Finding the Perfect Match: Practical Advice on Column and Mobile Phase Selection

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When a method is not rugged and robust

- Method fails unexpectedly, halting production
- “Method creep”
- Risk of redeveloping method after validation
- Compromise quality

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

Examples of Common Separation Goals and Method Performance Criteria

Good System Suitability Parameters

- Resolution: ≥2
- Peak shape: USP Tf close to 1 (<2)
- Injection Repeatability: areas, Tf, etc. (RSD 0.1 - 0.25%)
- Absolute retention factors: 1< k<10
- Relative Retention: α or k2/k1
- 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)

These inhibit the ability to speed up your method in the future!

What Makes a Good Starting Point for RP Method Development?

1. Smaller particles and superficially porous particles offer fast, efficient analysis
2. C18 column – most general purpose column choice
3. Simple mobile phase
   a) Formic acid or other additive in aqueous portion (buffer salts only if necessary)
   b) Acetonitrile or methanol as organic modifier
4. Start with a linear gradient (5% organic to 95% organic) for reversed-phase methods
5. Adjust mobile phase to get the desired retention and resolution
   a) Adequate resolution of all peaks, Rs ≥ 2.0
   b) Retention of first peak at least k=1
   c) Fastest analysis time with required resolution

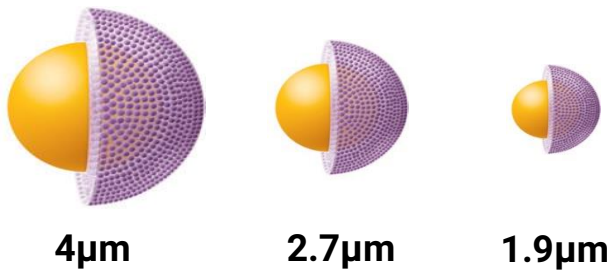
Newer shorter, columns with small particle sizes can provide more efficiency and resolution in a very short time, speeding up method development

The Future – Higher Efficiencies using SPP

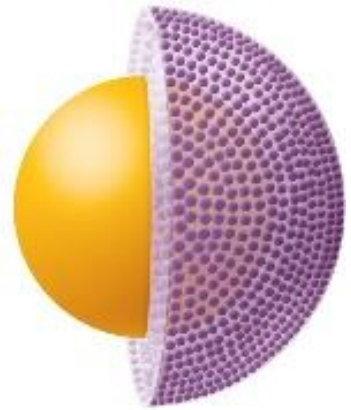
InfinityLab Poroshell 120

Additional efficiency can be generated through the use of superficially porous particles (SPP) rather than a totally porous particle (TPP)

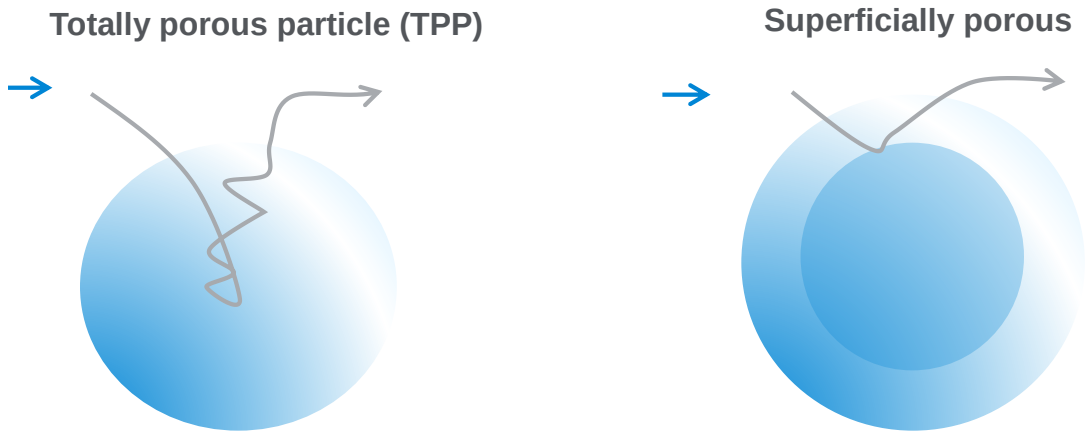
SPP particle	For	Maximum pressure	Typical pressure	Efficiency
1.9 µm	Highest UHPLC performance	1300 bar	Similar to sub-2 µm totally porous	~120% of sub-2 µm totally porous
2.7 µm	UHPLC performance at lower pressures	600 bar	50% of sub-2 µm totally porous	~90% of sub-2 µm totally porous
4 µm	Improved HPLC performance	600 bar	Typically < 200 bar	~200% of 5 µm totally porous



