Maximizing Resolution and Selectivity: Superficially Porous Column Chromatography Options

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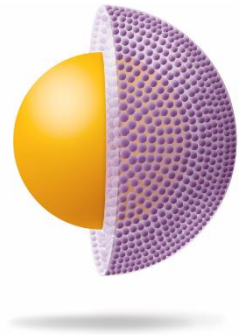
Agenda

- Superficially porous particle (SPP) specifications and benefits
- Method development challenges and objectives
- Method development with selectivity
  - Bonded phase
  - Mobile phase pH
- How to ensure the best performance from your SPP column
- When you need more help choosing a column…



SUPERFICIALLY POROUS PARTICLES SPECIFICATIONS AND BENEFITS

Current Status of Superficially Porous Particles

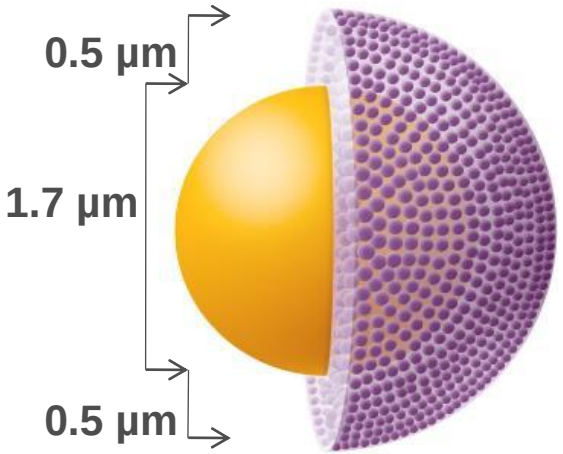
	Status in 2000	Status in 2010	Status in 2015			
	# of Vendors	# of Vendors	# of Vendors	Particle size range	Pore size range	# of Phase chemistries
Small molecules	0	3	16	1.3 µm to 5 µm	80Å to 120Å	>12 chemistries
Large molecules	1 (Agilent)	1 (Agilent)	9	2.6 µm to 5 µm	160Å to 450Å	>8 chemistries

Bell, LC-GC, 2015 June
Majors, LC-GC, 2014 Nov

Superficially Porous Column Technology

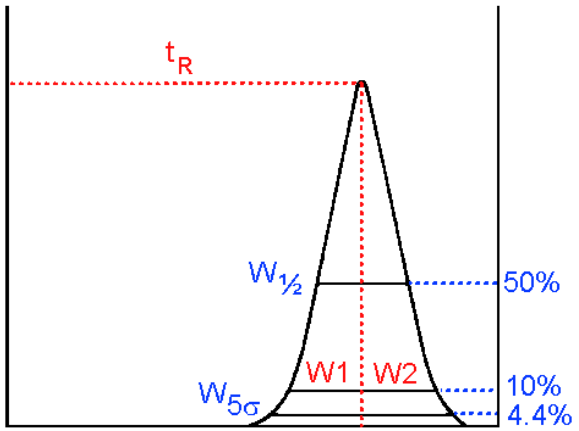
Poroshell 120 2.7 µm

- d_p = 2.7 µm
- Particles
  - 1.7 µm solid core
  - 0.5 µm diffusion path
  - 2.7 µm total diameter



- Efficiency (N) ≈ 90% of sub-2 µm
- N ≈ 2X 3.5 µm (totally porous)
- Pressure ≈ 40-50% of sub-2 µm

- 2 µm frit to reduce clogging
- P_{limit} = 600 bar for HPLC or UHPLC



Comparing Efficiency and Pressure with Different Types of Columns

Particle Size/Type	Pressure	Efficiency	LC Compatibility
3.5 µm Totally Porous	123 bar	7,800	All 400 bar instruments
2.7 µm Poroshell 120	180 bar	12,000	All LCs/UHPLCs (up to 600 bar)
1.8 µm Totally Porous	285 bar	12,500	All LCs/UHPLCs (up to 1200 bar)

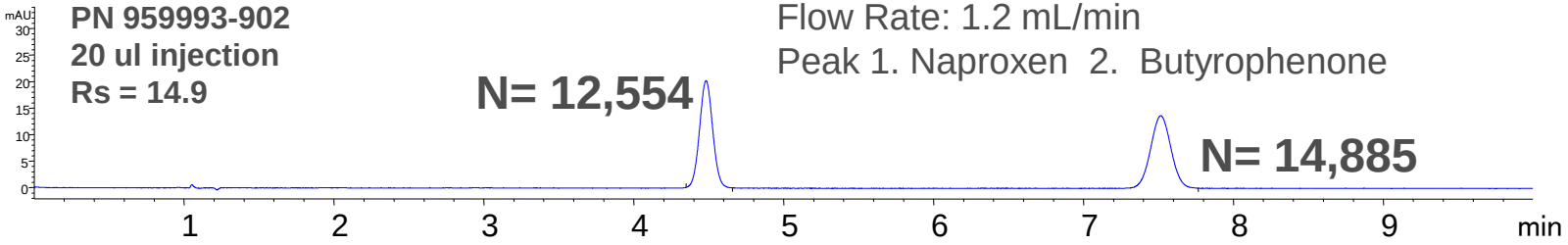
Columns: 4.6 x 50mm, Mobile Phase: 60% ACN:40% Water Flow Rate: 2 mL/min

USP Method for Naproxen Tablets

Method Requirement N > 4000, Rs better than 11.5

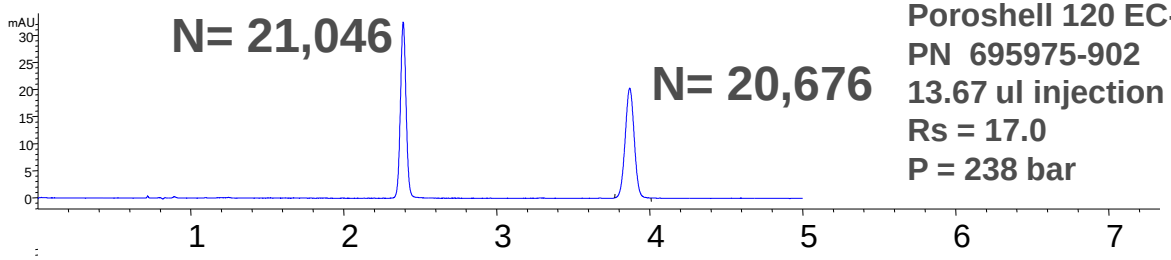
4.6 x 150 mm (L1)
Eclipse Plus C18, 5um (TPP)
PN 959993-902
20 ul injection
Rs = 14.9

Common Conditions:
Mobile Phase: 50:49:1 MeCN:H2O Acetic Acid
Flow Rate: 1.2 mL/min
Peak 1. Naproxen 2. Butyrophenone



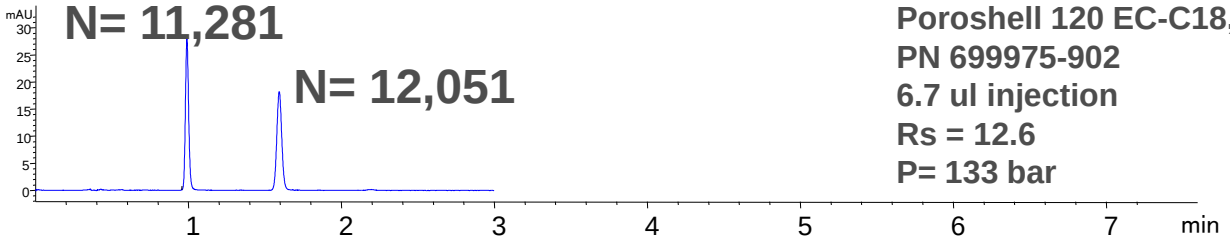
4.6 x 100 mm (L1)
Poroshell 120 EC-C18, 2.7 um
PN 695975-902
13.67 ul injection
Rs = 17.0
P = 238 bar

2X Faster

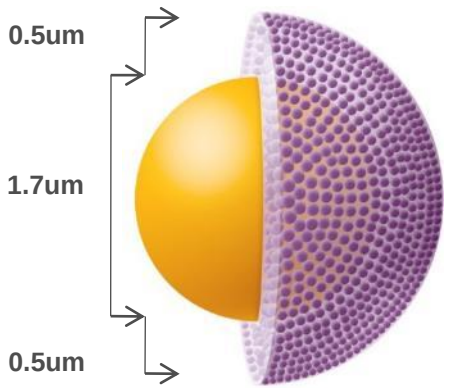


4.6 x 50 mm (L1)
Poroshell 120 EC-C18, 2.7 um
PN 699975-902
6.7 ul injection
Rs = 12.6
P= 133 bar

