

In Search of Orthogonal Selectivity: Maximizing Peak Capacity in Reversed-phase Chromatography

Pamela Iraneta, Bonnie Alden, Claude Mallet, Jonathan
Turner, Scott McCall, Chris Hudalla, and Martin Gilar

Waters Corporation, 34 Maple Street, Milford, MA,
01757-3696, USA

- Goal of the study
 - Reversed-phase stationary phases that exhibit orthogonal selectivity in pH 3 mobile phases
 - Intended for use in 2-D RPxRP separations of peptides
 - Used gradient elution in the study
 - Necessary for the rapid analysis of complex samples
 - Provide higher peak capacity than isocratic separations
 - Used a diverse set of small molecules to predict orthogonality for peptides
- Review equations for peak capacities in 2-D chromatography
- Assessing orthogonal selectivity
 - Selectivity tools
 - Small molecule examples
- Maximizing peak capacity for gradient optimization
 - Peptide examples

Maximizing 2-D Peak Capacity

$$2\text{-D Peak Capacity} = P_{c1\text{st-D}} P_{c2\text{nd-D}}$$

Peak Capacity in 1st or 2nd D

$$P_c = 1 + \frac{\sqrt{N}}{4} \frac{S\Delta\varphi * t_G}{t_G + S\Delta\varphi * t_0}$$

**Assumes
Orthogonal Selectivity**

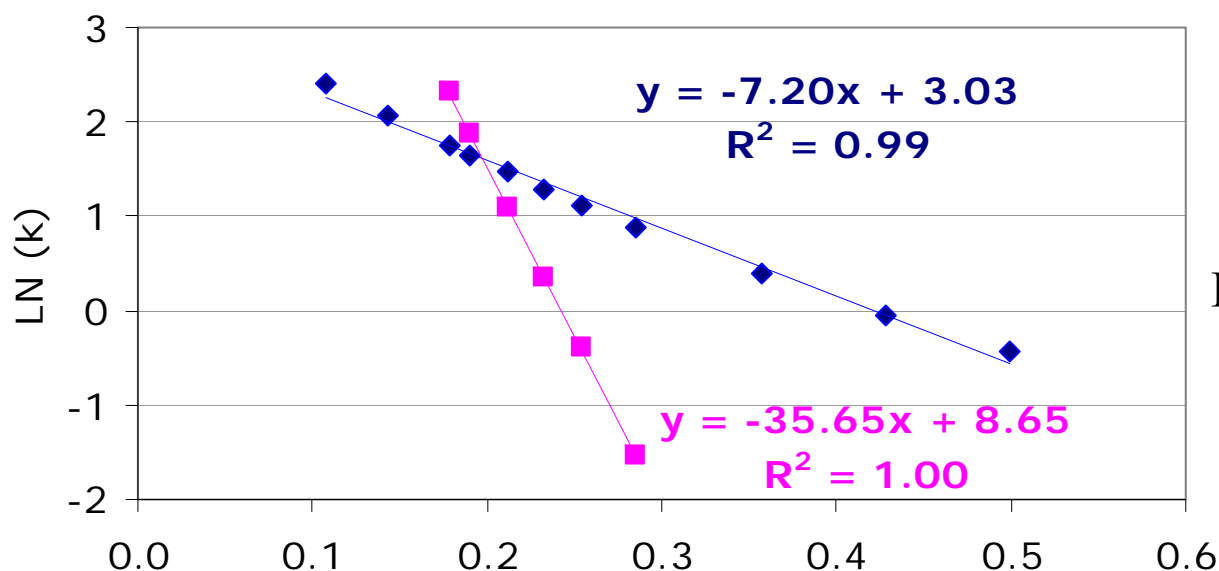
- N = column efficiency
- S = slope of ln k vs. fraction of organic solvent (LSS)
- $\Delta\varphi$ = change in organic during gradient
- t_G = gradient time (min)
- t_0 = retention time for an unretained peak (min)

$$P_c = 1 + \frac{\sqrt{N}}{4 \left(1 + S\Delta\varphi * \frac{t_0}{t_G} \right)} \ln \frac{\left(S\Delta\varphi * \frac{t_0}{t_G} + 1 \right) e^{S\Delta\varphi} - 1}{S\Delta\varphi * \frac{t_0}{t_G}}$$

S Terms for Angiotensin II and Acetophenone

$$P = 1 + \frac{\sqrt{N}}{4} \left(\frac{1}{t_0 \Delta \phi \frac{S}{t_G} + 1} \right) \ln \left(\frac{t_0 \Delta \phi \frac{S}{t_G} + 1}{t_0 \Delta \phi \frac{S}{t_G}} e^{S \Delta \phi} - \frac{1}{t_0 \Delta \phi \frac{S}{t_G}} \right)$$

Ref 1,2



Simplified for $S > 40$

$$P_c = 1 + \frac{\sqrt{N}}{4} \frac{S \Delta \phi * t_G}{t_G + S \Delta \phi * t_0}$$

Acetonitrile/H₂O with 0.1%TFA (v/v)

◆ Acetophenone ■ Angiotensin II

1. U.D. Neue, J. Chromatogr. A 1079 (2005) 153.
2. U.D. Neue, J. Chromatogr. A 1184 (2008) 107-130

Outline

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

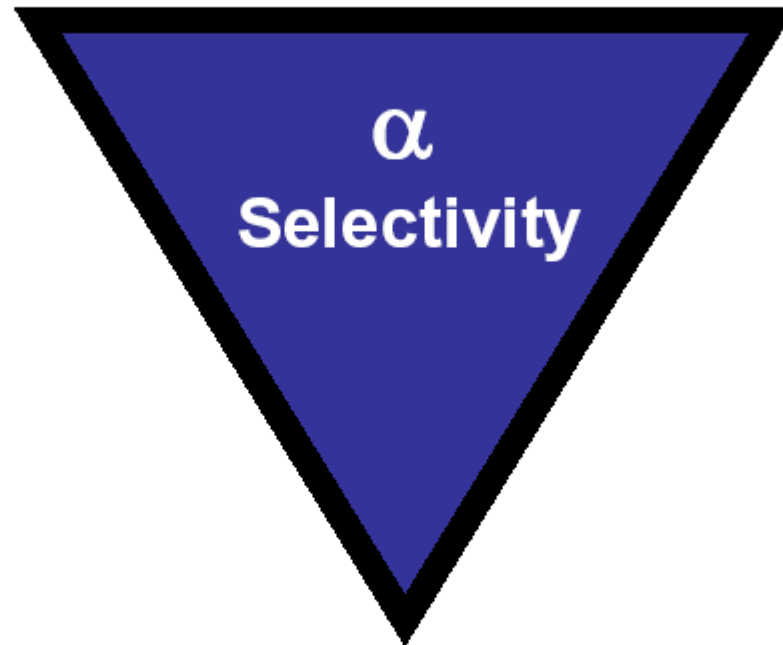
- Selectivity tools
 - Stationary phases
 - Solvents
 - pH
 - Particle characteristics

Selectivity Tools

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Solvent

pH



Column Chemistry

- Ligand
- Base particle

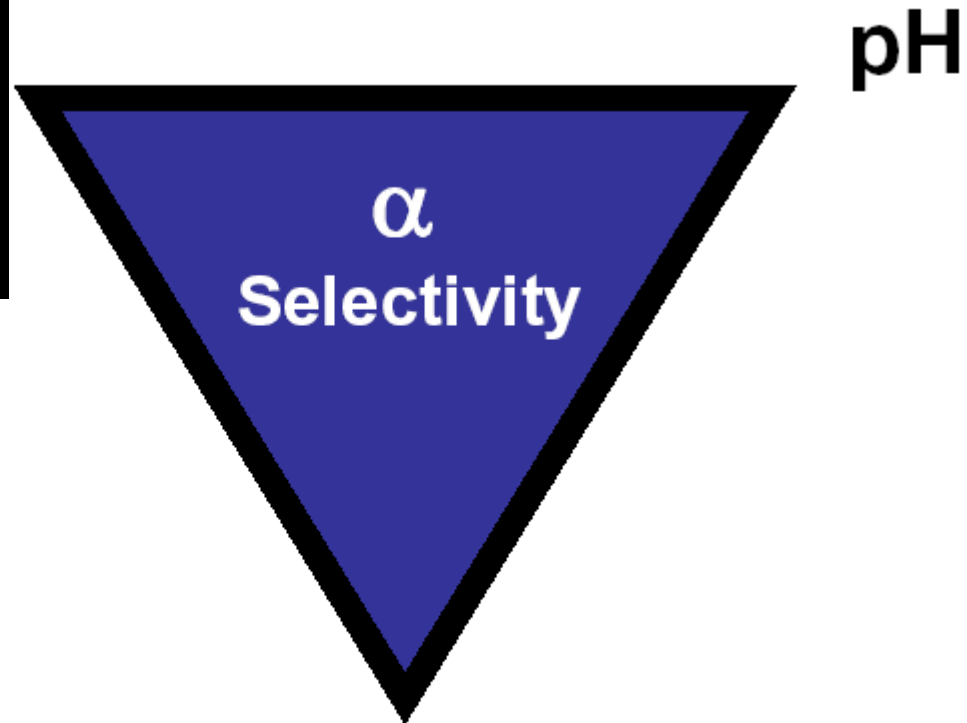
Selectivity Tools: Column Chemistry

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Provide:
 - Differences in hydrophobicity
 - longer alkyl chains provide longer retention
 - Differences in particle substrate
 - silanol activity affects peak shape through secondary interactions
 - silanols affect selectivity through H-bonding or ion-exchange
 - Others like graphite, titania, zirconia...
 - Differences in ligand functional groups
 - Embedded polar groups (EPG) enhance H-bonding
 - Phenyl groups enhance π - π interactions

Solvents

- Methanol
- Acetonitrile

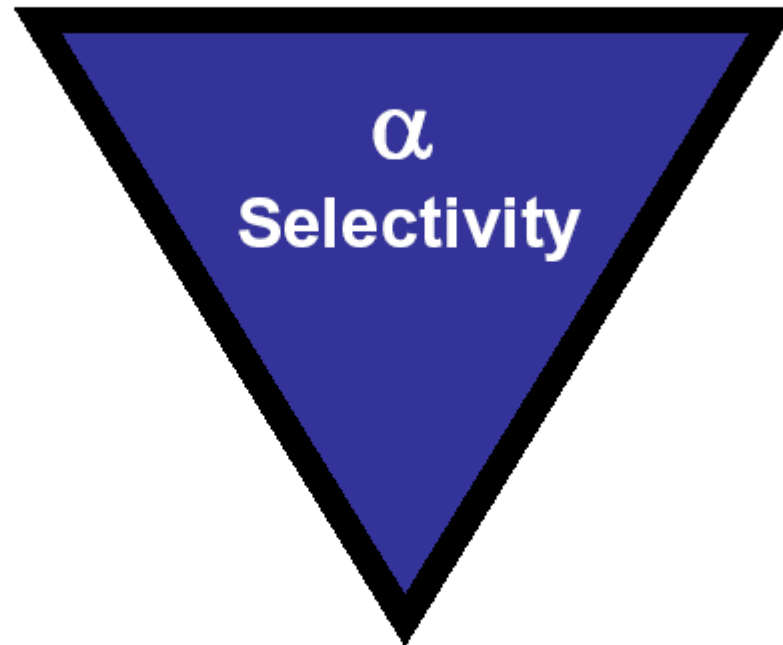


Column Chemistry

Selectivity Tools: Solvent Properties

- Methanol (MeOH)
 - weaker elution strength
 - H-bonding solvent
 - Dielectric constant 32.6
 - UV cutoff: 1 AU at 205 nm
 - Viscosity for 50% MeOH at 30°C = 1.32 cP
- Acetonitrile (ACN)
 - stronger elution strength
 - aprotic solvent
 - Dielectric constant 37.5
 - UV cutoff: 1 AU at 190 nm
 - Viscosity for 25% ACN at 30°C = 0.90 cP

