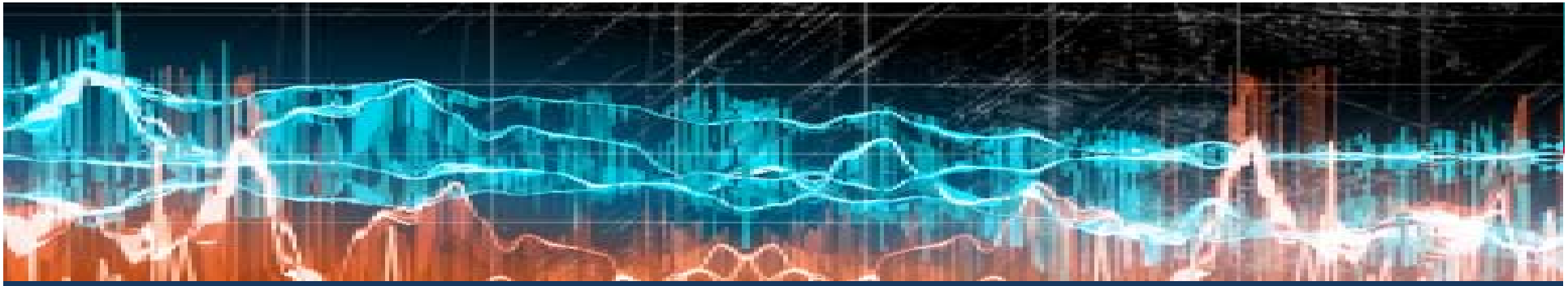
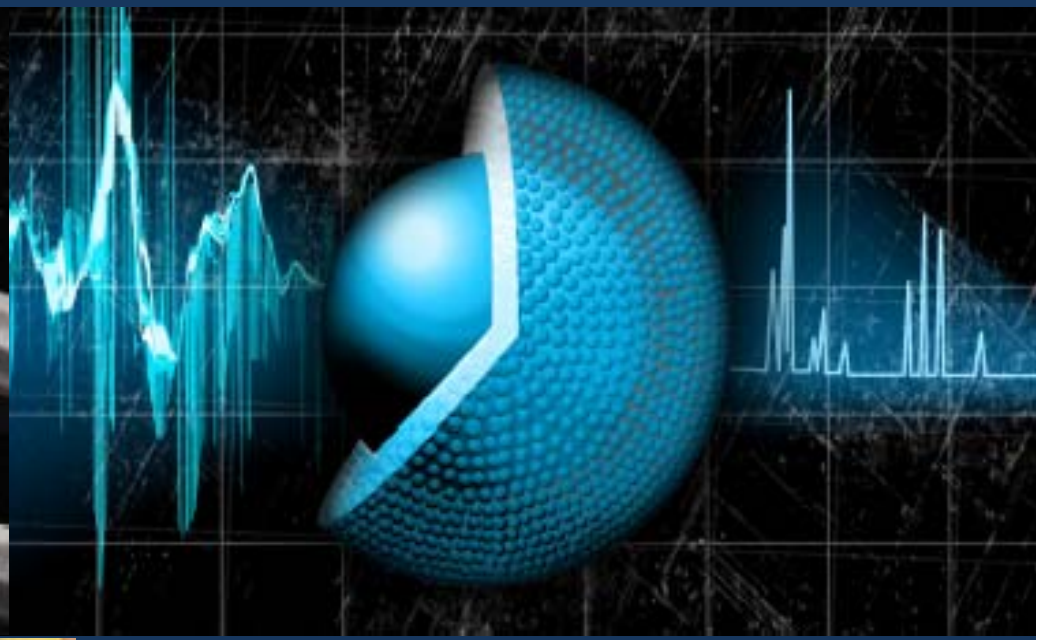
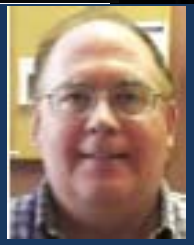


Exploiting pH as Part of Your RPLC Method Development Strategy



Presented By:
Thomas Waeghe, Ph.D.
Senior Scientist
MAC-MOD Analytical



Sponsored By:

LC GC north america

ACE

macmod

Key Learning Objectives

- **Review the importance of selectivity on resolution in reversed-phase separations and learn which LC parameters affect selectivity most**
- **Discover the features and advantages of the ACE solid-core columns with their broad pH range for UHPLC/UPLC and HPLC separations**
- **Learn how to use pH, the most powerful parameter for ionizable analytes, and other parameters as part of an effective RPLC method development strategy**
- **A discussion of how to incorporate the various parameters into a method development strategy and an opportunity for questions will follow.**
- **This webcast will also provide chromatographers and analysts with information and guidance regarding method development strategies and an opportunity for questions about how these new solid-core products can benefit them.**

Outline of Presentation

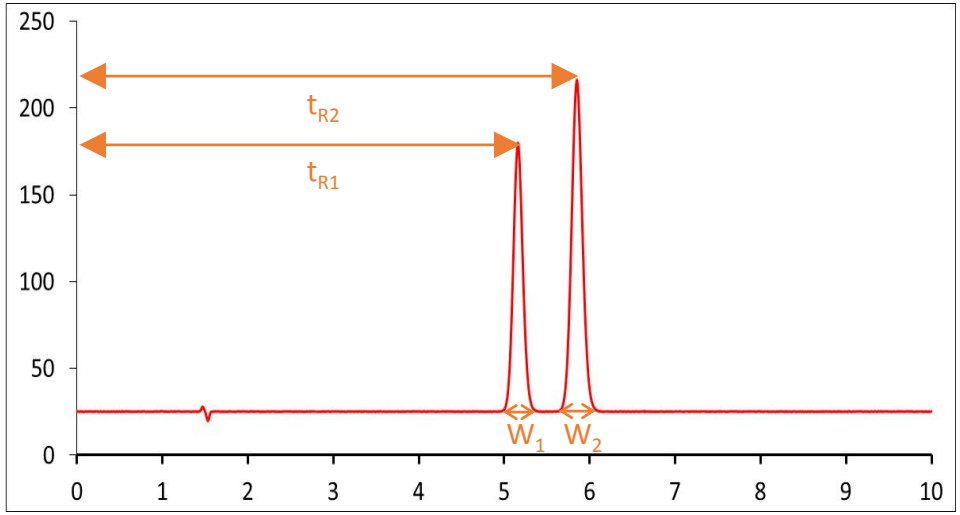
- Resolution and Selectivity
- Key Parameters Affecting Selectivity
- Parameter prioritization ala Snyder, Dolan and Carr
- Method development strategies: screening and optimization
- UltraCore SuperC18 and SuperPhenylHexyl
- What is EBT?
- Orthogonality of UltraCore phases and conditions
- Method development strategy with UltraCore phases
- Examples from Pittcon 2015
  - Appetite Suppressants
  - Drugs of Abuse
- UltraCore Phases with LC-MS
- Summary
- Questions and Answers

Resolution

- **Resolving analytes is the principal goal for most tasks.**

- **Chromatographic resolution is a relationship with retention and peak width:**

$$R_s = \frac{2\left(t_{R2} - t_{R1}\right)}{\left(W_1 + W_2\right)}$$



- **A resolution value of 1.5 indicates baseline separation although most analysts aim for at least 1.7 – 2.0.**

Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

The Resolution Equation

- **From a practical perspective we need a way to understand how changes in conditions affect separation.**