4.5X Faster

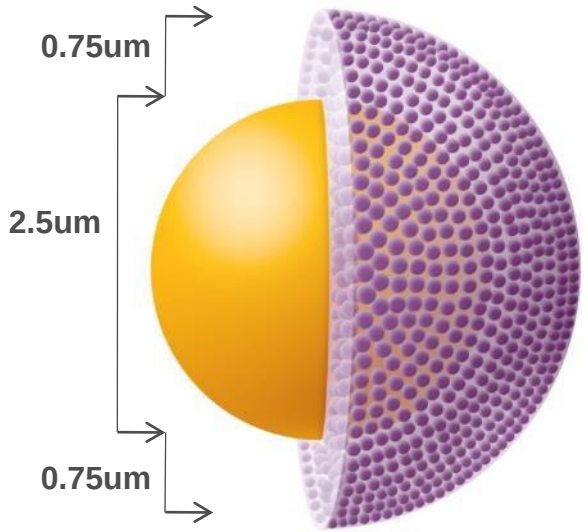


Larger Diameter SPP



Poroshell 120 2.7 µm

SA = 120 m^2/g
Pore size = 120-140Å

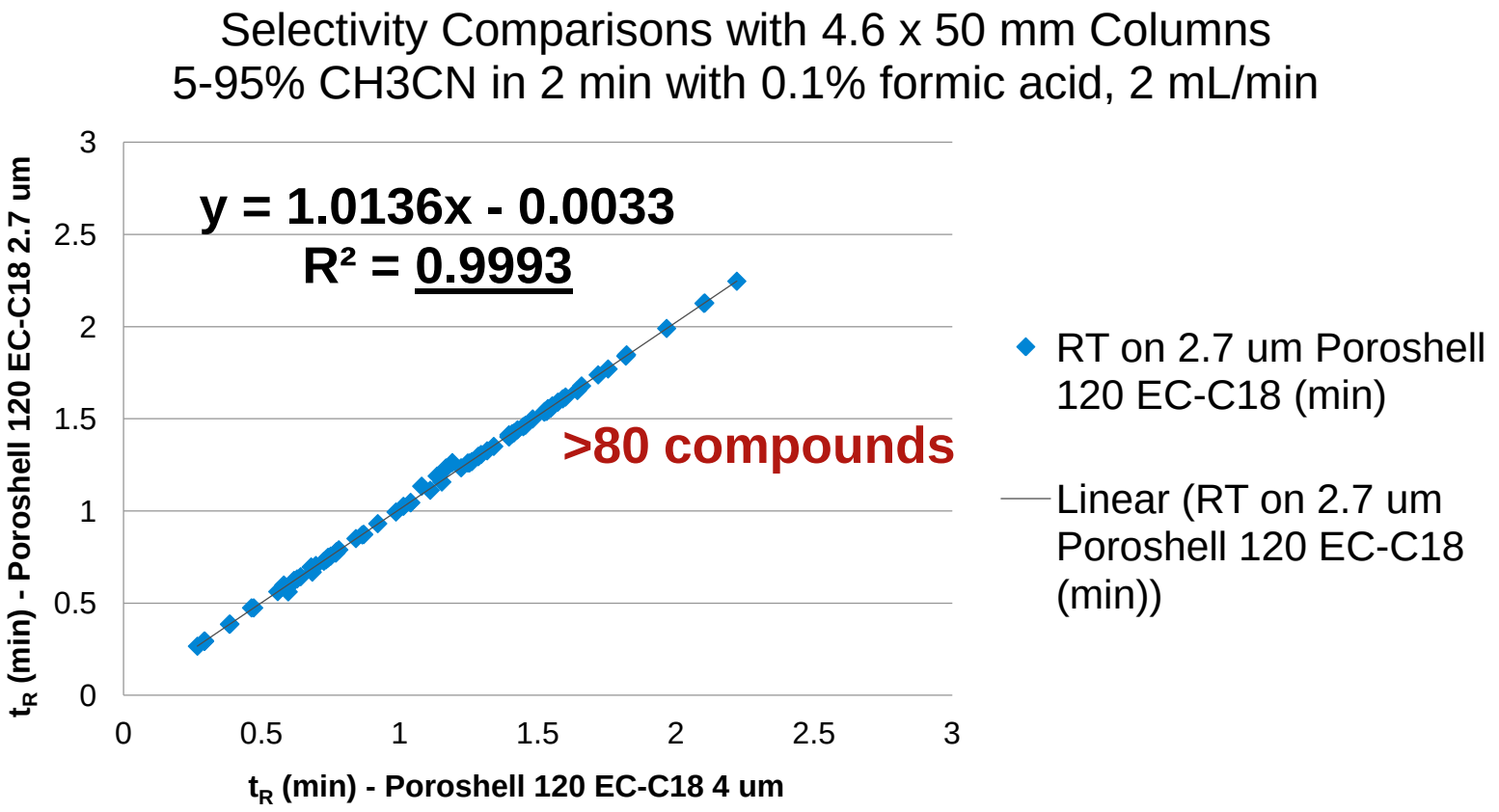


Poroshell 120 4 µm

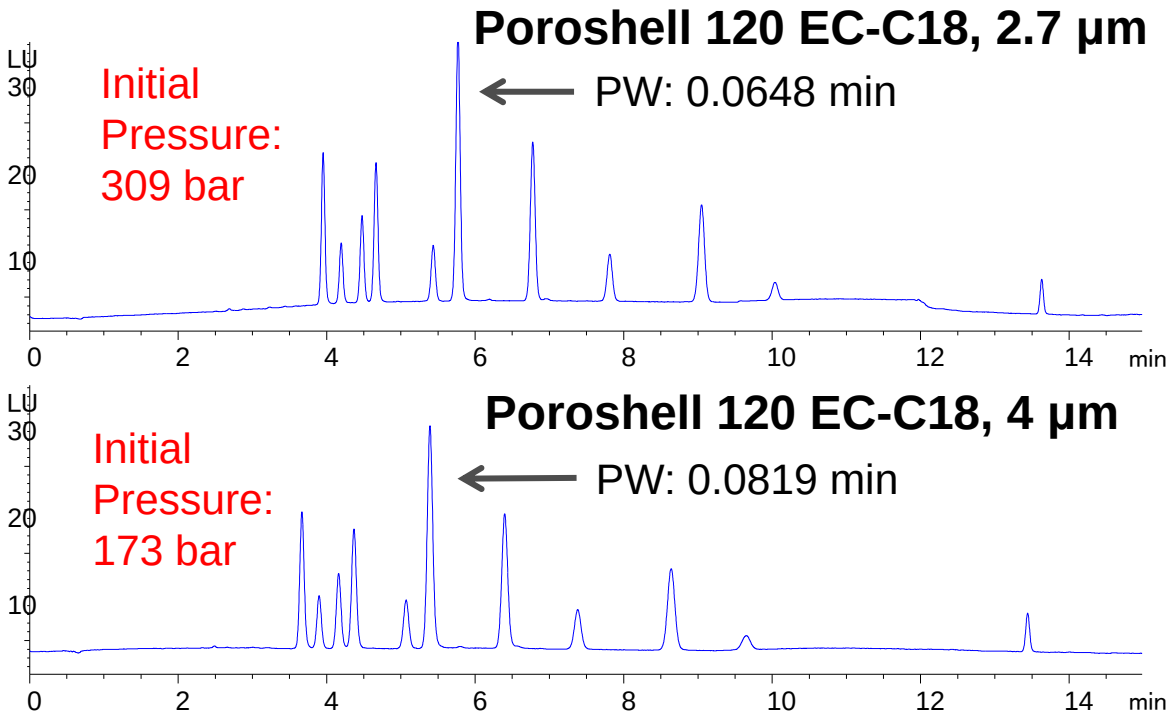
SA = 120 m^2/g
Pore size = 120-140Å

- Offers nearly 2X the performance of traditional 5 µm columns with the easy drop-in replacement for current methods

2.7 and 4 µm Poroshell 120 Have Similar Selectivity for Easy Method Transfer and Scaling



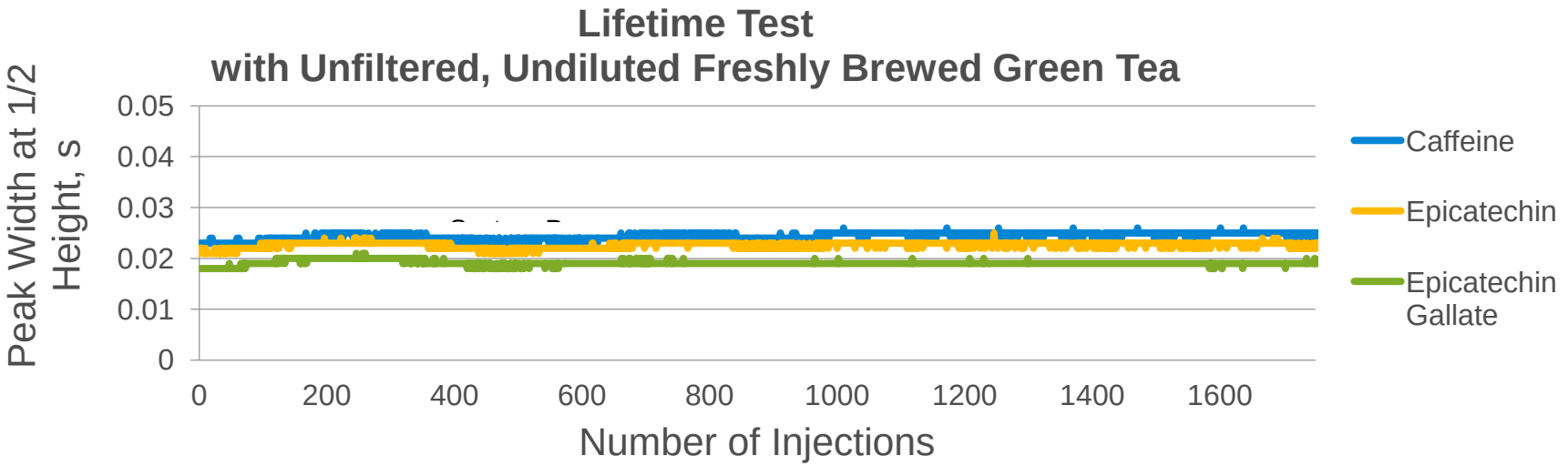
Comparison between Poroshell 120 2.7 µm and 4 µm for 4-quinolones in Milk

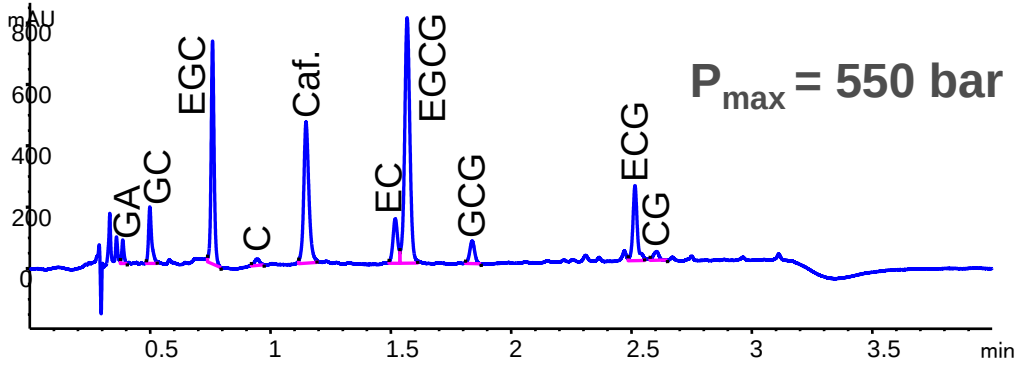


Norfloxacin
Ofloxacin
Ciprofloxacin
Pefloxacin
Lomefloxacin
Danofloxain
Enrofloxacin
Sarafloxacin
Difloxacin
Oxilinic acid
Flumequine

- $PW_{1/2}$ with 4 µm column increased by 30% compared to 2.7 µm
- Pressure on 4 µm decreased by 45% compare to 2.7 µm column. It is more suitable to use on a < 400 bar LC, while 2.7 µm column is suitable for 600 bar LC.

Long Lifetime with Poroshell 120 2.7 µm Column >1800 Injections at 550 bar - No Performance Change





P_{max} = 550 bar

A: 0.2% HCOOH in H_2O, B: 0.2% HCOOH in CH_3CN

0.833 mL/min

Time	0.00	1.25	2.50
%B	10	15	27

40 °C

Agilent Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm

Sig=210,4nm, Ref=Off

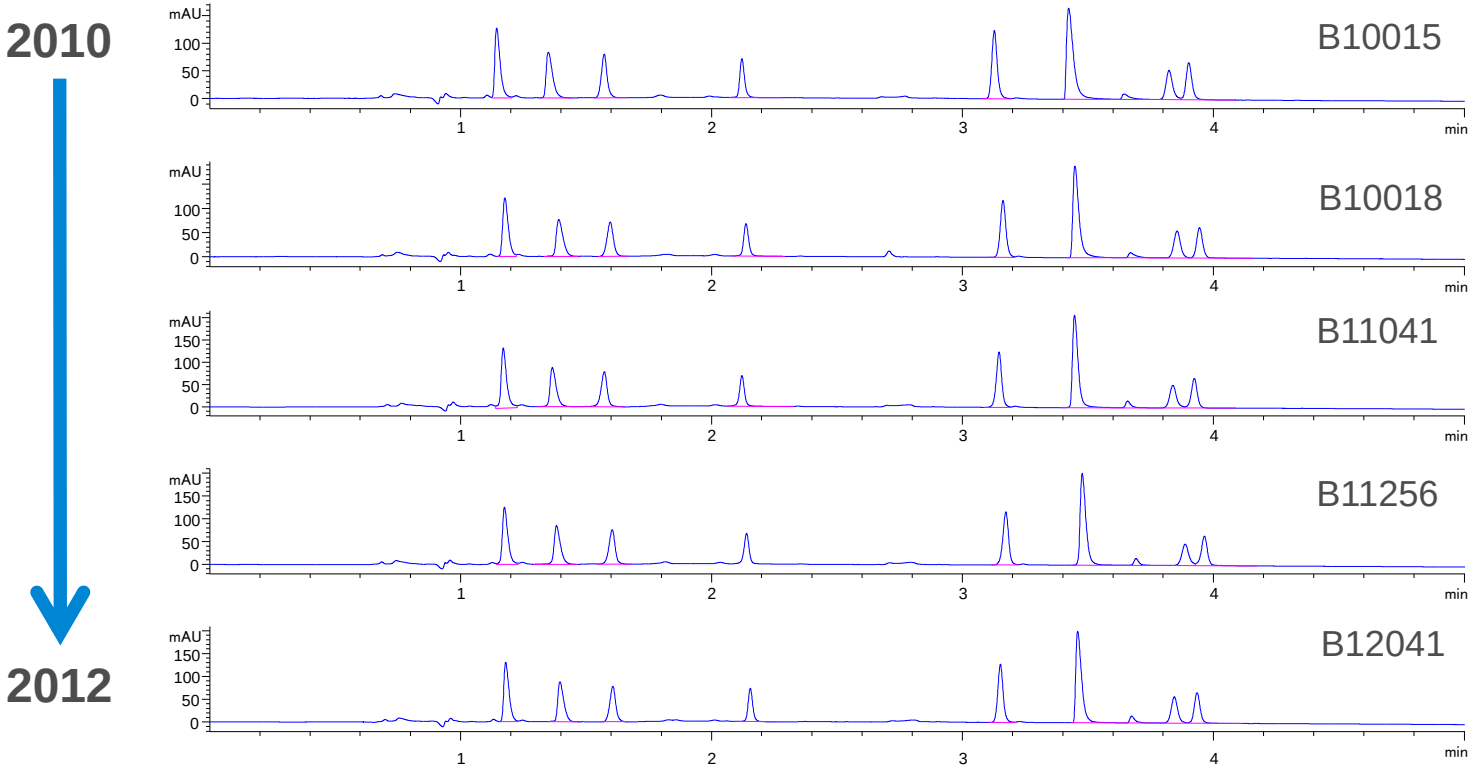
2-µL, 3-mm micro flow cell (PN G1315-60024)

Sample: 2 µL of freshly brewed green tea

(brewed from a commercial tea bag in 6 oz of initially boiling water for six minutes)

Other Considerations when Selecting a Column

- **Robustness and batch-to-batch reproducibility**



METHOD DEVELOPMENT CHALLENGES AND OBJECTIVES

Challenges In Method Development

- Worldwide method transfers
  - Instruments and configurations differ from lab to lab
  - More contract labs
- Many chromatographic mode choices
  - RP, SEC, IEX, HILIC, Chiral…
  - RP most common
- Too many columns to choose from
  - Endcapped C18 is a good starting point

Defining the Objective

- How complex is the sample?
- Is high efficiency important?
- Is speed important?
- What are the instrument limitations?
- What is the skillset of the operator?

