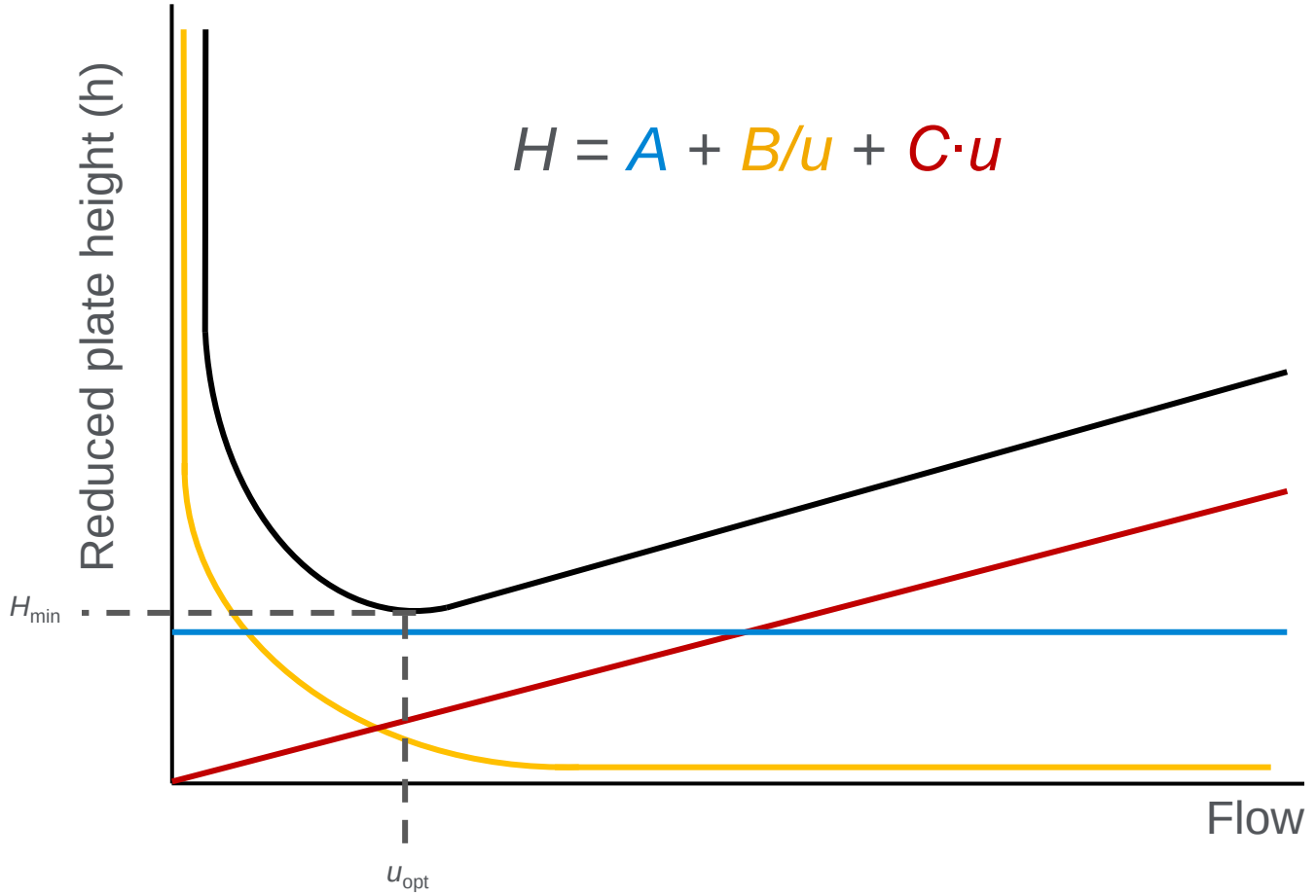
Parameters influencing column efficiency:

- Column length (increasing column length increases efficiency)
- Particle size (decreasing particle size increases efficiency)

Key Terminology

Efficiency: Van Deemter equation

- The Van Deemter curve describes the relationship between the mean linear velocity u of the mobile phase and the plate height H or plate number N.
- Minimum (H_{min}) of the curve represents the optimal linear velocity u_{opt}.
- Van-Deemter curve is always analyte and method specific.

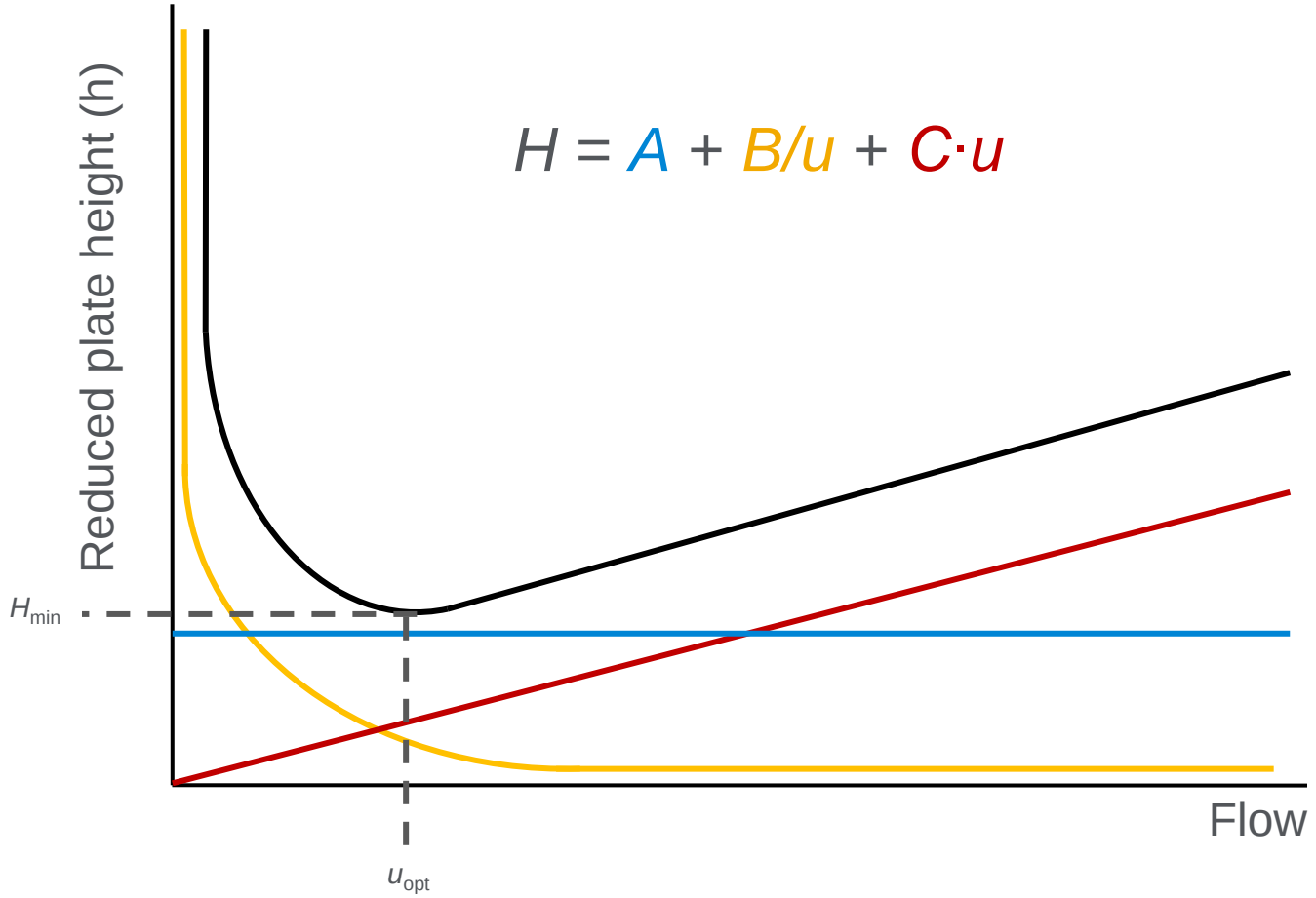


Lower *h (reduced plate height)* = higher efficiency $h = L/N$

Key Terminology

Efficiency: Van Deemter equation

- **A term:** eddy diffusion and flow distribution
  - particle size & packing quality important
  - narrow particle size distribution
- **B term:** longitudinal diffusion
  - Diffusion in the mobile phase
- **C term**: mass transfer
  - shorter diffusion paths
  - better with superficially porous particles
  - more effect on large molecules
- *u*: linear velocity
  - velocity of mobile phase through column
  - $u = L/t_0$ in cm/sec

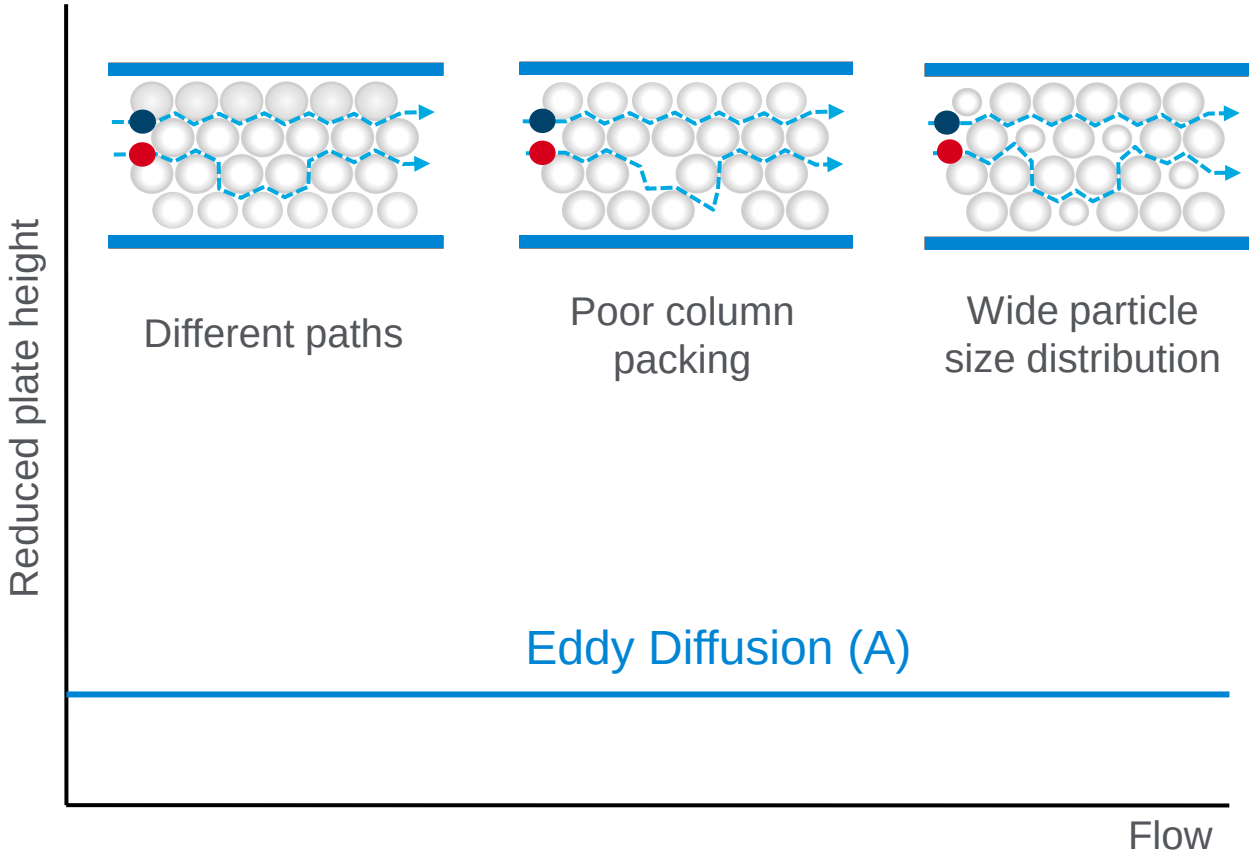


H	*A*	*B*	*C*	*u*
m	m	$m^2 \cdot s^{-1}$	s	$m \cdot s^{-1}$

Key Terminology

Efficiency: Van Deemter

Eddy Diffusion



Note:

By flowing around the packaging vortices (eddy) are created.

→ Analyte molecules are carried through the column in different ways.

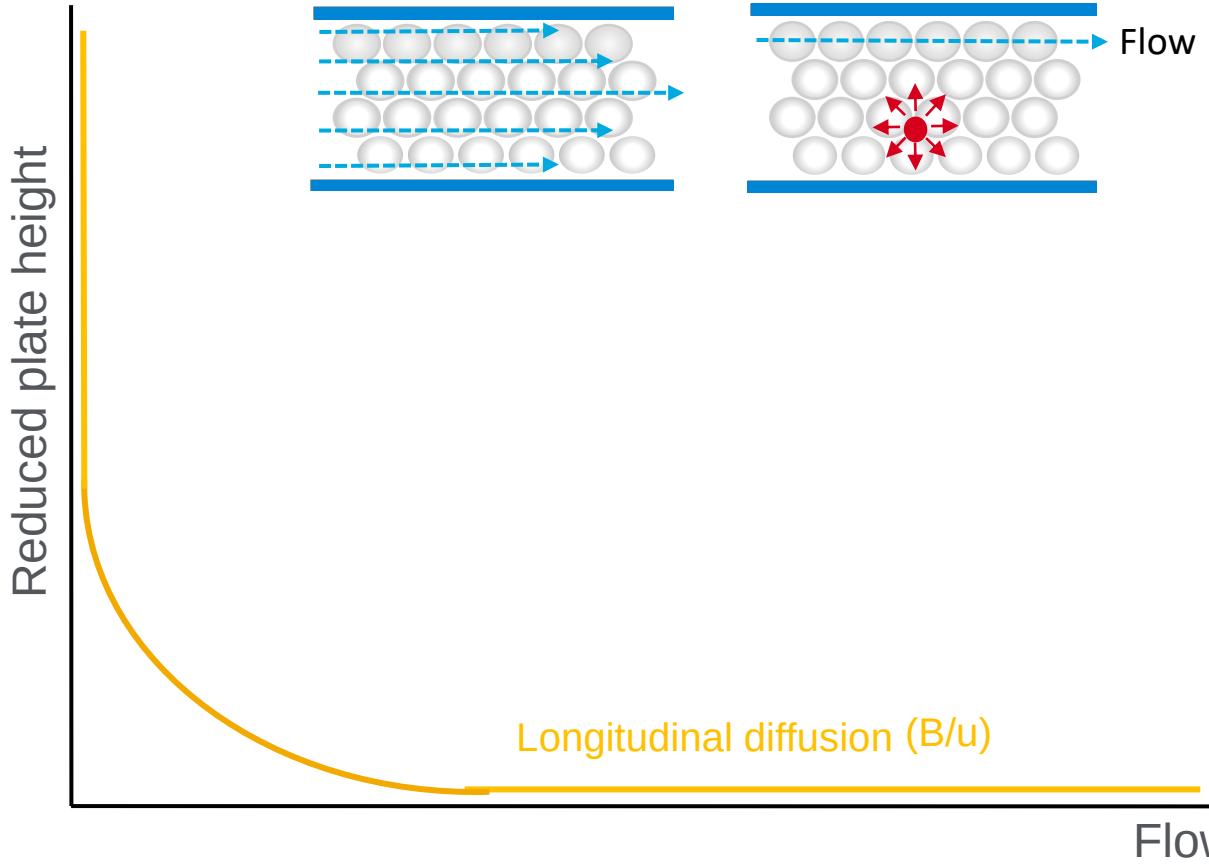
→ Molecules will reach detector at different time points!

Van Deemter JJ, Zuiderweg FJ and Klinkenberg A (**1956**): *Longitudinal diffusion and resistance to mass transfer as causes of non ideality in chromatography*. Chem. Eng. Sc. 5: 271–289. doi:10.1016/0009-2509(56)80003-1.

Key Terminology

Efficiency: Van Deemter

Longitudinal Diffusion



Note:

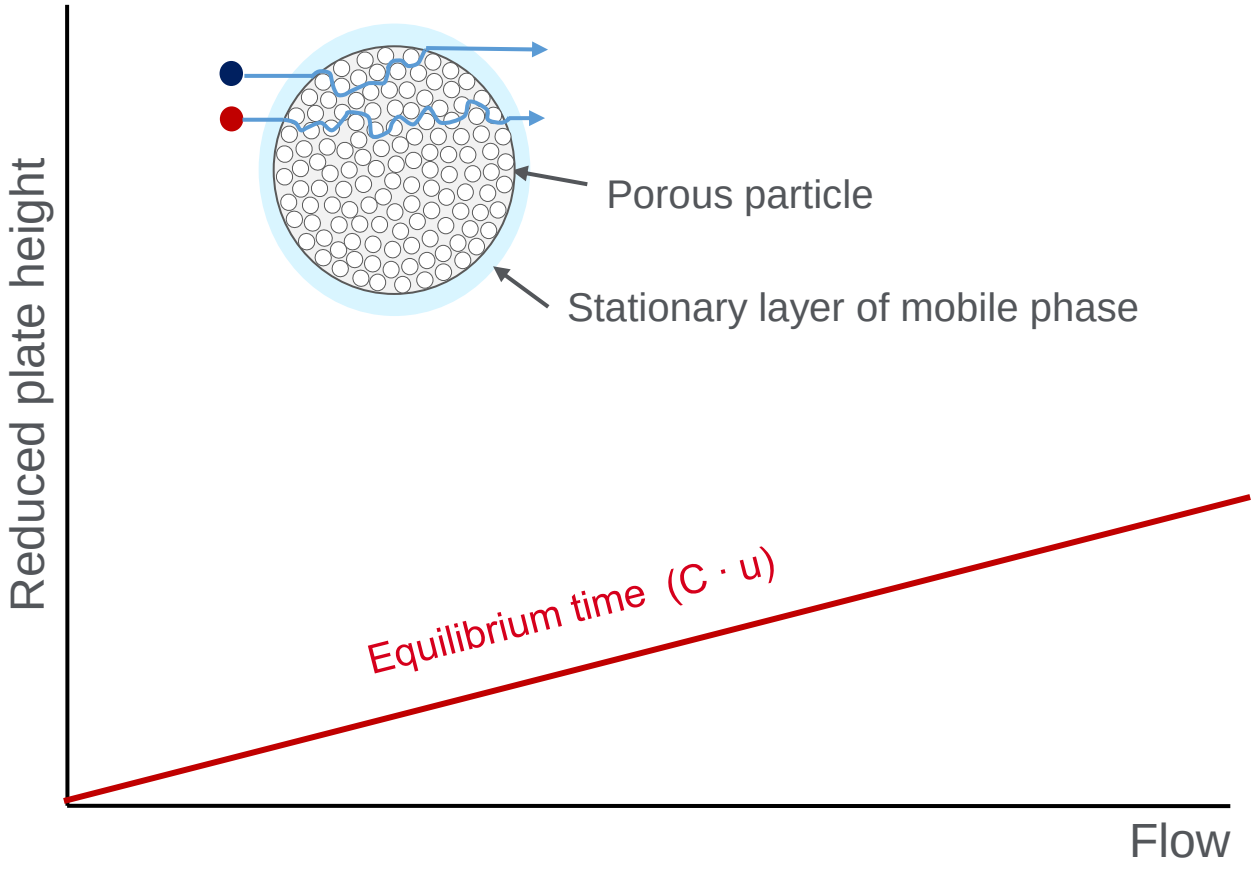
- Can be observed in any pipeline with a laminar flowing fluid!
- Flow velocity is is higher in the center than at the edges.
- Molecules migrating in the center are eluted faster than those migrating at the edge .
- Diffusion of the sample zone!