Solvent



pH

- pH 3
- pH 10

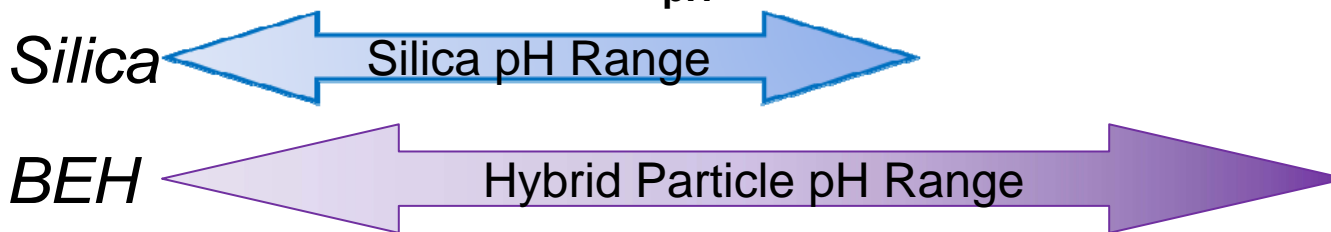
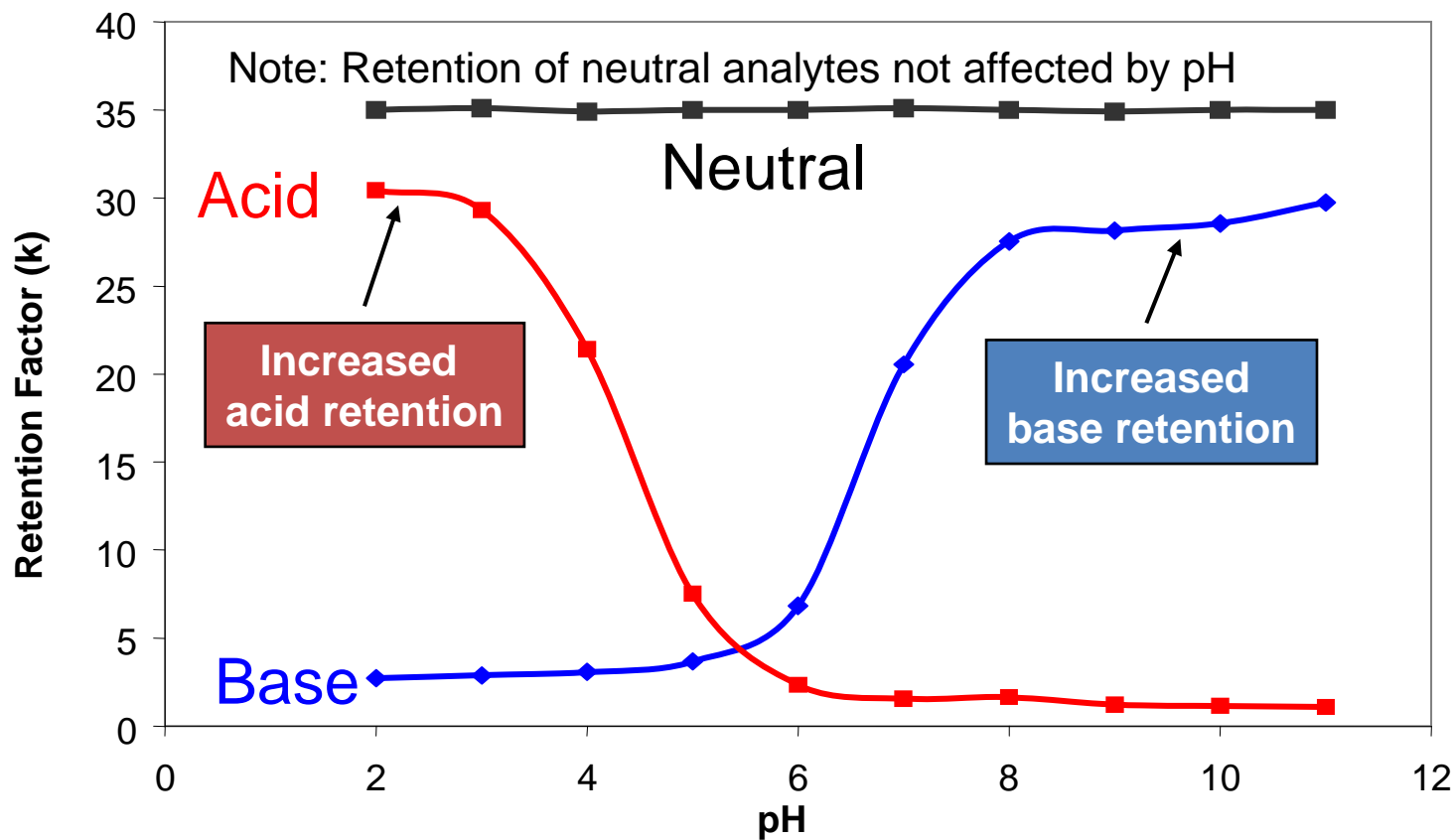
Column Chemistry

Selectivity Tools:

pH

Waters

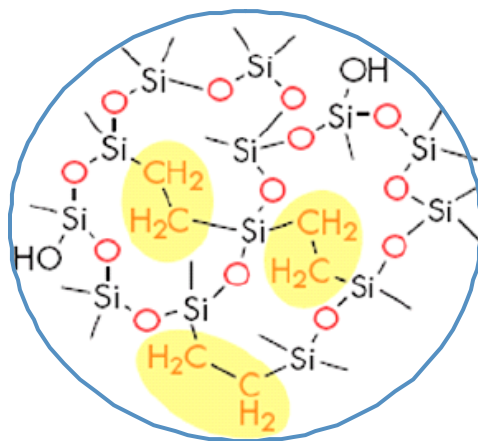
THE SCIENCE OF WHAT'S POSSIBLE.™



ACQUITY UPLC® BEH and Experimental Phases: pH Stability

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- The Bridged Ethyl Hybrid particle was selected as the base particle in this study due to its increased thermal and pH stability relative to silica.

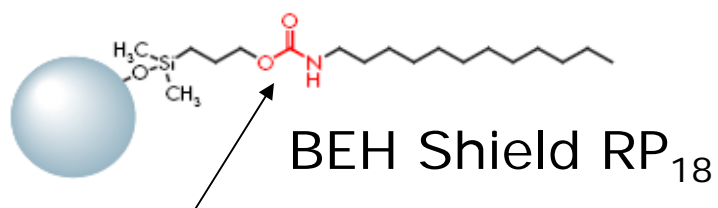
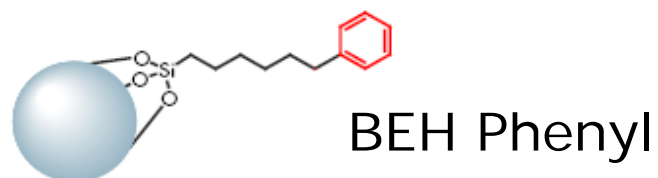
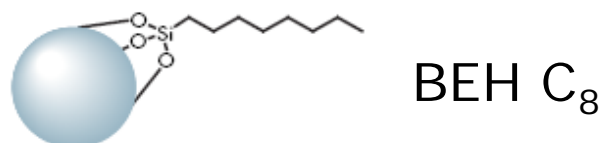
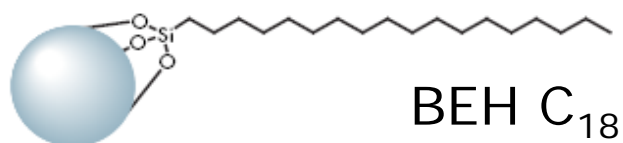


Bridged Ethyl Hybrid Particles

ACQUITY UPLC® BEH 1.7µm Family of Columns

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Bonded Phase



Polar
Group

Step 1 %C	Step 1 Coverage	Endcap	Final %C
16.1 %C	3.2 µmol/m ²	Proprietary	17.5 %C
11.1 %C	3.1 µmol/m ²	Proprietary	12.8 %C
13.1 %C	3.2 µmol/m ²	Proprietary	14.6 %C
16.6 %C	3.3 µmol/m ²	TMS	16.7 %C

Experimental Family of columns

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

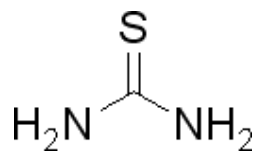
- Based on 3-4 μ m BEH particles
 - C₁₈
 - Embedded Polar Group (EPG) 1
 - Embedded Polar Group (EPG) 2
 - Embedded Polar Group (EPG) 3

Scouting Gradient Test Conditions

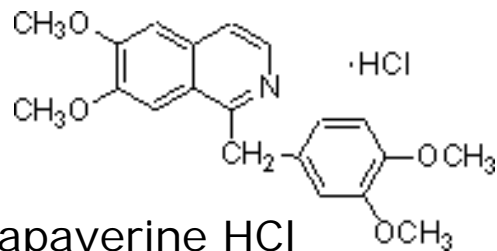
Scouting Gradient Conditions Using 2.1 x 50mm Columns		
Column Temperature	30°C	
Injection Volume	5 µL	
Flow rate	500 µL/min	
Gradient:	5 to 90% B (MeOH or ACN) using a 5 min linear gradient 0.5 minute hold at 90% B Total run time = 7.5 minutes	
Mobile Phases:	A1: 10mM Ammonium Bicarbonate, pH 10	B1: Methanol
	A2: 10mM Ammonium Formate, pH 3	B2: Acetonitrile
System:	ACQUITY UPLC® System TUVe (V 1.40) at 254nm, 50µL Mixer, 30 µL PeekSIL sample Needle, 5 µL injection Loop	
Acquisition	Empower™ 2 Software	

Gradient Separation of 17 Analytes Basic Test Compounds with V_0

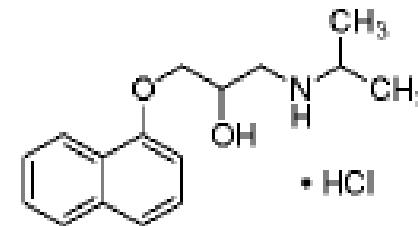
Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



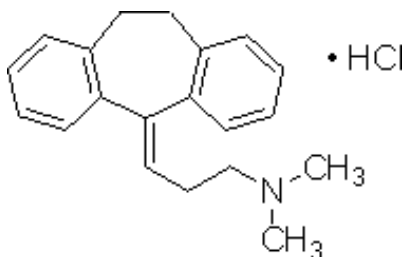
Thiourea



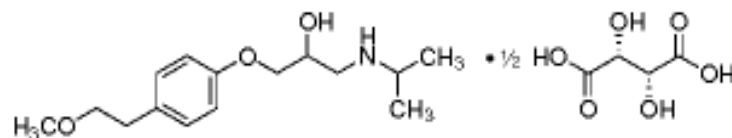
Papaverine HCl



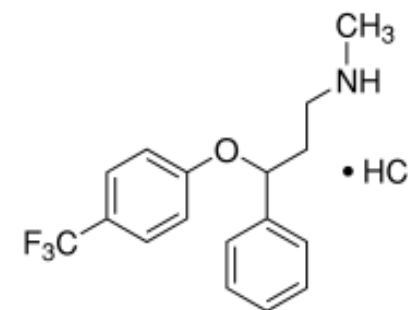
Propranolol



Amitriptyline HCl



Metoprolol tartrate

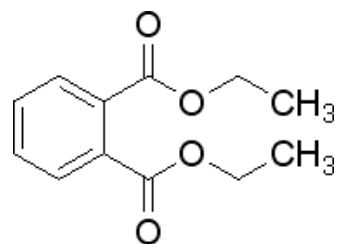


Fluoxetine

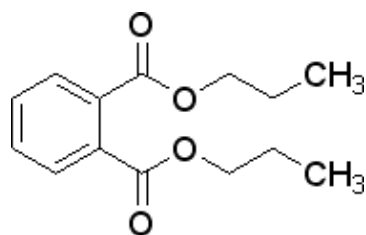
Gradient Separation of 17 Analytes

Neutral and Acidic Test Compounds

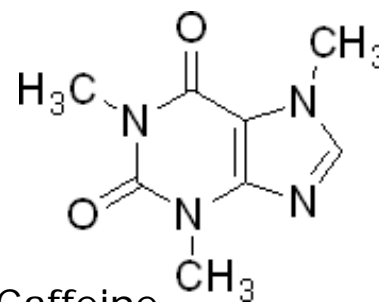
Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