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

Method Performance Criteria

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

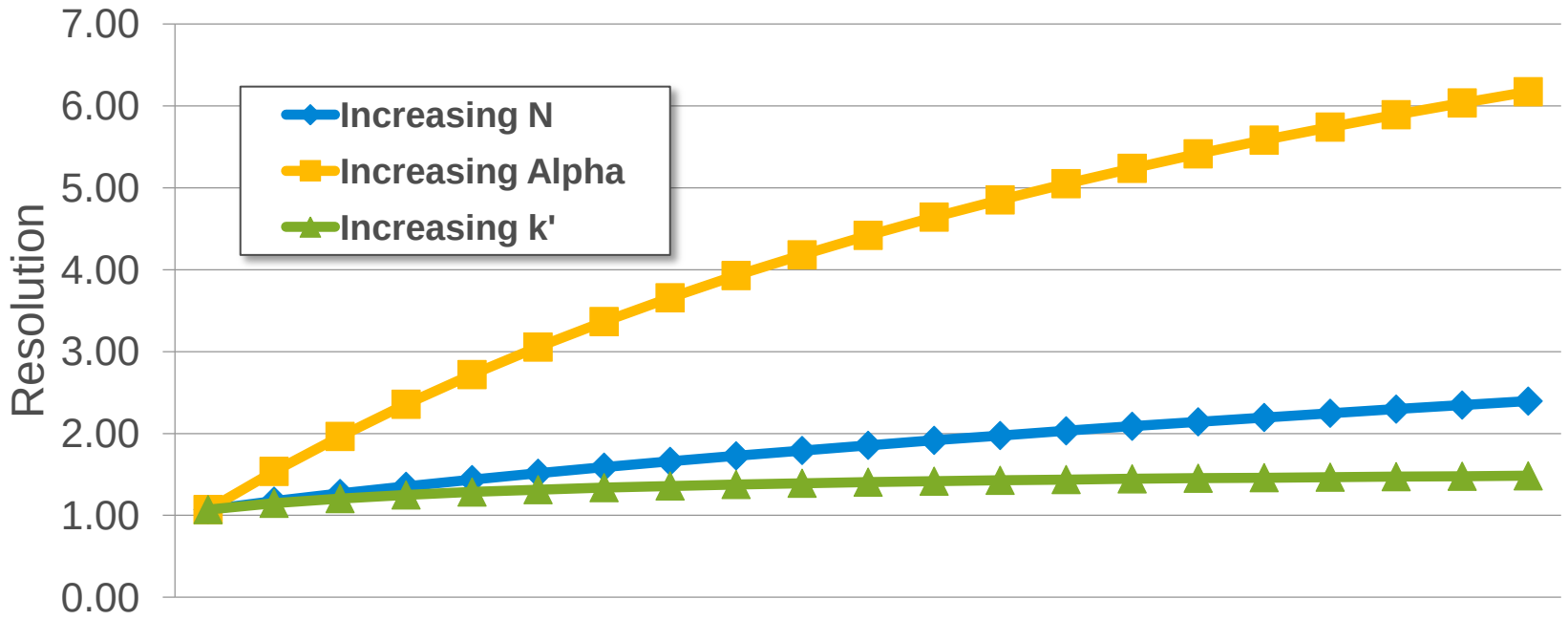
AVOID THESE for System Suitability Criteria:

Column efficiency (theoretical plates) & Absolute retention time

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

Where to Begin?

$$R_s = N^{1/2}/4 \bullet (\alpha-1)/\alpha \bullet k'/(k'+1)$$



Plates:	5000	10000	15000	20000	25000
Alpha:	1.10	1.35	1.60	1.85	2.1
k	2.0	4.5	7.0	9.5	12.0

Selectivity Impacts Resolution Most

- Change bonded phase
- Change mobile phase

} Typical Method Development Parameters

Plates are easiest to increase

METHOD DEVELOPMENT WITH SELECTIVITY – BONDED PHASE

Why is Changing the Bonded Phase Effective?

- 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
- Having numerous different bonded phases available on the same particle makes development easier

  - Fast SPP methods make development faster

Poroshell 120 Column Chemistries

Multiple bonded phases for flexibility in method development

Poroshell 120 EC-C18 and C8

- Robust endcapped C18 for best peak shape at pH 2-9

Poroshell 120 StableBond C18 and C8

- Robust chemistries for pH<2

Poroshell HPH-C18 and HPH-C8

- Long lifetime at high pH

Poroshell 120 Phenyl-Hexyl

- Excellent choice for pi-pi interactions
- Selectivity similar to phenyl, diphenyl, or other phenyl-hexyl columns

Poroshell 120 SB-Aq

- Proprietary bonding phase is an excellent choice for polar analytes

Poroshell 120 Bonus-RP

- Embedded polar group provides unique selectivity for polar compounds

Poroshell 120 EC-CN

- Flexible endcapped CN chemistry with Normal and Reversed Phase character

Poroshell 120 HILIC

- Bare silica HILIC for use in hydrophilic interaction chromatography of polar molecules

Poroshell 120 PFP

- Perfluorophenyl chemistry

HSM a Way to Look at Column Orthogonality

Poroshell 120	H	S*	A	B	C	k'	F
EC-C18	1.020	0.008	-0.130	-0.004	0.161	6.920	0
EC-C8	0.877	0.011	-0.232	0.023	0.127	4.840	6
EC-CN	0.421	-0.057	-0.476	0.002	0.045	0.950	17
Phenyl-Hexyl	0.752	-0.083	-0.394	0.018	0.136	3.590	13
Bonus RP	0.686	-0.030	-0.573	0.180	-0.670	3.980	75
SB-C18	0.956	-0.041	0.168	0.025	0.210	5.440	12
SB-C8	0.726	-0.087	0.068	0.044	0.087	3.560	15
SB-Aq	0.581	-0.120	-0.133	0.051	-0.014	2.150	22
PFP	0.630	-0.520	-0.520	0.430	-0.110	2.300	85

Data provide by Dwight Stoll

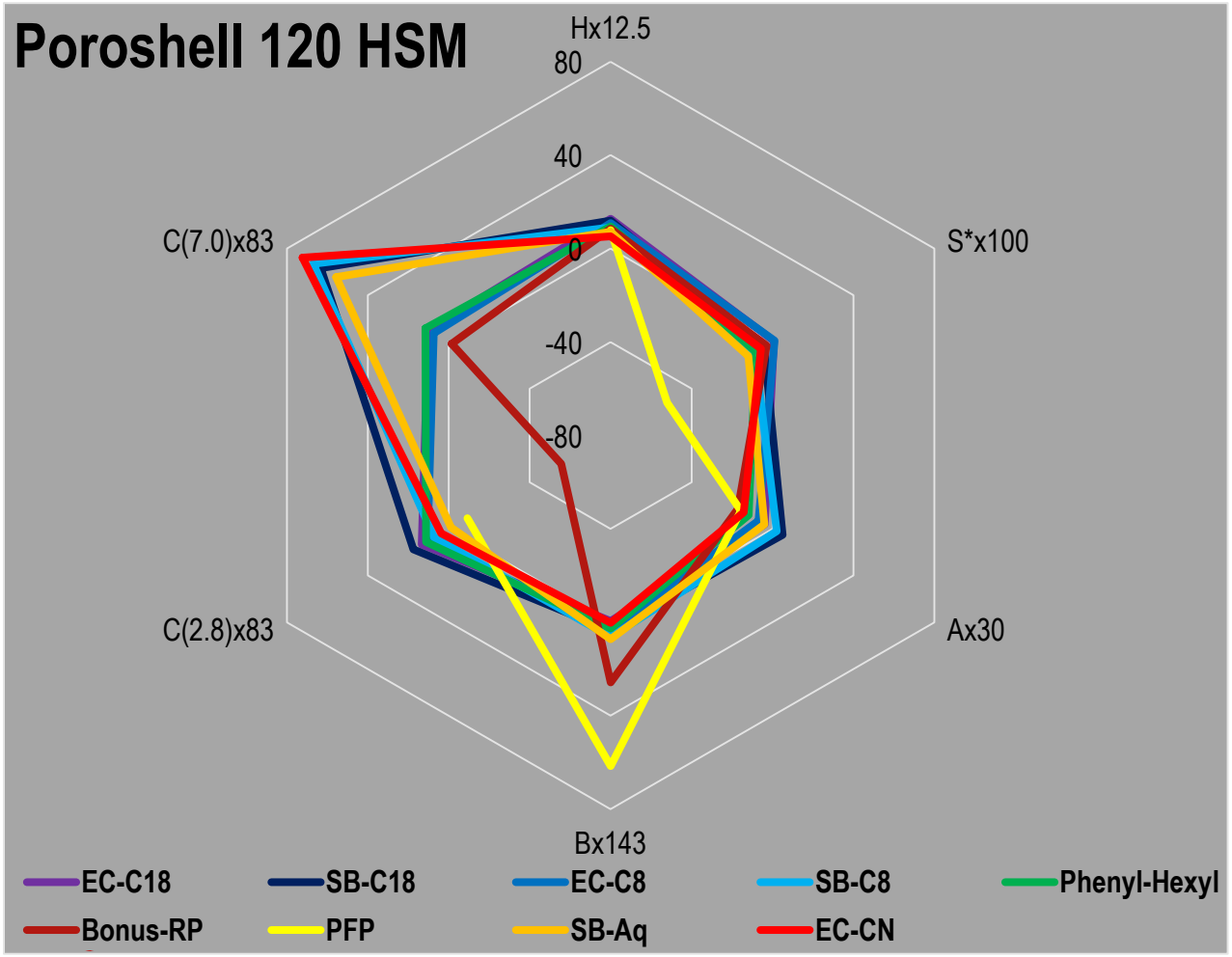
F_s factor describes the similarity of two columns. A small F_s indicates that two columns are very similar, while a large factor indicates that two columns are very different. Calculated according to the following equation:

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{\frac{1}{2}}$$

Further details at:

http://www.hplccolumns.org

HSM Data for Poroshell 120



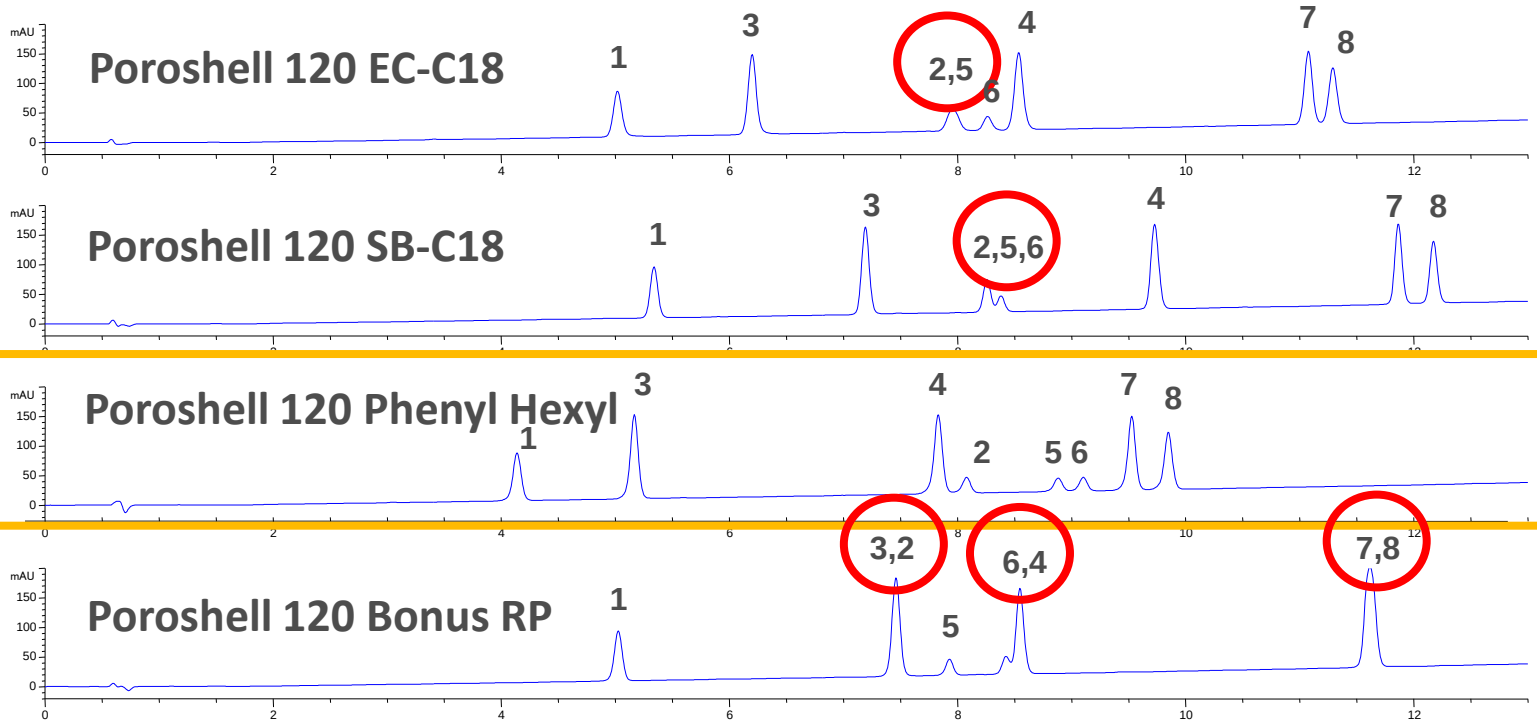
Poroshell 120	F
EC-C18	0
EC-C8	6
EC-CN	17
Phenyl-Hexyl	13
Bonus RP	75
SB-C18	12
SB-C8	15
SB-Aq	22
PFP	85