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

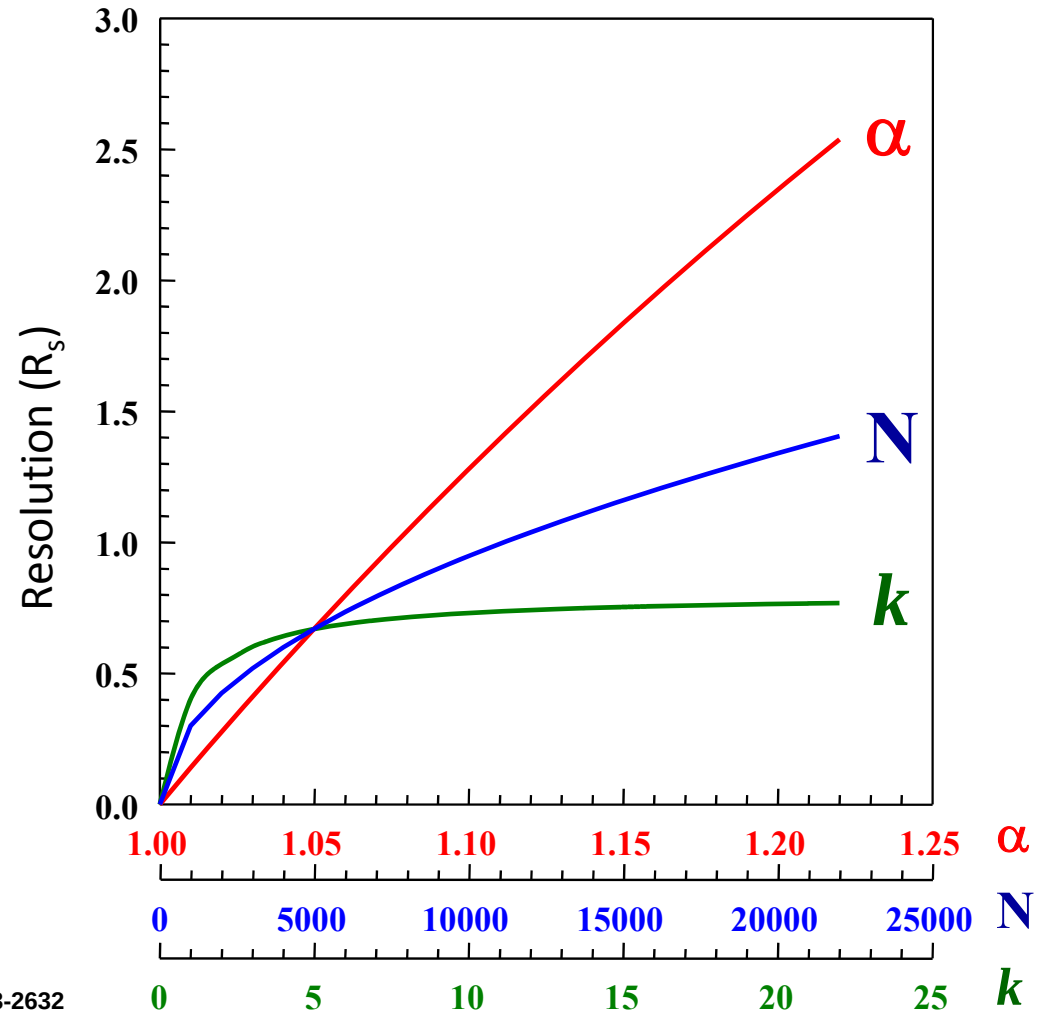
This brings together resolution, efficiency, selectivity and retention and is very useful in method development

Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

The Resolution Equation (2)

Efficiency Selectivity Retention

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



Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Which Factors[1] Affect Selectivity (α) Most?

Isocratic Separations

- Column stationary phase type
- pH (ionisable analytes only)
- Organic modifier type
- % Organic modifier
- Buffer choice
- Column temperature
- Buffer concentration

Limited pH range for many solid core columns (2-8)

MOST Influence

LEAST Influence

Gradient Separations

- All parameters for isocratic separations PLUS
- Gradient steepness ($b = 1/k^*$)
- k^* is f (t_G, F, V_M, $\Delta\Phi$, MW)

$$\frac{1}{1.15 \times b} = k^* = \frac{87 \times t_G \times F}{\Delta\Phi \times V_M \times S}$$

$$S = 0.48 \times MW^{0.44}$$

- V_M is function of column length, ID and porosity
- Ratio of gradient volume to column volume (dwell time)

[1] Adapted from 'Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & sons

Most Powerful Factors Affecting Selectivity (per Snyder, Dolan, Carr)

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

Relative Impact of Different Changes in RPLC Parameters on Selectivity[1]

Parameter	Change in Parameter	Maximum $\lvert\delta \log \alpha\rvert$
pH	5 pH units	0.70
Organic Modifier	$CH_3CN \Leftrightarrow CH_3OH$	0.20
Gradient Time (t_G)	10-fold	0.20
Orthogonal Column	$\Delta F_s \geq$ ~65	0.19
% Organic Modifier	10% (v/v)	0.08
Column Temperature	20 °C	0.07
Buffer Concentration	2-fold	0.02

For R_s = 1.5

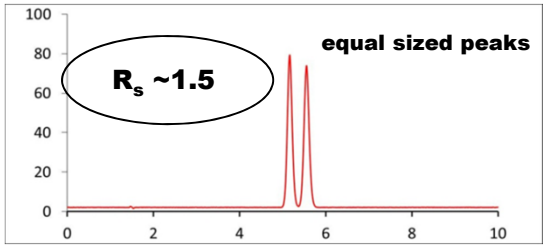
N = 10,000 (2.1 x 50 mm UHPLC)

k ≥ 1

$(\alpha - 1)$ = 0.12 and α = 1.12

log α = 0.05

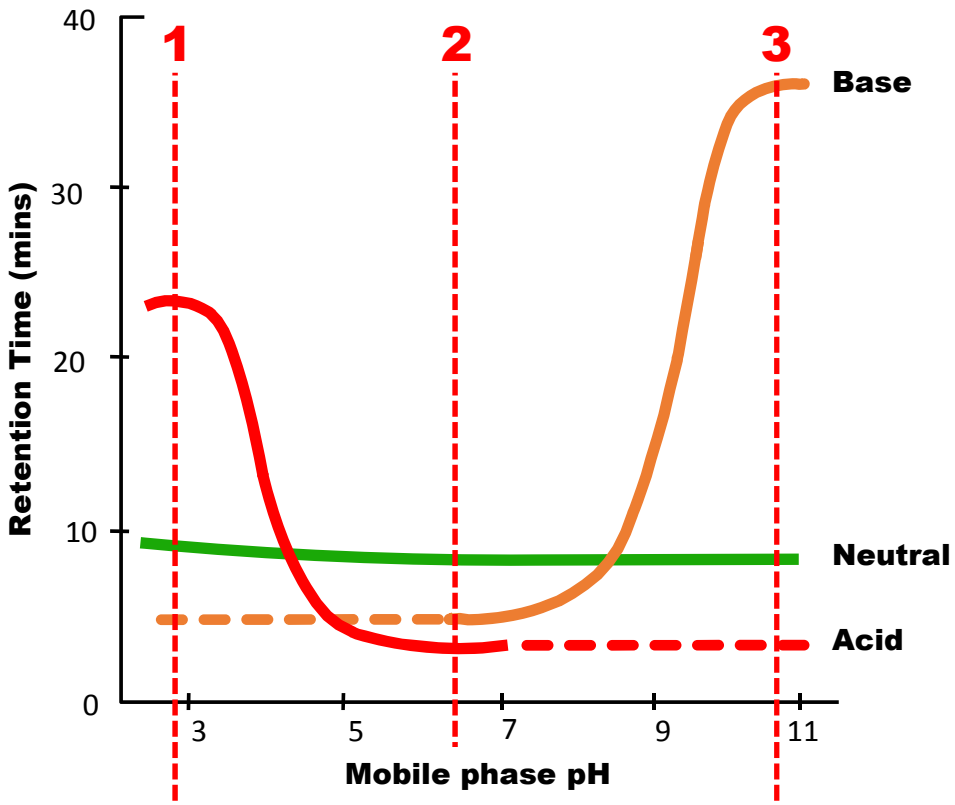
Snyder proposed $\lvert\delta \log \alpha\rvert_{avg} \geq 0.10$

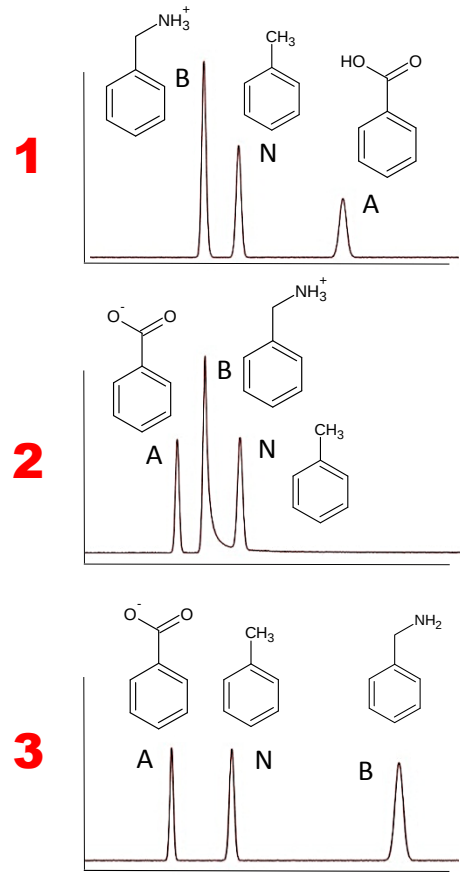


Effects are magnified as square root of sum of the squares when changed together

Exploring Selectivity: Eluent pH

- **Eluent pH effects can be large…and multimodal.**





- **Eluent pH is Powerful For Selectivity and Retention**

Different Strategies of Varying Complexity for Systematic Method Development

Increasing Sample Complexity

Screening 1st

- 1 Column Phase
- 1 Temperature
- 1 pH
- 2 Organic modifiers
- 1 Gradient time

- 1 Column Phase
- 1 Temperature
- 2 pHs
- 1 Organic modifier
- 1 Gradient time

- 1 Column Phase
- 1 Temperature
- 2 pHs
- 2 Organic modifiers
- 1 Gradient time

- Multiple Phases
- 1 Temperature
- 2–3 pHs
- 2 Organic modifiers
- 1 gradient time