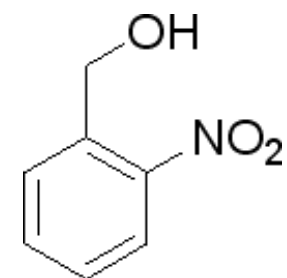
Diethylphthalate



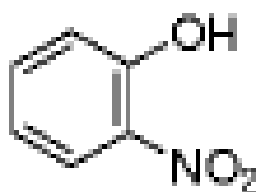
Dipropylphthalate



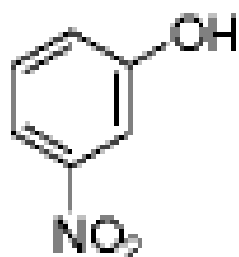
Caffeine



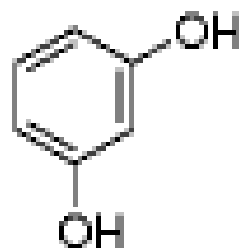
2-Nitrobenzyl alcohol



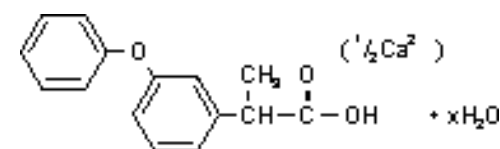
2-Nitrophenol



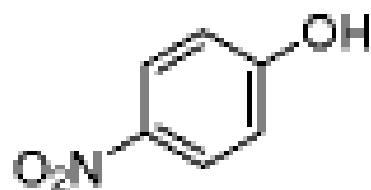
3-Nitrophenol



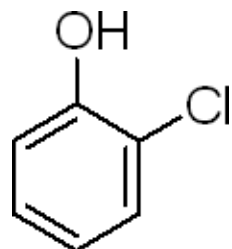
Resorcinol



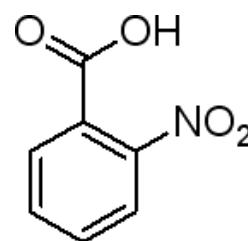
Fenopropfen



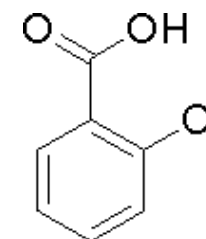
4-Nitrophenol



2-Chlorophenol

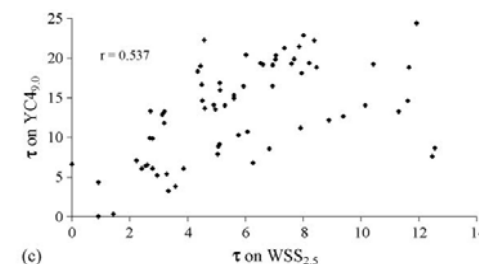
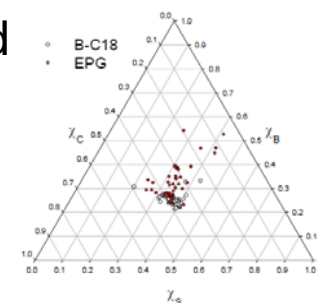
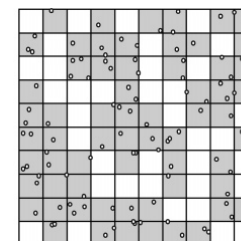


2-Nitrobenzoic acid



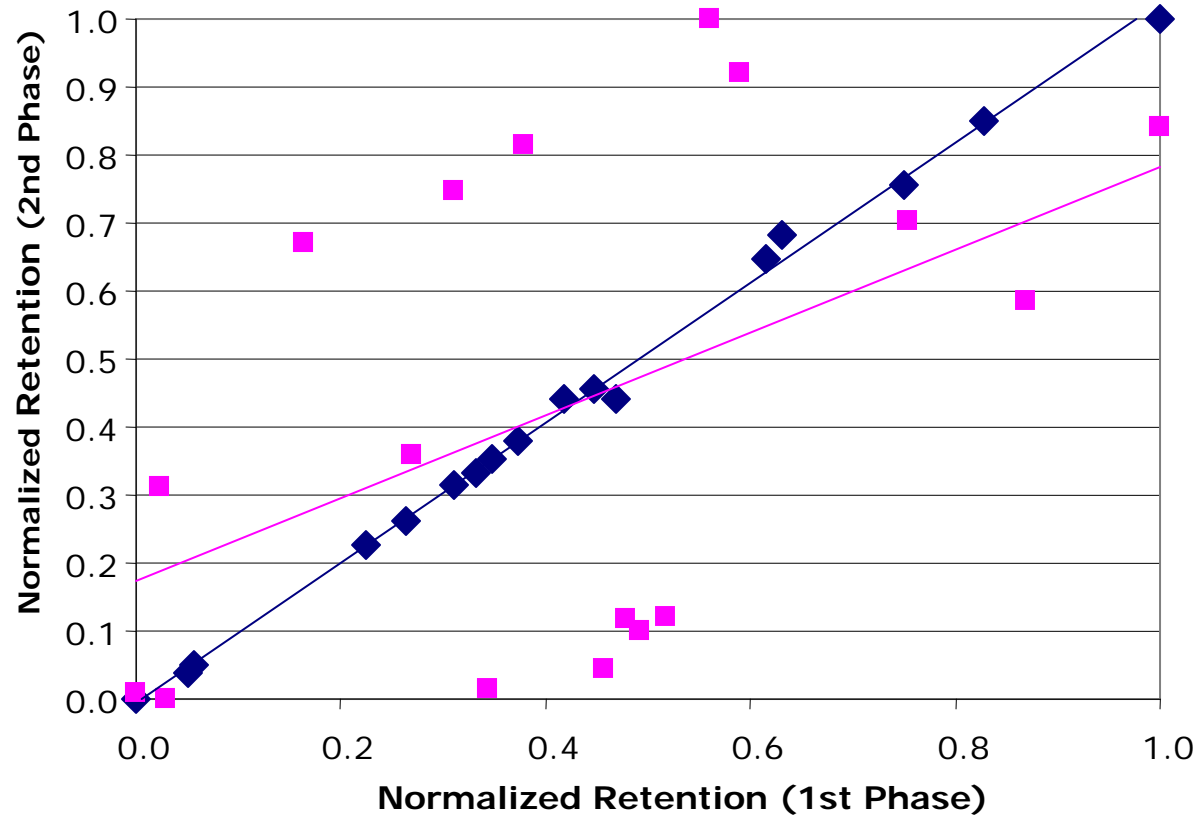
2-Chlorobenzoic acid

- Geometric method
 - Martin Gilar, Petra Olivova, Amy E. Daly, and John C. Gebler, *Anal. Chem.*, **2005**, 77 (19), 6426-6434
- Selectivity triangles – Visual approach using adjusted parameters from the hydrophobic subtraction model
 - Y. Zhang, P.W. Carr, In Press, *Journal of Chromatography A* (2009)
- Pearson-correlation coefficients r between normalized retention times t_R
 - E. Van Gyseghem, M. Jimidar, R. Sneyers, D. Redlich, E. Verhoeven, D.L. Massart, Y. Vander Heyden, *Journal of Chromatography A*, 1074 (2005) 117–131
 - S. Zhu, *Journal of Chromatography A*, 1216 (2009) 3312–3317



Similar versus Orthogonal Selectivity

Examples of Similar and Orthogonal Selectivity



◆ *Similar selectivity*

$r = 0.998$; 3° spreading angle

■ r values near 1.0

■ *Orthogonal selectivity*

$r = 0.476$; 62° spreading angle

■ low r values

Outline

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Background
- Assessing orthogonal selectivity
 - tools
 - Small molecule selectivity
- Maximizing peak capacity
 - Peptide examples

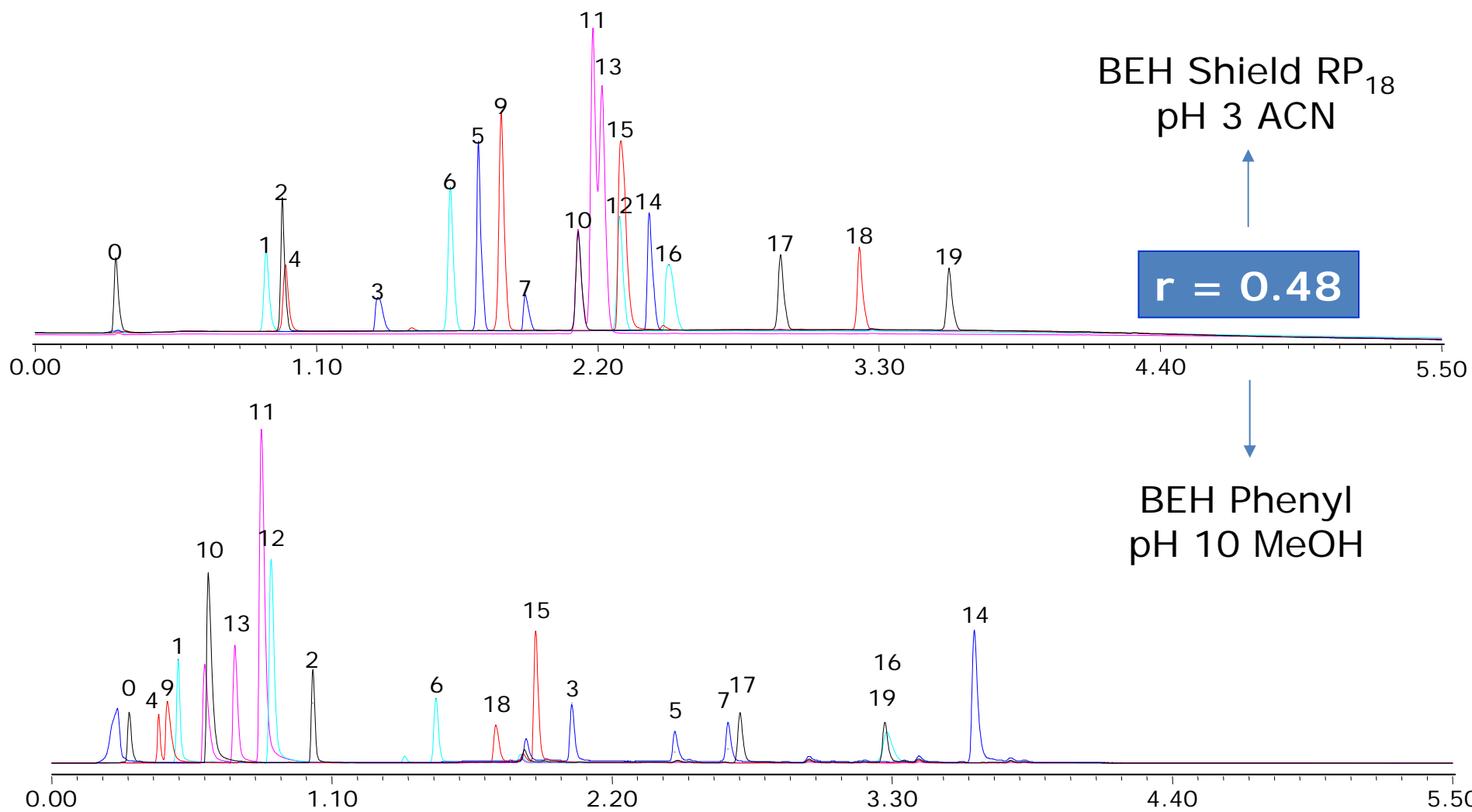
Selectivity Tool: pH

BEH Family			r at pH 3							
			Acetonitrile				Methanol			
			C18	RP18	Ph	C8	C18	RP18	Ph	C8
r pH 10	ACN	C18	0.68	0.56	0.72	0.70	0.77	0.63	0.81	0.77
		RP18	0.67	0.55	0.71	0.69	0.76	0.63	0.80	0.77
		Ph	0.66	0.54	0.70	0.68	0.76	0.62	0.80	0.76
		C8	0.69	0.57	0.73	0.71	0.78	0.64	0.81	0.78
	MeOH	C18	0.65	0.52	0.69	0.67	0.77	0.62	0.81	0.77
		RP18	0.64	0.52	0.69	0.66	0.76	0.62	0.81	0.77
		Ph	0.60	0.48	0.65	0.63	0.73	0.58	0.78	0.73
		C8	0.65	0.53	0.70	0.67	0.77	0.62	0.81	0.77

Selectivity differences mostly result due to the **change in the analyte's ionization state!**
At pH 10, bases become neutral and acids become ionized.