HSM	
Hydrophobicity	H
Steric interaction	S*
Hydrogen-bond acidity	A
Hydrogen-bond basicity	B
Ion-exchange capacity	C
K ethyl benzene	EB

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{\frac{1}{2}}$$

Separation of 8 Steroids with Methanol Gradient

Best Resolution of all analytes with Poroshell 120 Phenyl-Hexyl

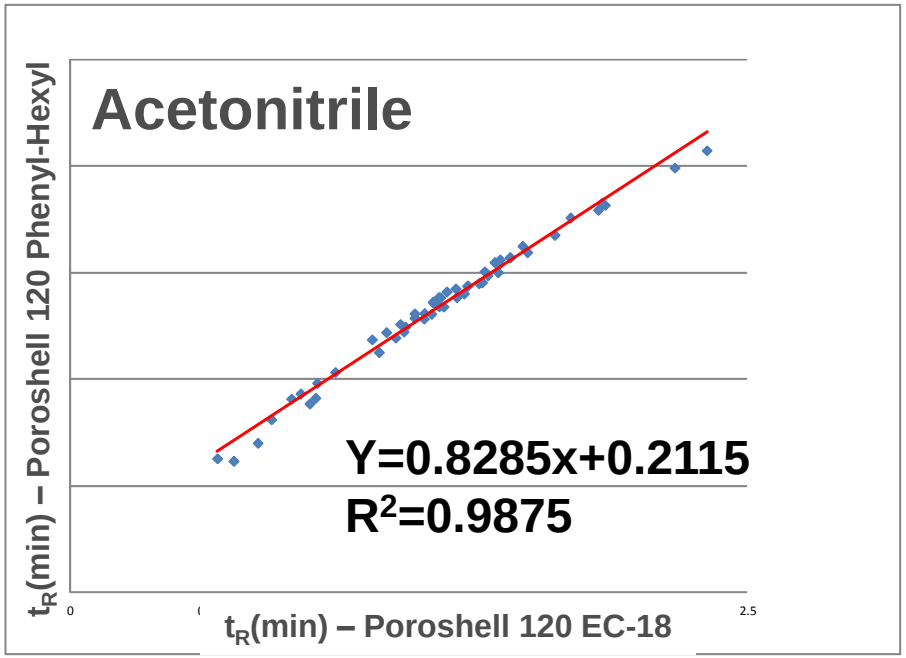


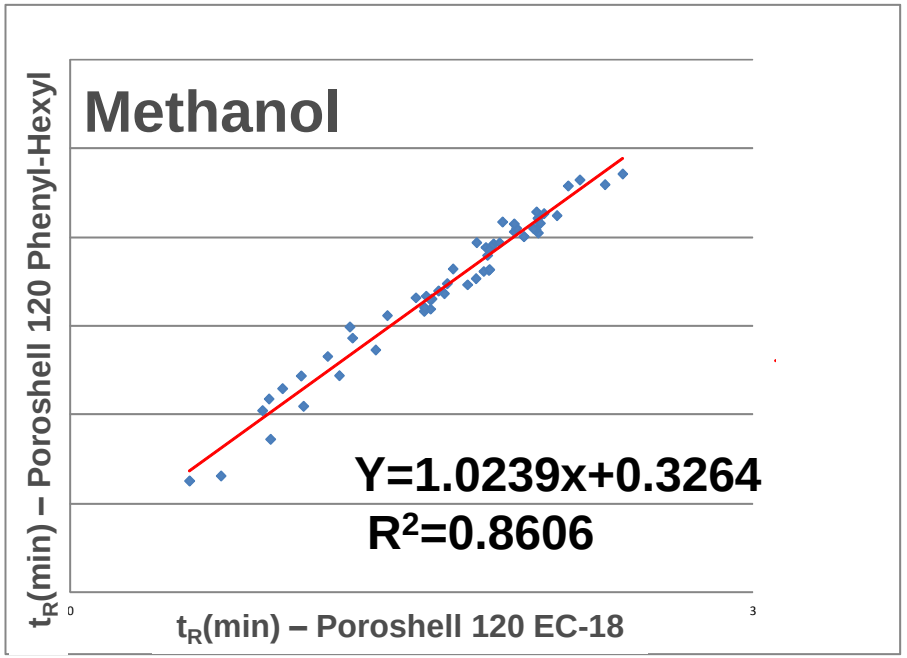
1.Hydrocortisone, 2.B Estradiole, 3. Andostadiene 3,17 dione, 4. Testosterone, 5. Ethyestradione, 6. Estrone, 7. Norethindone acetate, 8. Progestreone

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

Poroshell 120 Phenyl-Hexyl vs Poroshell 120 EC-C18

Phenyl-Hexyl alternative selectivity to C18
- recommended for aromatics, especially with methanol
- compatible with highly aqueous mobile for polar compounds.

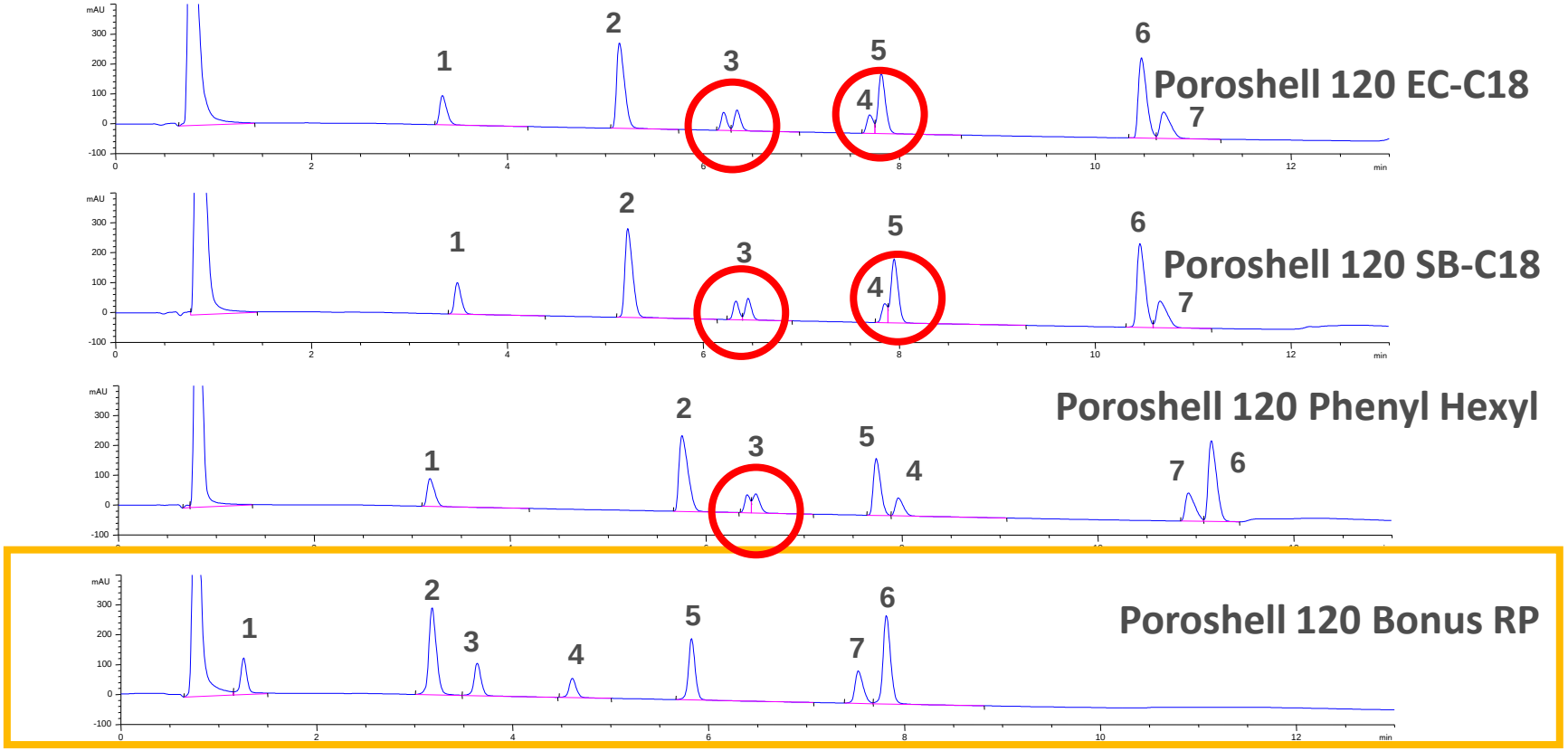




Buffer-Organic Gradients

Beta Blockers with Methanol Gradient

Best Resolution of all analytes with Poroshell Bonus-RP

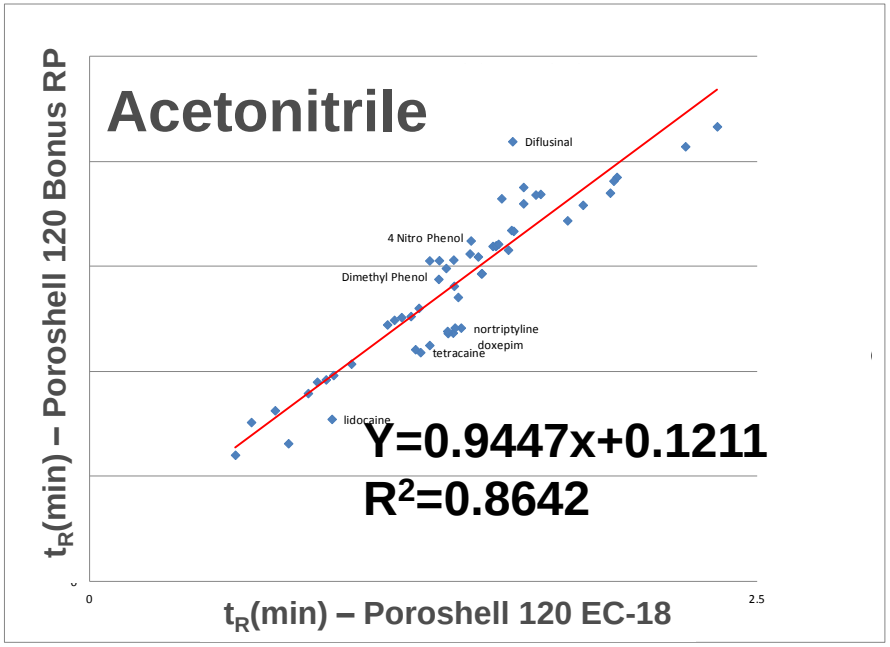


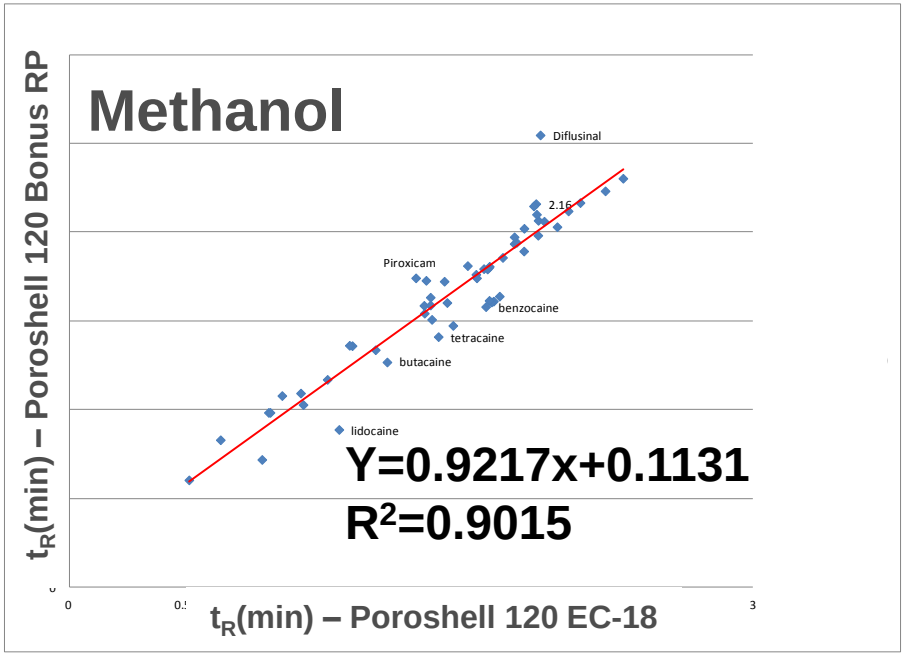
1.Atenolol, 2. Pindolol, 3. Naldolol, 4. Metoprolol, 5. Acebutolol, 6. Propranolol, 7. Alprenolol

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

Poroshell 120 Bonus-RP vs Poroshell 120 EC-C18

- Embedded polar group gives unique selectivity for polar compounds compared to C18.