Optimization 2nd

- 1 Column Phase
- 1 Temperature
- 1 pH
- 1 Organic modifier
- 2–3 Gradient times

- 1 Column Phase
- 1 Temperature
- 1 pH
- 2 Organic modifiers
- 2–3 Gradient times

- 1 Column Phase
- 1 Temperature
- 2–3 pHs
- 1 Organic modifier
- 2–3 Gradient times

- 1 Column
- 2–3 Temperatures
- 1 pH
- 2 Organic modifiers + 1:1 blend of each
- 2–3 gradient times

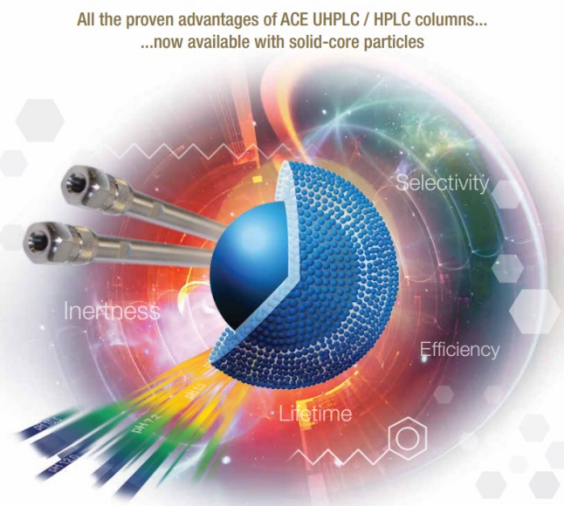
Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

ACE UltraCore™ Solid-Core Columns

ACE UltraCore 2.5 µm:

Total particle diameter = 2.5 µm

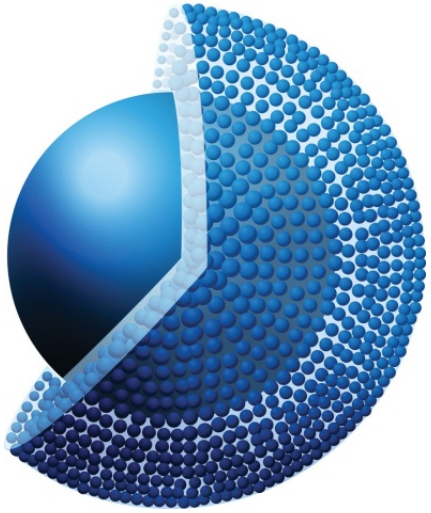
Shell thickness = 0.45 µm



ACE UltraCore 5 µm:

Total particle diameter = 5 µm

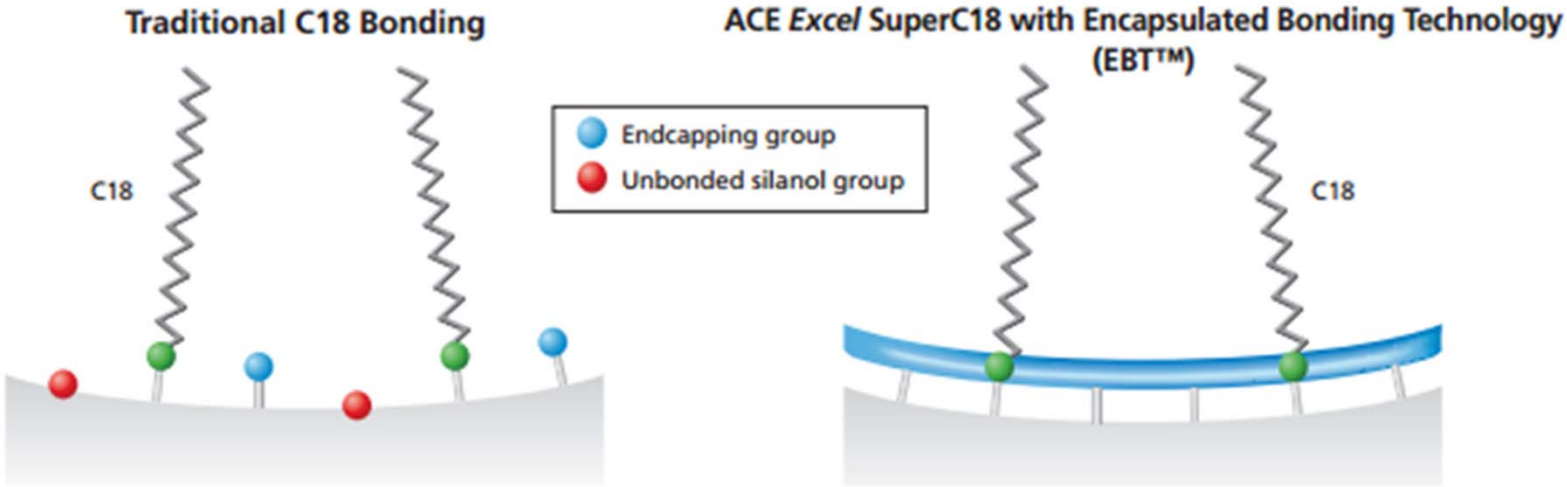
Shell thickness = 0.7 µm



UltraCore SuperC18 and SuperPhenylHexyl Columns

- **Alternate selectivities:**
  - **hydrophobic and π-π interactions**
- **stable to 1000 bar (14,500 psi)**
- **2 µm frits for improved ruggedness and uptime**
- **20,000 column volume lifetime minimum (≤ 40 °C, pH 8–11.0)**

Advantages of Encapsulated Bonding Technology (EBT™)



Encapsulated Bonding Technology

- **Uniquely developed for ACE UltraCore SuperC18 and UltraCore SuperPhenylHexyl***
- **EBT bonding and endcapping**
  - **dramatically higher ligand coverage**
  - **effectively eliminates the negative effects of unbonded silanol groups**

*and totally porous ACE Excel SuperC18)

Benefits of EBT

- **Inertness—superb peak shape**
  - **for bases, acids, and neutrals (pH 1.5–11.0)**
- **Stability**
  - **silica protected from eluent at mid and high pH**
  - **use with volatile buffers for max. stability, ideal for LC and LC-MS (NH_4OH, NH_4OAc, etc.)**
- **Versatility**
  - **no memory effects from switching among eluents at different pHs**

Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

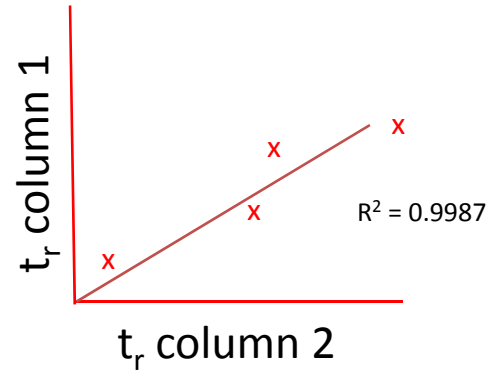
Measuring Orthogonality: Neue Selectivity Factor, S

Uwe Neue Selectivity Descriptor, S, as measure of orthogonality

$$S = 100 \times \sqrt{(1 - R^2)}$$

For collection of representative, diverse analytes,

Plot analyte gradient retention times (obtained under the same conditions) for different stationary phases, or you can plot gradient RTs for different combinations of analysis conditions and columns) versus each other.