BEH Shield RP₁₈ pH 3 ACN
BEH Phenyl pH 10 MeOH

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



ACQUITY UPLC® BEH Family: Selectivity comparisons at the same pH

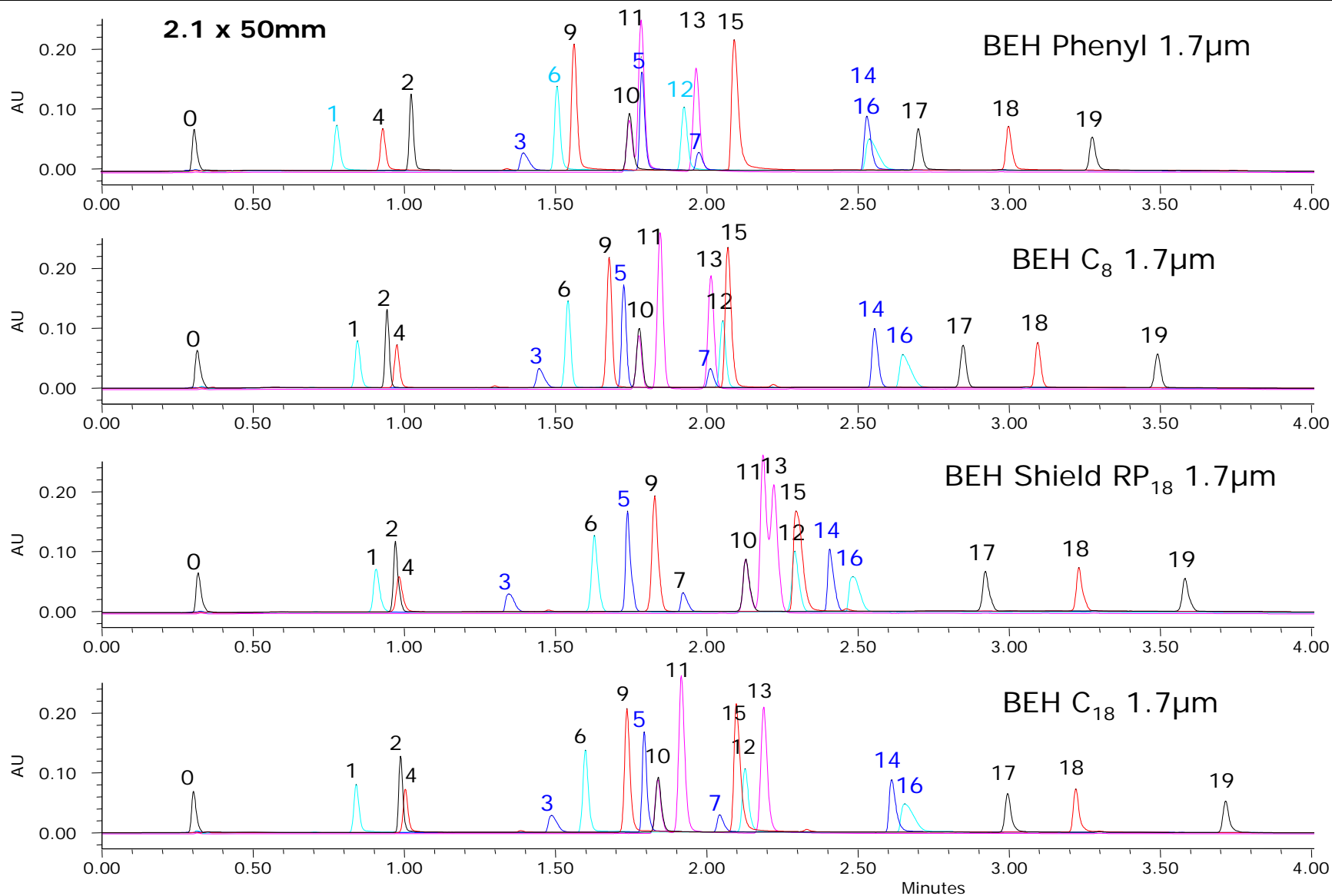
Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

BEH Family			r at pH 10							
			Acetonitrile				Methanol			
			C18	RP18	Ph	C8	C18	RP18	Ph	C8
r pH 10	ACN	C18	--							
		RP18	1.00	--						
		Ph	1.00	1.00	--					
		C8	1.00	1.00	1.00	--				
	MeOH	C18	0.97	0.98	0.98	0.98	--			
		RP18	0.98	0.98	0.98	0.98	1.00	--		
		Ph	0.97	0.97	0.98	0.97	1.00	1.00	--	
		C8	0.97	0.98	0.98	0.98	1.00	1.00	1.00	--

BEH Family			r at pH 3							
			Acetonitrile				Methanol			
			C18	RP18	Ph	C8	C18	RP18	Ph	C8
r pH 3	ACN	C18	--							
		RP18	0.98	--						
		Ph	1.00	0.97	--					
		C8	1.00	0.98	1.00	--				
	MeOH	C18	0.96	0.92	0.98	0.97	--			
		RP18	0.97	0.98	0.98	0.98	0.97	--		
		Ph	0.94	0.90	0.97	0.95	0.99	0.95	--	
		C8	0.96	0.92	0.98	0.97	1.00	0.97	0.99	--

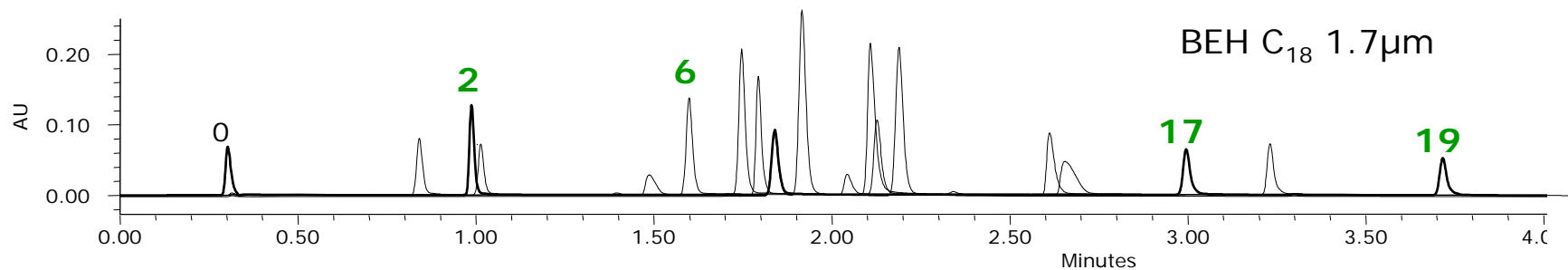
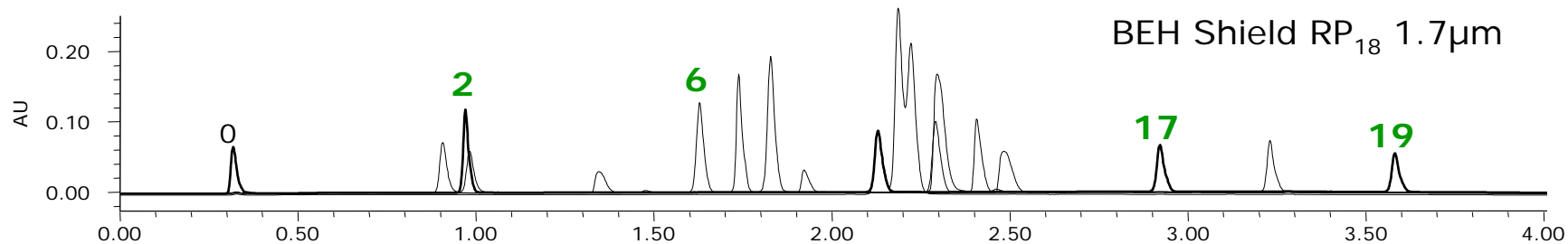
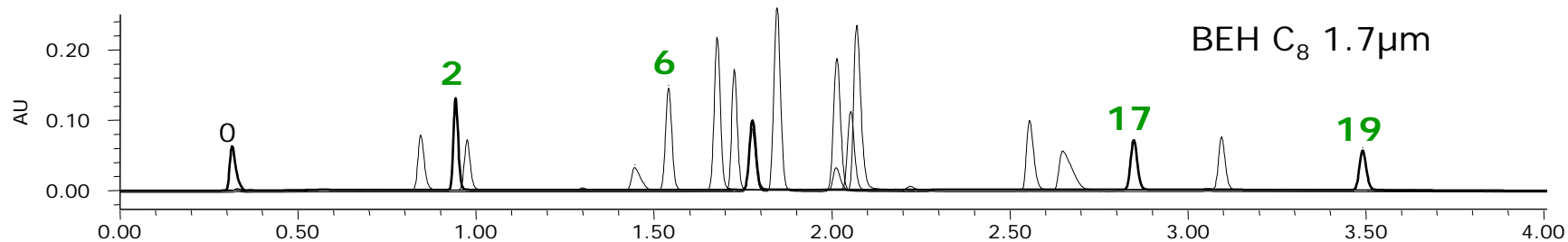
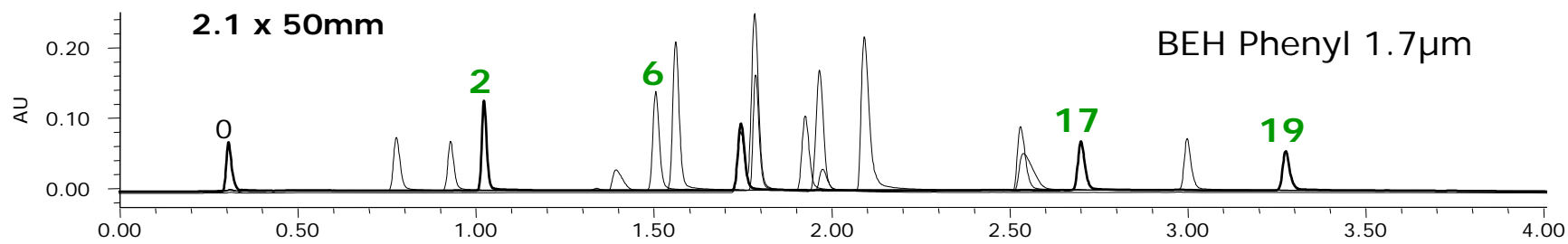
Scouting Gradients at 30°C Acetonitrile-10mM Ammonium Formate, pH 3

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



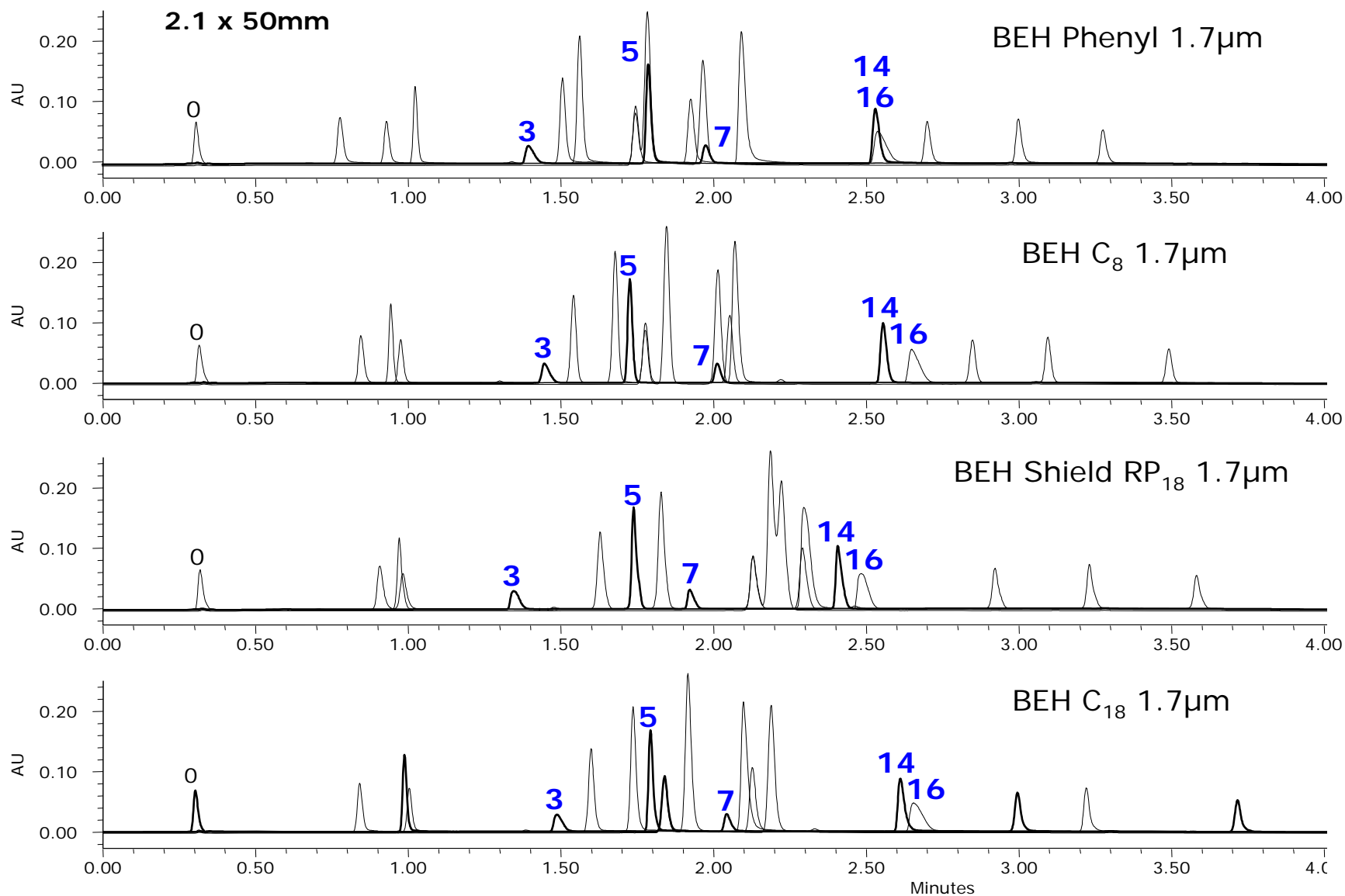
Scouting Gradients at 30°C Acetonitrile-10mM Ammonium Formate, pH 3