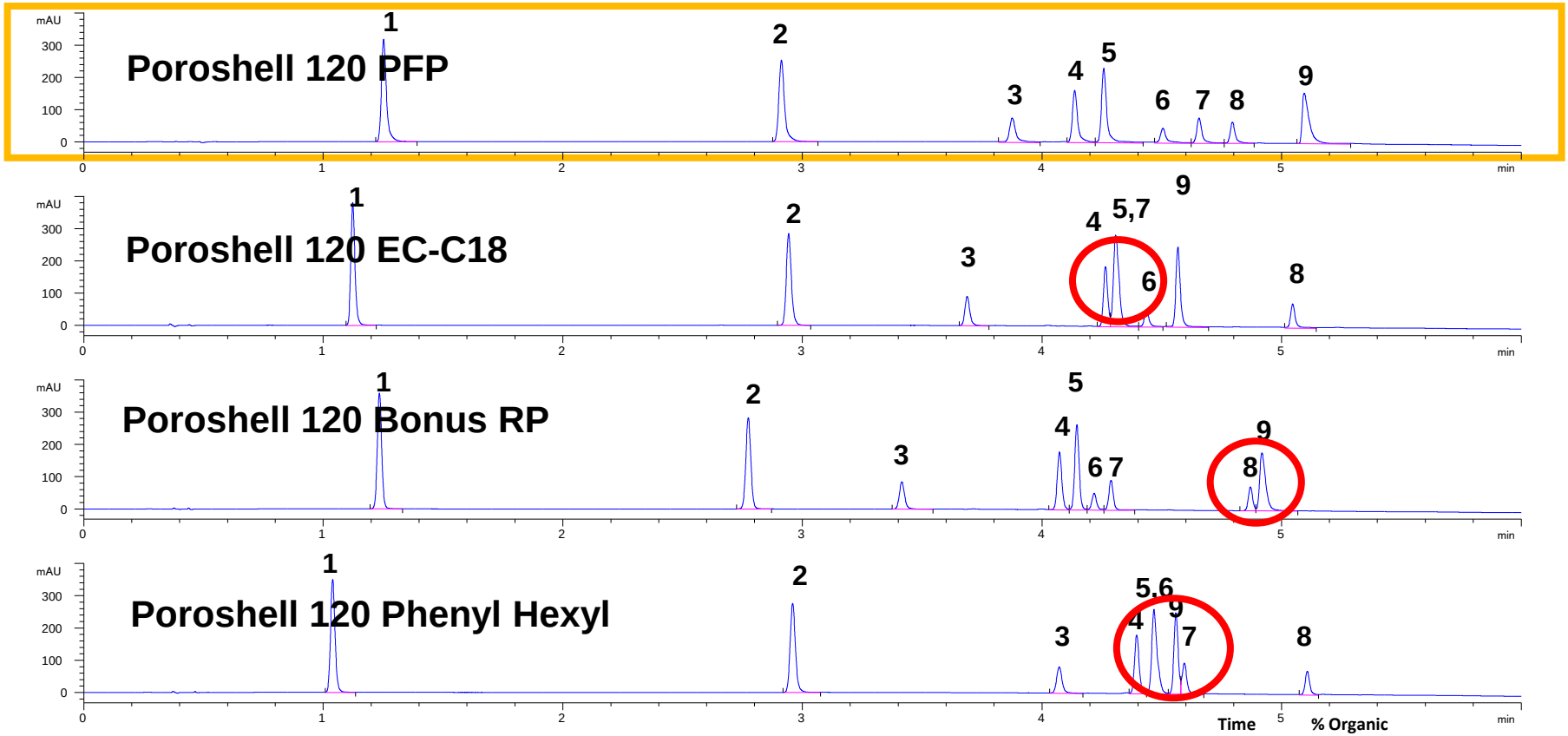




Buffer-Organic Gradients

NSAID Separation with a Methanol Gradient

Best Resolution of all analytes with Poroshell 120 PFP



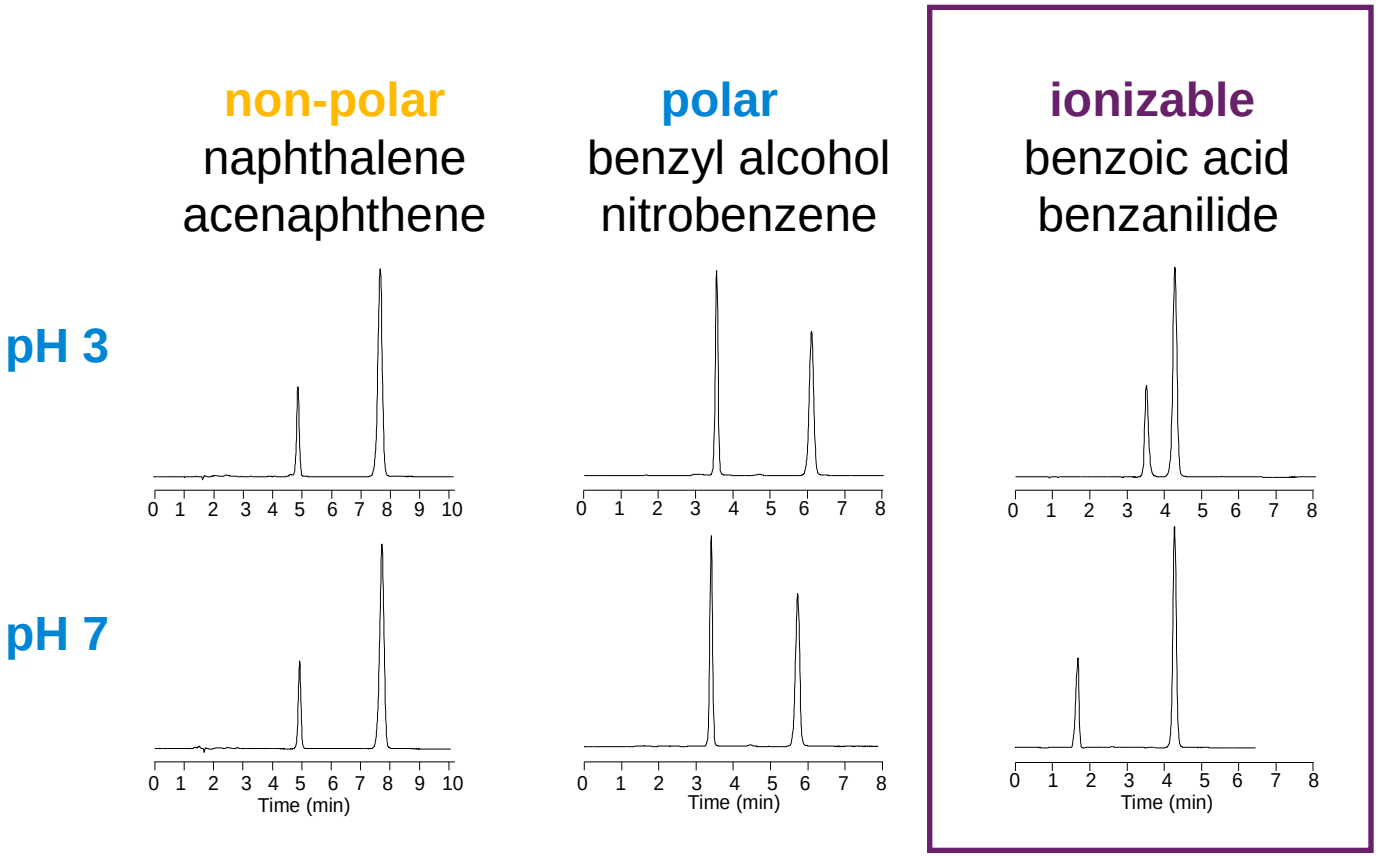
1. APAP, 2. Phenacetin, 3. Piroxicam, 4. Tolmetin, 5. Ketoprofen, 6. Naproxen, 7. Sulindac, 8. Diclofenac, 9. Diflunisal

Time	% Organic
0	8
6	100
7	100
8	8

2mL/min 254 nm

METHOD DEVELOPMENT WITH SELECTIVITY – MOBILE PHASE PH

When Does pH Affect Selectivity and Resolution? Compound Type Comparison

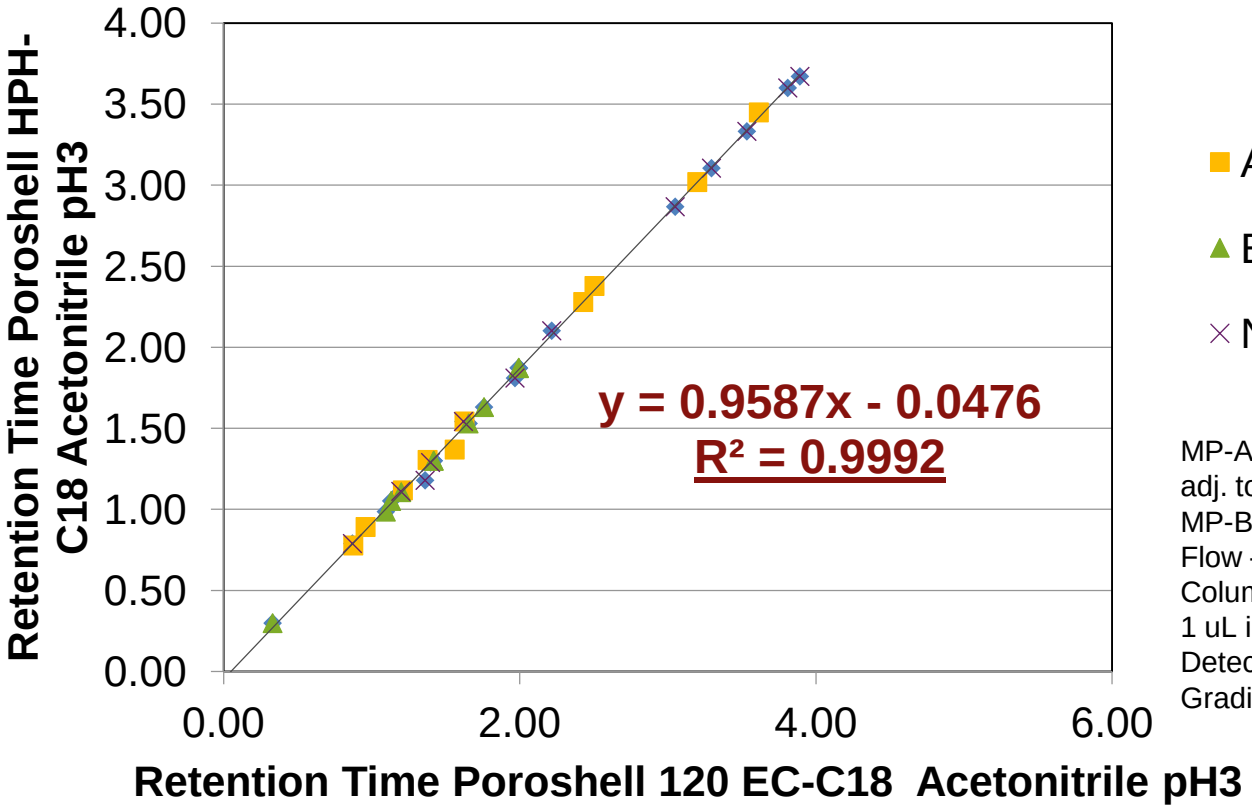


- **Ionizable compounds – acids and bases can change retention and selectivity most with changes in pH**