Poroshell Technology – What makes it better?



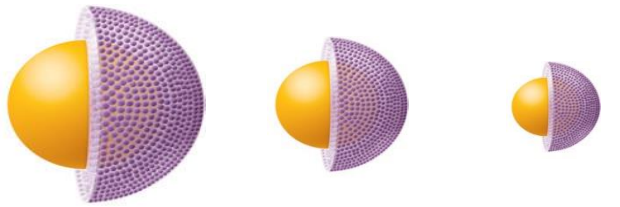
Poroshell is made of a solid core with a porous outer layer



- 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. 2.7um) for nearly equivalent performance to a smaller TPP column (ie. sub-2um)
- Using a larger particle allows for lower backpressure than comparable TPP columns, and flexible use on HPLC or UHPLC systems

Agilent InfinityLab Poroshell 120 Portfolio

Best all around	Best for low pH mobile phases	Best for high pH mobile phases	Best for alternative selectivity	Best for polar Analytes	Best for Chiral
InfinityLab Poroshell **EC-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C18** 2.7 µm	InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Bonus-RP** 2.7 µm	InfinityLab Poroshell **HILIC** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Chiral-V** 2.7 µm
InfinityLab Poroshell **EC-C8** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C8** 2.7 µm	InfinityLab Poroshell **HPH-C8** 2.7 µm, 4 µm	InfinityLab Poroshell **PFP** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-Z** 2.7 µm	InfinityLab Poroshell **Chiral-T** 2.7 µm
			InfinityLab Poroshell **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-OH5** 2.7 µm	InfinityLab Poroshell **Chiral-CD** 2.7 µm
			InfinityLab Poroshell **SB-Aq** 2.7 µm		InfinityLab Poroshell **Chiral-CF** 2.7 µm
			InfinityLab Poroshell **EC-CN** 2.7 µm		