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



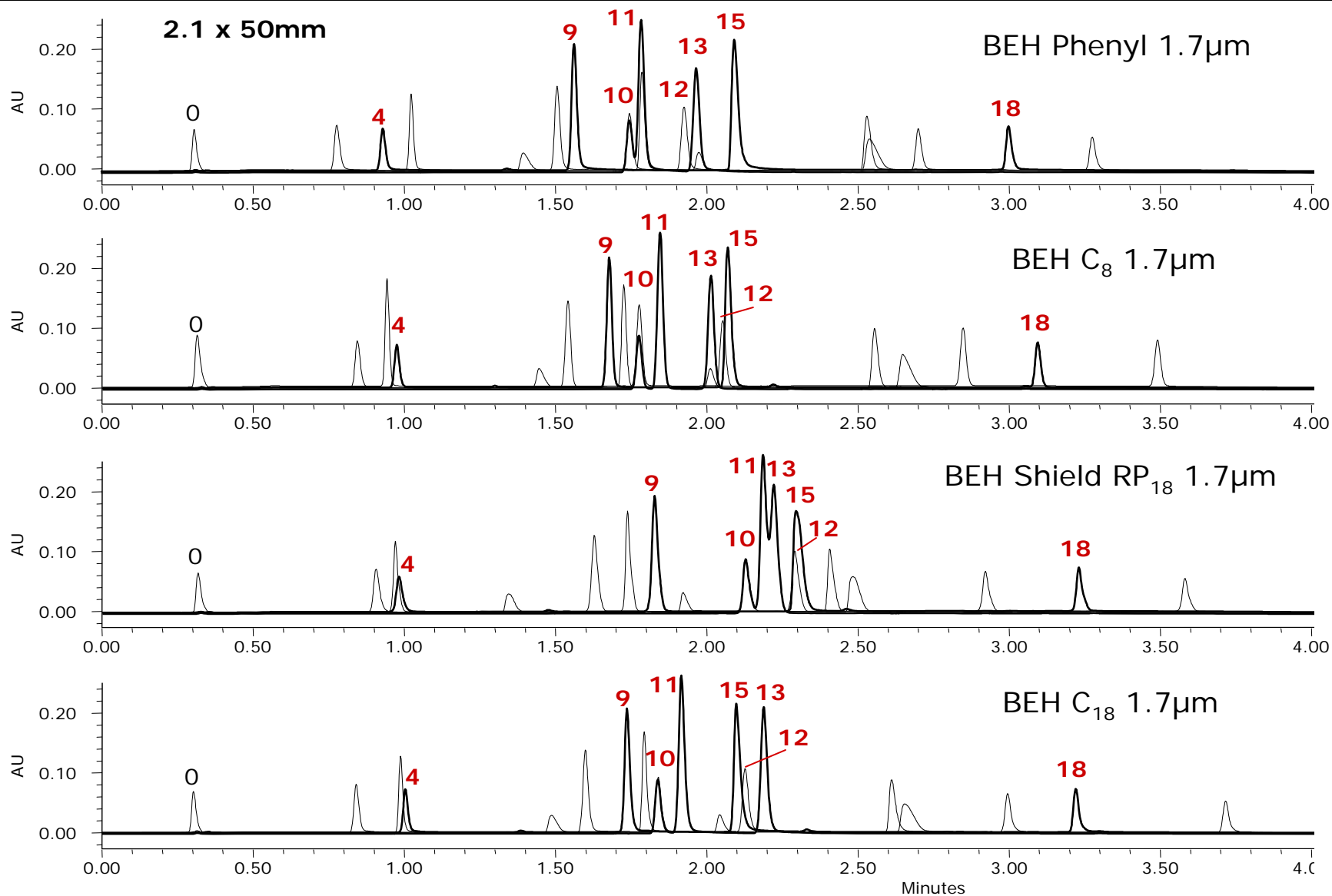
Scouting Gradients at 30°C Acetonitrile-10mM Ammonium Formate, pH 3

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



Scouting Gradients at 30°C Acetonitrile-10mM Ammonium Formate, pH 3

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



Looking for Orthogonal Selectivity at pH 3

BEH Family			r at pH 3			
			Acetonitrile			
			C18	RP18	Ph	C8
r pH 3	ACN	C18	--			
		RP18	0.98	--		
		Ph	1.00	0.97	--	
		C8	1.00	0.98	1.00	--
	MeOH	C18	0.96	0.92	0.98	0.97
RP18		0.97	0.98	0.98	0.98	
Ph		0.94	0.90	0.97	0.95	
C8		0.96	0.92	0.98	0.97	

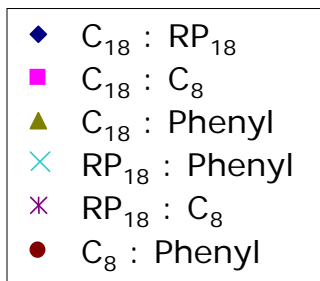
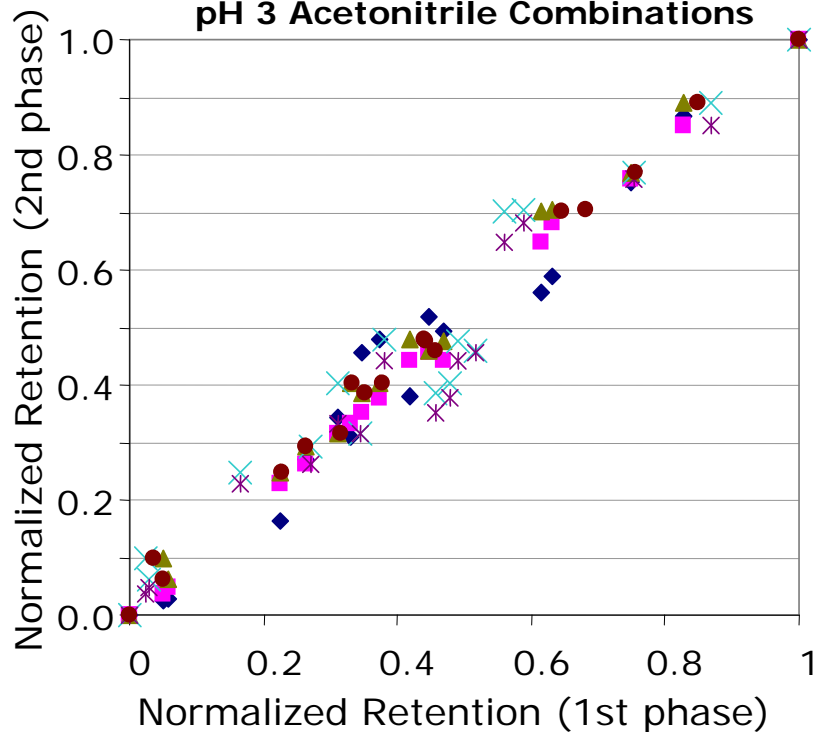
Experimental Family			r at pH 3			
			Acetonitrile			
			C18	EPG 1	EPG 2	EPG 3
r pH 3	ACN	C18				
		EPG 1	1.00			
		EPG 2	0.75	0.76		
		EPG 3	0.43	0.45	0.91	
	MeOH	C18	0.95	0.96	0.88	0.64
EPG 1		0.96	0.97	0.86	0.61	
EPG 2		0.61	0.63	0.96	0.92	
EPG 3		0.36	0.38	0.88	0.99	

Because we are interested in peptide separations
we will *focus on pH 3 acetonitrile gradients*

Comparison of Selectivity pH 3 Acetonitrile Gradients

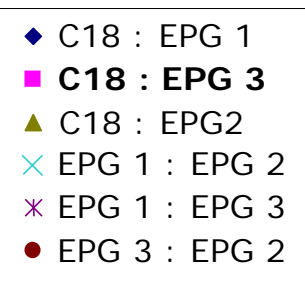
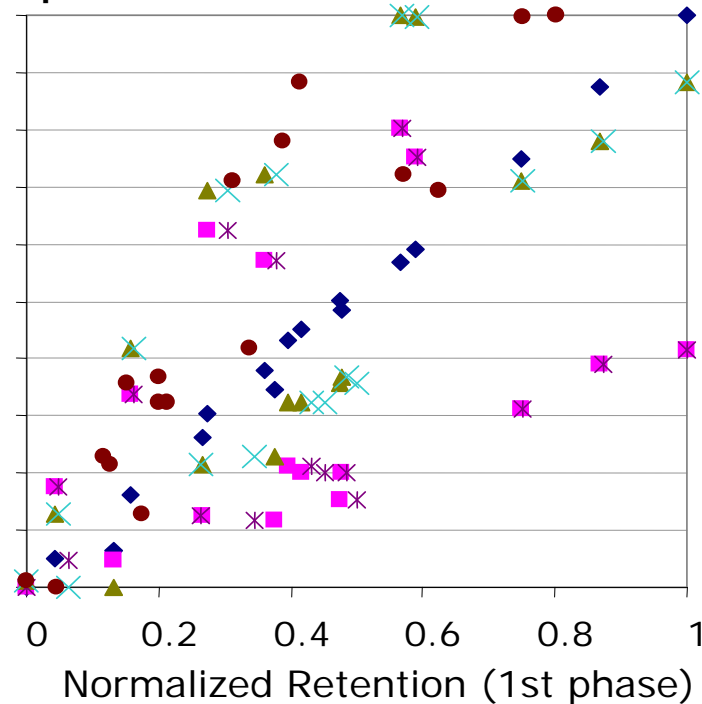
BEH Family:

pH 3 Acetonitrile Combinations

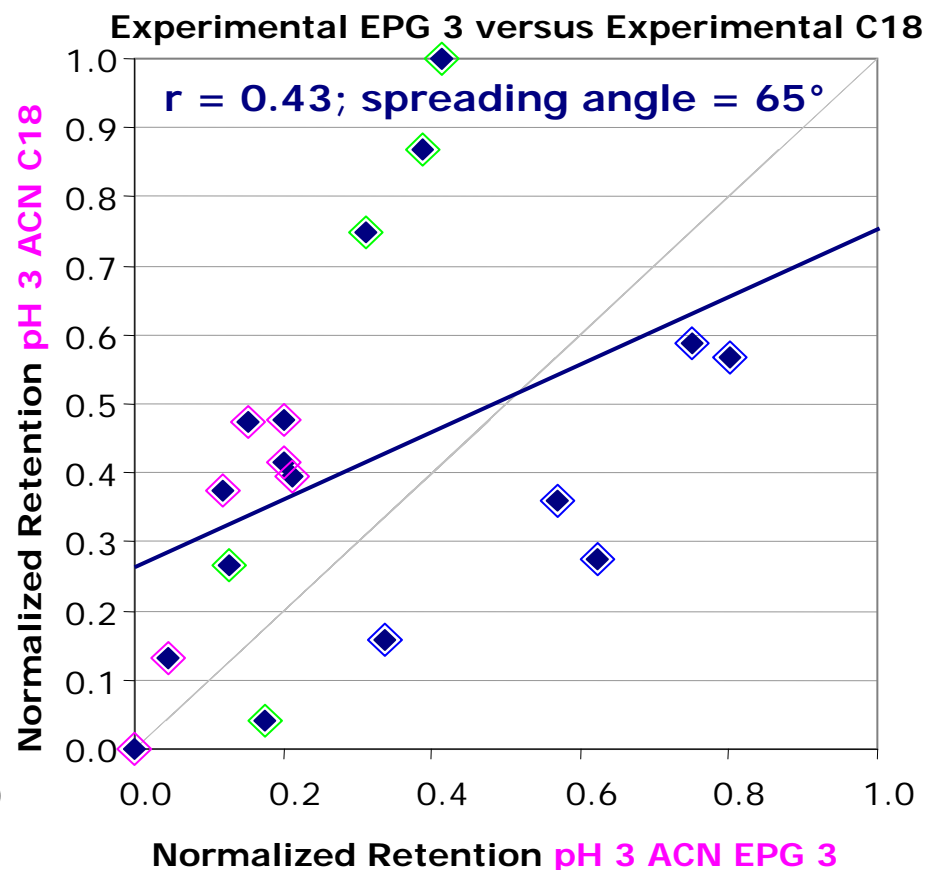
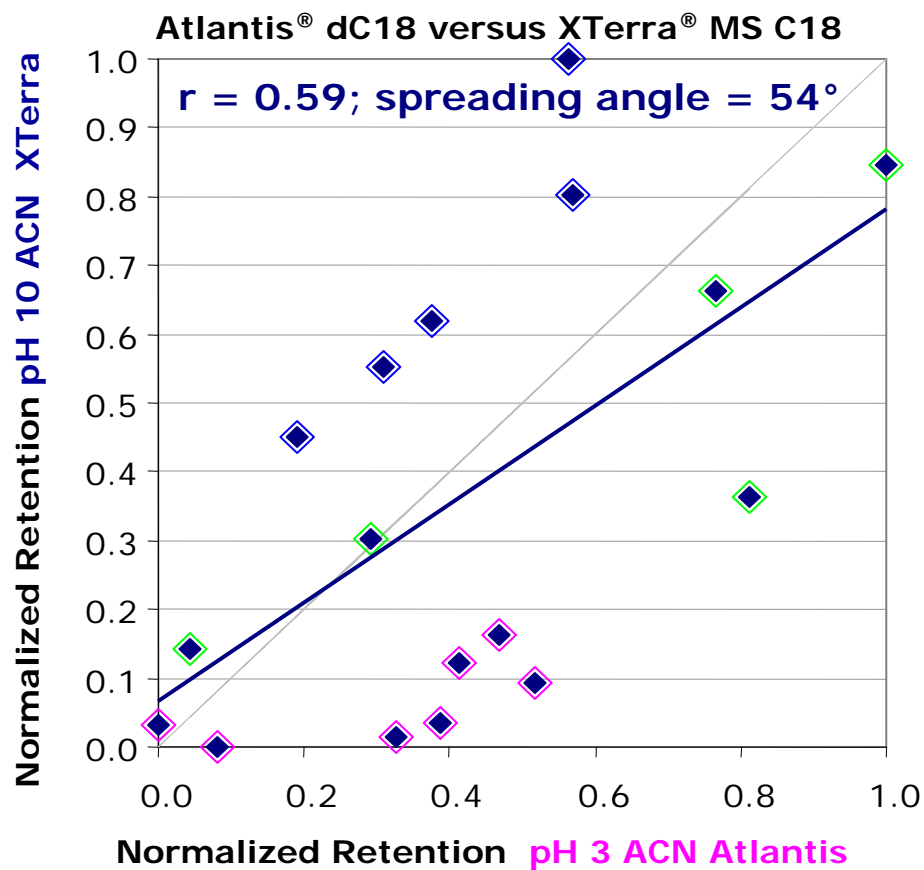


Experimental Family:

pH 3 Acetonitrile Combinations



Comparison to Literature Reference



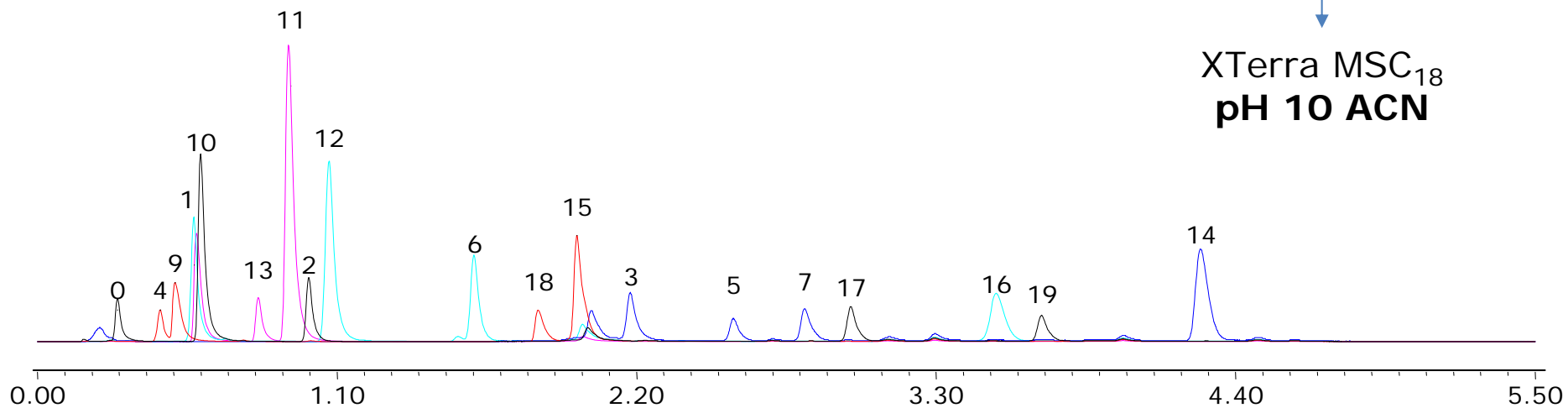
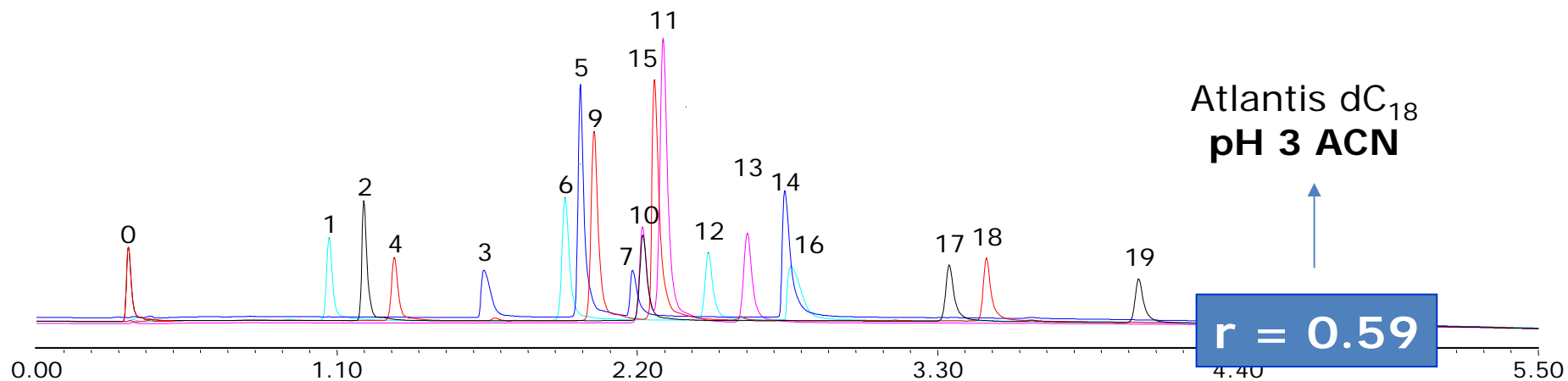
Orthogonality of Separation in Two-Dimensional Liquid Chromatography

Martin Gilar, Petra Olivova, Amy E. Daly, and John C. Gebler
Anal. Chem., **2005**, 77 (19), 6426-6434

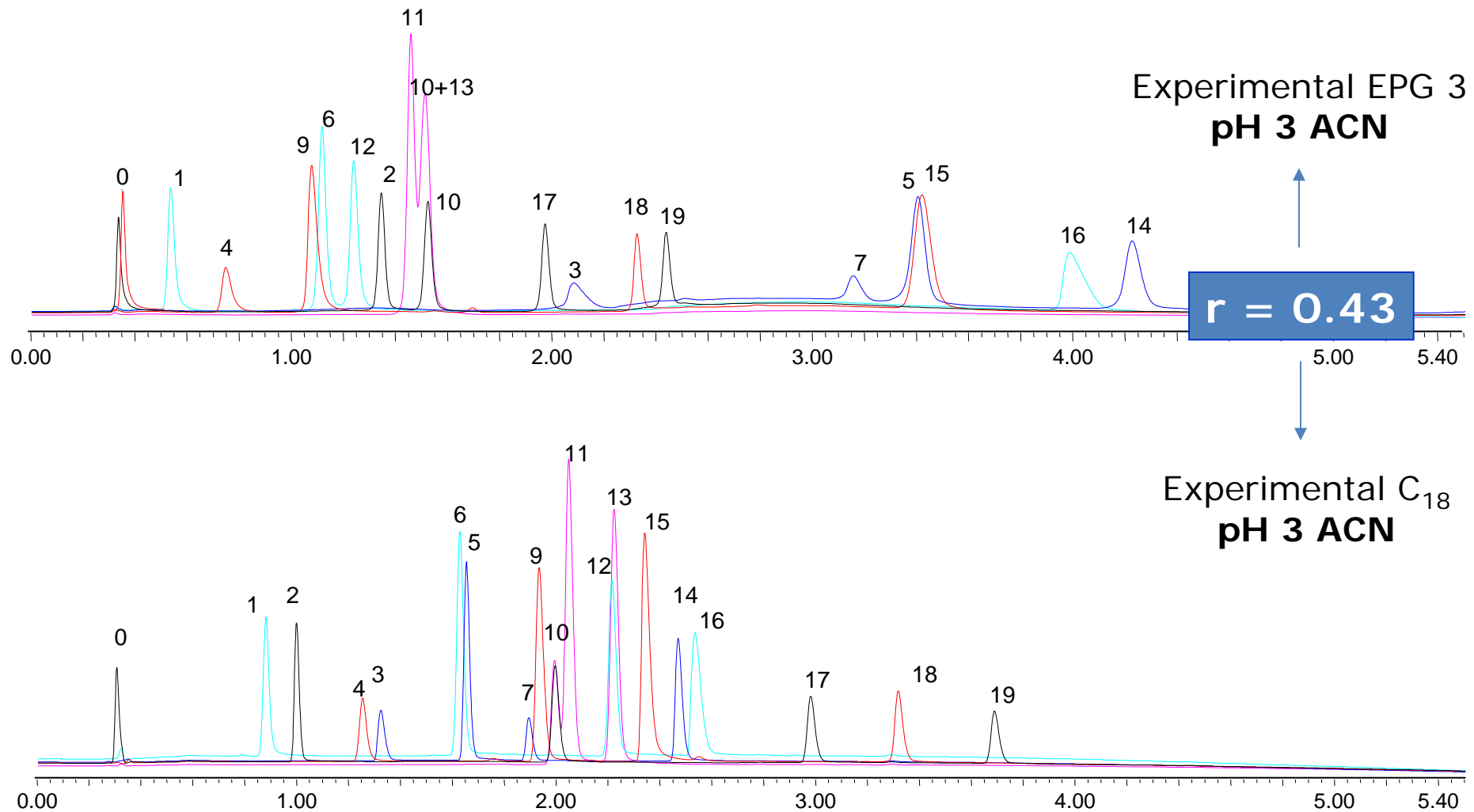
- ◇ Basic analytes
- ◇ Acidic analytes
- ◇ Neutral analytes

Atlantis[®] dC₁₈ versus XTerra[®] MSC₁₈

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™



Experimental EPG 3 versus C₁₈



Using exactly the same mobile phases and gradients!