Change in Retention with pH for Ionizable Compounds is Key to Method Development

- Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)
- Silanols on silica ionize at mid-pH, increasing retention of basic analytes (i.e possible ion-exchange interactions)
- Choose mobile phase pH to optimize retention and selectivity during method development
- Ensure that your column is compatible with and stable in the mobile phase pH you select

Poroshell HPH-C18 vs Poroshell 120 EC-C18

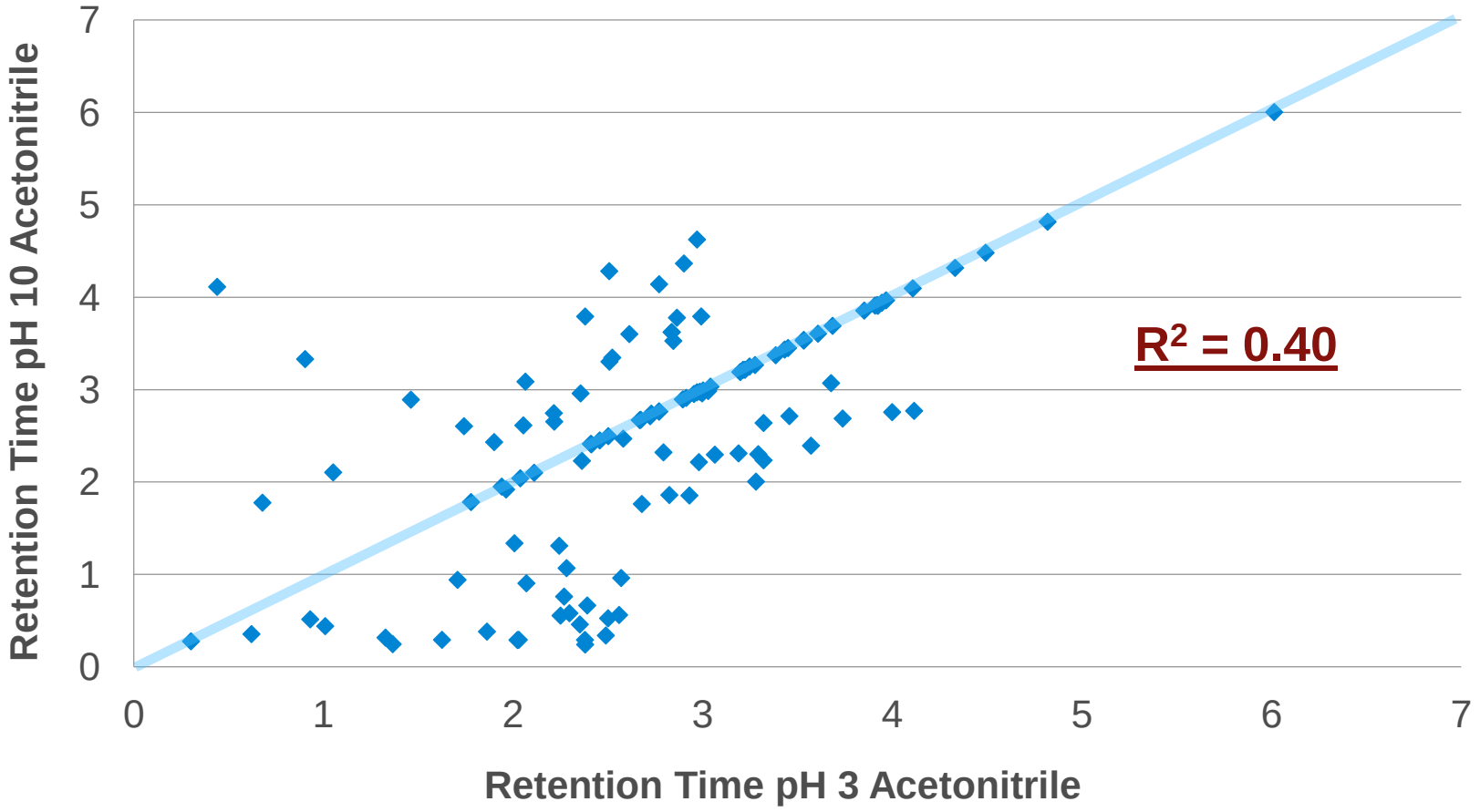


MP-A 10mM Ammonium Formate/water adj. to pH3 or 7 using Formic Acid
MP-B ACN or MeOH
Flow – 0.42 mL/min
Column Temp. Ambient
1 uL injection
Detection 254 nm

Gradient:

time, min	%B
0	5
4	95
5	95
6	5
7	stop run

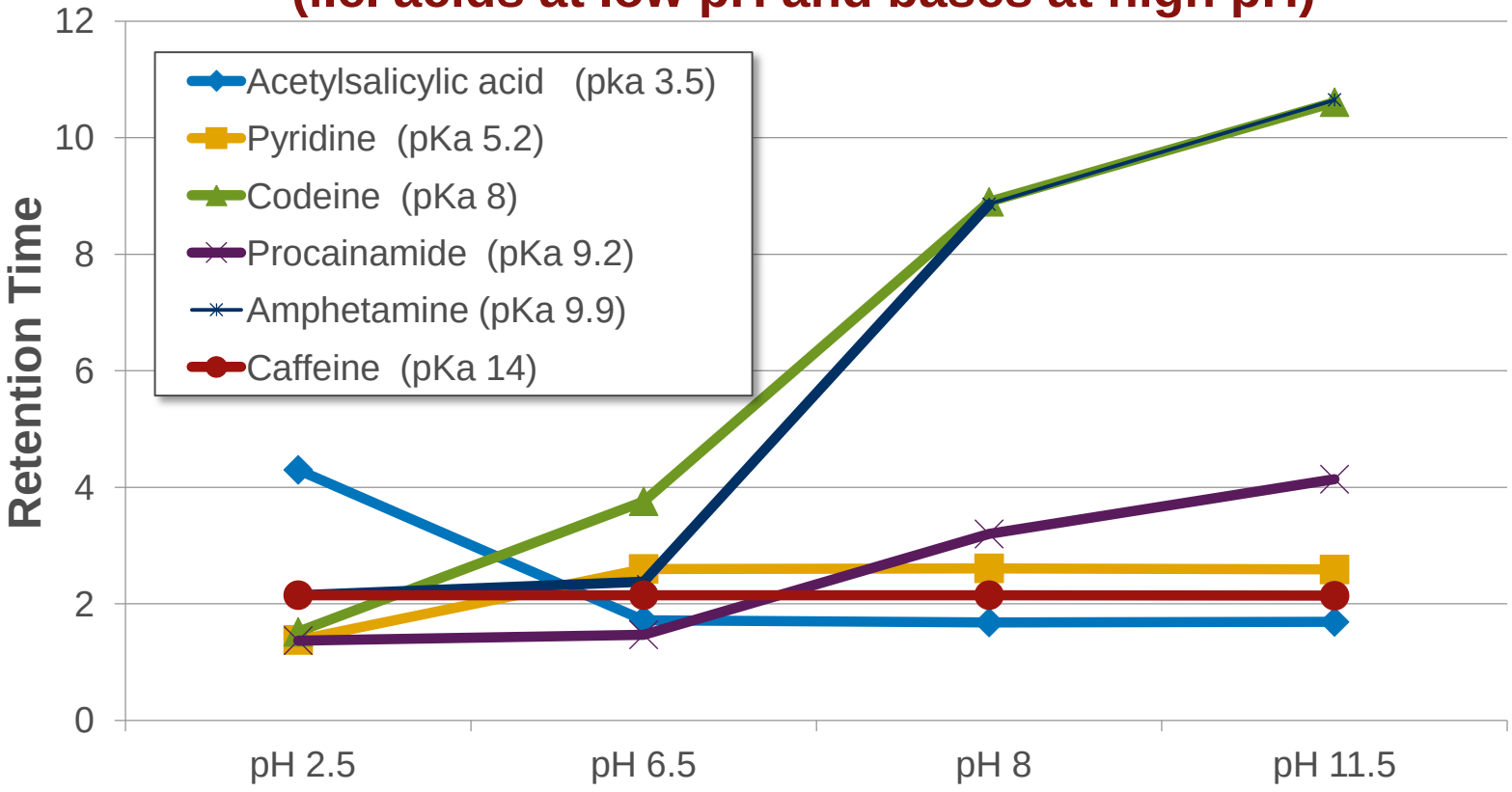
Use of Varied pH can Help Build Separations that are Very Different (poorly correlated)



Column: Poroshell HPH-C18 2.7 µm

Change in Retention with pH for Ionizable Compounds is Compound Dependent

More retention for non-charged analytes (i.e. acids at low pH and bases at high pH)



Column: Poroshell HPH-C18 2.7 µm

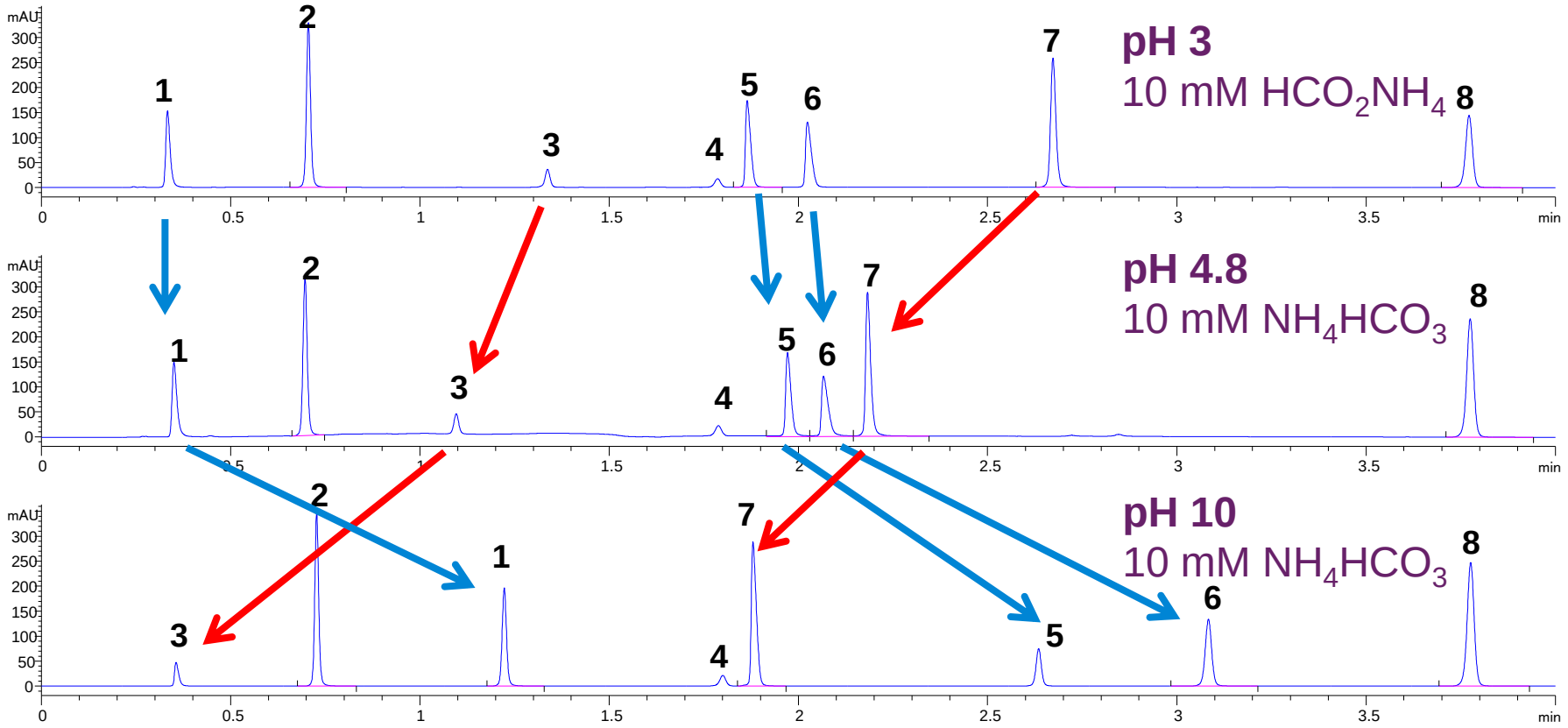
Mobile Phase: 45% Methanol, 55% 20 mM Phosphate Buffer