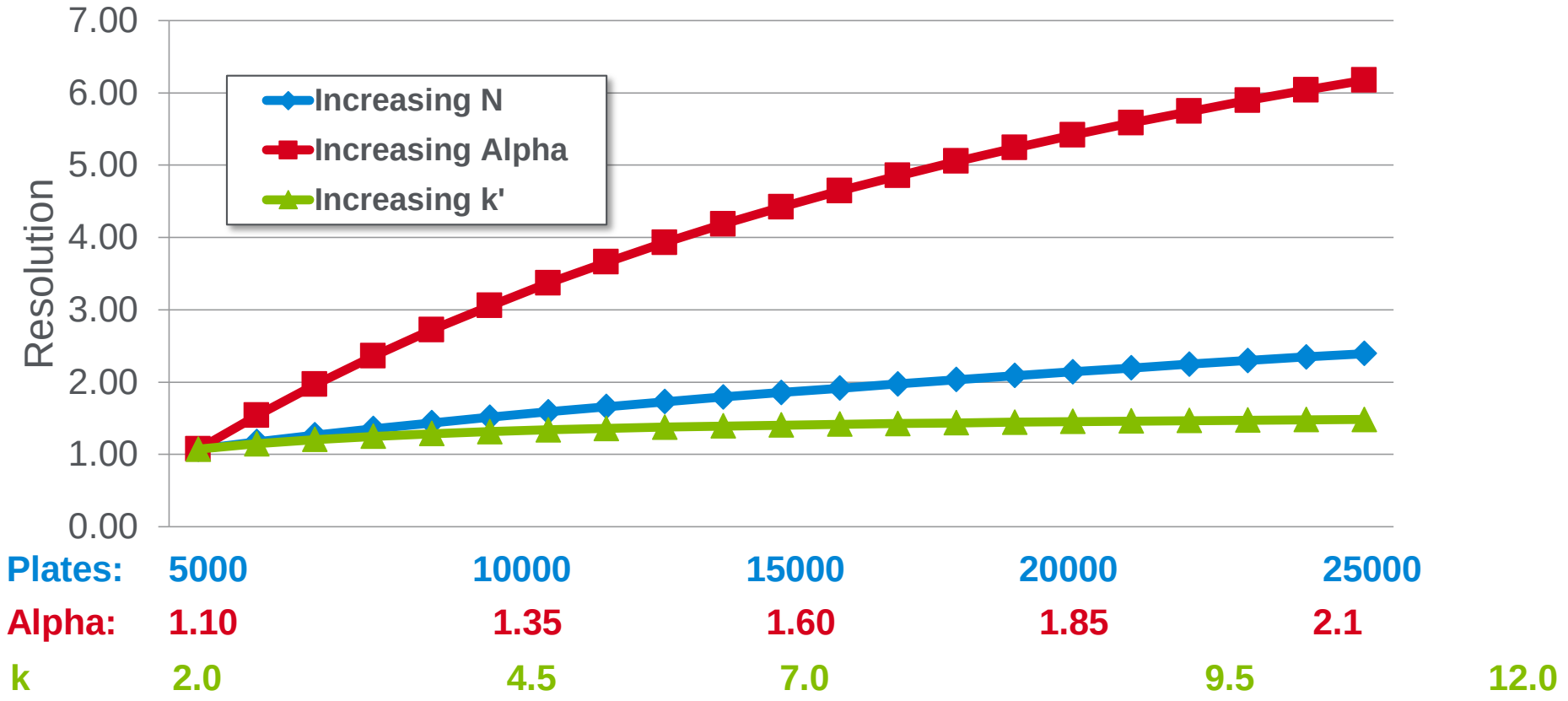
4 µm **2.7 µm** **1.9 µm**

Reversed-phase chemistries

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**

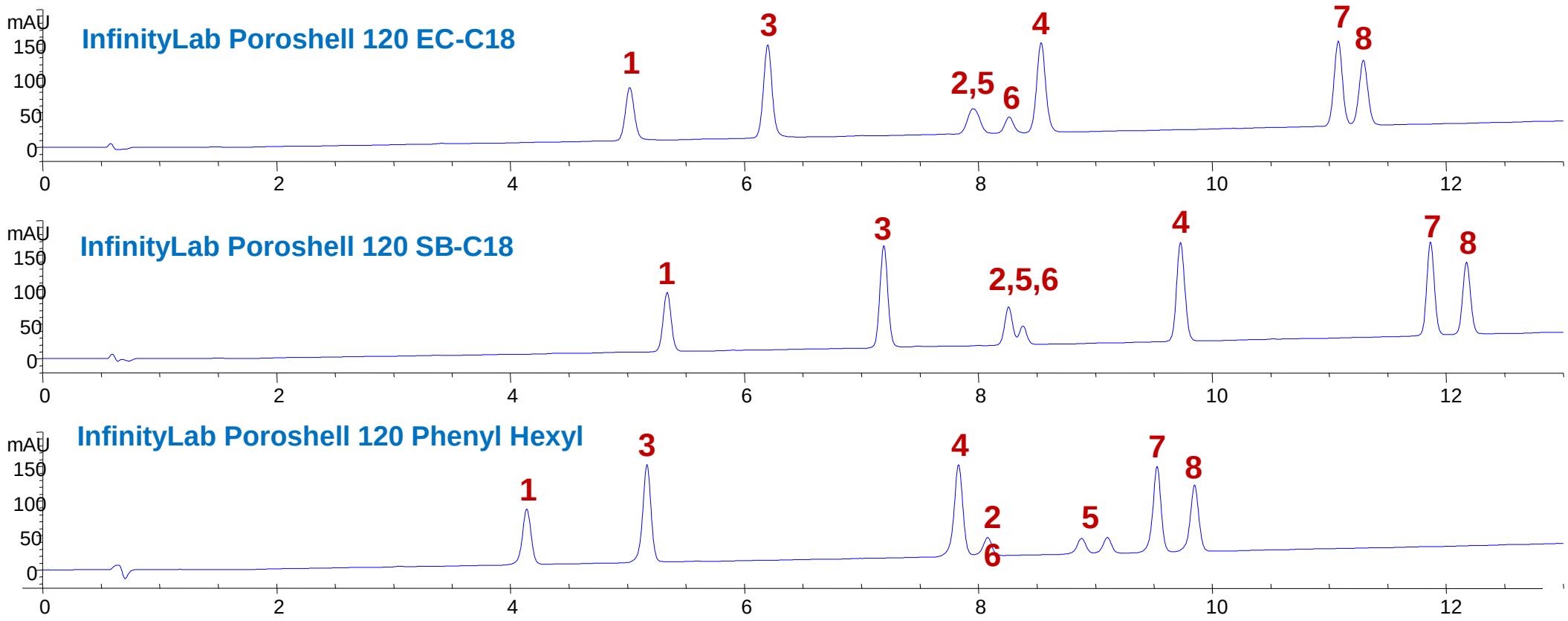


Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} **Typical Analytical Method Development Parameters**

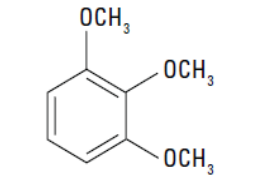
Selectivity Differences Across InfinityLab Poroshell Bonded Phases



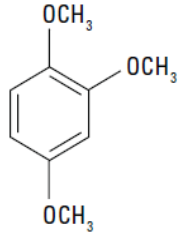
1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone
5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

40–80% Methanol in 14 min, DAD 260, 80 nm 0.4 ml/min, 2.1 x 100 mm column, 40º C, 0.1% formic acid in water and methanol, Agilent 1260 method development solution

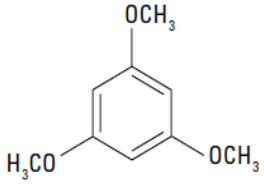
Importance of Alternate Selectivity Chemistries