Optimizing Peptides Gradient Separations

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Selected phases that show orthogonal selectivity at pH 3
 - Experimental C₁₈
 - Experimental EPG 3
- Investigate the operational parameters for peptide gradient separations
 - Temperature
 - Flow rate
 - Sensitivity

Optimizing Peptides Gradient Separations

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

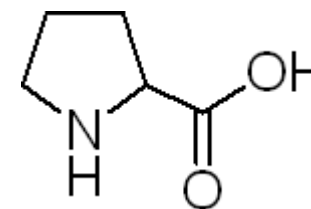
- Effect of temperature on peak capacity
- Effect of flow rate on peak capacity
- Effect of mobile phase modifier on MS sensitivity

Effect of Temperature on Peptide Peak Capacity

Peptide Mixtures	Amino Acid Length	# of Prolines	%Proline	%Higher Pc (40°C to 80°C)	
				BEH C ₁₈ 1.7µm	XBridge C ₁₈ 3.5µm
MassPREP Peptide Mix	123	13	11	42	22
<i>Tryptic digest of:</i>					
Bovine Hemoglobin	143	6	4.2	22	12
Enolase	437	14	3.2	9	7

- Pc increased significantly at 80°C relative to 40°C for proline containing peptides due to slow kinetics of proline cis-trans isomerization.

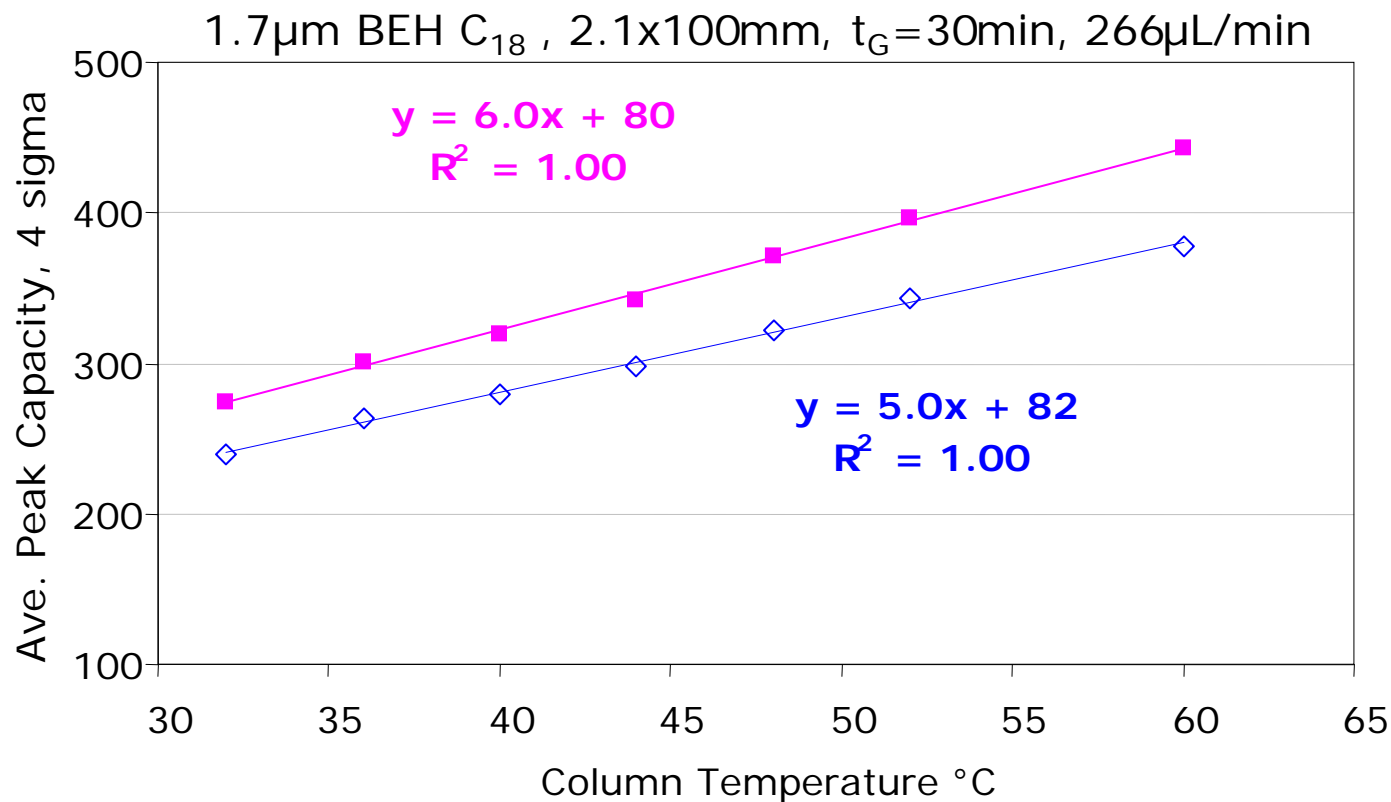
- Wayne R. Melander, Jana Jacobson, and Csaba Horvath, *Journal of Chromatography*, 234 (1982) 269-276: *Peak shape due to isomerization studied*
- Jana Jacobson, Wayne Melander, Gintaras Vaisnys, and Csaba Horvath, *J. Phys. Chem.* 1984, 88, 4542-4541: *Calculation of Damkohler number*



MassPREP Peptide Mix

MassPREP Peptide Mix	MW	pI	Sequence (Basic AA, Proline)
RASG-1	1000	9.34	R GD S PASS K P
Ang frag. 1-7	898	7.35	DRVYI H P
Bradykinin	1060	12.00	R PPGF S P F R
Ang II	1046	7.35	DRVYI H P F
Ang I	1296	7.51	DRVYI H P F HL
Re sub	1758	7.61	DRVYI H P F HLLVYS
Eno T35	1872	7.34	WLTG P QLADLY H S L M K
Eno T37	2827	3.97	Y P IVSIED P FAEDDWEAWS H FF K
Melittin	2846	12.06	GIGAVL K VLTTGL P ALISWIK R K R Q Q

Effect of Temperature on Proline Containing Peptide Peak Capacities



■ Using t_G

$$Pc = 1 + \frac{t_G}{W_{13.4\%}}$$

◇ Using retention window

$$*n_c = 1 + \frac{t_{last} - t_0}{W_{13.4\%}}$$

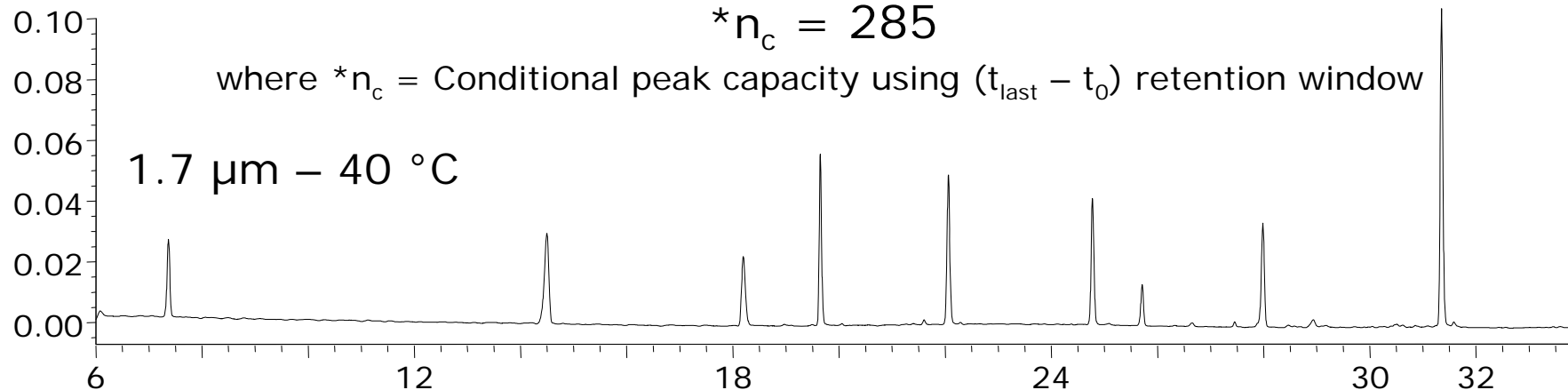
Comparison of MassPREP™ Peptide Mix on BEH C₁₈ 1.7 μm at 40 °C vs. 80 °C

Waters
THE SCIENCE OF WHAT'S POSSIBLE™

All peptides contain at least one proline

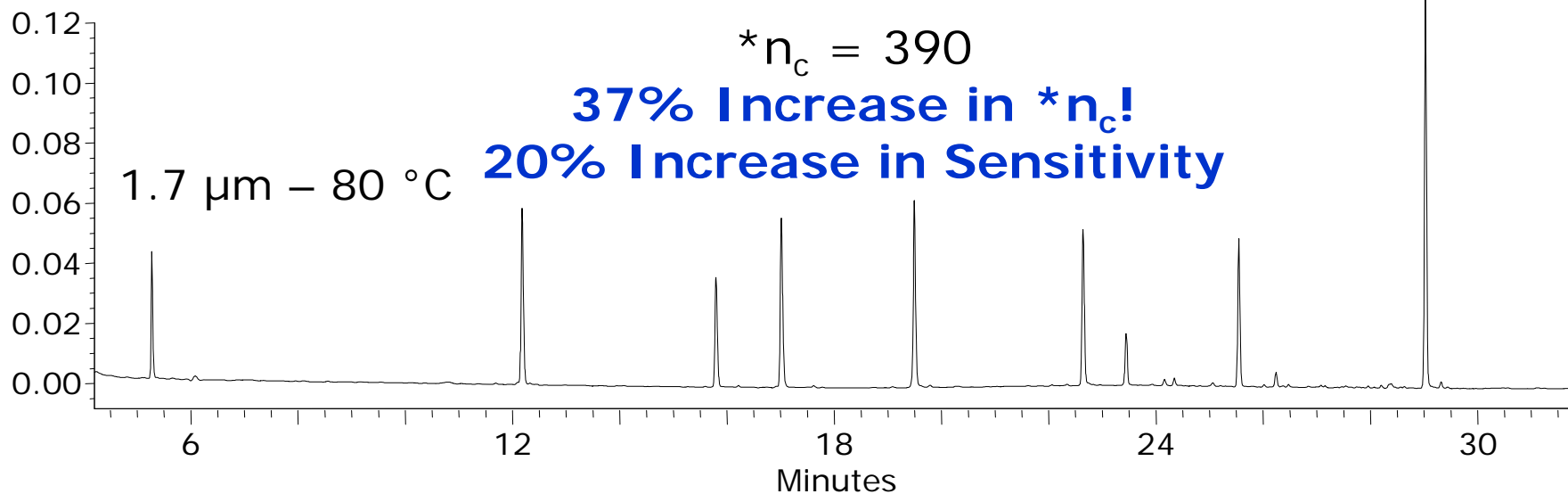
$$*n_c = 285$$

where $*n_c$ = Conditional peak capacity using $(t_{last} - t_0)$ retention window