Selectivity Can be Controlled by Changing pH

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

Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm



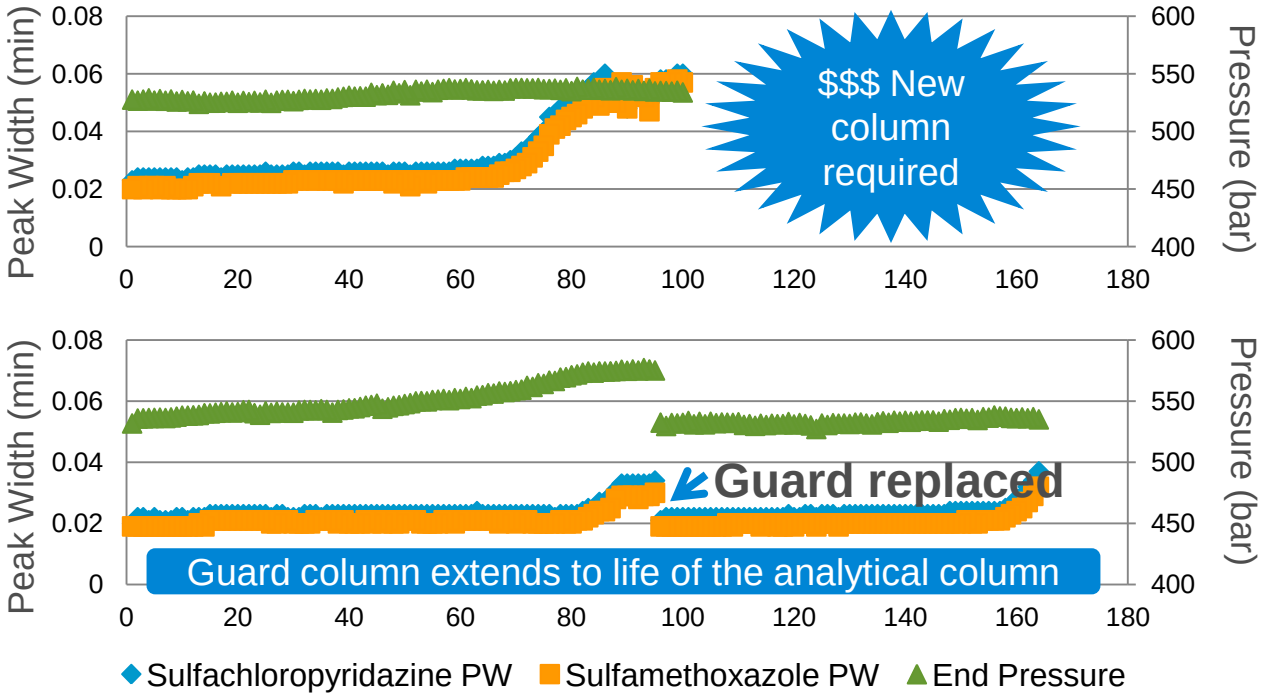
HOW TO ENSURE THE BEST PERFORMANCE FROM YOUR SPP COLUMN

Benefits of Installing a Guard Column

Accelerated Lifetime Test

Similac sample (milk substitute diluted 300:1) containing 2 sulfa drugs

Peak width change indicating column failure



No Guard

- Column failure @ inj. 70; new column required

With Guard

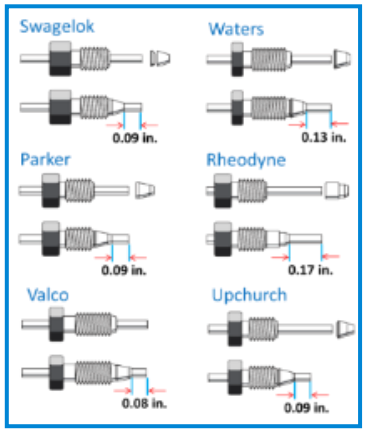
- Guard failure @ inj. 80; guard replaced
- Same column used throughout analysis

By installing a guard column when using dirtier samples, one can extend the life of their column, and utilize more inexpensive guard columns rather than analytical column replacements

Agilent A-Line Fittings

Importance of the Spring Loaded Feature

Most commonly used fittings in UHPLC are non-adjustable 2-piece or 3-piece metallic fittings. Since different manufacturers of column hardware have different design in column end fittings, as shown in Figure 1, a new set of tubing and fittings needs to be installed for every brand of column to guarantee that the stem length, namely the length between the bottom of the ferrule and the end of tubing, fits the column end fitting.



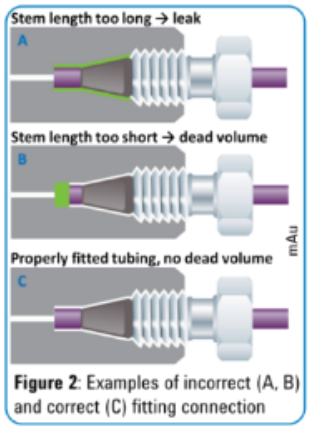


Figure 2: Examples of incorrect (A, B) and correct (C) fitting connection

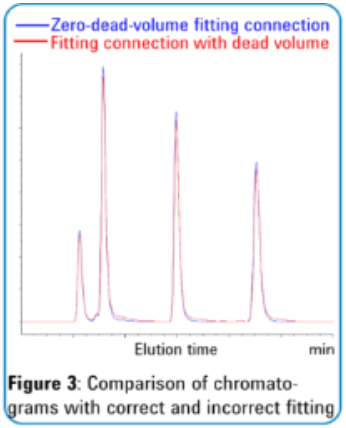
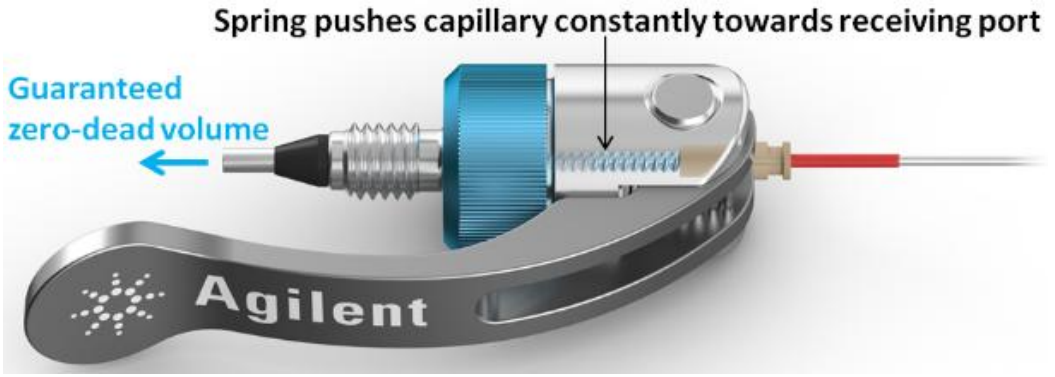


Figure 3: Comparison of chromatograms with correct and incorrect fitting

The spring-loaded design constantly pushes the tubing against the receiving port, delivering a reproducible connection with no dead volume for consistent chromatographic performance

Stem length is adjustable through the spring, which makes the fitting compatible with all types of LC columns.



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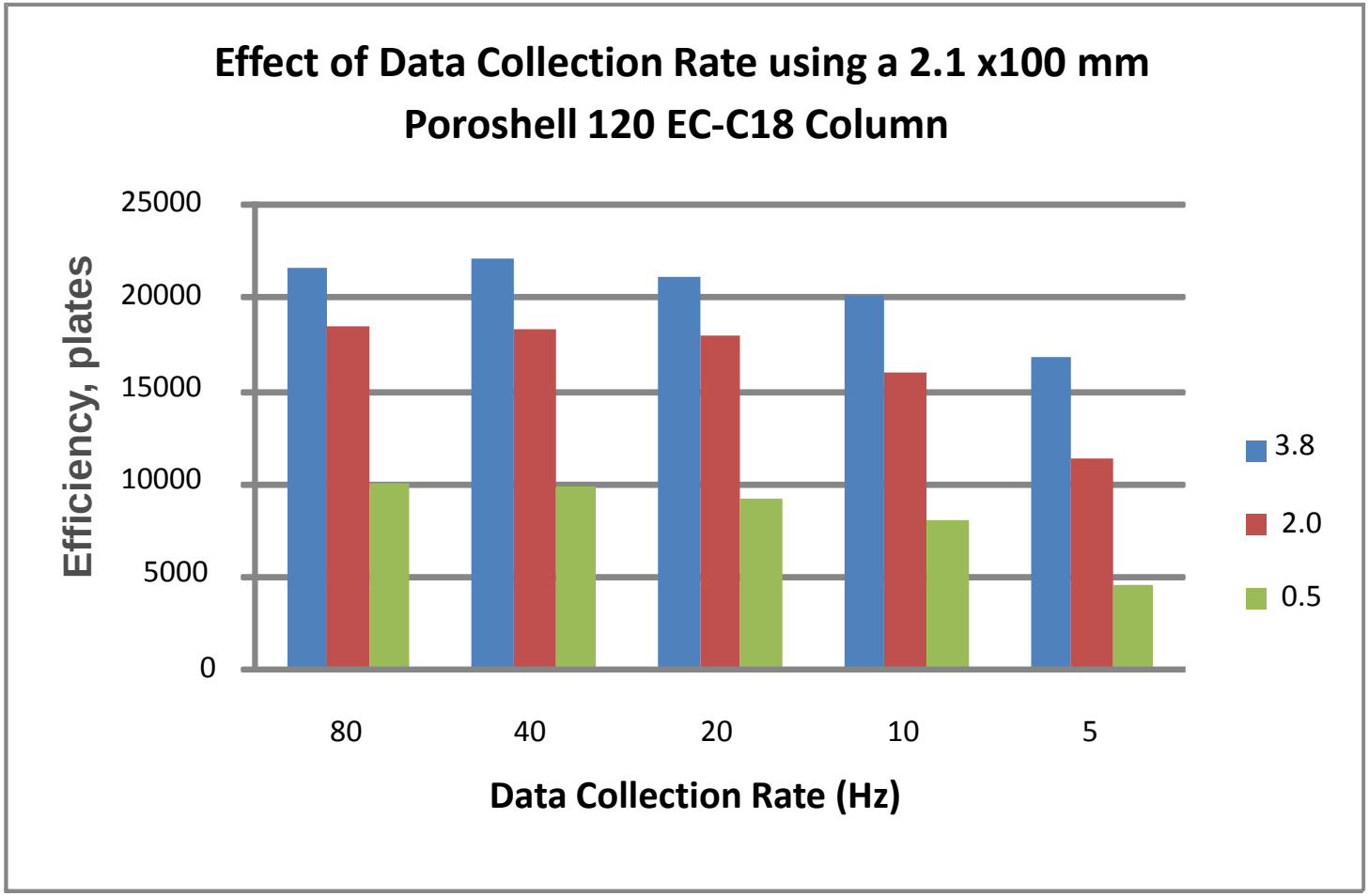
Agilent A-Line LC supplies