Key Terminology

Efficiency: Van Deemter

Mass Transfer

Diffusion path differences



Note:

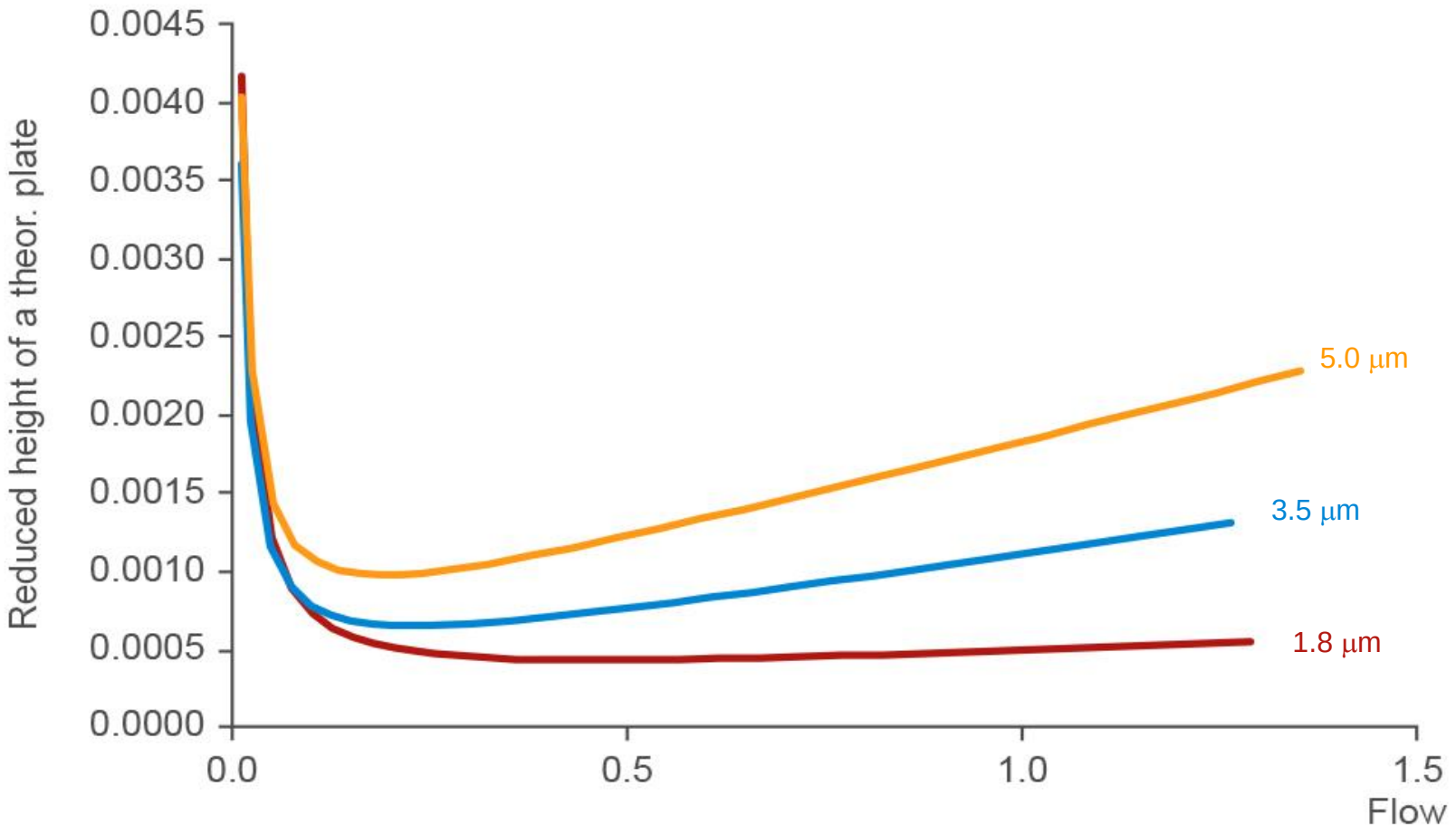
- Equilibrium/mass transfer between mobile and stationary phase!
- Mass exchange takes time, so it is favored by low flow rates..
- Band broadening due to low flow through pores.

Key Terminology

Efficiency: Van Deemter

Mass Transfer

- Small particles lead to lower plate heights and therefore higher separation efficiency
- For smaller particles, the separation efficiency suffers less when increasing the flow



Key Terminology

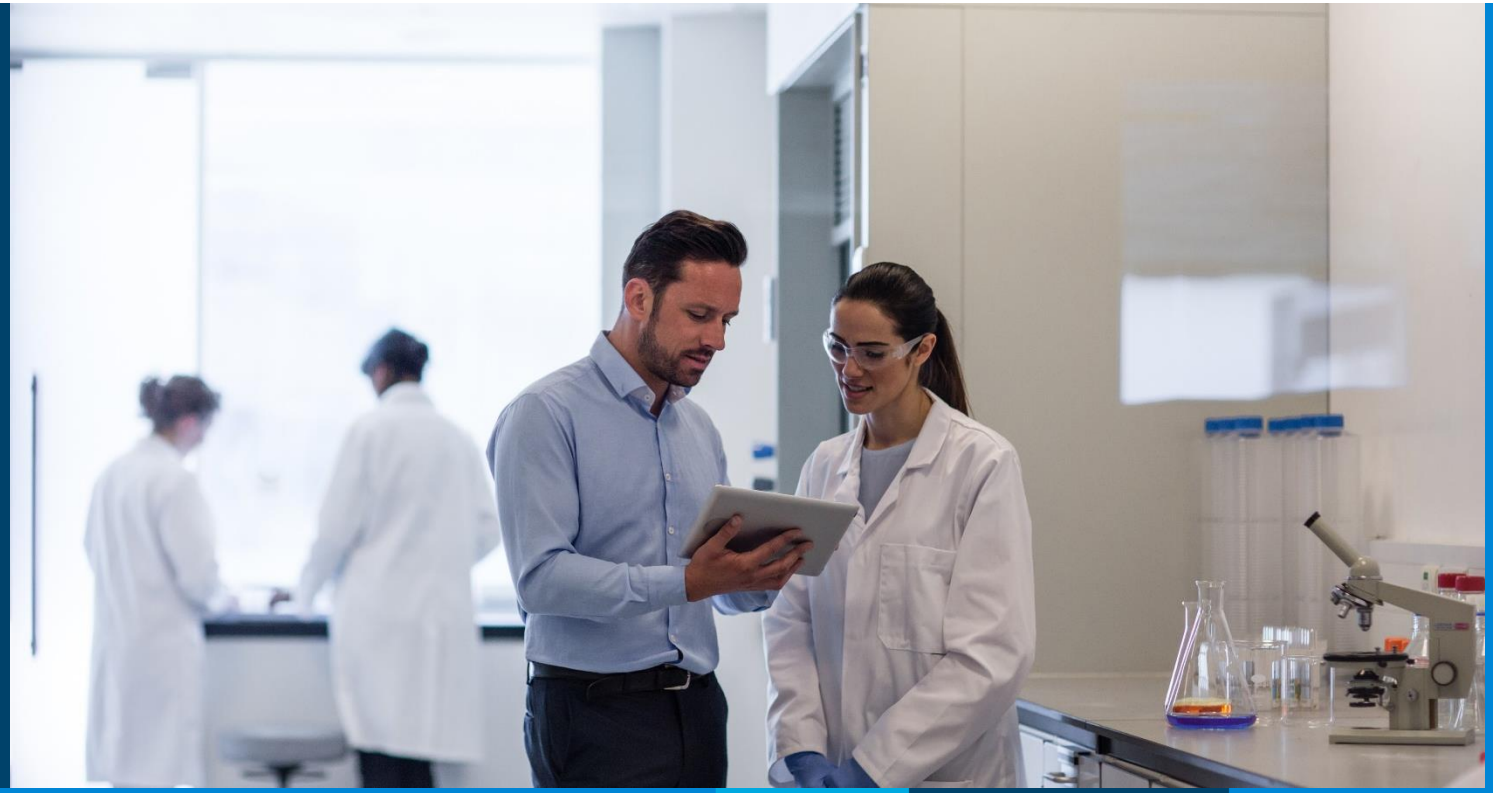
Summary

Condition	Retention (κ)	Selectivity (α)	Efficiency (N)
% B	●●	●	—
B-solvent (acetonitrile, methanol, etc.)	●	●●	—
Temperature	●	●	●
Column type (C18, phenol, etc.)	●	●●	—
Mobile phase pH	●●	●●	●
Buffer concentration	●	●	—
Ion-pair-reagent concentration	●●	●●	●
Column length	NA	NA	●●
Particle size	NA	NA	●●
Flow rate	NA	NA	●

Symbol	Meaning
●●	Major effect
●	Minor effect
—	Relatively small effect
blue	Conditions that are primarily used to control variable

Method Development 101:

Establishing Method Goals



Defining Method Objectives

- Is the primary goal detection, characterization or isolation of purified material?
- Is it necessary to resolve all sample components?
- What levels of accuracy and precision are required.
- How many samples will be analyzed at one time (Throughput)
- What HPLC equipment and operator skills are present in the laboratory that will use the final method?

Defining Method Objectives

Examples of common separation goals and method performance criteria

Good System Suitability Parameters

- Resolution: ≥2
- Peak shape: USP T_f close to 1 (<2)
- Injection Repeatability: areas, T_f, etc. (RSD 0.1 - 0.25%)
- Absolute retention factors: 1< k<10
- Relative Retention: α or k_2/k_1
- Signal-to-Noise Ratio: >10

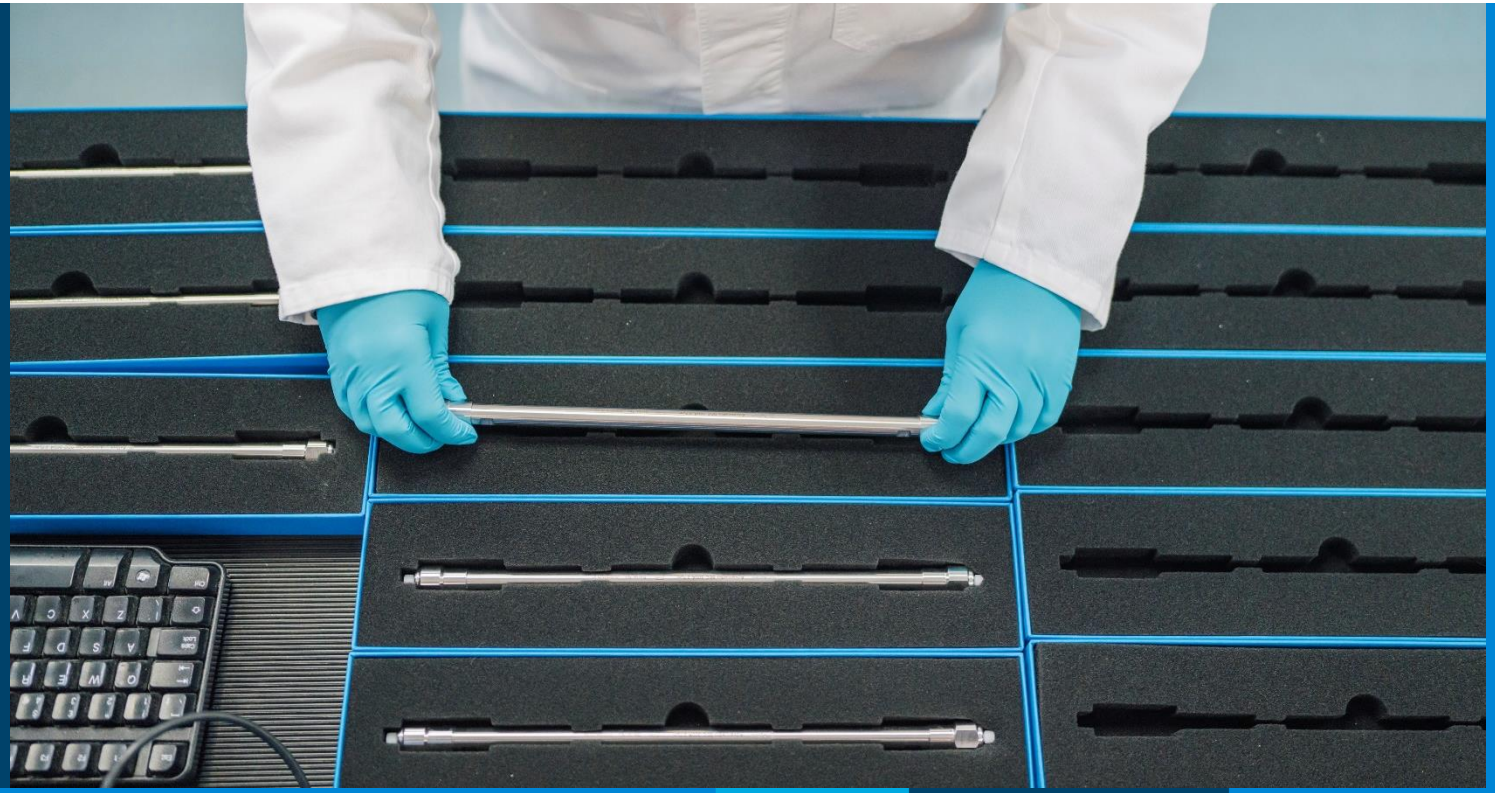
Avoid these for system suitability criteria:
- Column efficiency (theoretical plates)
- Absolute retention time

Method Performance Criteria

- Accuracy
- Precision
  - Ruggedness
  - Robustness
- Selectivity/Specificity
- Linearity
- Range
- Quantitation Limit (LOQ, 10x S/N)
- Detection Limit (LOD, 3x S/N)

Method Development 101:

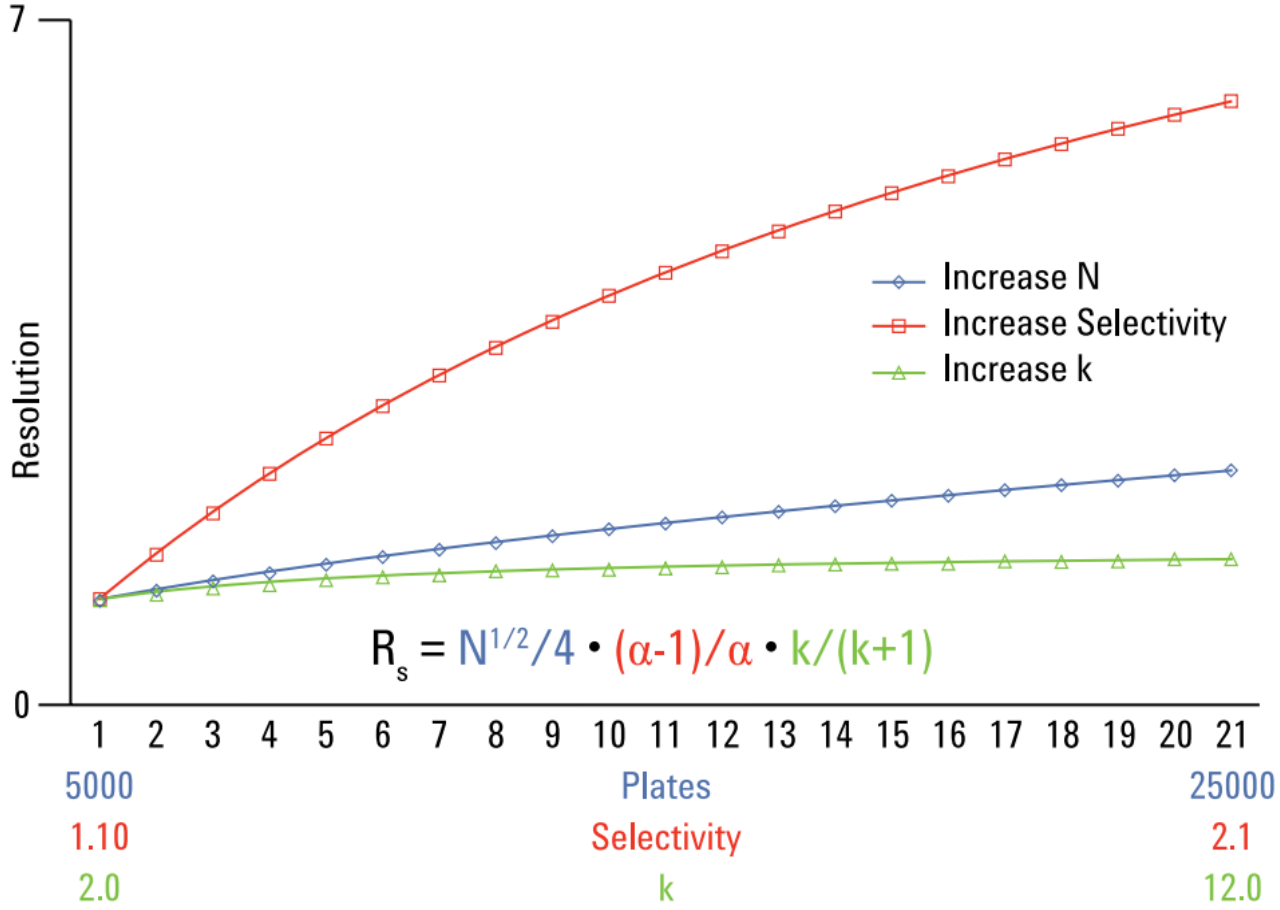
Column Selection



Column Selection

Selectivity

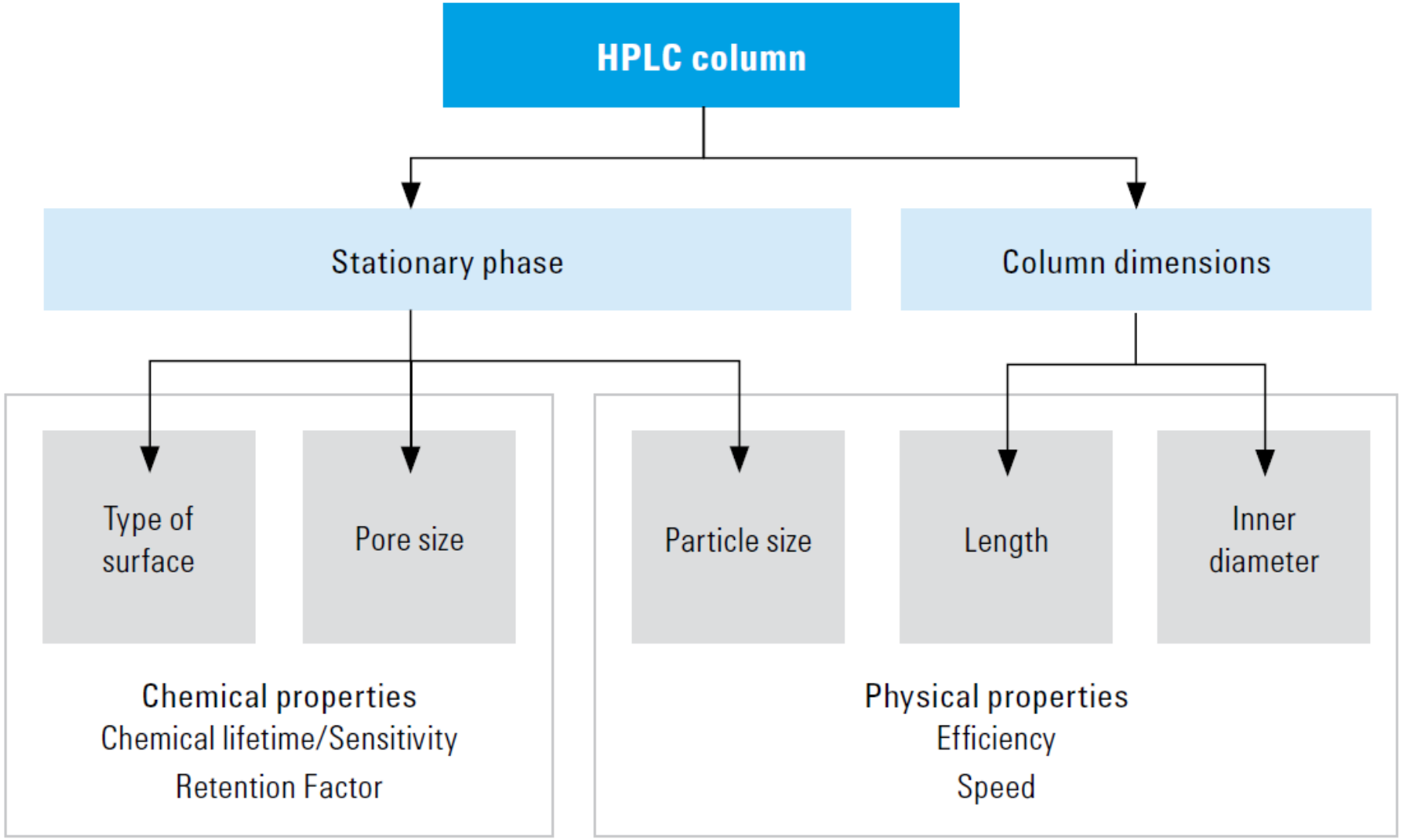
- Bonded phase affects selectivity (α)
- Different interactions for polar and non-polar compounds.
- Exploit other interactions with bonded phase (e.g., pi-pi)
- Changing the bonded phase can improve selectivity/resolution, reduce analysis time
- *Evaluating different bonded phase chemistries early can save time in optimization and generate a more robust method*



LC-Handbook-Complete-2.pdf (agilent.com)

Column Selection

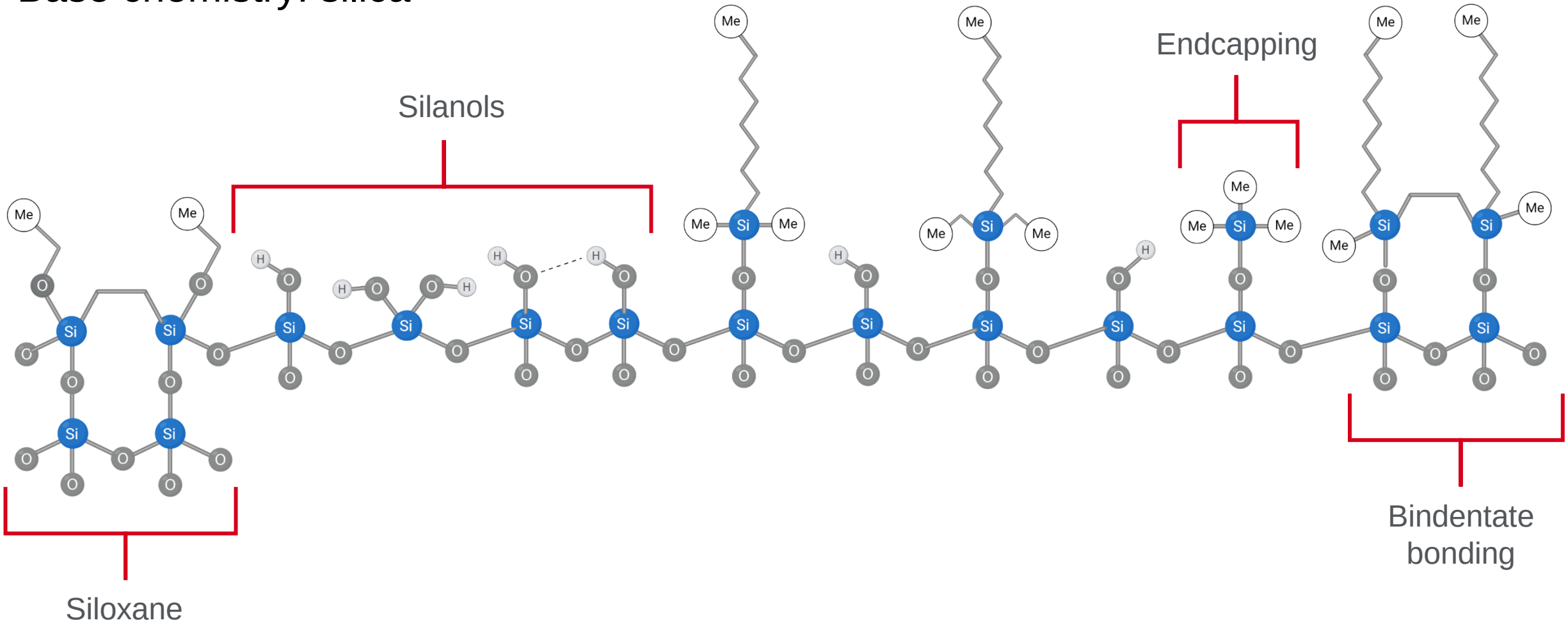
Overview



LC-Handbook-Complete-2.pdf (agilent.com)

Column Selection

Base chemistry: silica

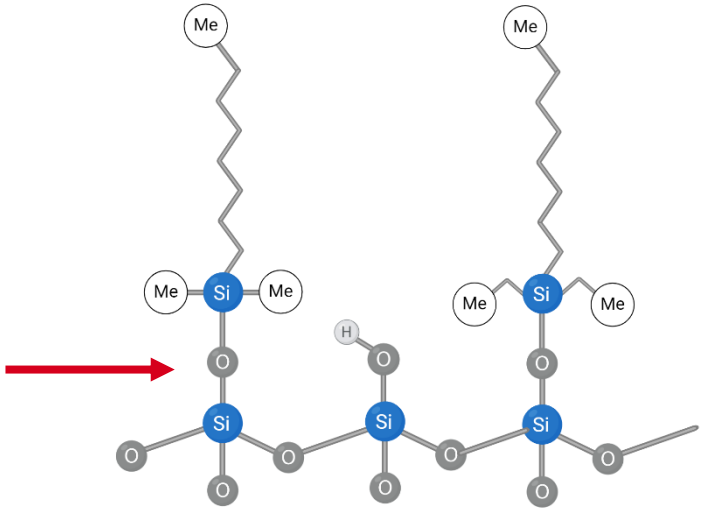


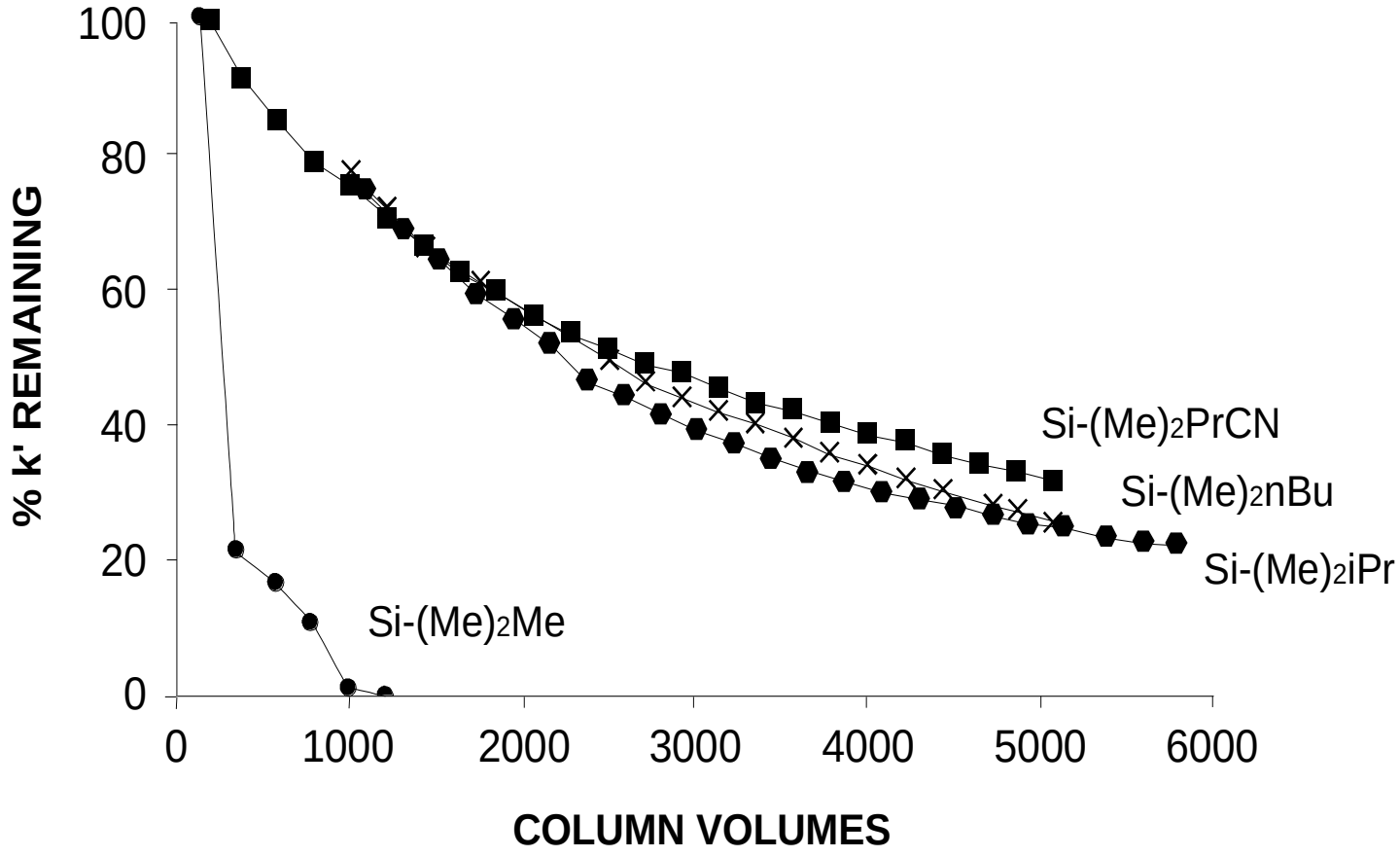
Column Selection

Column lifetime: low pH

Low pH methods

- Breaking of siloxane bond reduces column lifetime, especially with short chain alkyl ligands





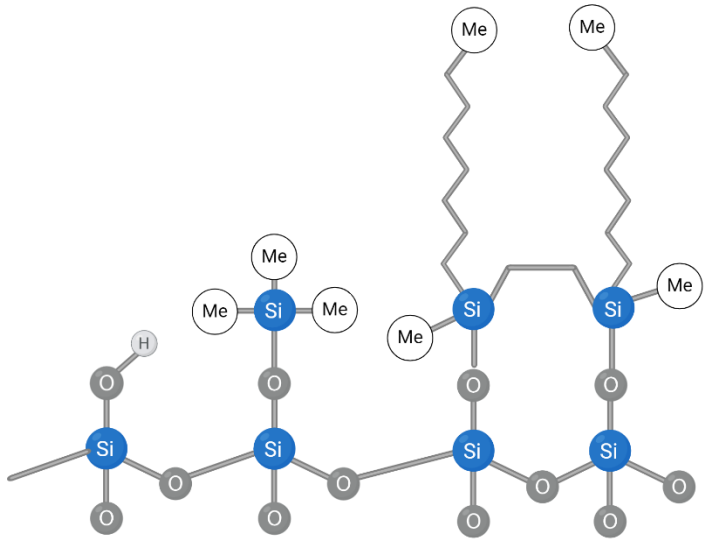
Kirkland, J.J., J.L. Glajch, and R.D. Farlee, *Analytical Chemistry* (1989), 61, 2.

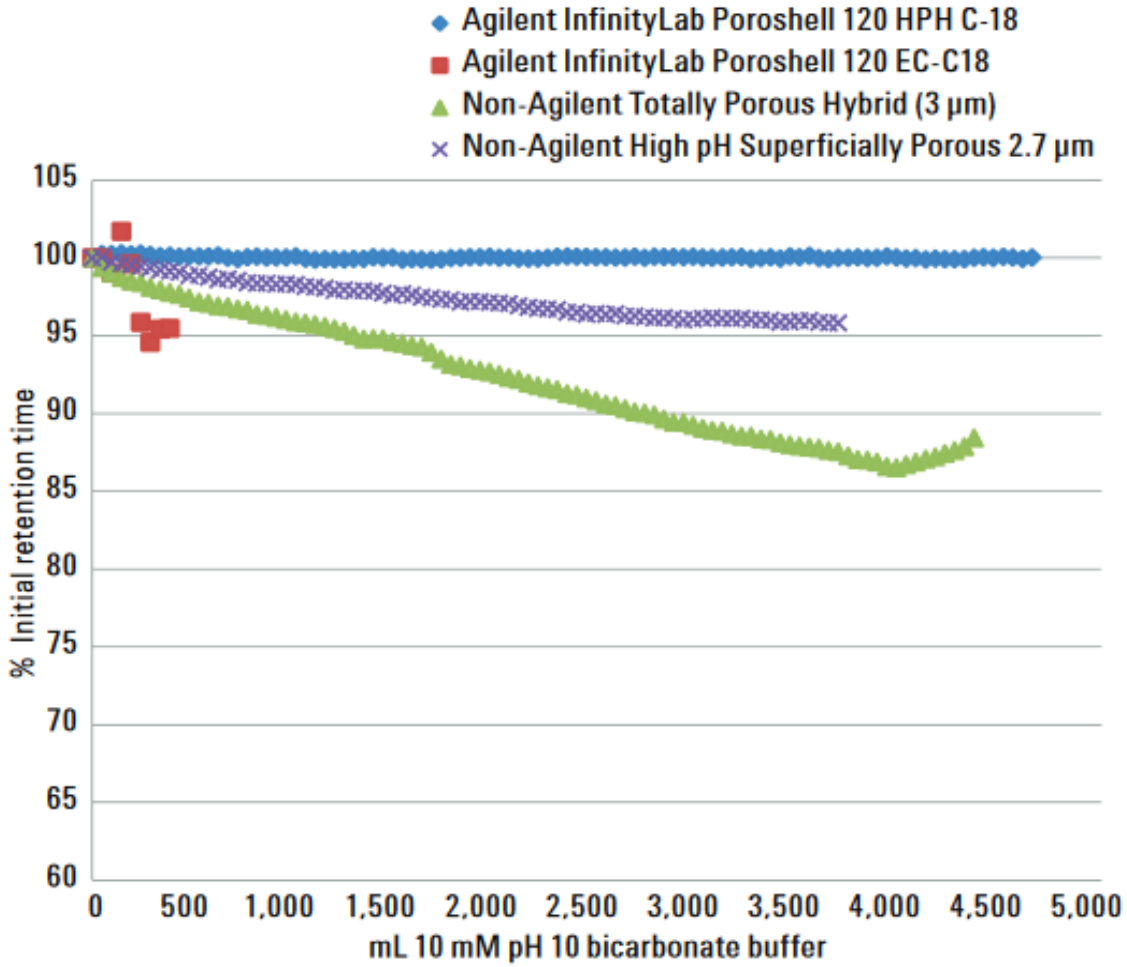
Column Selection

Column lifetime: high pH

High pH methods

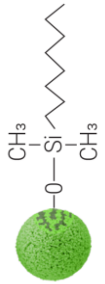
- High pH methods can lead to the dissolution of the silica stationary phase
- Double end-capping and/or bidentate bonding improves peak shape of basic compounds and high pH stability

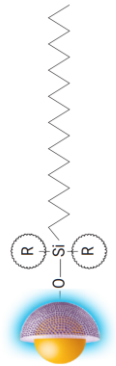


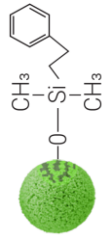


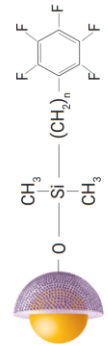
Column Selection

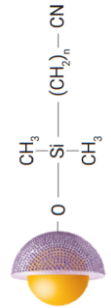
Stationary phase chemistry











Chemistry	Alkyl	Alkyl polar	Phenyl, diphenyl, phenyl hexyl	Pentafluoro phenyl (PFP)	Cyano
Example	SB-C18, EC-C18	Bonus RP, Aq-C18	Phenyl, Phenyl hexyl	PFP	EC-CN
Hydrophobicity	●●●●	●●●●	●●●●	●●●●	●●●
π-π interaction	—	—	●●● (donor)	●●● (acceptor)	●
Dipole-dipole	—	●●	●	●●●●	●●●
Hydrogen bonding	●	●●●●	●●	●●●	●●
Applicability	• General purpose	• Enhanced retention of polar analytes while also separating non-polar analytes	• Alternate selectivity with aromatic and moderately polar groups	• Alternate selectivity for halogenated, polar, and isomeric analytes • Excellent peak shape for polar and non-polar compounds	• Alternate selectivity for polar and mid-polar compounds