1,2,3-Trimethoxybenzene

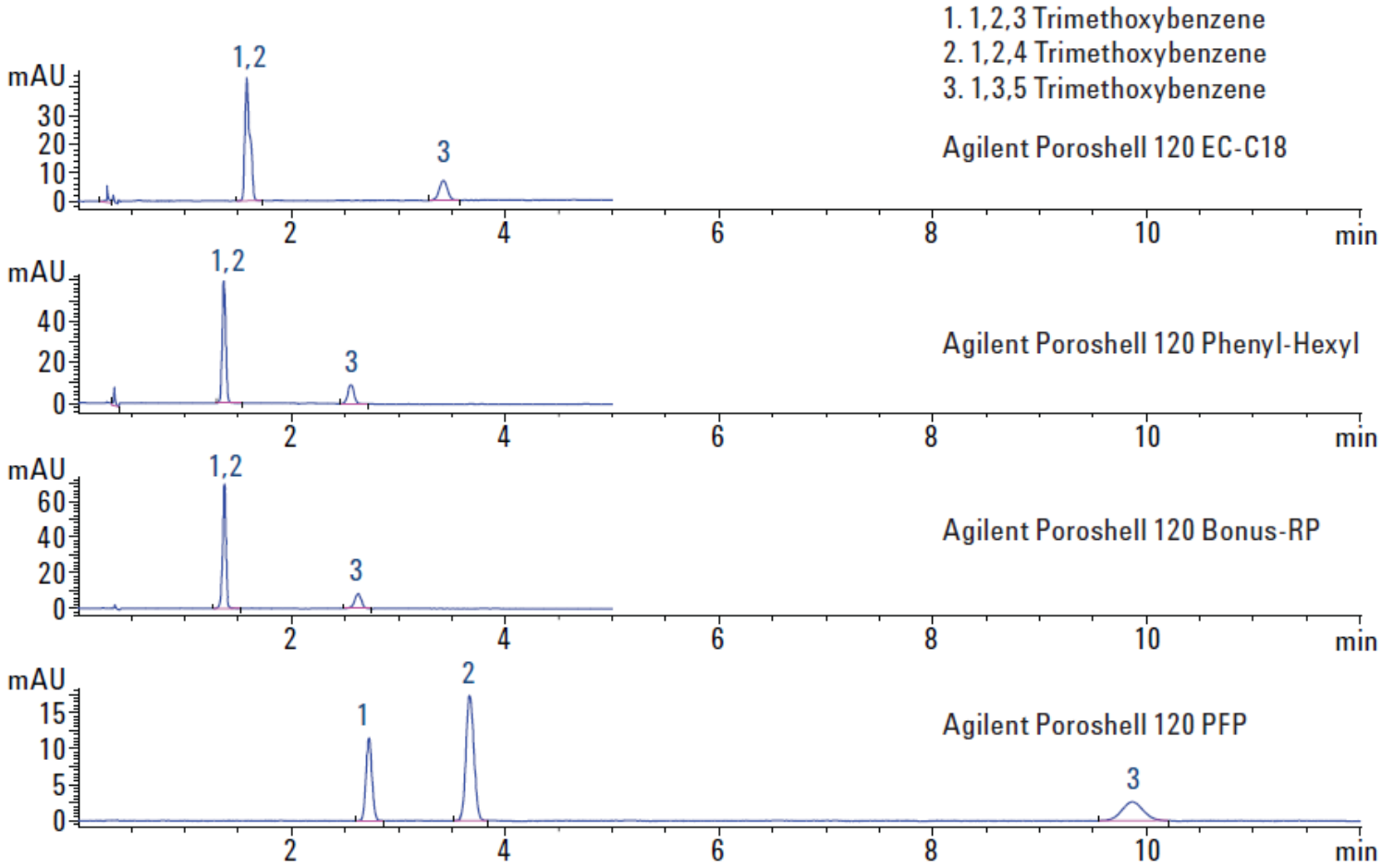


1,2,4-Trimethoxybenzene



1,3,5-Trimethoxybenzene

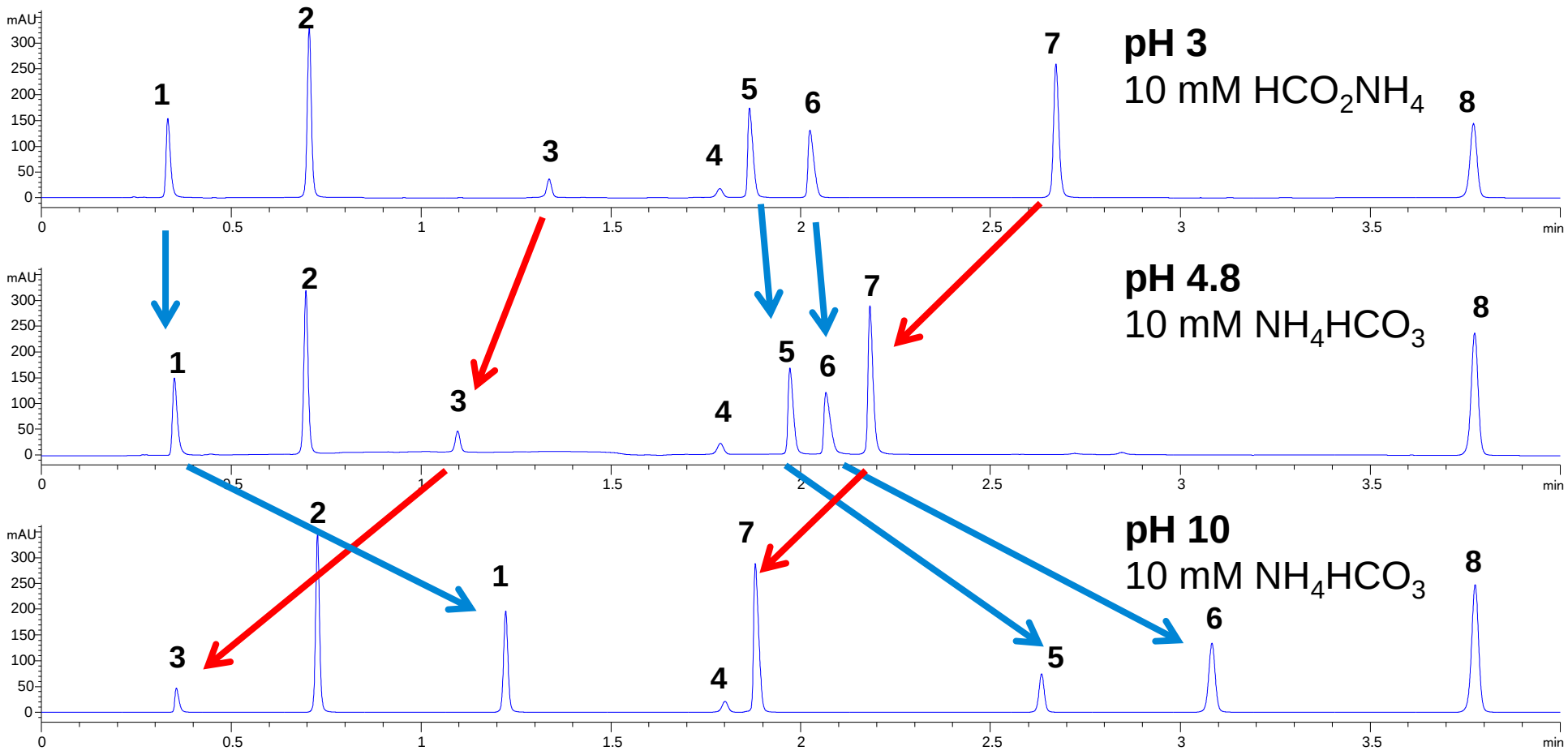
- 3 compounds
  - Same molecular weight
  - Only differ by positional location of the functionality



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

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

Agilent Pub No. 5991-4893

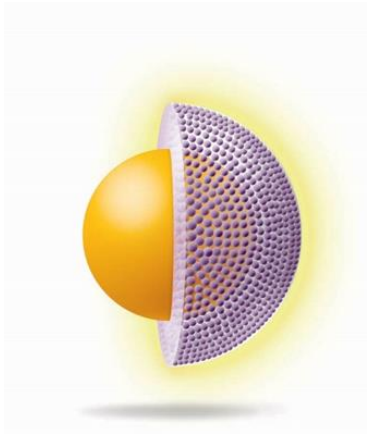
Acids
Bases

pH – A Method Development Tool for Ionizable Compounds

- Ionizable compounds will be in a charged or uncharged state based on pH
- Choose mobile phase pH to optimize retention and selectivity
- Non-charged analytes have better retention
  - i.e. acids at low pH and bases at mid or high pH
- Silanols on silica ionize at mid-pH, possible ion-exchange interaction of basic analytes
- Ensure that your column is compatible with and stable in the mobile phase pH you select

Agilent InfinityLab Poroshell HPH particles

Hybridized Poroshell 120 silica offers more rugged silica particle and enhanced stability up to pH 11



Best for high pH
InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm
InfinityLab Poroshell **HPH-C8** 2.7 µm, 4 µm