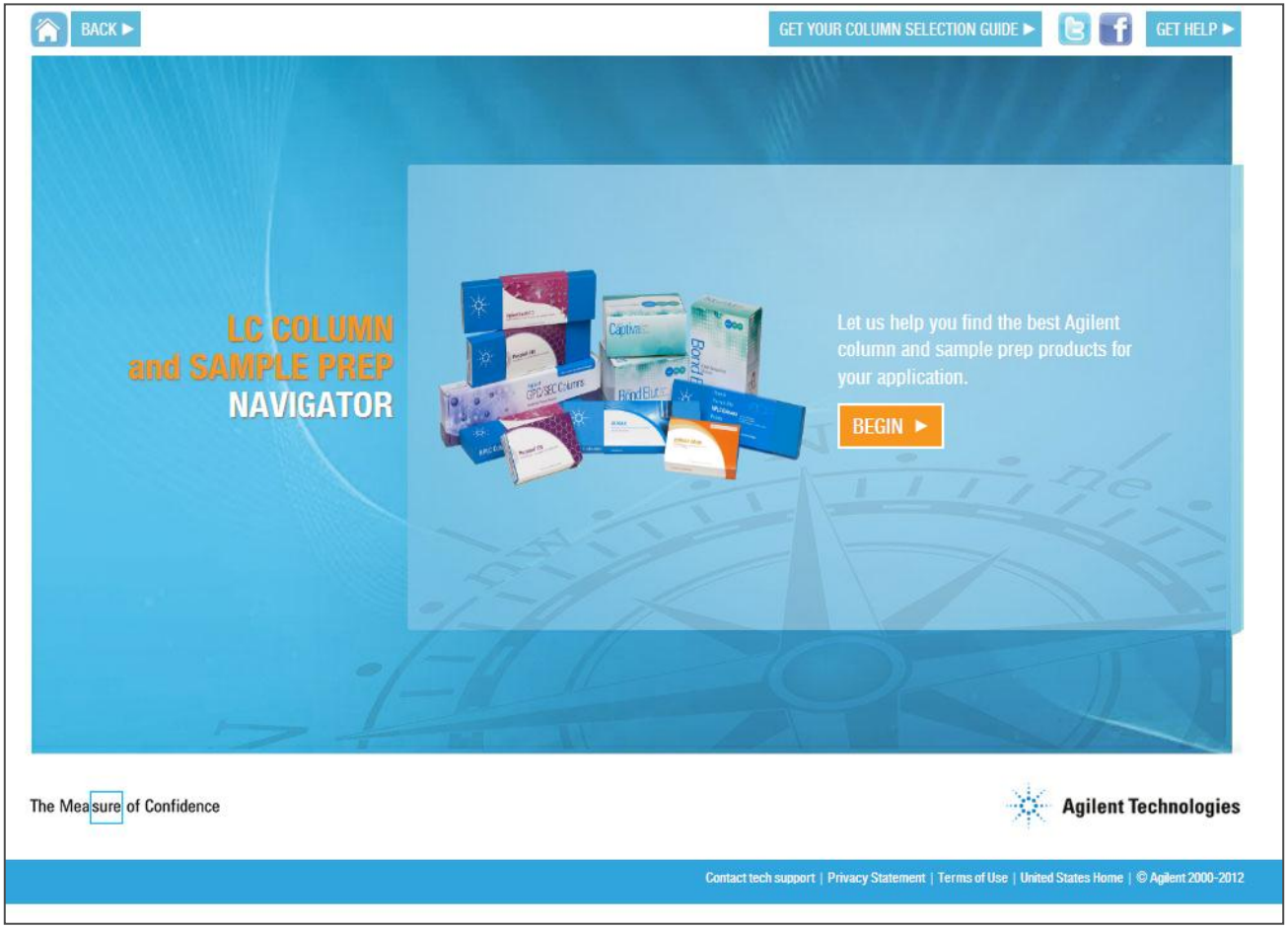
Data Collection Rate



1 ul QC Mix, Uracil, Phenol (k=0.5), 4-Chloronitrobenzene(k=2), Napthalene(k=3.8)
55% MeCN 45 % Water 0.55 ml/min micro flow cell

WHEN YOU NEED MORE HELP CHOOSING A COLUMN…

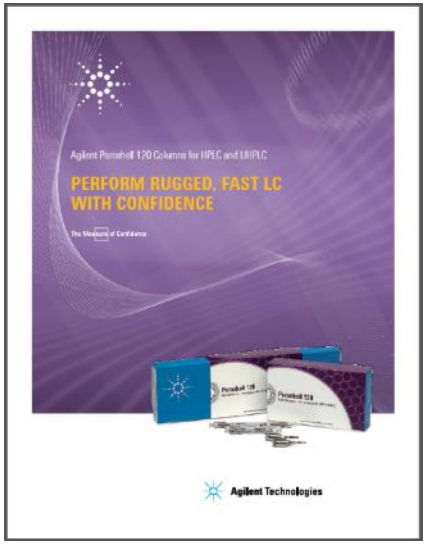
On-Line Tool "The Navigator"
A Column and Sample Prep Selection Tool



http://navigator.chem.agilent.com

Literature on Poroshell 120 Columns

- There are continuous updates and additions to Poroshell 120 Columns Literature.
- Brochures, app notes, flyers and other documents are updated and added often!



5990-5951EN

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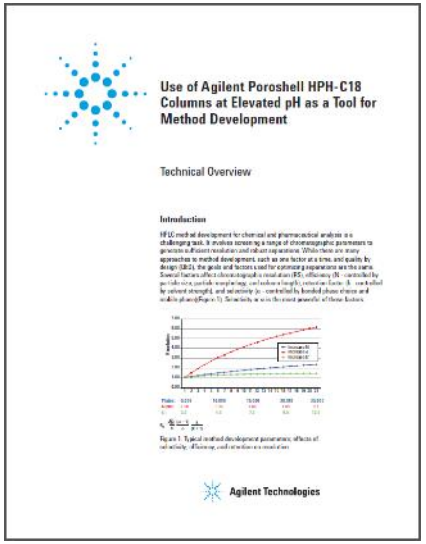
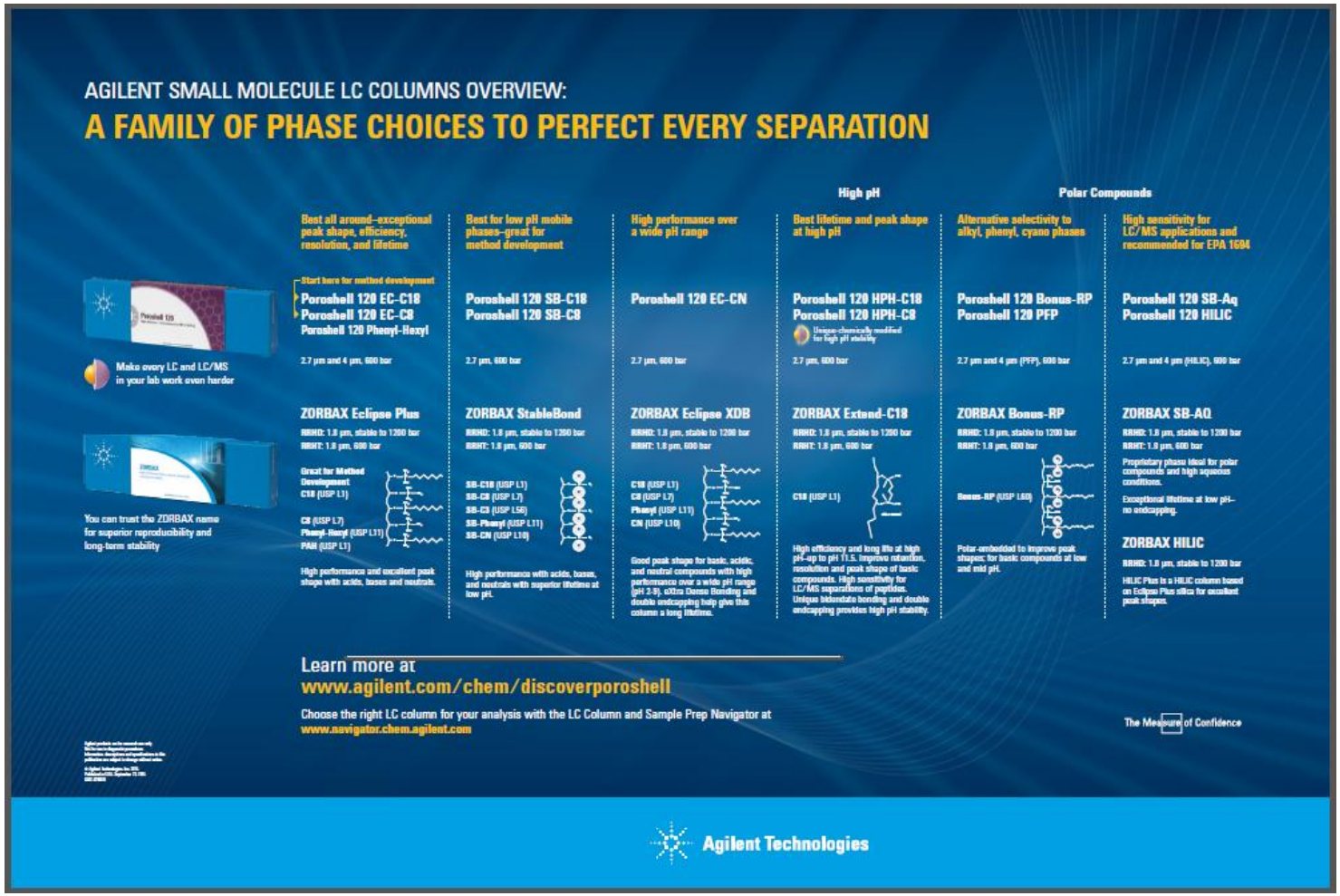


5991-4893EN

Phase Selection Wall Chart
5991-6240EN



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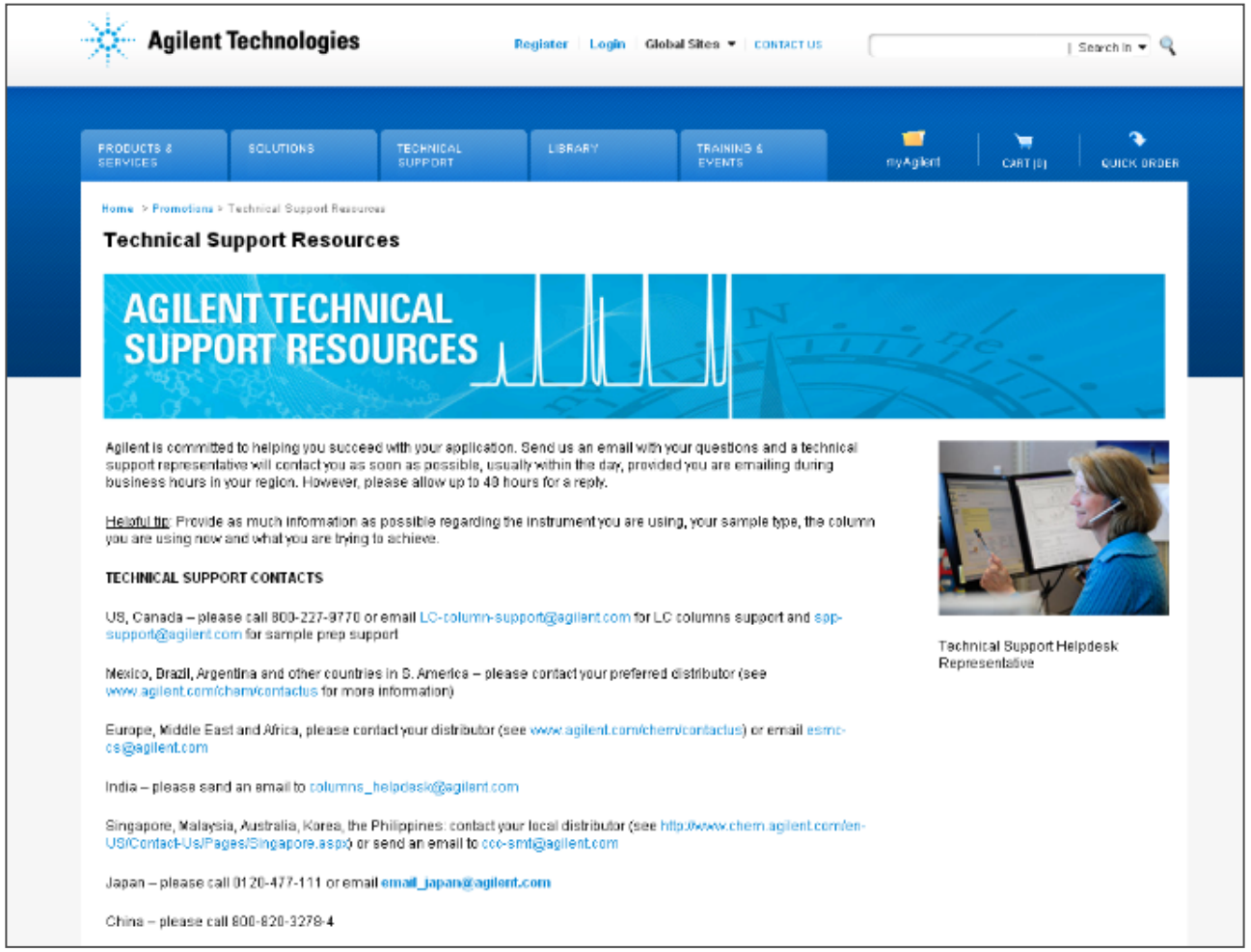
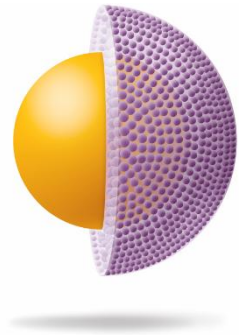
Visit www.agilent.com/chem/cstechsupport



Conclusions

- Resolution is a common goal during method development
- Selectivity is a main driver of resolution
- Superficially porous particle columns (e.g., Poroshell 120) offer…
  - 12 chemistries, including high pH stable options
  - Faster method development
  - Higher sample throughput
  - Maintain method ruggedness
  - Compatibility with any LC system



Thank You

Questions?