Column Selection

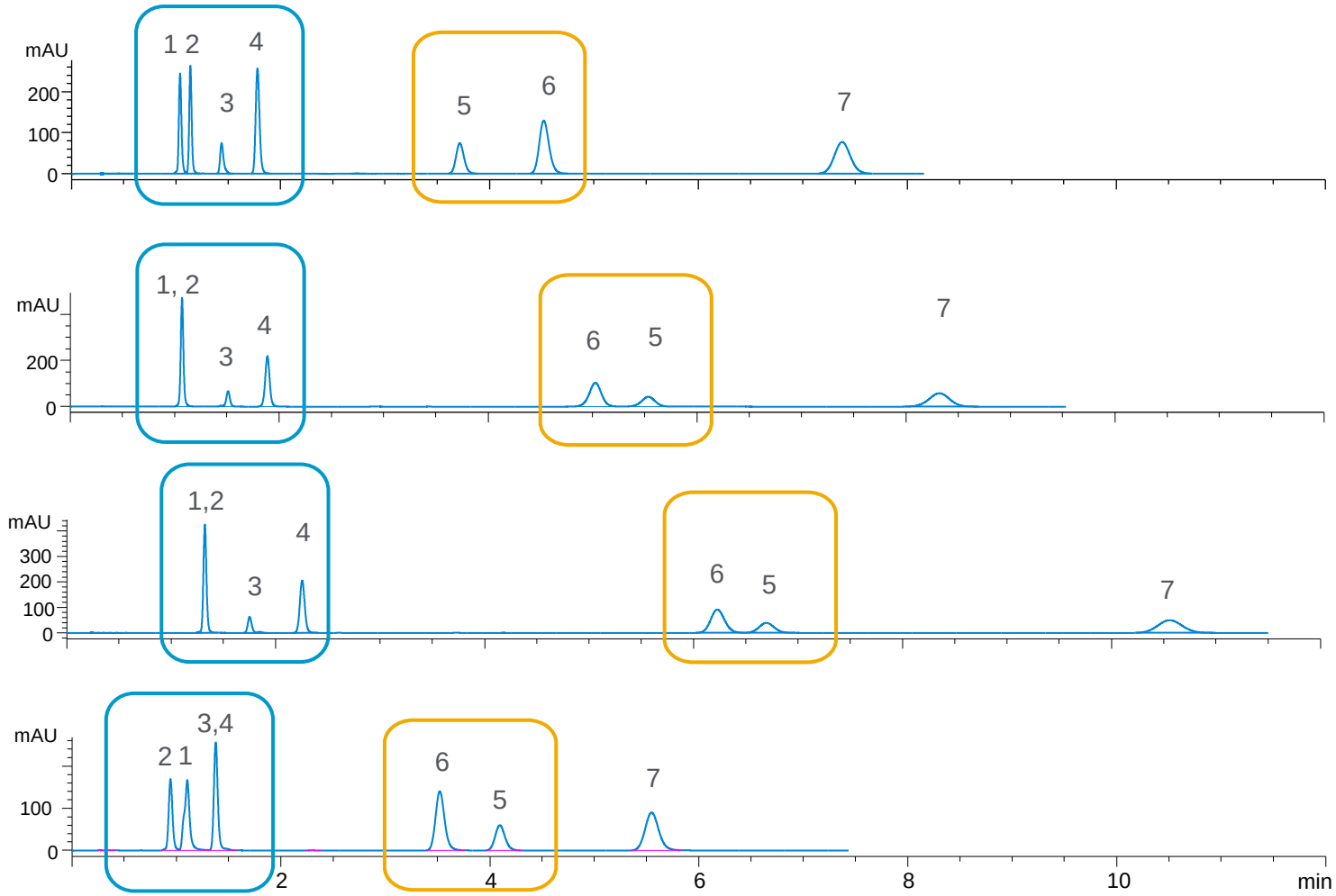
Stationary phase chemistry

Eclipse Plus Phenyl Hexyl

Eclipse Plus C8

Eclipse Plus C18

Eclipse PAH



1. Piroxicam
2. Sulindac
3. Tolmetin
4. Naproxen
5. Ibuprofen
6. Diclofenac
7. Celebrex

Mobile Phase 40 % ACN 60 % 25 mM Sodium Phosphate Buffer pH= 2.4 Flow Rate= 1.5 ml/min 4.6 x 50mm UV 210 nm

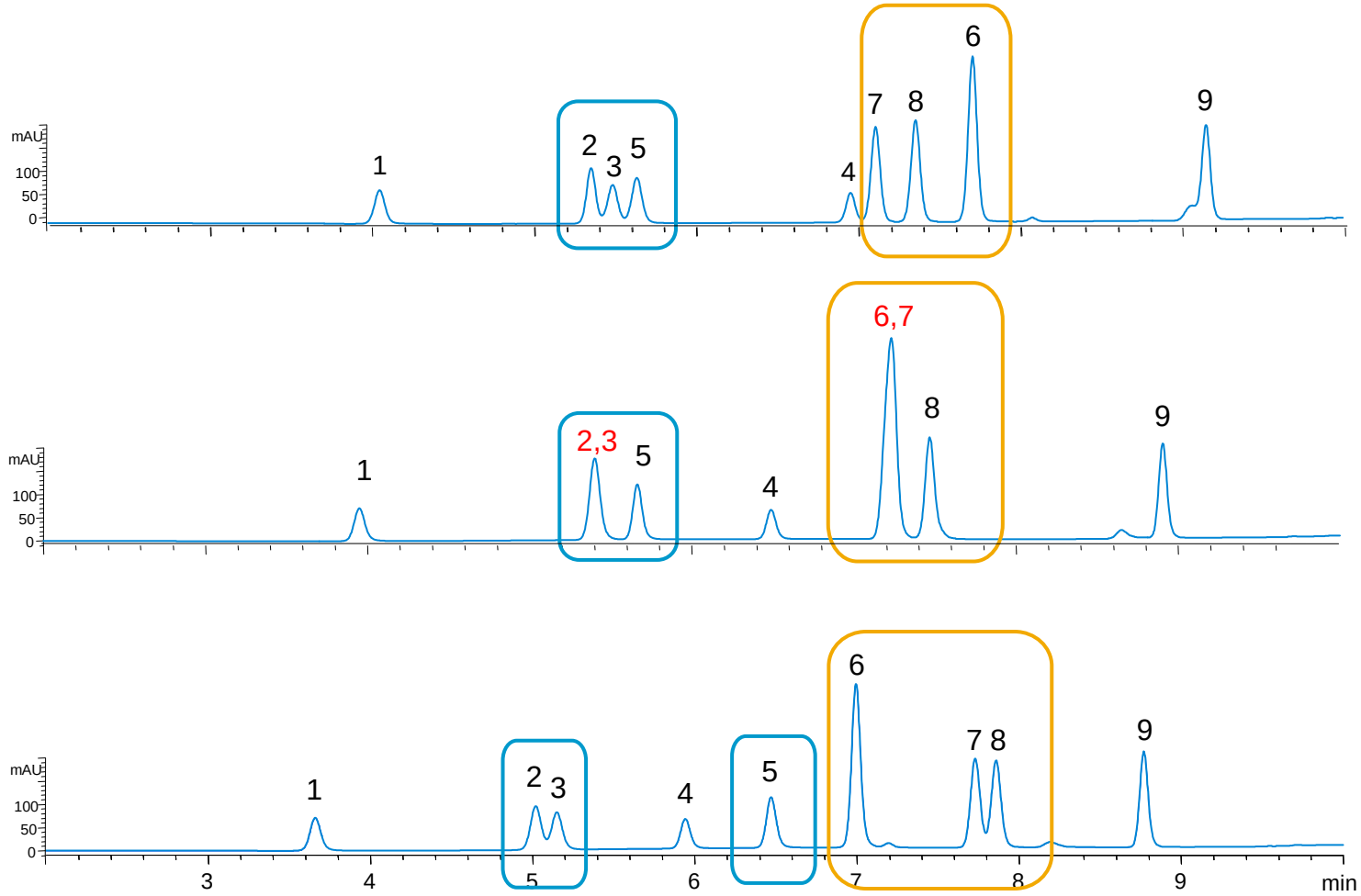
Column Selection

Stationary phase chemistry

Eclipse Plus Phenyl-Hexyl

Eclipse XDB-Phenyl

Zorbax SB-Phenyl



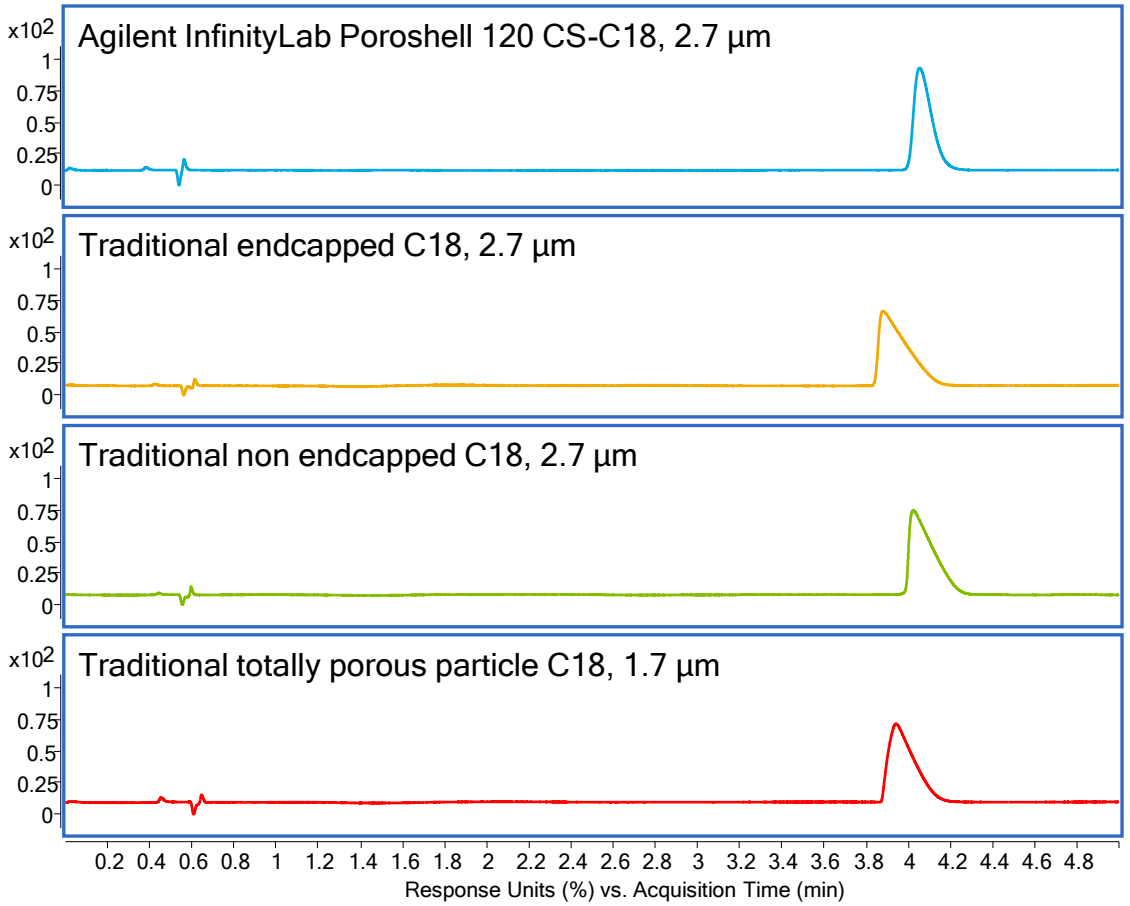
1. Analine
2. o-toluidine
3. Anisidine
4. Chloroaniline
5. Benzidine
6. Naphthylamine
7. o-tolidine
8. Dimethoxybenzidine
9. Dichlorobenzidine

Mobile phase A: 10 mM ammonium acetate, pH 4.7, Mobile phase B: methanol. 25% to 90% MPB in 9 minutes

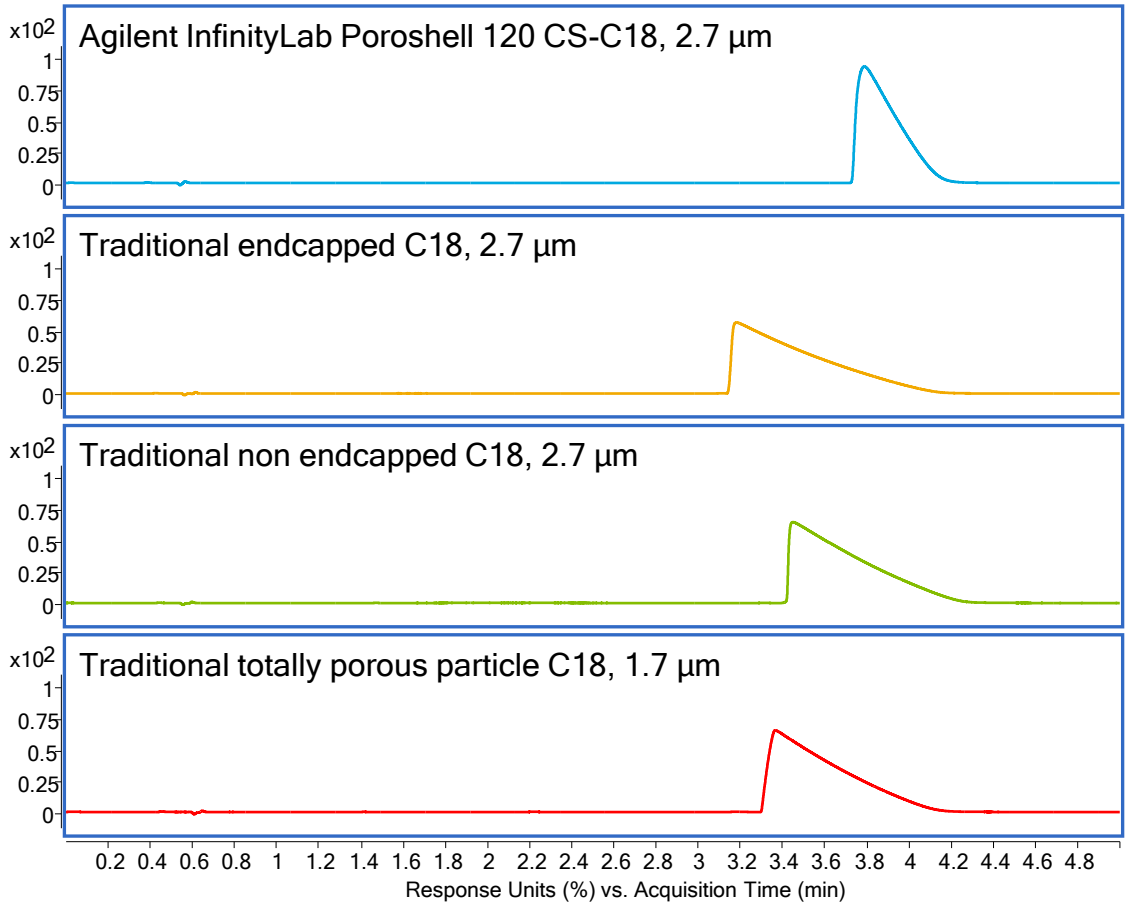
Column Selection

Stationary phase chemistry

Moderate Sample Load: 30 ng amitriptyline



High Sample Load: 500 ng amitriptyline



Improved Sample Loading and Peak Shape with InfinityLab Poroshell 120 CS-C18 Columns (agilent.com)

Column Selection

Pore size

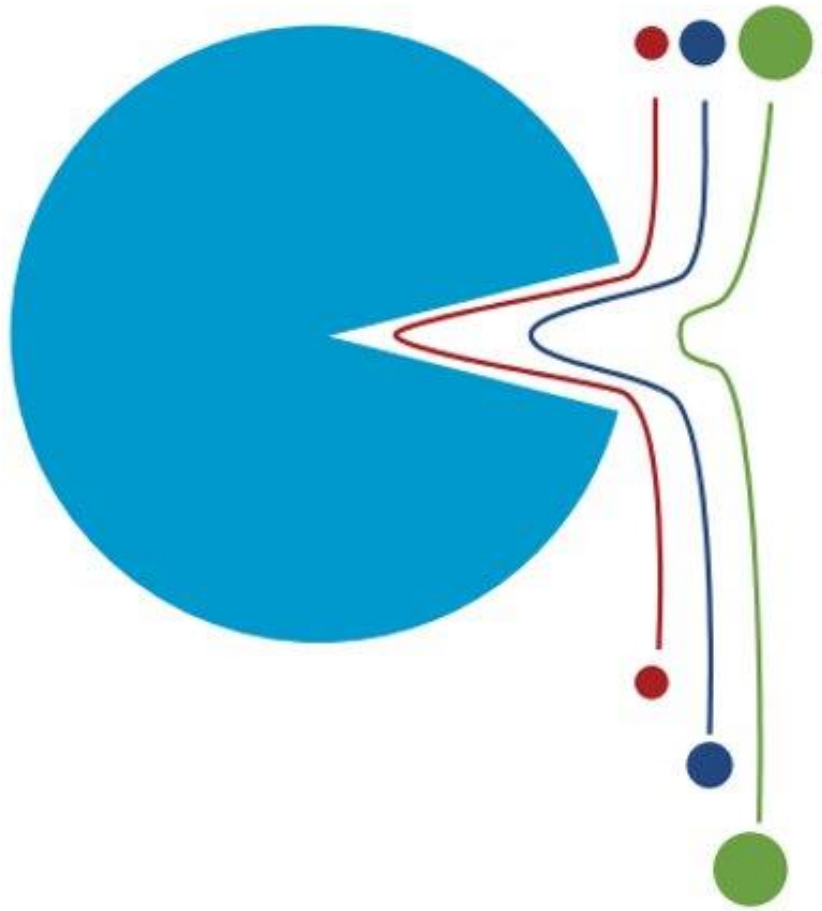
As a general rule, the pore size should be 3X the hydrodynamic radius of your analyte