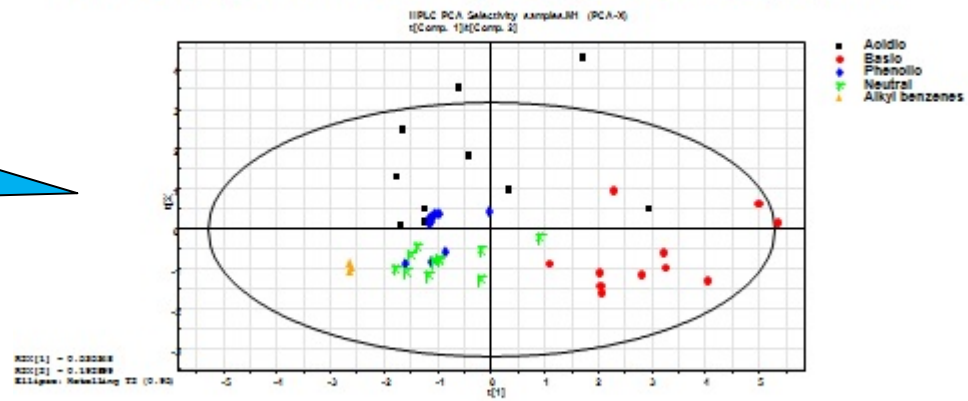


R^2 is coefficient of determination (a measure of how close the data are to the fitted regression line.

Principal Component Analysis

Important to assess orthogonality using diverse set of analytes

- A mixture of 50 acidic, basic & neutral small molecule analytes were used that represent a broad range of physico-chemical properties.



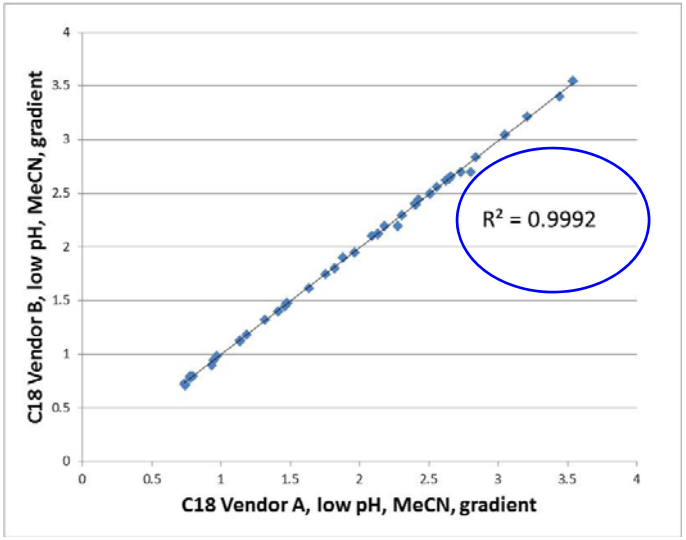
- Challenging stationary phases with these probes gives an indication of chromatographic selectivity for each phase and between phases.

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

Low Selectivity Differences

- **Selectivity values 0 to < 10**
- **Little difference in analyte retention on the 2 phases therefore little scatter seen. High correlation.**

C18 Columns
- **Vendor A vs. Vendor B**
- **CH_3CN**
- **Low pH**



Selectivity = 3

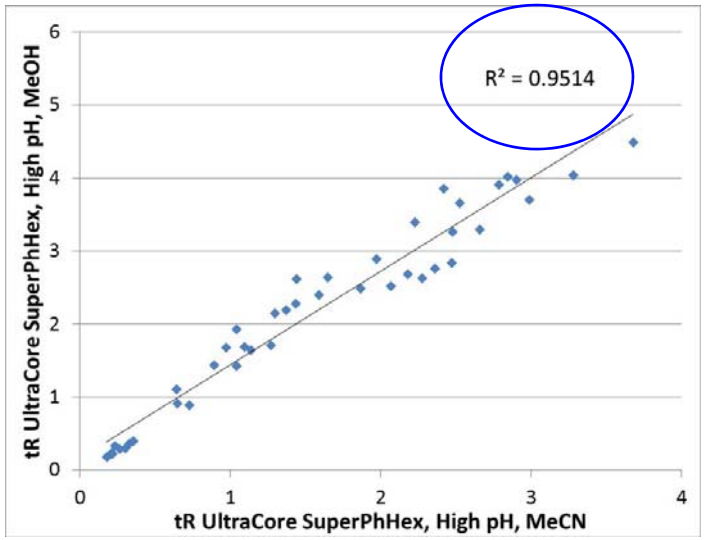
- **Typically observed when comparing same phase type (i.e., same mechanism) from different vendors.**

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

Moderate Selectivity Differences

- **Selectivity values > 10 and < 30.**
- **Changes in analyte retention between the 2 phases observed. may include elution order changes. Scatter observed and correlation decreases.**

UltraCore SuperPhenHexyl
- **CH_3CN vs. CH_3OH**
- **high pH**



Selectivity = 22

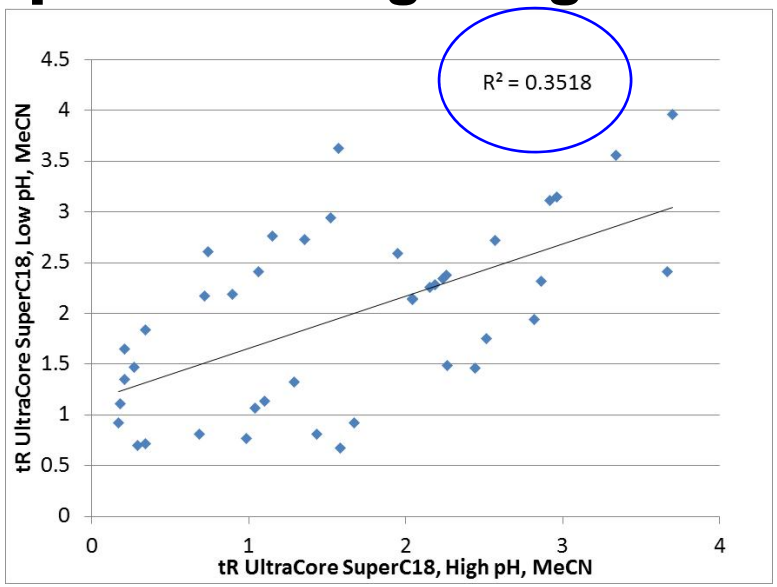
- **Typically observed when changing a variable, e.g., <u>comparing different phases</u> OR <u>changing solvent</u>.**

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

High Selectivity Differences

- **Selectivity values > 30.**
- **Significant changes in analyte retention and elution order between the 2 phases. Large degree of scatter observed.**

UltraCore SuperC18
- **CH3CN**
- **low pH vs. high pH**



Selectivity, S = 81

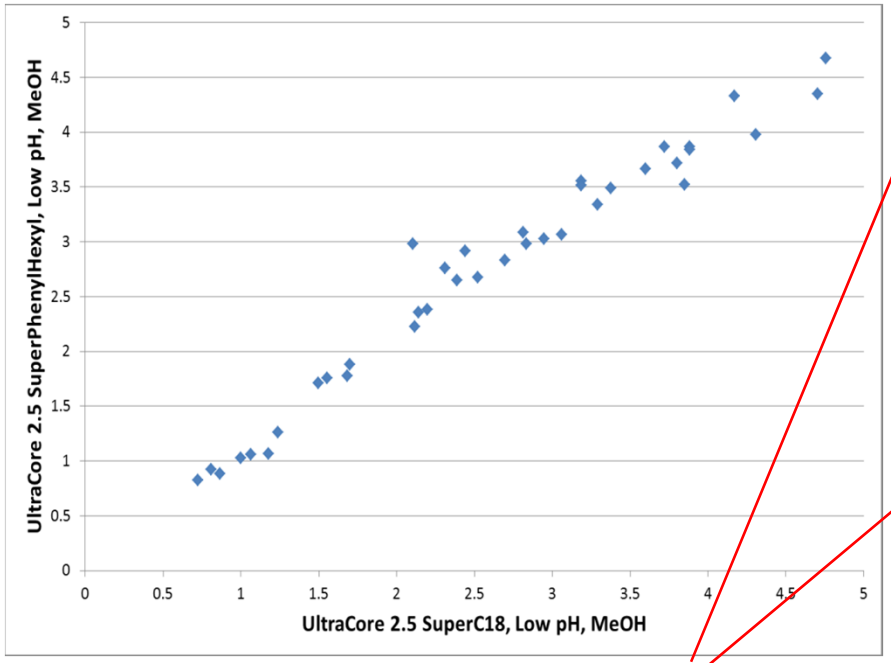
- **Typically observed when <u>changing multiple variables</u>.**
- **<u>Or changing eluent pH</u> (e.g., low vs high) with ionizable analytes in the sample. ← Column pH range limitations.**