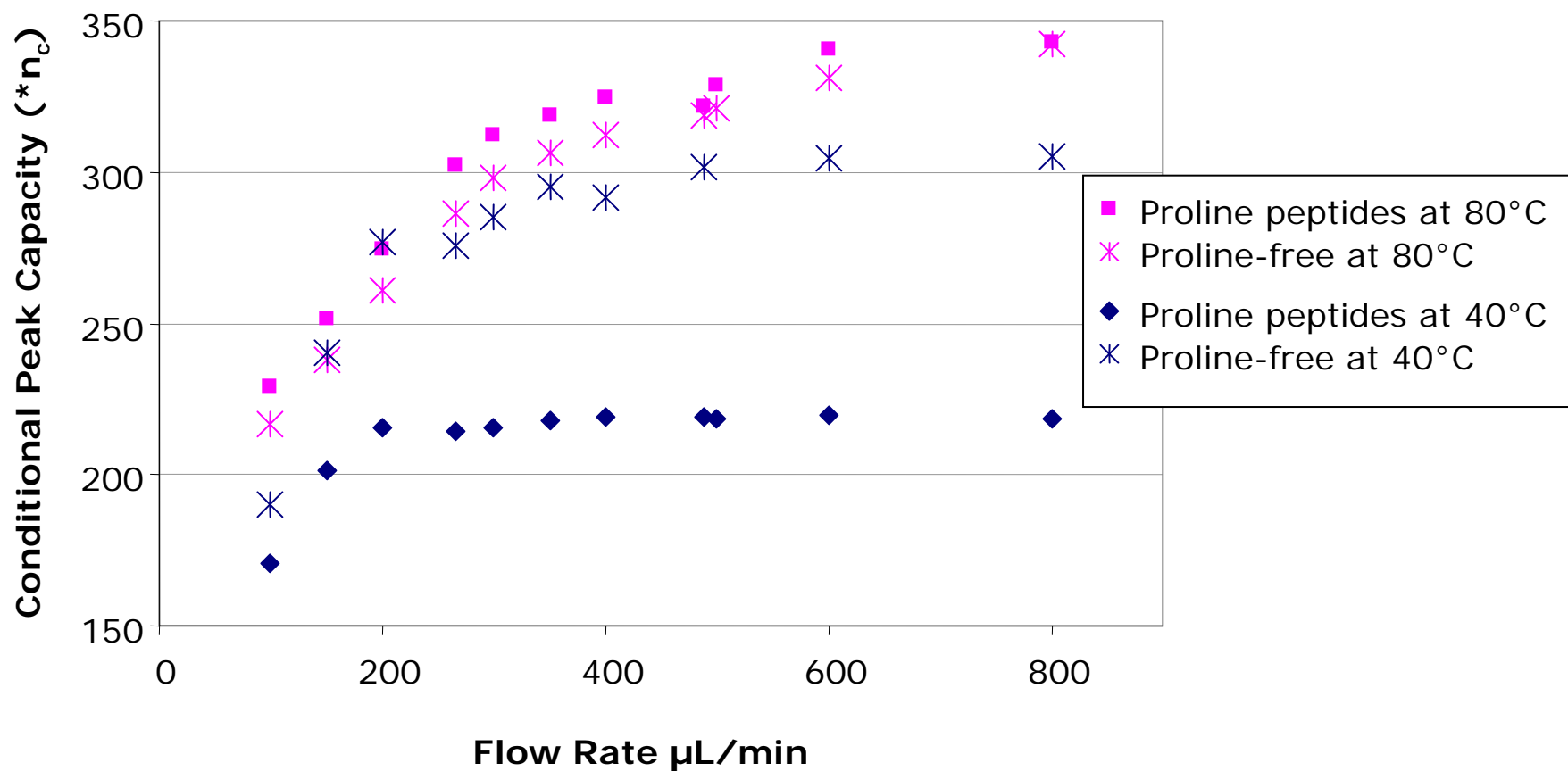
$$*n_c = 390$$

37% Increase in $*n_c$!
20% Increase in Sensitivity



Comparison of Proline Containing and Proline-free Peptides

$t_G = 20$ min on 1.7 μm BEH C_{18} in 2.1x50mm



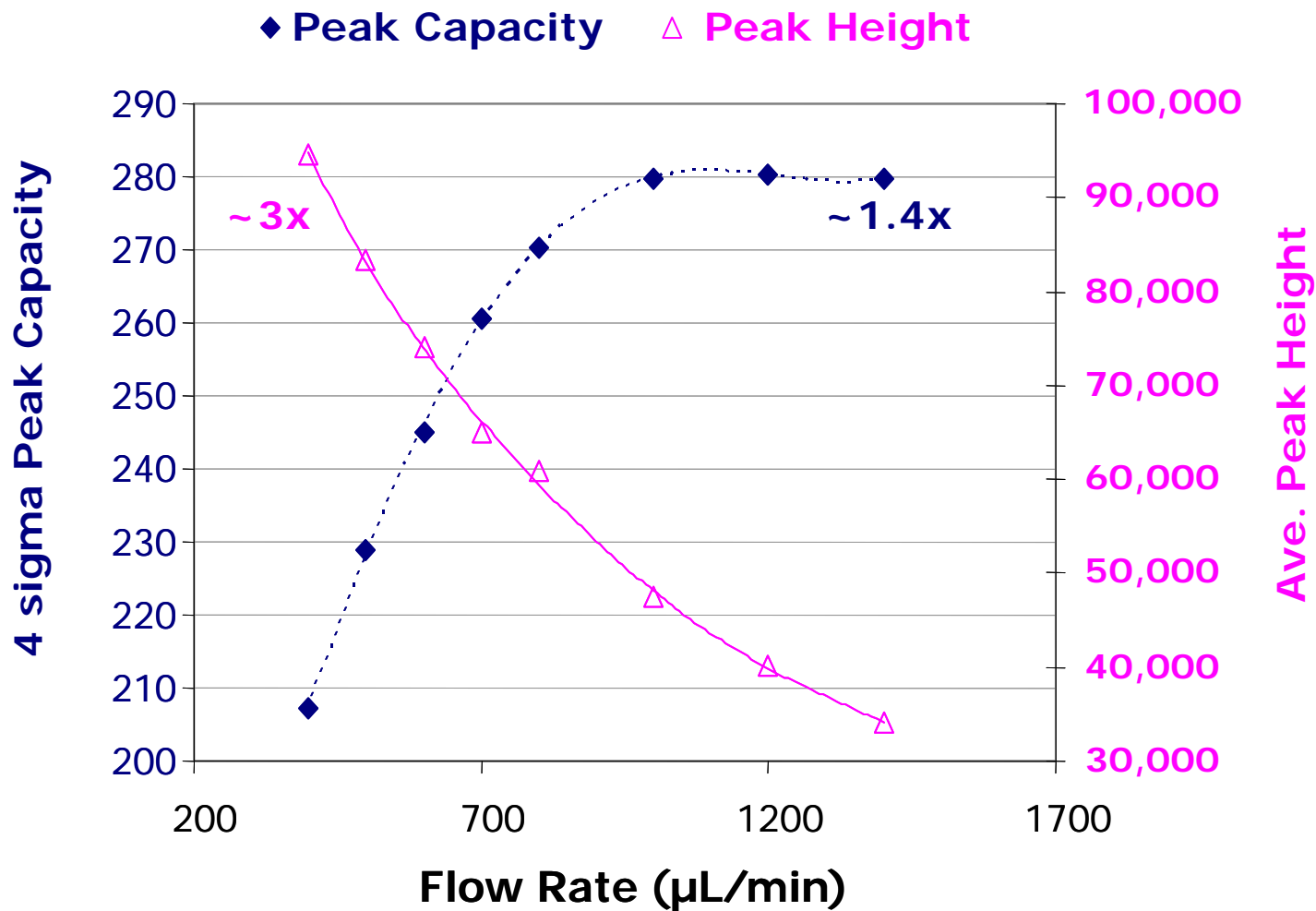
Optimizing Gradients

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Effect of temperature on peak capacity
- Effect of flow rate on peak capacity and sensitivity
- Effect of mobile phase modifier on MS sensitivity

Sensitivity and Peak Capacity versus Flow Rate with $t_G = 5$ min

$t_G = 5$ min on 1.7 μm BEH C_{18} in 2.1x50mm at 80 °C



Optimizing Gradients

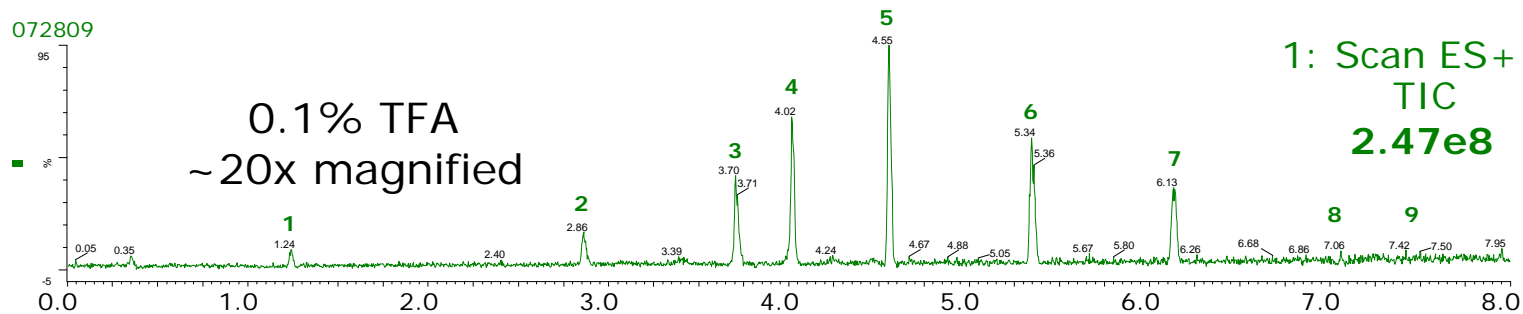
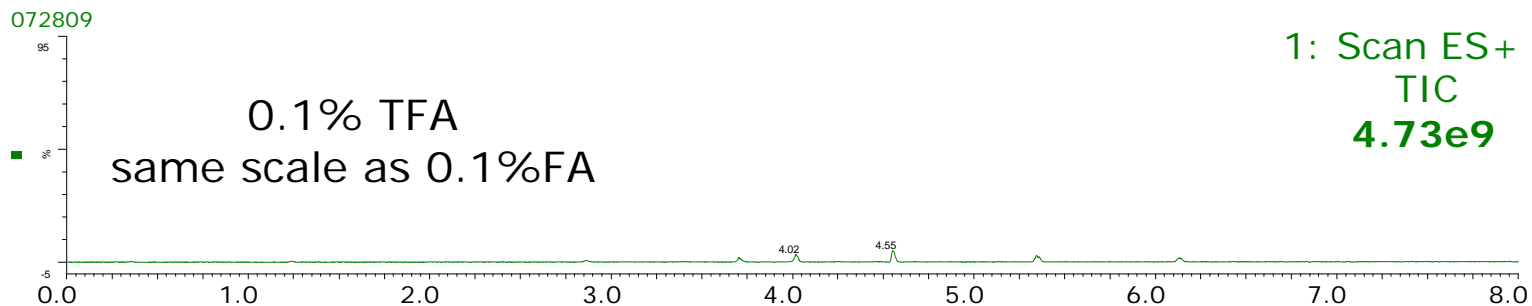
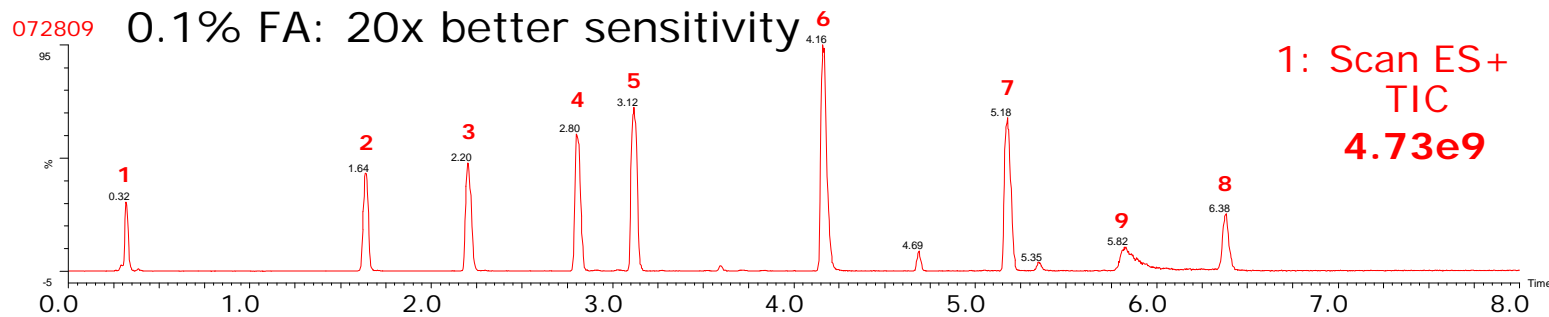
Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

- Effect of temperature on peak capacity
- Effect of flow rate on peak capacity
- Effect of mobile phase modifier on MS sensitivity

Effect of 0.1% TFA versus 0.1%FA on ES+ MS Sensitivity

10min Gradient at 350 µL/min, 80°C

- 1) RASG-1
- 2) Angiotensin Frag. 1-7
- 3) Bradykinin
- 4) Angiotensin II
- 5) Angiotensin I
- 6) Renin substrate
- 7) Enolase T35
- 8) Enolase T37
- 9) Melittin



- Successfully identified reasonably orthogonal phases in pH 3 acetonitrile gradients
 - Experimental EPG 3 and C₁₈ stationary phases
- Investigated parameters to maximize peak capacities for peptides
 - Increasing temperature increases peak capacity especially on sub 2 μm columns for proline containing peptides
 - Increasing flow rate can increase peak capacity but at the expense of sensitivity
- **Sensitivity is the next optimization challenge.** Will evaporation be needed between the 1st dimension EPG 3 column and the 2nd dimension Experimental C₁₈ column?
 - Use 80 °C to increase peak capacity without diluting the sample
 - Use lower flow rates, especially in the 1st dimension, at the expense of peak capacity to achieve sensitivity goals
 - Focus on maximizing peak capacity in the 2nd dimension
 - Formic acid is the modifier of choice for the best sensitivity in the 2nd dimension

- Waters Chemistry Organization Synthesis Group
 - Kevin Wyndham
 - Dan Walsh
 - Christina Wong
 - Jim Cook