Small molecules

- 80 – 120 Å
- Maximizes loading and retention

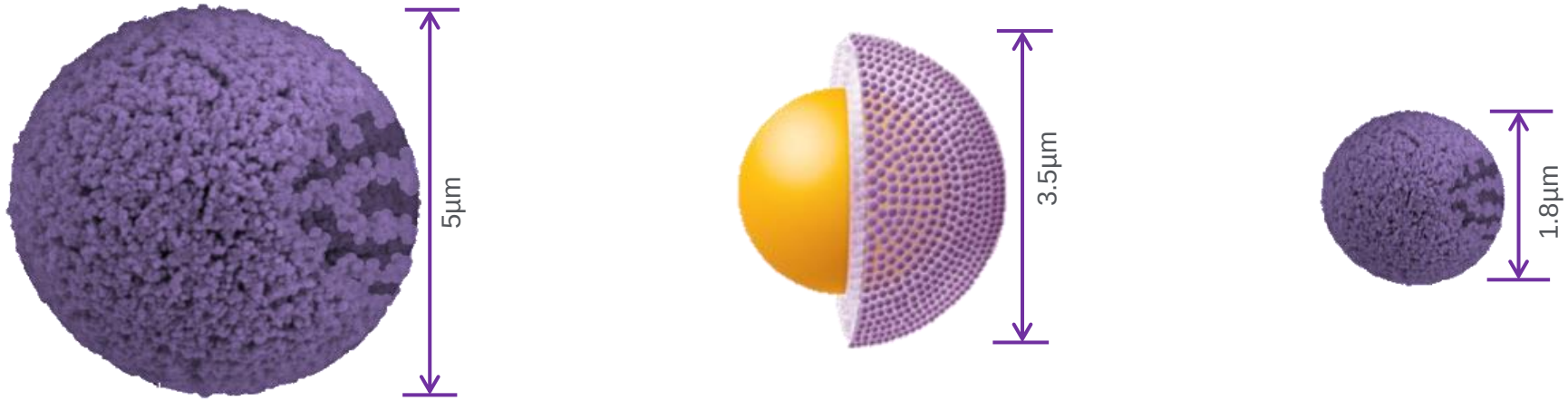
Peptides, proteins, other large biomolecules

- 120 Å (Peptides, small oligonucleotides)
- 300 Å to 450 Å (Proteins, mAb)
- 1000 Å (Larger proteins, larger oligonucleotides)
- 4000 Å (mRNA, pDNA, VLP)
- Maintain high efficiency



Column Selection

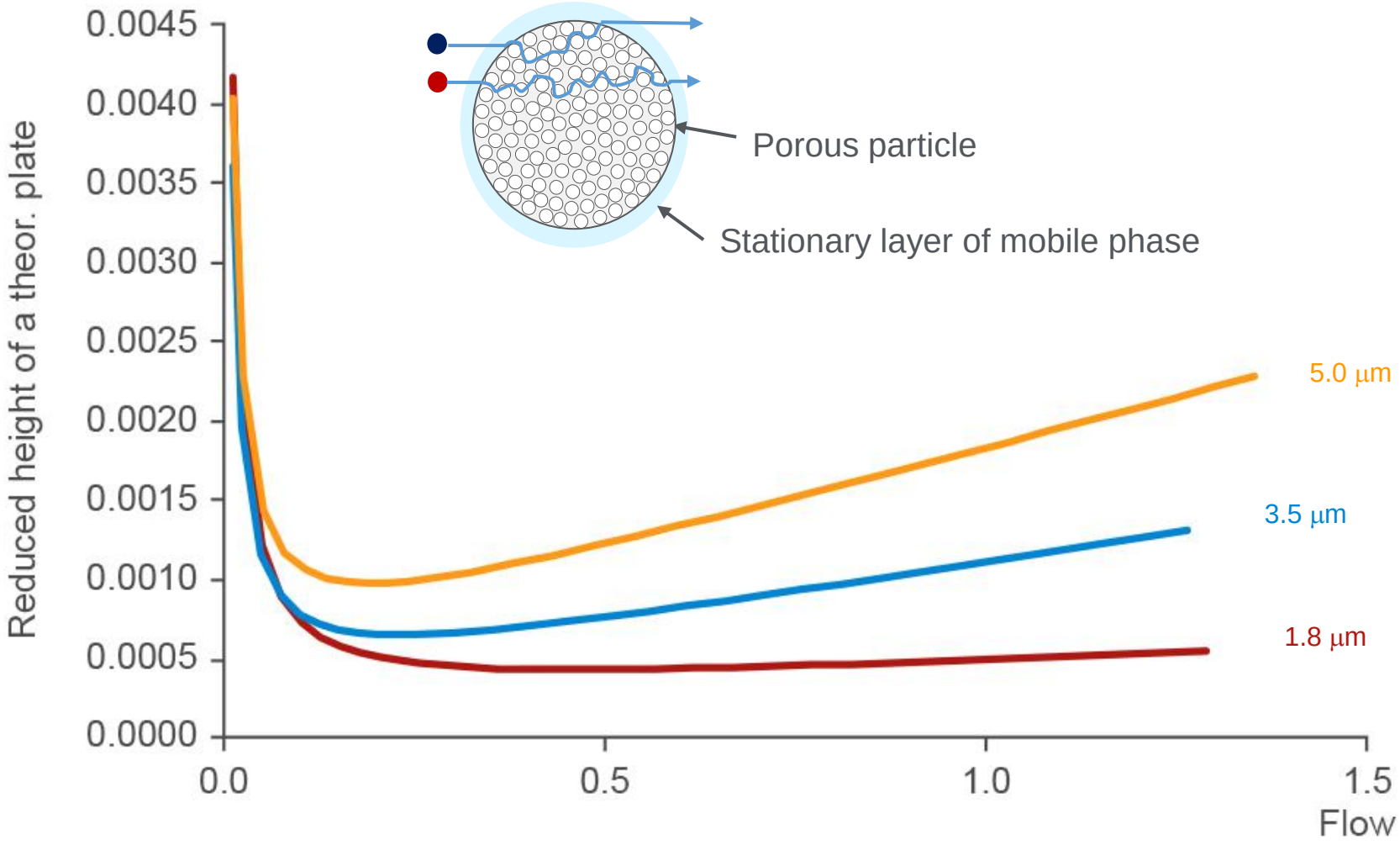
Particle size



Increasing resolution, pressure

Column Selection

Particle size



Column Selection

Particle Size

Original method

ZORBAX LC column Extend-C18

4.6 x 150 mm, **5 µm**

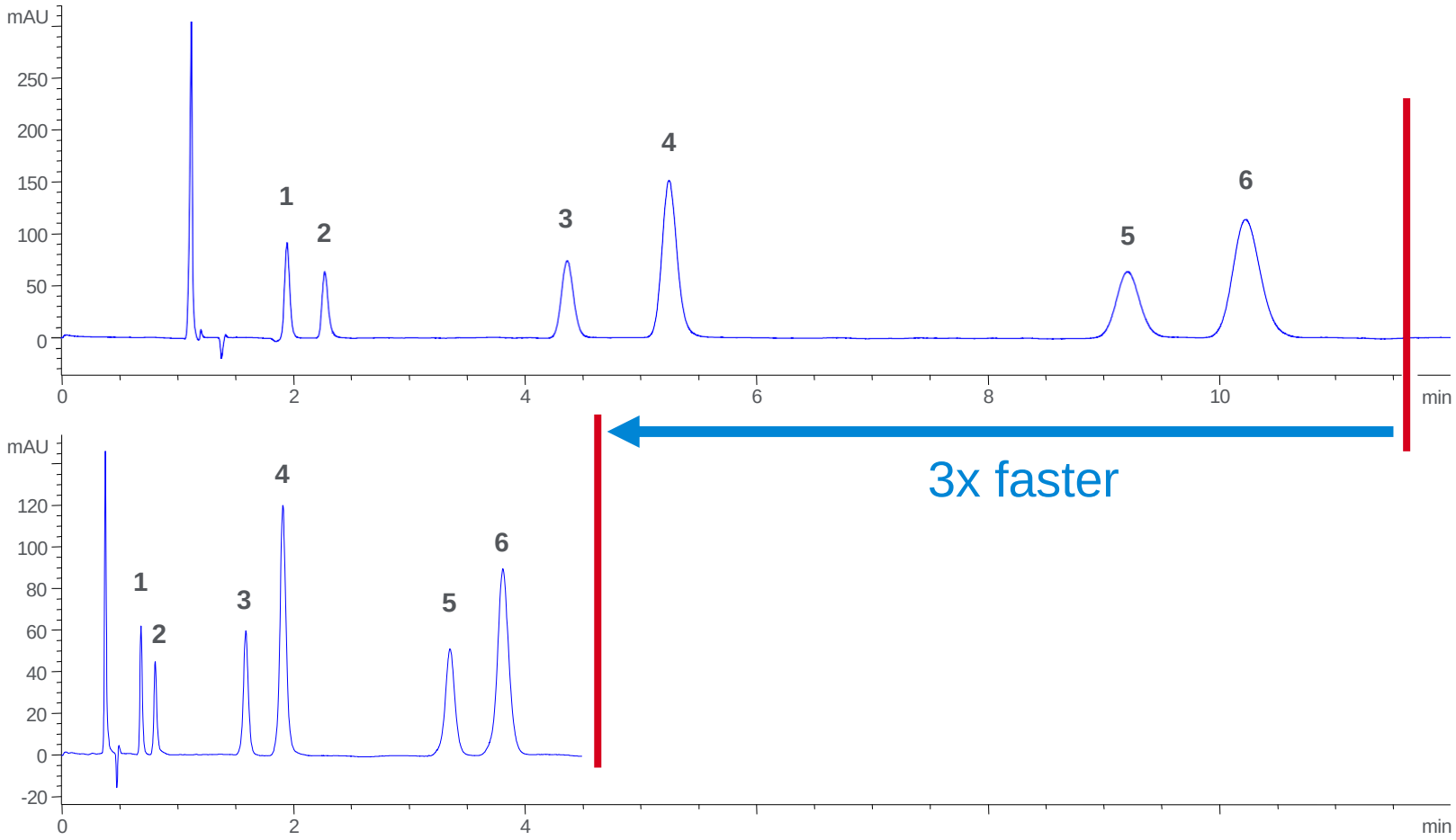
3 µL inj

Updated method

ZORBAX **RRHT** column Extend C18

4.6 x 50 mm, **1.8 µm**

1 µL inj.



Mobile phase: (70:30) MeOH: 50 mM pyrrolidine buffer Flow = 1.0 mL/min, Temp. : ambient

Column Selection

Particle Size

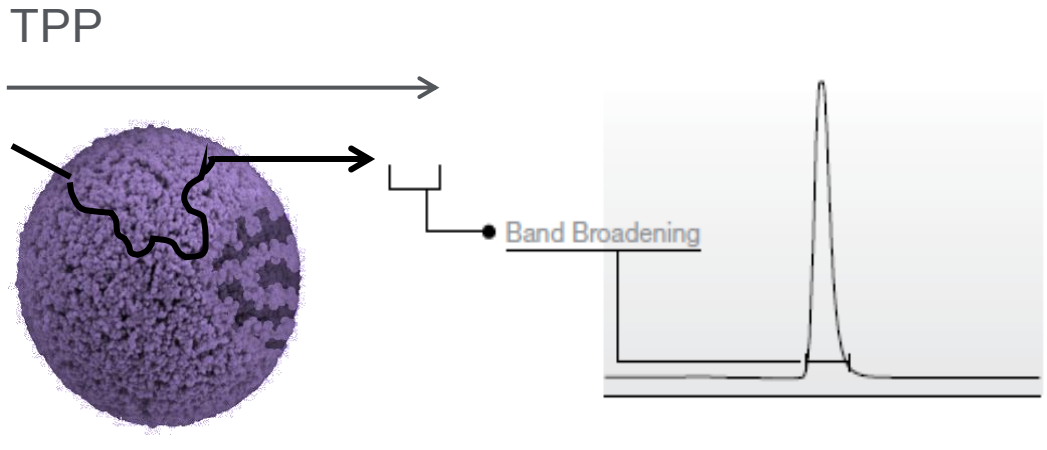
Separation Efficiency

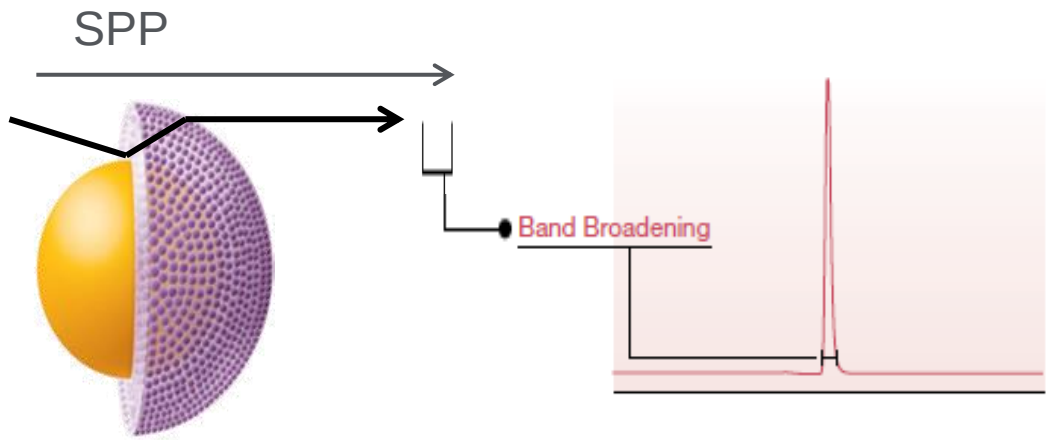
< 400 bar	400-800 bar	800-1300 bar
InfinityLab Poroshell 120 4 µm	InfinityLab Poroshell 120 2.7 µm	Infinity Lab Poroshell 120 1.9 µm
ZORBAX 3.5 & 5 µm	ZORBAX RRHT 1.8 µm	ZORBAX RRHD 1.8 µm
HPLC	**UHPLC**	**Low dispersion UHPLC**
Pressure: low	Pressure: Moderate	Pressure: High
Sample prep: low	Sample prep: SPP low / FPP moderate	Sample prep: High
Loadability: High	Loadability: SPP medium / FPP high	Loadability: SPP Low / FPP medium

Column Selection

Totally porous particles (TPP) vs. superficially porous particles (SPP)

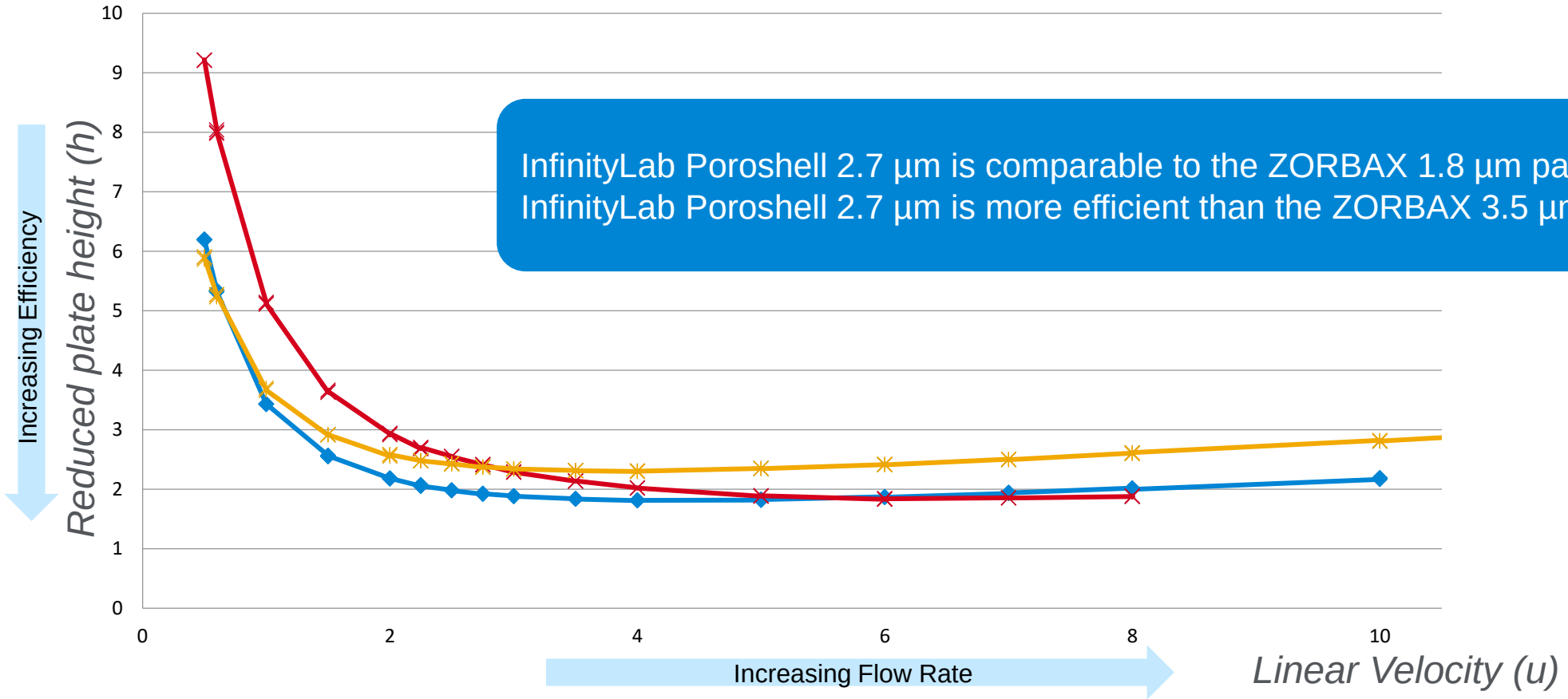
- Analytes travel though the particle more efficiently
- High efficiency allows you to use a larger SPP (i.e., 2.7um) for nearly equivalent performance to a smaller sub-2 um (STM) TPP column
- Using a larger particle allows for lower backpressure than comparably efficient totally porous STM columns and flexible use on HPLC or UHPLC systems





Column Selection

TPP vs. SPP



InfinityLab Poroshell 2.7 µm is comparable to the ZORBAX 1.8 µm particle
InfinityLab Poroshell 2.7 µm is more efficient than the ZORBAX 3.5 µm particle

Column Selection

TPP vs. SPP

SPP particle	For	Maximum pressure	Typical pressure	Efficiency	Target system
1.9 µm	Highest UHPLC performance	1300 bar	Similar to sub-2 µm totally porous	~120% of sub-2 µm totally porous	1290 Infinity II
2.7 µm	UHPLC performance at lower pressures	600 bar / 1000 bar	50% of sub-2 µm totally porous	~90% of sub-2 µm totally porous	1290 Infinity II 1260 Infinity II
4 µm	Improved HPLC performance	600 bar	Typically < 200 bar	~200% of 5 µm totally porous	1260 Infinity II VL 1220 Infinity II (VL)

Highend LC-Systems

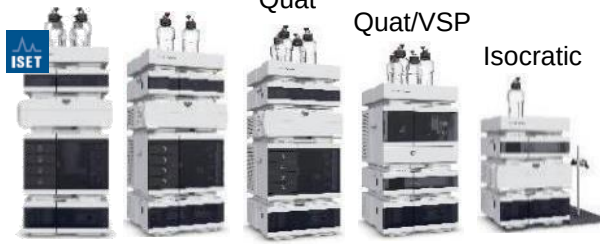
1290 Infinity II LC

High-Speed Flexible

Core LC Modular

1260 Infinity II LC

Prime LC Binary Quat Quat/VSP Isocratic



Core LC Integr.