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

Selectivity Plot: Exploring The Effect Of Solid Core Phase

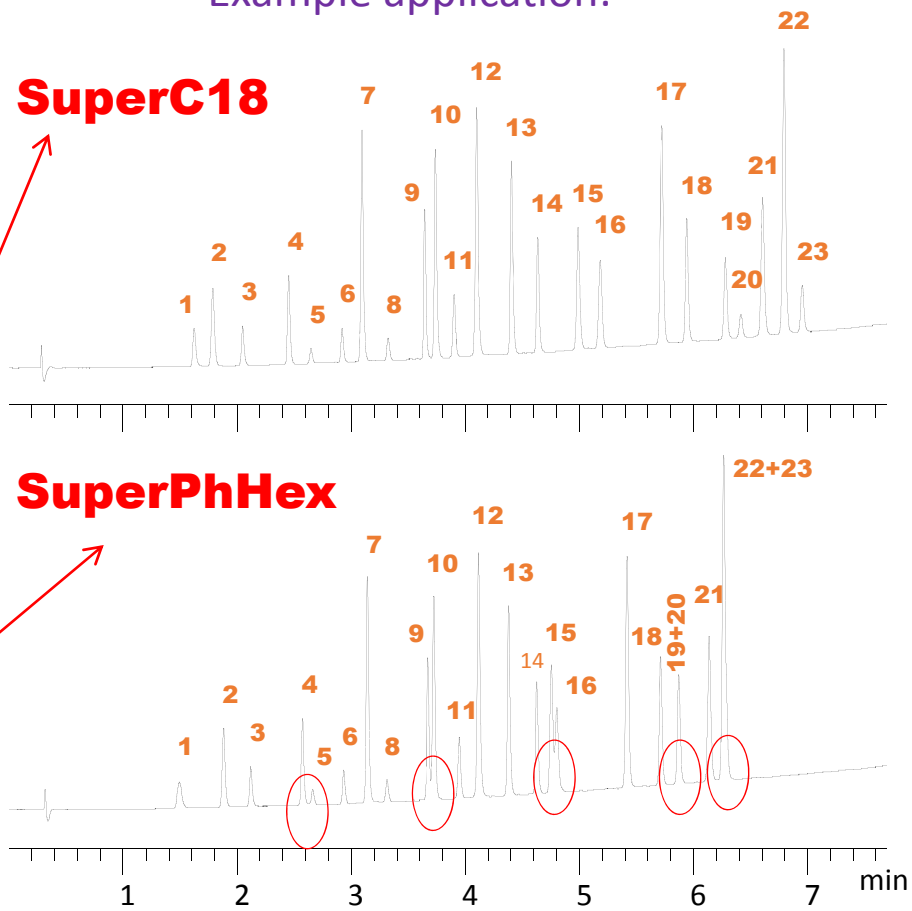
SuperC18, low pH, MeOH vs SuperPhenylHexyl, low pH, MeOH

Changes in peak spacing noted



Selectivity, S = 19

Example application:



50x2.1mm, 2.5µm, gradient analysis, A= 20mM $HCOONH_4$, pH3 (aq), B= 20mM $HCOONH_4$, pH 3 in MeCN/water 9:1 v/v, 3-100%B in 7.5 mins, hold 100%B for 1.5 mins, 40°C, 0.40 mL/min, 254 nm.

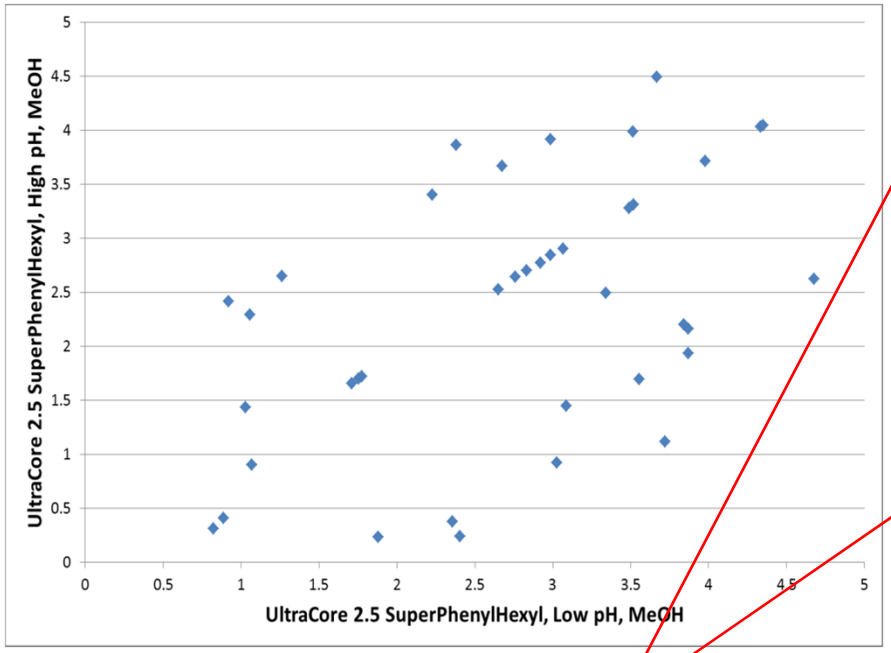
1 amiloride, 2 benzamide, 3 3-hydroxybenzoic acid, 4 vanillin, 5 2-hydroxybenzoic acid, 6 benzoic acid, 7 methyl paraben, 8 p-cresol, 9 cortisone, 10 ethyl paraben, 11 dimethylphthalate, 12 piroxicam, 13 hydro cortisone-21-acetate, 14 ketoprofen, 15 ethyl benzoate, 16 toluene, 17 valerophenone, 18 mefenamic acid, 19 hexanophenone, 20 propylbenzene, 21 phenanthrene, 22 heptanophenone, 23 butylbenzene

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

Selectivity Plot: Exploring Eluent pH With SuperPhenylHexyl

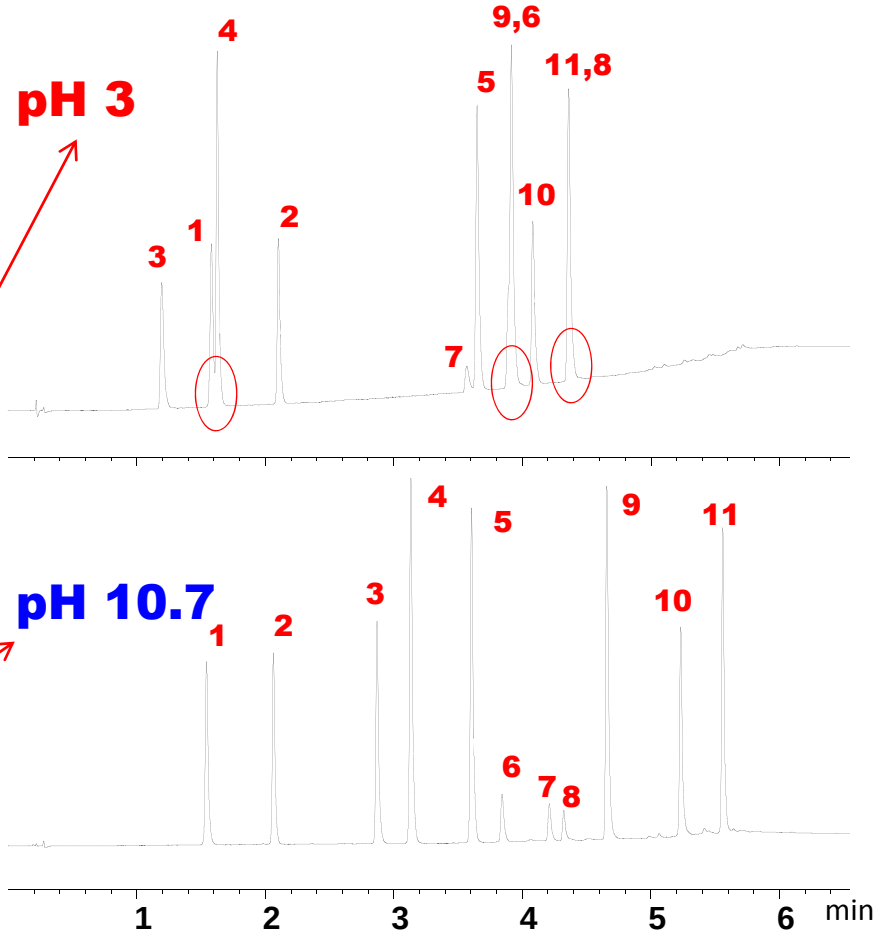
SuperPhenylHexyl, low pH, MeOH vs SuperPhenylHexyl, high pH, MeOH