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion
- Dipole-Dipole
- Hydrogen Bonding
- Ionic (Coulombic) Interactions
- Charge Transfer (π – π interactions)

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion – due to the instantaneous positions of electrons around the solute and solvent. The strength of dispersion increases with polarizability. But, the critical point is that dispersive effects are non-specific, significant in both mobile- and stationary-phases and therefore tend to cancel out. **They have little impact on selective partitioning.**
- Dipole-Dipole
- Hydrogen Bonding
- Ionic (Coulombic) Interactions
- Charge Transfer (π – π interactions)

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion
- Dipole-Dipole – Large, permanent dipole moments will drive molecules to align for maximum electrostatic interaction. As an example, acetonitrile with the functional group -C≡N would be expected to have a dipole-dipole interaction with the –R-NO_2 group of a nitroalkane. The strength of this interaction is related to the strength of the dipole moments of the two functional groups, not the dipole moment of the entire molecule.
- Hydrogen Bonding
- Ionic (Coulombic) Interactions
- Charge Transfer (π – π interactions)

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion
- Dipole-Dipole
- Hydrogen Bonding – these interactions occur either when a proton donor (acidic) solvent interacts with a proton acceptor (basic) solute or when a basic solvent interacts with an acidic solute. Example: MeOH (acidic) and amines (basic)
- Ionic (Coulombic) Interactions
- Charge Transfer (π – π interactions)

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion
- Dipole-Dipole
- Hydrogen Bonding
- Ionic (Coulombic) Interactions – occurs with charged (ionizable) compounds and solvents with large dielectric constants. Can also occur between charged analytes and charged bonded phase – as in ion pair chromatography
- Charge Transfer (π – π interactions)

Solvent Selection

Hydrophobicity and more

Solvent/Solute Interactions

- Dispersion
- Dipole-Dipole
- Hydrogen Bonding
- Ionic (Coulombic) Interactions
- Charge Transfer (π – π interactions) – aromatic or saturated compounds may be either π-electron rich (a π-base) or π-electron poor (a π-acid). Phenyl groups are electron rich, whereas rings with electron withdrawing functional groups, such as nitro groups, tend to be electron poor. Interactions between π-acids and π-bases can drive selective partitioning.

Gradients

Solvent Selection and Rate of Change

$$R \approx \frac{\sqrt{N}}{4} \alpha^{*} k^{*}$$

k* - represents the fact that k changes constantly during a gradient

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

$\Delta\%B$ =	difference between initial and final % B values
S =	constant
F =	flow rate (mL/min.)
t_g =	gradient time (min.)
V_m =	column void volume (mL)