1220 Infinity II LC

Gradient Isocratic

Bio-inert LC

1260 Infinity II Bio-inert
1260 Infinity II Bio-SEC

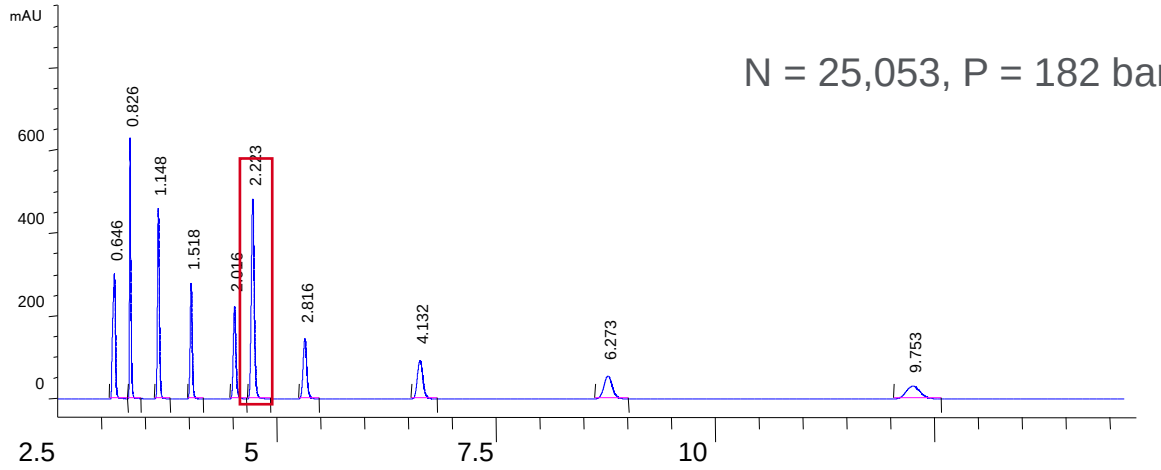
Column Selection

TPP vs. SPP

The InfinityLab Poroshell column has 90 percent the efficiency and half the pressure of the ZORBAX column

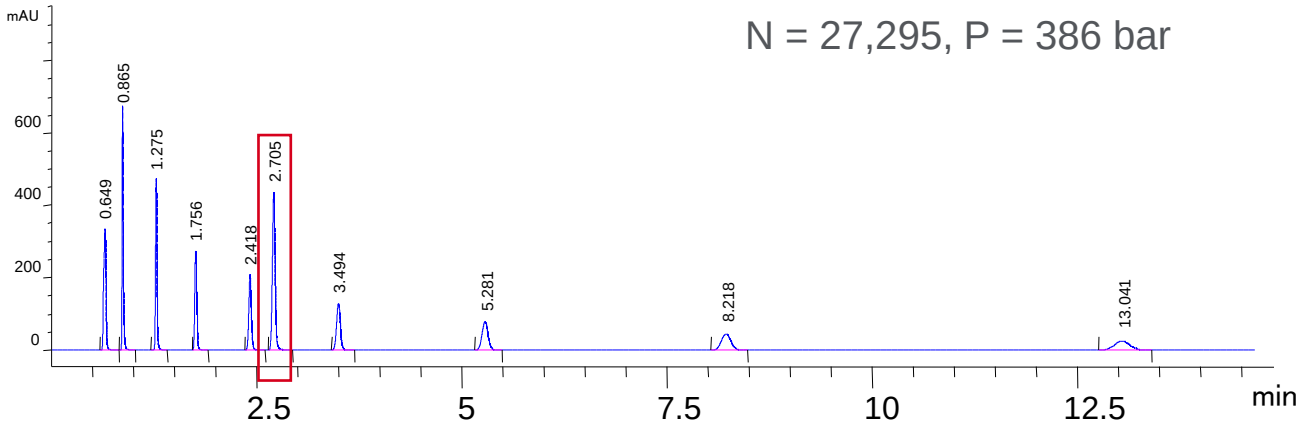
InfinityLab Poroshell 120 EC-C18
2.7 µm, 3.0 x 100mm

N = 25,053, P = 182 bar



ZORBAX Eclipse Plus C18
1.8 µm, 3.0 x 100mm

N = 27,295, P = 386 bar



Column Selection

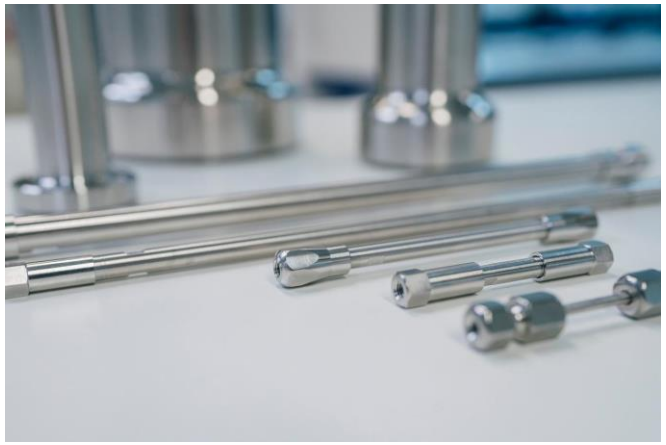
Column dimensions

Inner diameter (ID)

ID	Optimum Flow Rate	Recommended Use
4.6 mm	1.00-1.25 mL/min	Legacy methods
3.0 mm	0.8-1.0 mL/min	Lower solvent use
2.1 mm	0.4-0.5 mL/min	MS applications, lowest solvent use

Column length

Column Length	Recommended Use
50 mm	High throughput
100 mm	High resolution
≥150 mm	Ultra-high resolution



Column Selection

Method develop kits

Column kits for method development

Simplify method development by offering one of **26** predefined method development kits that make method development easy!

- Particle Technology: Poroshell / Zorbax
- Different selectivities (classic RP / aqueous)
- pH ranges
- Column size options: 2.1 x 50 mm, 4.6 x 50 mm, and 4.6 x 100 mm with 1.8, 3.5 or 5 µm particle sizes

Method development column kit Webpage

Method development column kit flyer

Method Development Kits for HPLC | Agilent

Column Selection

Method develop kits

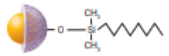
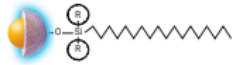
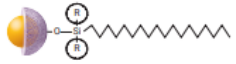
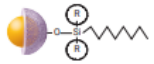
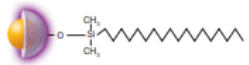
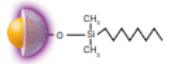
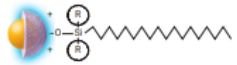
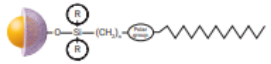
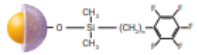
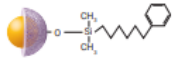
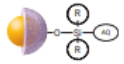
Poroshell Method Development Column Kits

Product number	Description	Particle size
5190-6155	Poroshell 120 Selectivity Method Development Kit, includes **Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns**, 2.1 x 50 mm	2.7 µm
5190-6156	Poroshell 120 Selectivity Method Development Kit, includes **Poroshell 120 EC-C18, Phenyl-Hexyl, Bonus RP columns**, 4.6 x 50 mm	2.7 µm
5190-6157	Poroshell 120 Aqueous Method Development Kit, includes **Poroshell 120 SB-Aq, Phenyl-Hexyl, Bonus RP columns**, 2.1 x 50 mm	2.7 µm
5190-6158	Poroshell 120 Aqueous Method Development Kit, **includes Poroshell 120 SB-Aq, Phenyl-Hexyl, and Bonus RP columns**, 4.6 x 50 mm	2.7 µm
5190-6159	Poroshell 120 L1, L7, and L10 USP Method Development Kit, includes **Poroshell 120 EC-C18, EC-C8, EC-CN columns**, 4.6 x 100 mm	2.7 µm
5190-6160	Poroshell 120 L1, L7, and L10 USP Method Development Kit, includes **Poroshell 120 EC-C18, EC-C8, EC-CN columns**, 3.0 x 100 mm	2.7 µm

Method Development Kits for HPLC | Agilent

Column Selection

Superficially porous column (SPP) selection poster

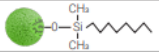
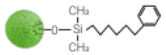
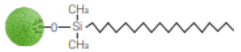
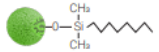
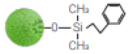
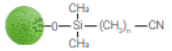
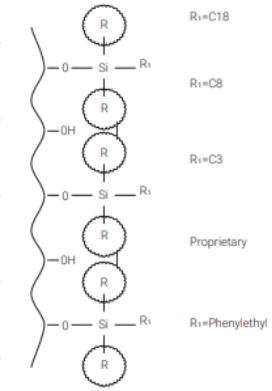
InfinityLab Poroshell 120	Chemistry	Particle Sizes	Pore Size	Temperature Limit	pH Range	Endcapped	Carbon Load	Surface Area	USP Designation	Benefits and Applications
EC-C18		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Yes	10%	130 m2/g	L1	General purpose Excellent peak shape and efficiency for acids, bases, and neutrals
EC-C8		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Yes	5%	130 m2/g	L7	General purpose Lower retention of hydrophobic analytes vs. C18
Aq-C18		2.7 µm	120 Å	90 °C	1.0–8.0	Yes	Proprietary	130 m2/g	L1	Enhanced retention for challenging polar compounds while also separating non-polar analytes 100% aqueous mobile phase compatibility and low pH stability
SB-C18		1.9 µm, 2.7 µm, 4 µm	120 Å	90 °C	1.0–8.0	No	9%	130 m2/g	L1	Excellent stability at low pH Great peak shape in highly acidic conditions
SB-C8		2.7 µm	120 Å	80 °C	1.0–8.0	No	5.5%	130 m2/g	L7	Excellent stability at low pH Lower retention of hydrophobic analytes vs. C18
HPH-C18		1.9 µm, 2.7 µm, 4 µm	100 Å	60 °C	2.0–11.0	Yes	Proprietary	95 m2/g	L1	High pH capability designed for longest lifetime, especially under high pH conditions Robust performance and long lifetimes Similar selectivity compared to EC-C18
HPH-C8		2.7 µm, 4 µm	100 Å	60 °C	2.0–11.0	Yes	Proprietary	95 m2/g	L7	High pH capability Robust performance and long lifetimes Lower retention of hydrophobic analytes vs. C18
CS-C18		2.7 µm	100 Å	90 °C	1.0–11.0	Yes	Proprietary	95 m2/g	L1	High pH capability with alternate selectivity Improved peak shape and sample capacity for basic compounds with low ionic strength mobile phases
Bonus-RP		2.7 µm	120 Å	60 °C	2.0–8.0	Yes	9.5%	130 m2/g	L60	Alternate selectivity to C18 Unique selectivity due to a polar embedded group, stable in 100% aqueous
PFP		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Yes	5.1%	130 m2/g	L43	Alternate selectivity Excellent peak shape for polar and nonpolar analytes Unique selectivity for aromatic and halogenated compounds
Phenyl-Hexyl		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Yes	9%	130 m2/g	L11	Alternate selectivity with aromatic groups Highly nonpolar bonded phase takes advantage of pi-pi interactions
SB-Aq		1.9 µm, 2.7 µm, 4 µm	120 Å	80 °C	1.0–8.0	No	Proprietary	130 m2/g	L96	Alternate selectivity Excellent peak shape and retention of polar compounds using reversed-phase LC Exceptional stability under high-aqueous conditions, including 100% water

5991-9013EN_InfinityLab_Poroshell120_poster (agilent.com)

InfinityLab Poroshell 120 | Agilent

Column Selection

Totally porous column (TPP) selection poster

Agilent ZORBAX	Chemistry	Particle Sizes	Pore Size (Å)	Temperature Limit	pH Range	Endcapped	Carbon Load (%)	Surface Area	USP Designation	Benefits and Applications
Eclipse Plus C18		1.8, 3.5, 5	95	60 °C	2–9	Double	9	160 m²/g	L1	General purpose Starting Point for LC method development
Eclipse Plus C8		1.8, 3.5, 5	95	60 °C	2–9	Double	7	160 m²/g	L7	General purpose Lower retention of hydrophobic analytes vs. C18
Eclipse Plus Phenyl-Hexyl		1.8, 3.5, 5	95	60 °C	2–8	Double	9	160 m²/g	L11	Alternative selectivity for aromatic compounds Enhanced pi-pi interactions when using methanol
Eclipse Plus PAH	Polymeric C18	1.8, 3.5, 5	95	60 °C	2–9	Double	14	160 m²/g	L1	Application-specific Designed for the separation of PAHs in LC
Eclipse XDB C18		1.8, 3.5, 5	80	60 °C	2–9	Double	10	180 m²/g	L1	General purpose, higher carbon load Higher hydrophobicity with alternative selectivity for lipophilic analytes
Eclipse XDB C8		1.8 (RRHT) 3.5, 5, 7	80	60 °C	2–9	Double	7.6	180 m²/g	L7	General purpose, higher carbon load Higher hydrophobicity with alternative selectivity for lipophilic analytes but reduced retention vs. XDB-C18
Eclipse XDB Phenyl		3.5, 5	80	60 °C	2–9	Double	7.2	180 m²/g	L11	Alternative selectivity for aromatic compounds Enhanced pi-pi interactions when using methanol
Eclipse XDB CN		3.5, 5	80	60 °C	2–9	Double	4.2	180 m²/g	L10	Polar analytes in RP, low bleed Excellent peak shape of polar and mid-polar compounds
StableBond C18	 R₁=C18	1.8, 3.5, 5, 7	80	90 °C	0.8–8	No	10	180 m²/g	L1	Low pH and high temperature Excellent stability and peak shape at highly acidic conditions
StableBond C8	R₁=C8	1.8, 3.5, 5, 7	80	80 °C	1–8	No	5.5	180 m²/g	L7	Low pH and high temperature Lower retention of hydrophobic analytes vs. C18
StableBond C3	R₁=C3	1.8, 3.5, 5	80	80 °C	1–8	No	4	180 m²/g	L56	Low pH and high temperature Reduced retention of hydrophobic analytes
StableBond Aq	Proprietary	1.8, 3.5, 5, 7	80	80 °C	1–8	No	Proprietary	180 m²/g	L96	Polar analytes in RP Excellent peak shape and retention of polar compounds using reversed-phase LC, stable at 100% aqueous mobile phases
StableBond Phenyl	R₁=Phenylethyl	1.8, 3.5, 5, 7	80	80 °C	1–8	No	5.5	180 m²/g	L11	Alternative selectivity for aromatic compounds Enhanced pi-pi interactions when using methanol
StableBond CN	R₁=(CH₂)₃-CN	1.8, 3.5, 5, 7	80	80 °C	1–8	No	4	180 m²/g	L10	Polar molecules at low pH or high temperature, low bleed Excellent peak shape of polar and mid-polar compounds

A proven and reliable portfolio of totally porous HPLC columns (agilent.com)

ZORBAX Reversed-Phase Columns | Agilent

Method Development 101:

Mobile Phase Selection

Mobile Phase Selection

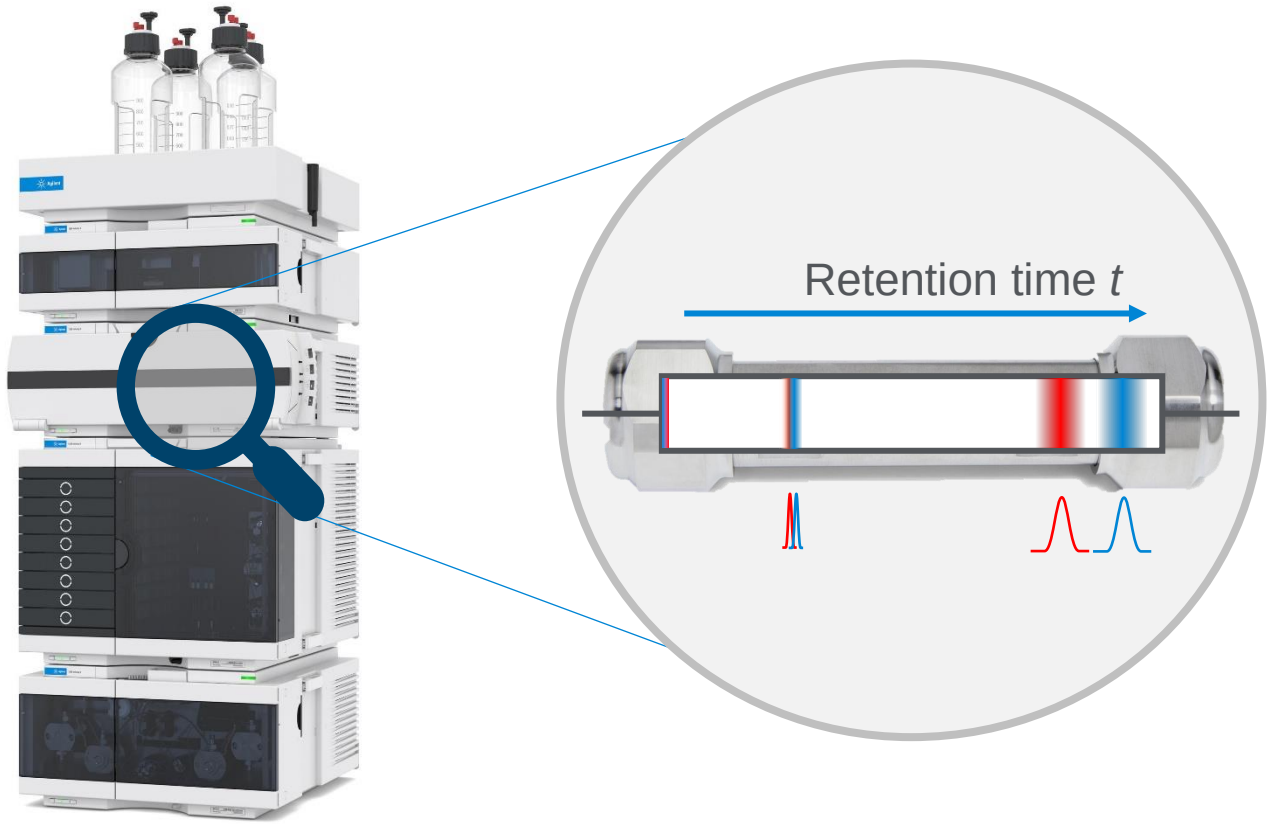
Retention and selectivity

Condition	Retention (κ)	Selectivity (α)	Efficiency (N)
% B	●●	●	—
B-solvent (acetonitrile, methanol, etc.)	●	●●	—
Temperature	●	●	●
Column type (C18, phenol, etc.)	●	●●	—
Mobile phase pH	●●	●●	●
Buffer concentration	●	●	—
Ion-pair-reagent concentration	●●	●●	●
Column length	NA	NA	●●
Particle size	NA	NA	●●
Flow rate	NA	NA	●