Significant changes in elution order noted



Selectivity = 83

Example application:

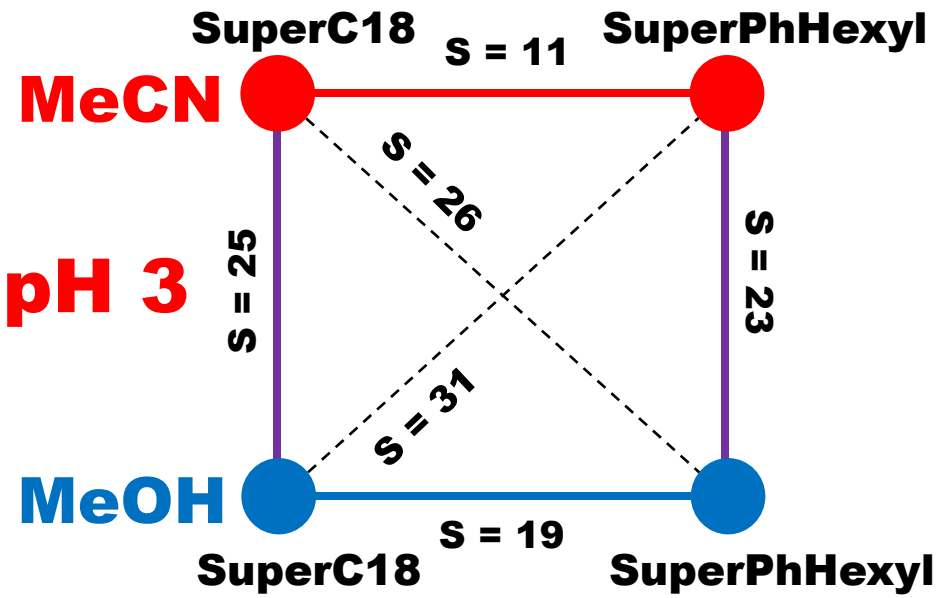


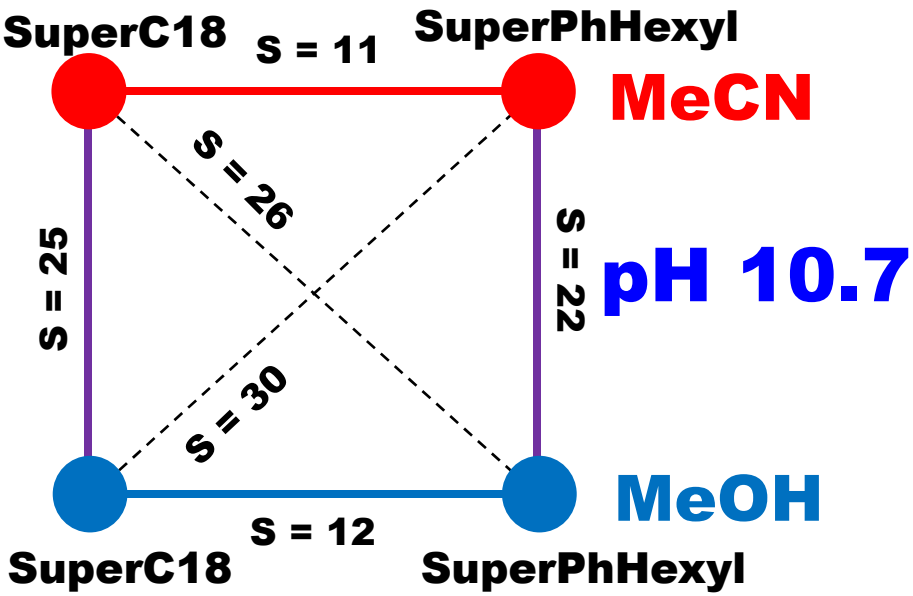
50x2.1mm, 2.5µm, gradient analysis, A1= 10mM $HCOONH_4$, pH3 (aq), B1= 10mM $HCOONH_4$, pH 3 in MeOH/water 9:1 v/v, A2= 0.1% NH_3, pH 10.7 (aq), B2= 0.1% NH_3, pH10.7 in MeOH/water 9:1 v/v, 3-100%B in 5mins, 100%B for 2mins, 40°C, 0.60 mL/min, 254 nm.
1. benzamide, 2 caffeine, 3 procainamide, 4 N-acetylprocainamide, 5 propiophenone, 6 toluene 7 remacemide, 8 ethylbenzene, 9 carvedilol, 10 nortriptyline, 11 clomipramine.

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

UltraCore Selectivity Diagrams: Stationary Phase, Solvent & pH (I)

- **SuperC18 & SuperPhHex, MeOH & MeCN, low or high pH**





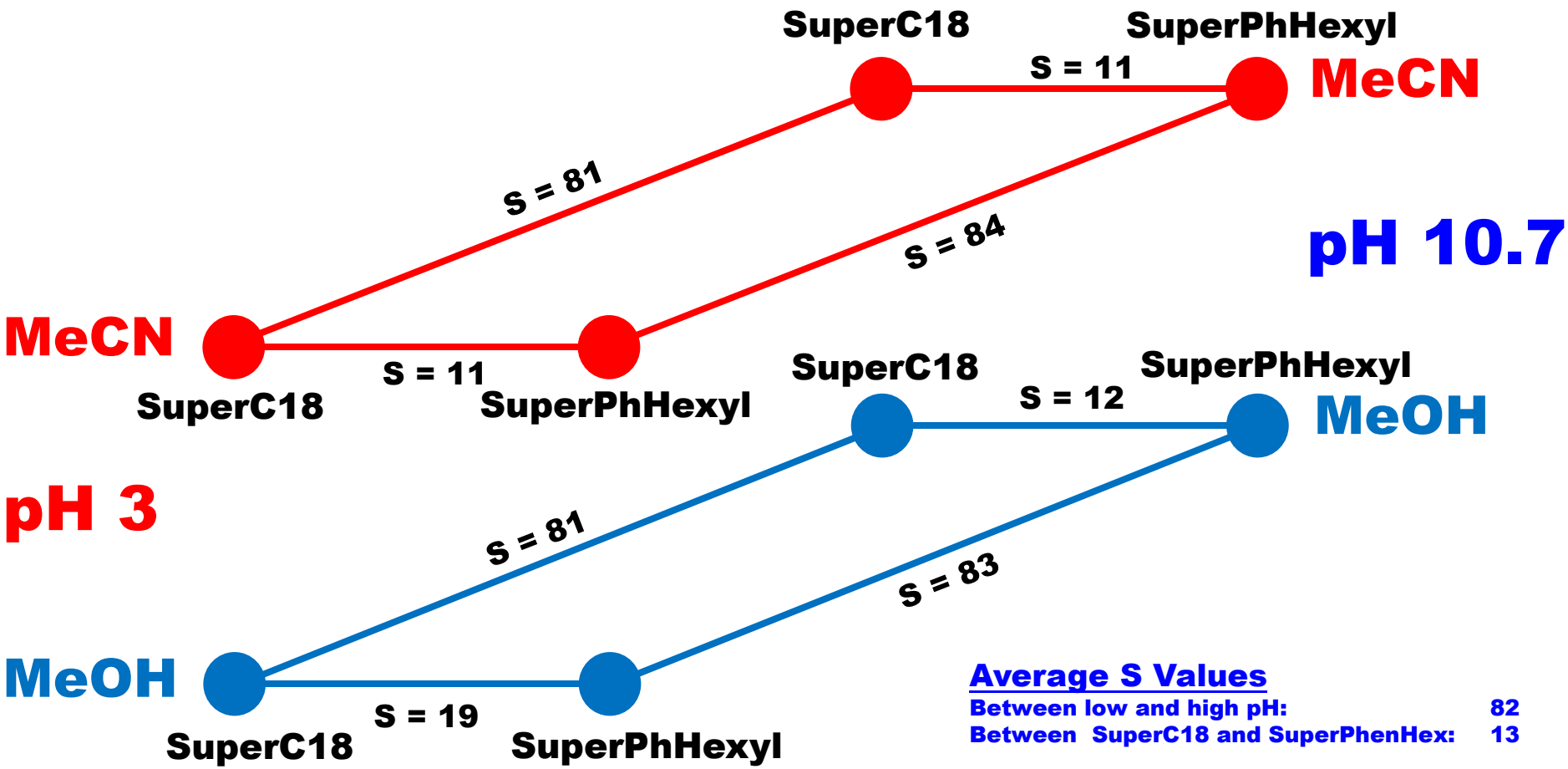
Average S Values

Between CH_3CN and CH_3OH:	24
Between SuperC18 and SuperPhenHex:	13

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

UltraCore Selectivity Diagrams: Phase, Solvent & pH (II)