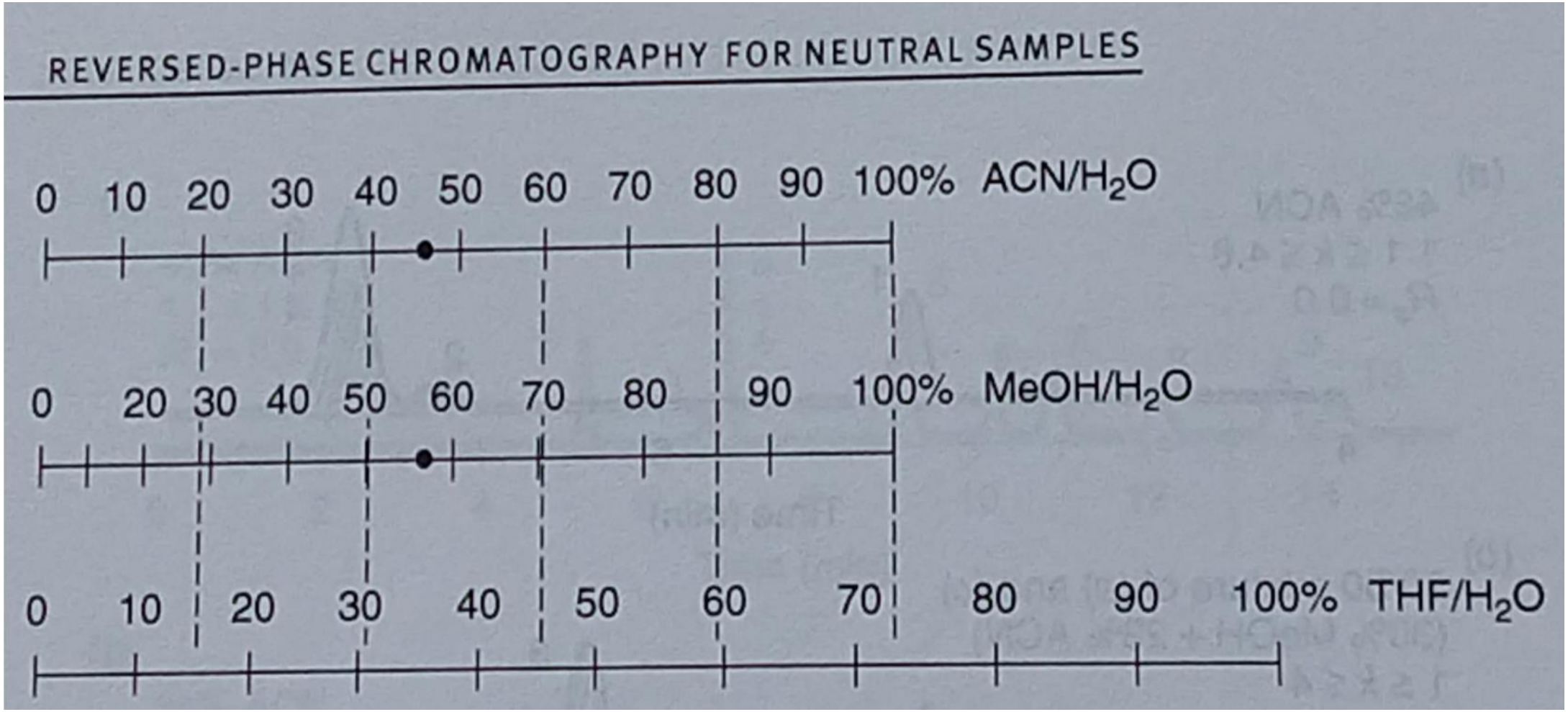
To Increase Gradient Resolution by Changing Retention (k*) Use:

t_G	• A longer gradient time
F	• A higher flow rate
Vm	• A shorter column
△%B	• A shorter organic range

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

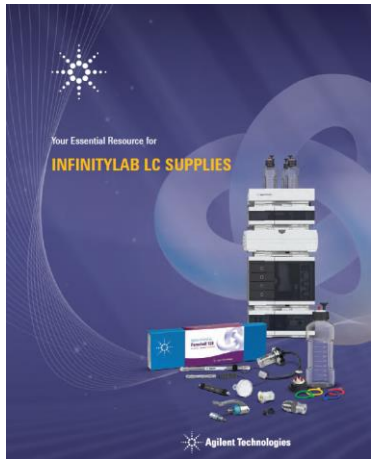
Solvent Selection

HPLC Nomograph



Resources for Support

- Agilent University http://www.agilent.com/crosslab/university
- Tech support http://www.agilent.com/chem/techsupport
- Resource page http://www.agilent.com/chem/agilentresources
  - Quick Reference Guides
  - Catalogs, Column User guides
  - Online Selection Tools, How-to Videos
- InfinityLab Supplies Catalog (5991-8031EN)
- Your local FSE and Specialists
- Youtube – Agilent Channel
- Agilent Service Contracts



Contact Agilent Chemistries and Supplies Technical Support

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

Option 1 for GC/GC/MS columns and supplies

Option 2 for LC/LC/MS columns and supplies

Option 3 for sample preparation, filtration and QuEChERS

Option 4 for spectroscopy supplies

Available in the USA & Canada 8-5 all time zones

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com