Symbol	Meaning
●●	Major effect
●	Minor effect
—	Relatively small effect
blue	Conditions that are primarily used to control variable

Mobile Phase Selection

Chromatographic process

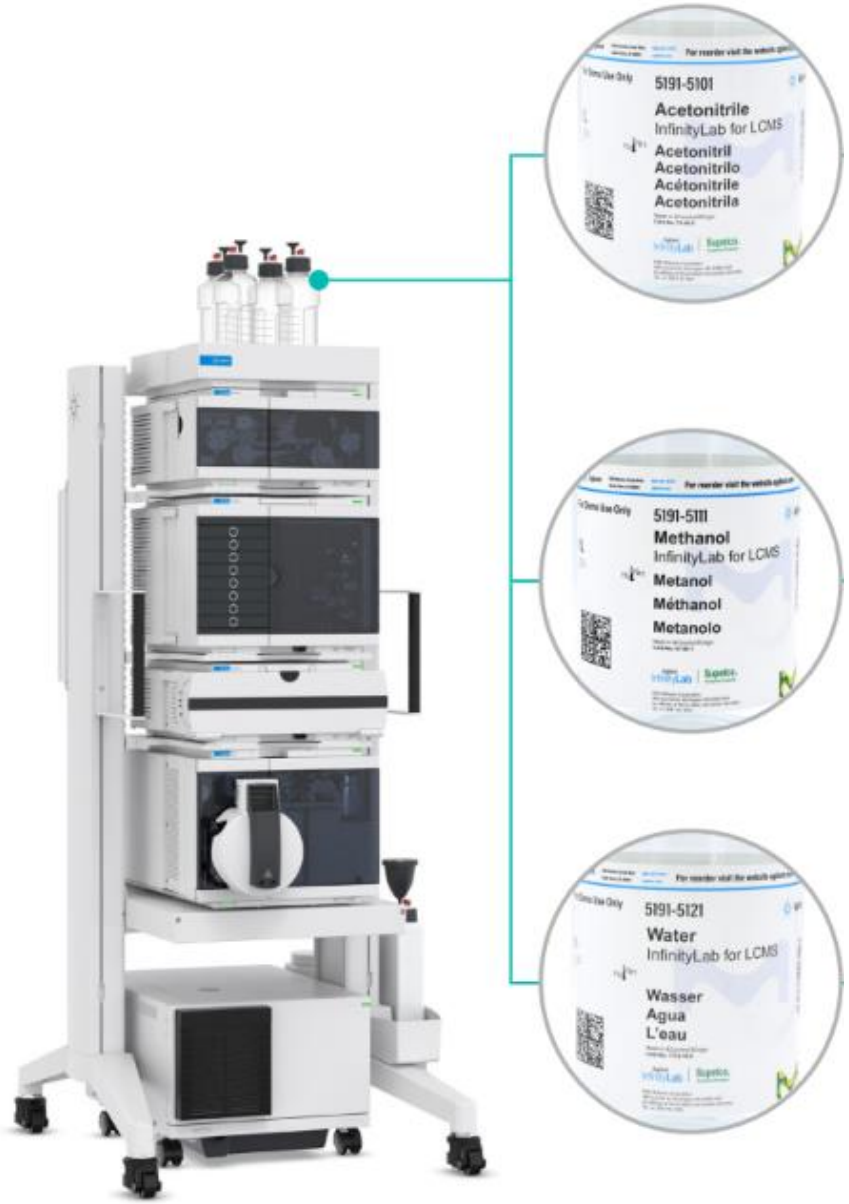


Reversed-Phase LC	
Polarity	Non-polar stationary phase (e.g., C18)
Mobile Phase	Polar mobile phase: H_2O/CH_3OH, H_2O/CH_3CN
Gradient	Decrease retention by decreasing polarity of mobile phase H2O ↓ = retention ↑ CH_3CN ↑ = retention ↓
Elution Order	polar to non-polar

Mobile Phase Selection

Reversed-phase mobile phases

- HPLC or LCMS grade solvents
- UV transparency
- Low viscosity
- High boiling point
- Sample Solubility
- Low cost, low toxicity, non-corrosive



Maximize Your Efficiency With Precision Solvents (agilent.com)

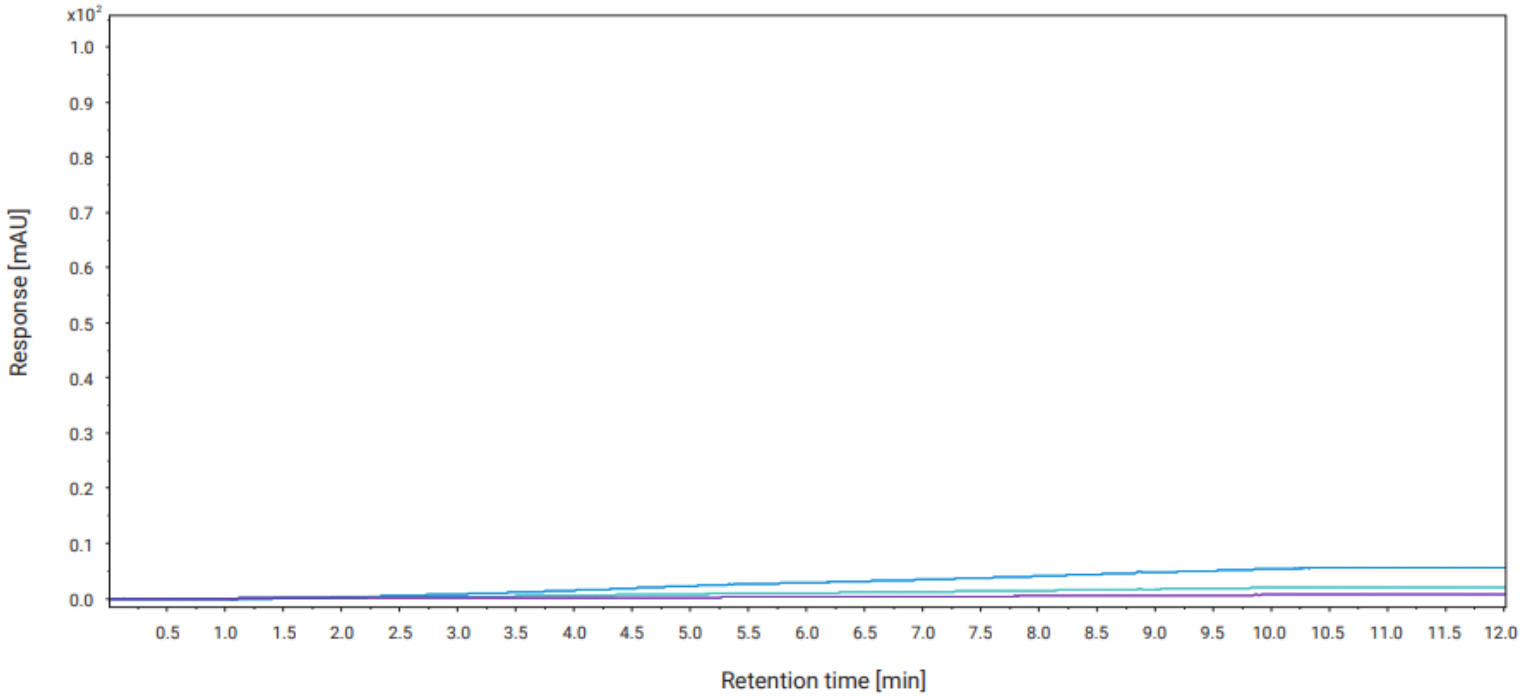
Mobile Phase Selection

Reversed-phase mobile phases

HPLC or LCMS grade solvents

- Lowest impurity levels, reducing ghost peaks in gradient runs
- 0.2 µm pre-filtering safeguards system from contaminants and clogging
- Highest lot-to-lot reproducibility

Water/Methanol Gradient Overlay at 210 nm, 225 nm, and 254 nm



Gradient from 5-95% ACN. Detection wavelengths 210 nm (blue), 225 nm (turquoise), and 254 nm (purple); Range: 0-100 mAU

Maximize Your Efficiency With Precision Solvents (agilent.com)

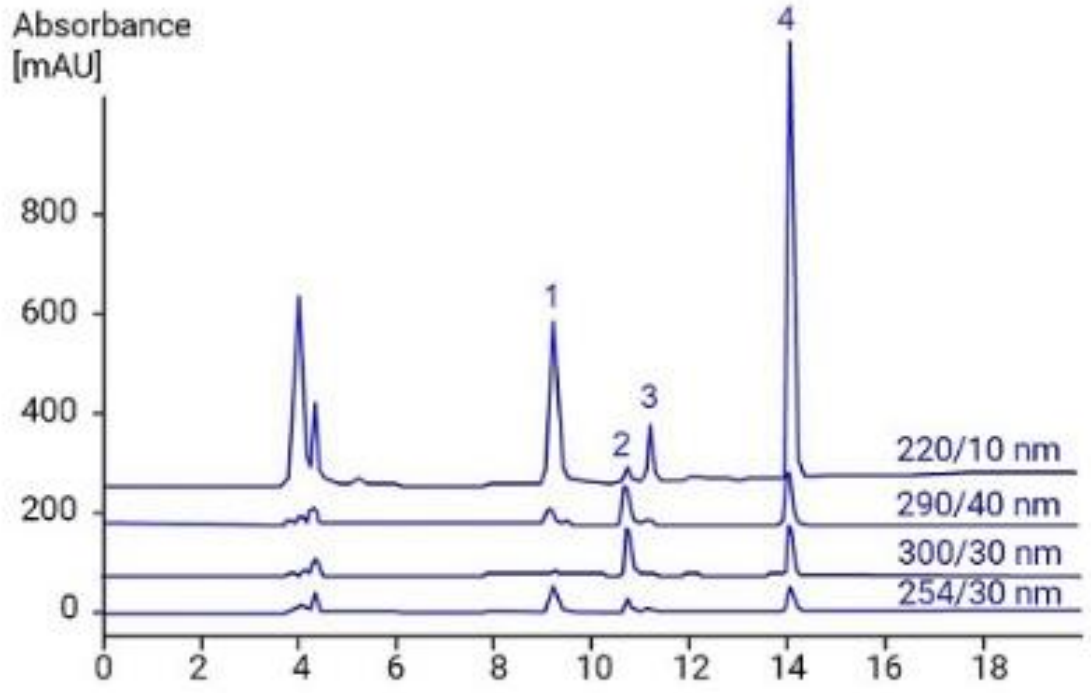
Mobile Phase Selection

Reversed-phase mobile phases

UV transparency

- Mobile phase will have an absorbance of A < 0.2 AU at the wavelength used for detection of the sample

The Effect of Changing Wavelength on Sensitivity



The effect of changing wavelength on sensitivity of the analysis of beta blocker molecules. At lower wavelengths, the background (baseline) signal rises as the UV cutoff point of the solvent is approached

Maximize Your Efficiency With Precision Solvents (agilent.com)

Mobile Phase Selection

Reversed-phase mobile phases

Low viscosity

- Pressure is directly proportional to the viscosity of solvents

Solvent	Boiling Point (°C)	Viscosity (cP)	UV cutoff (nm)
N-Hexane	69	0.31	190
Toluene	78	0.59	285
Methylene chloride	40	0.44	233
Tetrahydrofuran	66	0.55	212
Acetonitrile	82	0.30	190
2-Propanol	82	2.30	205
Methanol	65	0.54	205
Water	100	1.00	<190

Maximize Your Efficiency With Precision Solvents (agilent.com)

Mobile Phase Selection

Mobile phase modifiers

Buffers	•	To stabilize pHs of mobile phase (Phosphate, acetate, citrate)
Acidifiers	•	To suppress ionization of acidic analytes (TFA, FA)
Ion-pairing reagents	•	For separation of ionic compounds with reversed-phase methods (HFIP)
Amine modifiers	•	To reduce tailing of basic analytes with reversed-phase methods (TEA)

Mobile Phase Selection

Buffer selection

Desired properties

- pK_a and buffer capacity
- Solubility
- UV absorbance (UV detection)
- Volatility (MS or ELSD)
- Ion-pairing properties
- Stability and compatibility with equipment

Mobile Phase Selection

Buffer selection

Desired properties

- pK_a and buffer capacity
  - Mobile phase pH should be ± 1.0 units from buffer pK_a
  - Concentration typically falls within 5 to 25 mM
  - pH of buffer should be at least one unit above or below the pK_a of the sample

Name of Buffer	Range of pH	MS Compatible
Phosphate: pK_1	1.1-3.1	No
Phosphate: pK_2	6.2-8.2	No
Phosphate: pK_3	11.3-13.3	No
Sodium acetate	3.8-5.8	No
Ammonium acetate (< 50 nM)	3.8-5.8	Yes
Trifluoro acetic acid (0.1%)	2.0	Yes
Phosphoric acid (0.1%)	2.0	No
Formic acid (0.1%)	2.7	Yes
Ammonium formate (< 50 nM)	2.7-4.7	Yes
Ammonium bicarbonate	6.6-8.6	Yes
TRIS	7.3-9.3	Yes

Mobile Phase Selection

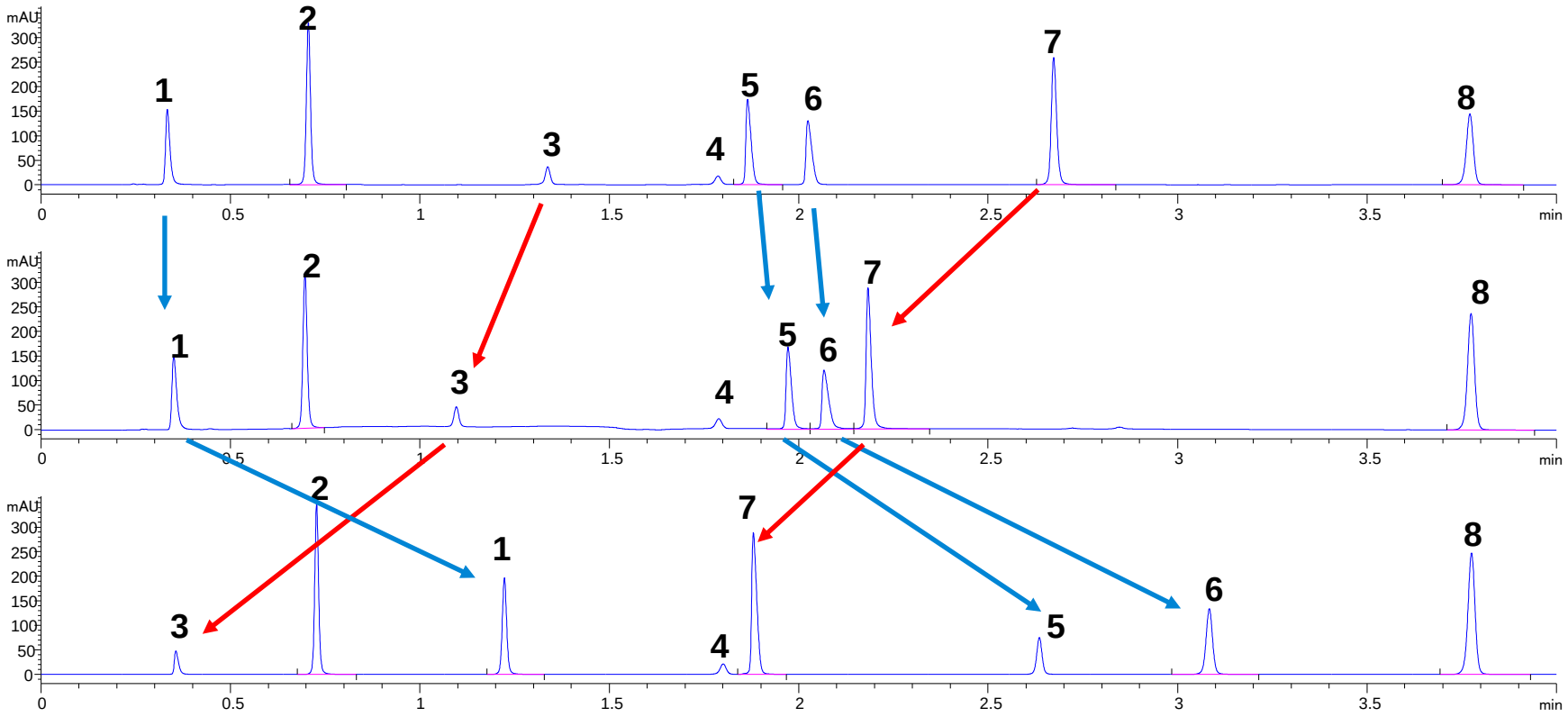
pH selectivity

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

pH 3
10 mM HCO_2NH_4

pH 4.8
10 mM NH_4HCO_3

pH 10
10 mM NH_4HCO_3



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

Method Development 101:

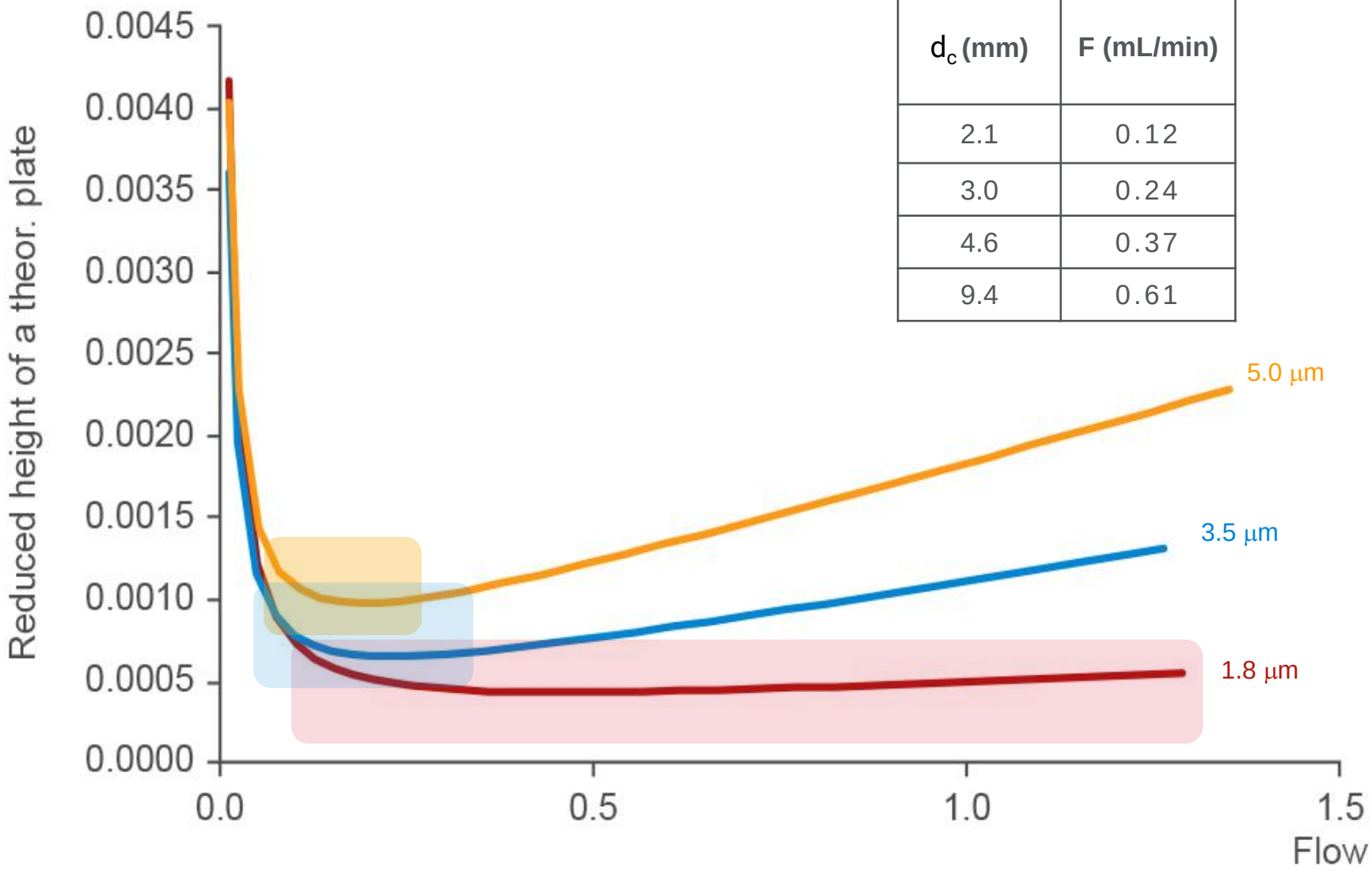
Flow Rates and Injection Volumes

Flow Rates and Injection Volumes

Flow rates

- The optimal flow rate depends on column diameter and particle size

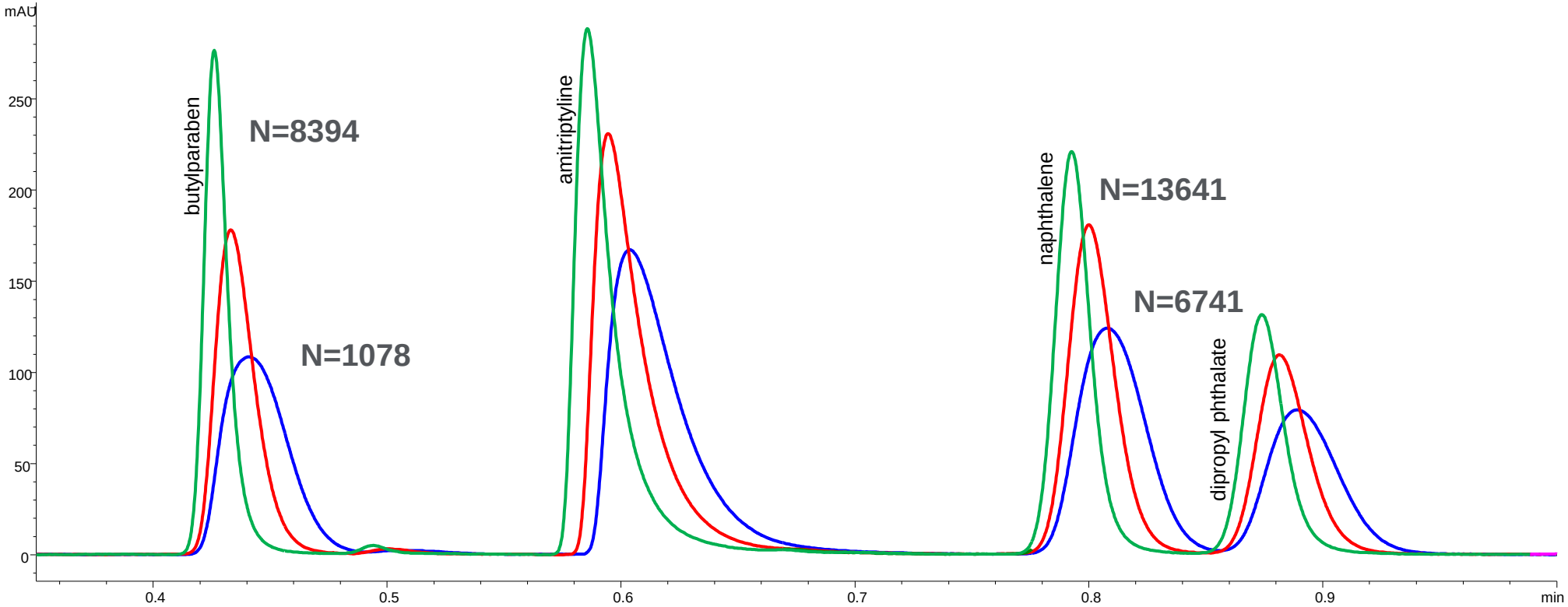
d_c (mm)	F (mL/min)
2.1	0.12
3.0	0.24
4.6	0.37
9.4	0.61



Flow Rates and Injection Volumes

Injection volumes

- Injection volumes contribute to overall system volume
- Keep injection volumes to a minimum, while retaining solubility



Flowrates and Injection Volumes

Injection volumes

$$V_m = \pi \cdot r^2 \cdot L \cdot \sim 0.6$$

Column volume is calculated as the volume of a cylinder less the space occupied by the packing material. As an example, Agilent ZORBAX Eclipse Plus C18 packing material occupies 40% of the column, the remaining 60% of the cylinder would be considered as column volume.

Column Dimensions (d_c x L, mm)	V_m (mL)
2.1 x 50	0.12
2.1 x 100	0.24
2.1 x 150	0.37
2.1 x 250	0.61
3.0 x 150	0.85
4.6 x 100	1.16
4.6 x 150	1.75
4.6 x 250	2.90

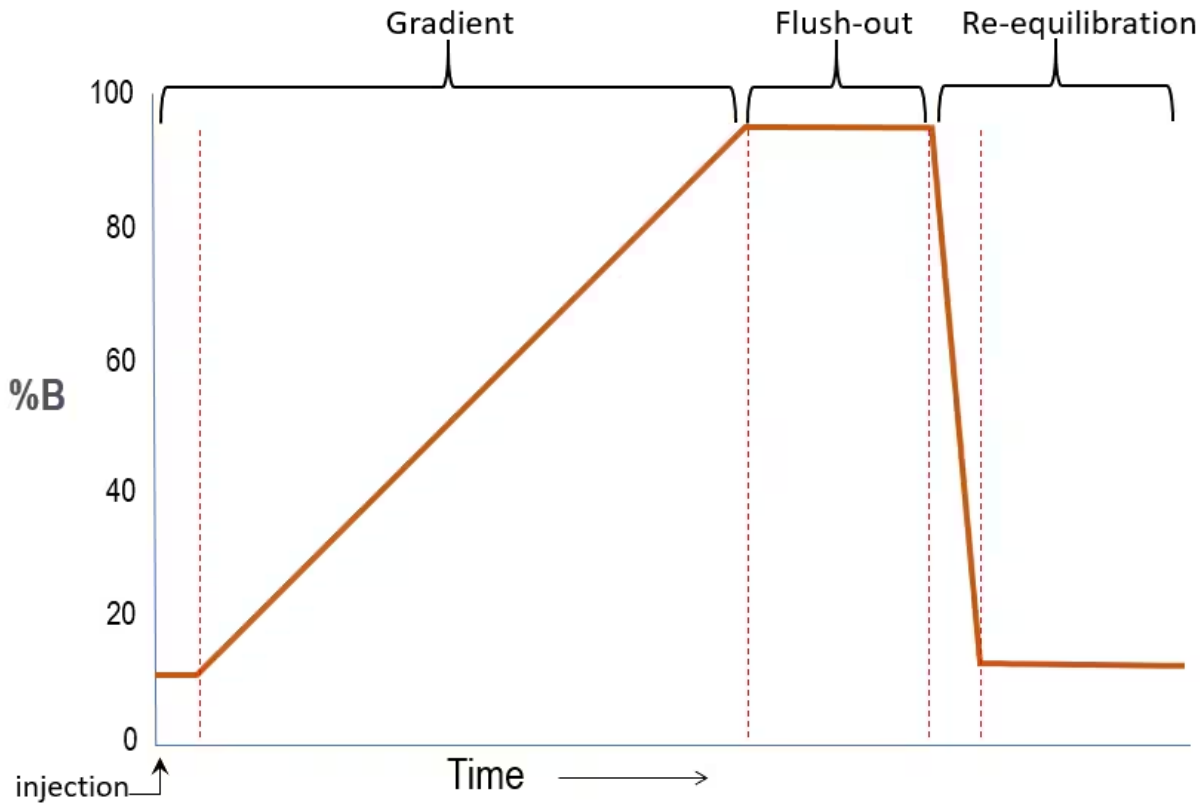
Method Development 101:

Scouting gradient

Method Development 101:

Scouting gradient

- A good starting point when developing a method is a scouting gradient.
- Recommended starting conditions are 5–95% MP B with a low pH
- Gradient length is dependent on the column length

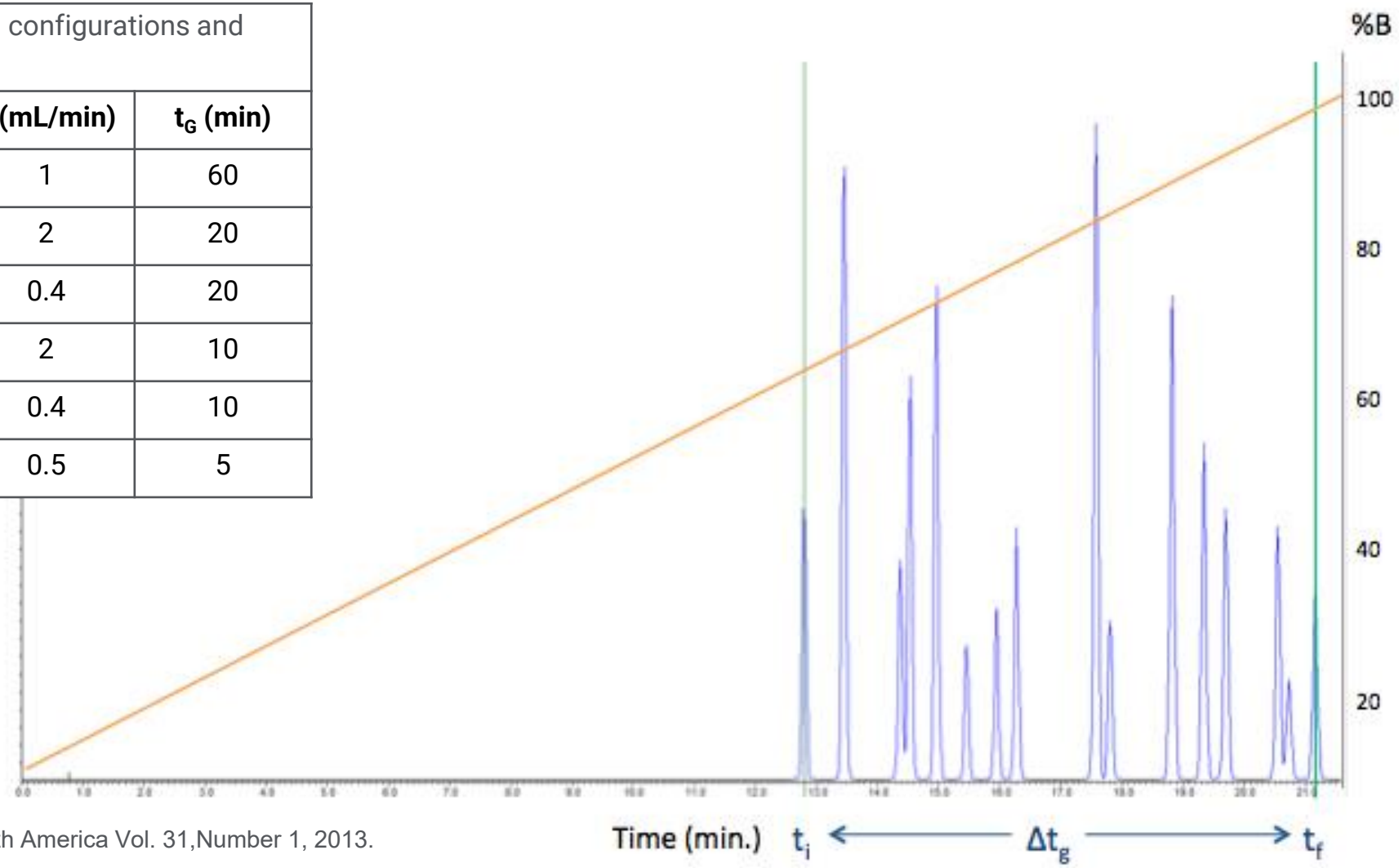


"Making to the most of a Gradient Scouting Run" LCGC North America Vol. 31,Number 1, 2013.

Method Development 101:

Scouting gradient

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



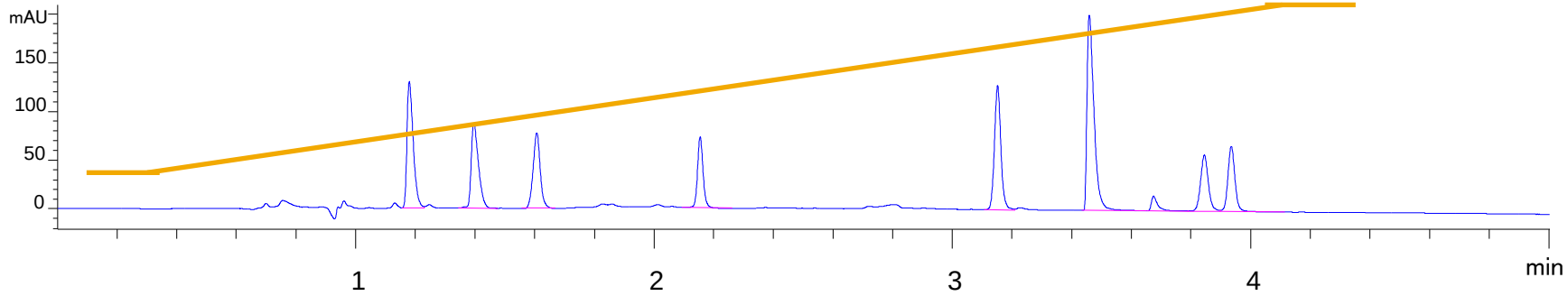
"Making to the most of a Gradient Scouting Run" LCGC North America Vol. 31,Number 1, 2013.

Method Development 101:

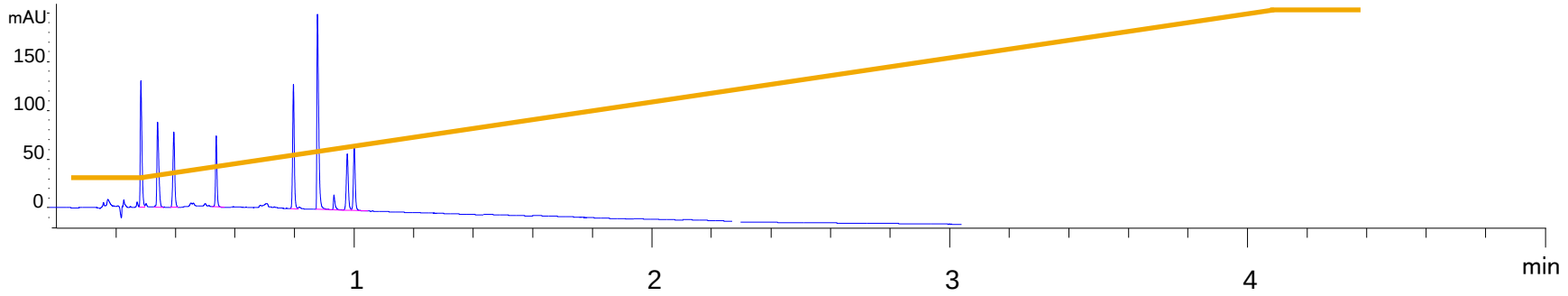
Scouting gradient

- 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

Ideal for Gradient Method



Ideal for Isocratic Method



Method Development 102

Title: HPLC Method Development: From Beginner to Expert, Part 2
Date: Thursday, March 28, 2024
Time: 11:00 AM Eastern Daylight Time
Duration: 1 hour

Jean Lane
Application Engineer
Agilent Technologies, Inc.

Method Development 102 will review and expand upon some of the 101 fundamentals as we cover advanced topics such as how to best transfer a method from one column dimension to another. We will explore reasons for why some methods are quite difficult to transfer to a different HPLC system. In addition, we will look at gradient method development and how to efficiently use a scouting gradient to quickly develop a good HPLC method.

HPLC Method Development: From Beginner to Expert, Part 2 (on24.com)

Method Development 101:
Conclusion

Conclusion

Tips for a robust method

- Always start method development with a new column
- Select columns with robust properties at pH of method
- Choose a quality column with long lifetimes
- Consider batch-to-batch reproducibility
- Consider scalability of particle sizes and chemistries for downstream method transfer
- Make sure mobile phase preparation is documented and transferrable

Agilent Resources for Support

- Resource page http://www.agilent.com/chem/agilentresources
  - Quick reference guides, product catalogs
  - Online selection tools, “How-to” videos
  - Column user guides - https://www.agilent.com/en-us/support/liquid-chromatography/kb005965
- Tech support: http://www.agilent.com/chem/techsupport
- InfinityLab LC Supplies catalog (5991-8031EN)
- Agilent University http://www.agilent.com/crosslab/university
- YouTube – Agilent Channel
- Your local product specialists

Contact Agilent Chemistries and Supplies Technical Support

Available in the USA 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

gc-column-support@agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

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Questions?

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