- **SuperC18 & SuperPhHex, low or high pH, MeOH or MeCN**



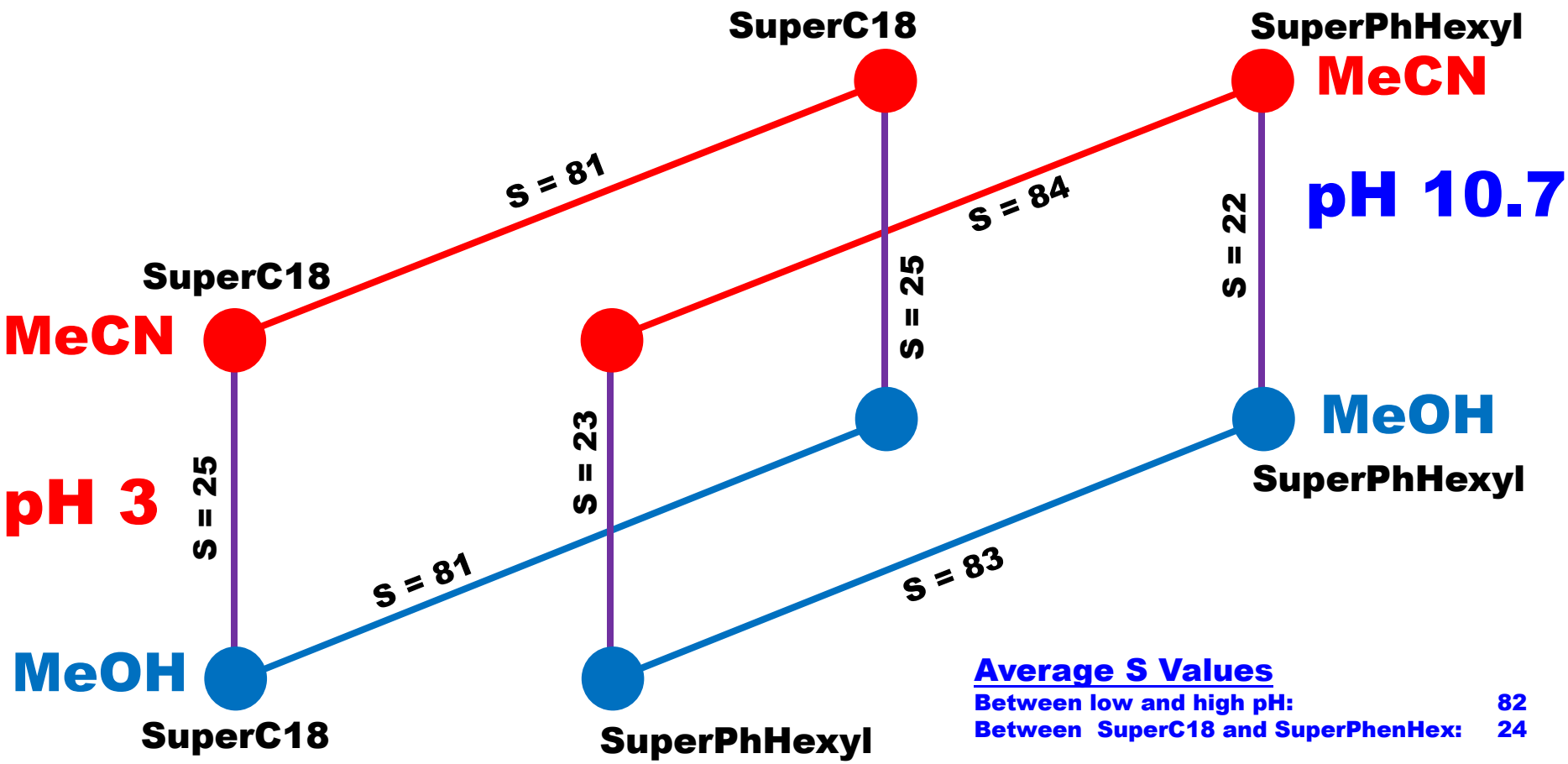
Average S Values

Between low and high pH:	82
Between SuperC18 and SuperPhenHex:	13

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

UltraCore Selectivity Diagrams: Phase, Solvent & pH (III)

- **Low & high pH, MeOH & MeCN, SuperC18 or SuperPhHex**

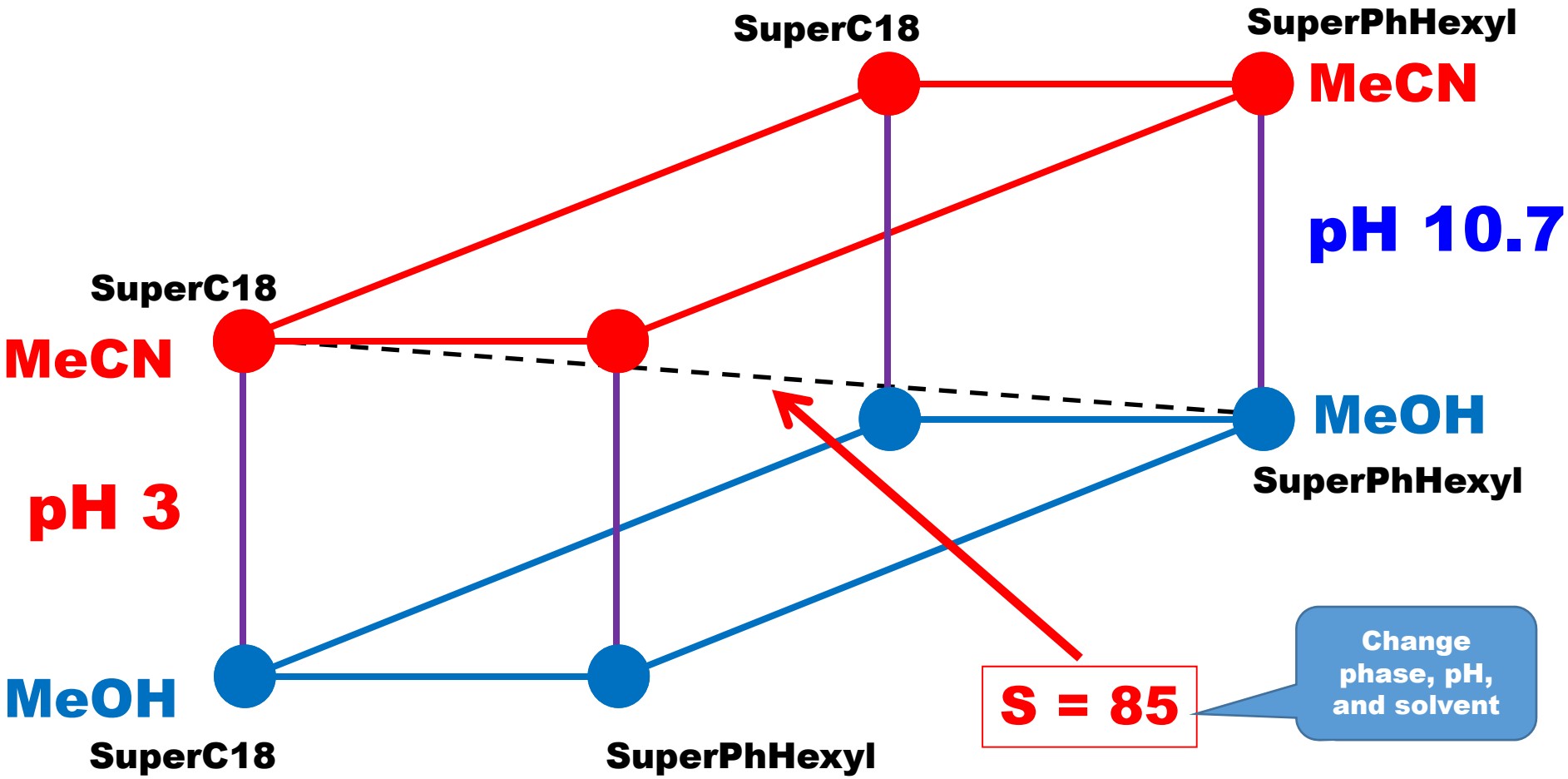


Average S Values

Between low and high pH:	82
Between SuperC18 and SuperPhenHex:	24

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

UltraCore Full Selectivity 'Space'



ACE® UltraCore™ Method Development Platform

2 phases, 2 solvents & 2 eluent pH values to fully explore selectivity

Data courtesy of Dr. Alan McKeown, Advanced Chromatography Technologies

Solid Core Method Development / Screening Workflow

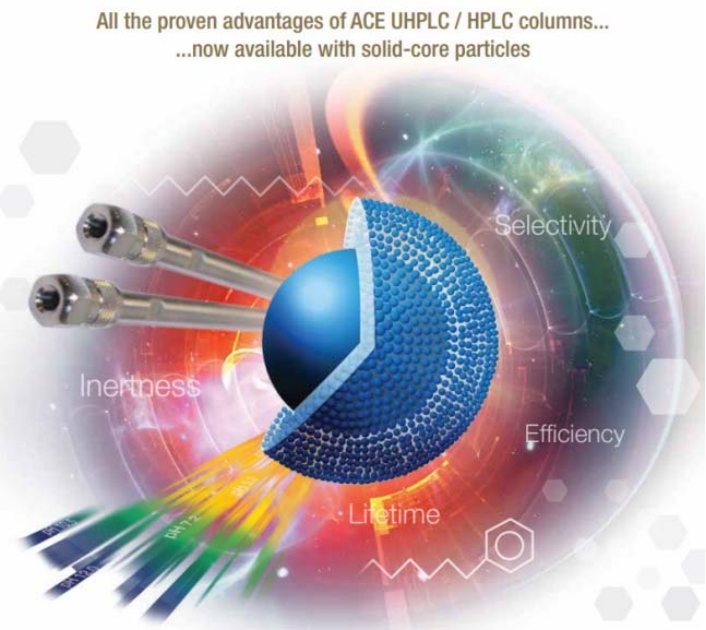
- 2 columns - 2 temperatures - 1 pH - 2 organic modifier - 2 x t_G	- 1 column - 2 temperatures - 1 pH - 2 organic modifiers - 2 x t_G	- ≥ 2 column - 2 temperatures - 2 pH values - 2 organic modifiers - 2 x t_G
SuperC18 SuperPhenylHexyl	MeOH MeCN	pH 3 pH 10.7

Information Rich Data <u>Based on Selectivity</u>

2 column method development / screening approach based on selectivity data

Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

Some Examples from Pittcon 2015



- **Multiple Phases**
- **1 Temperature**
- **2–3 pHs**
- **2 Organic modifiers**
- **1 gradient time**

Objective: Explore performance of ACE UltraCore columns for basic analytes

Parameters

- Stationary phases
  - ACE UltraCore SuperC18 and SuperPhenylHexyl
- Organic modifier
  - CH_3CN, CH_3OH
- pH
  - 2.8, 3.8, 8.2, 9.2, 10.2; 9.7 and 10.7

Instrumentation

- Shimadzu Prominence 600 bar system
- Delay volume ~ 0.4 mL

Samples

- 9 Appetite Suppressants
- 16 Drugs of Abuse

Sample 1: 9 Appetite suppressants

1. methamphetamine
2. amphetamine
3. ephedrine
4. fluoxetine
5. caffeine
6. phentermine
7. fenfluramine
8. chlordiazepoxide
9. phenylpropanolamine

Approach

Columns

- UltraCore SuperC18 and SuperPhenylHexyl, 2.1 x 50 mm, 2.5 μm

Organic modifiers

- ACN
- MeOH

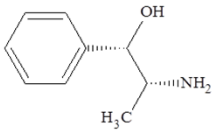
Aqueous buffers

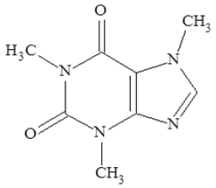
- ammonium formate, pH 2.7
- ammonium acetate/NH_4OH
- pH 8.2, 8.7, 9.2, 10.2, 10.7

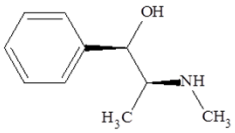
Gradients

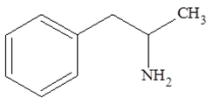
- from 5 to 95% organic
- from 2 to 50, 60, 70 or 95%

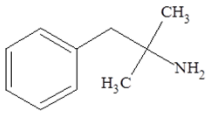
Sample: Appetite Suppressants

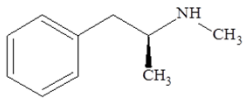

Phenylpropanolamine

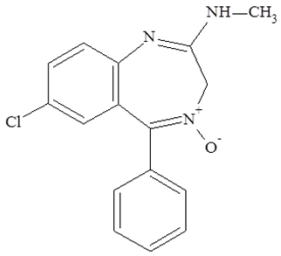

Caffeine

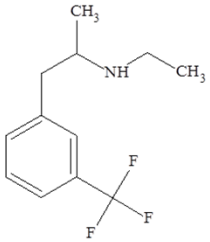

Ephedrine

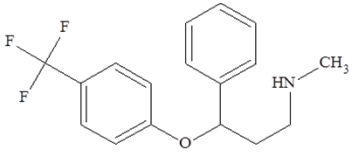

Amphetamine


Phentermine


Methamphetamine


Chlordiazepoxide


Fenfluramine


Fluoxetine

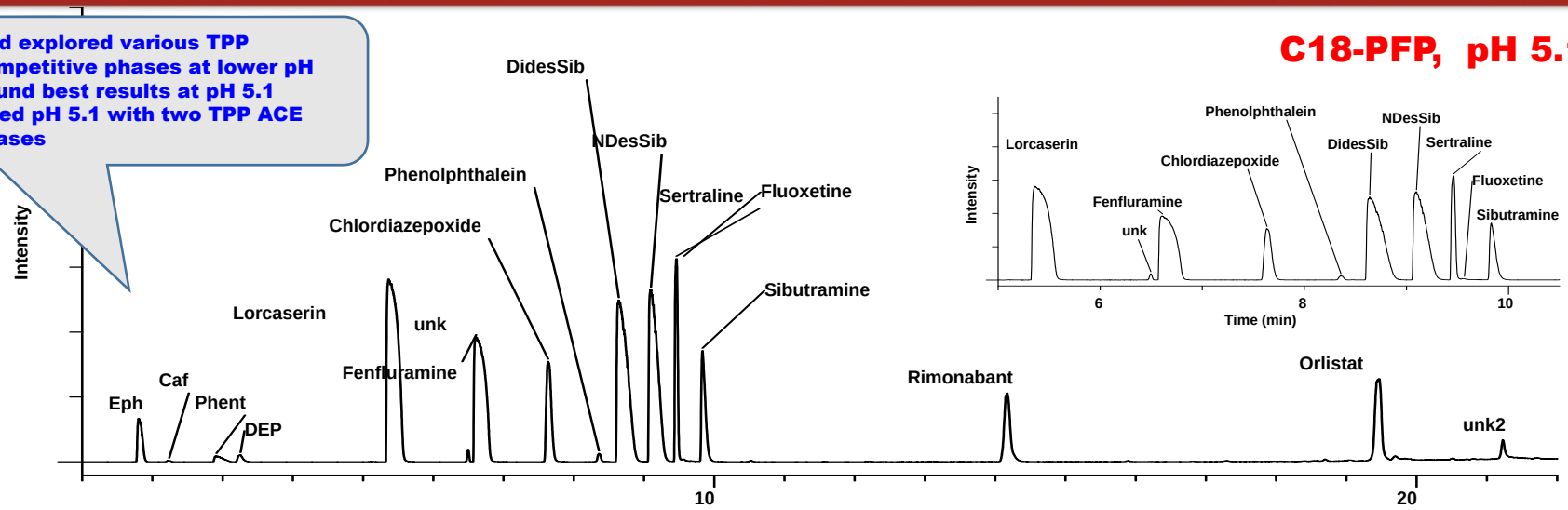
Compound	pKa	log P	pI	RT ACN pH 10.2 2-70% 9.71 min 0.6 mL/min
phenylpropanolamine	9.37	0.89	11.63	2.61
caffeine	14.0	-0.55	none	2.66
ephedrine	9.52	1.32	11.71	3.44
amphetamine	10.10	1.8	none	4.31
phentermine	10.25	2.08	none	4.85
methamphetamine	9.9	2.24	none	4.96
chlordiazepoxide	6.43	3.05	12.47	5.88
fenfluramine	10.22	3.47	none	7.18
fluoxetine	9.8	4.17	none	8.55

Log P, pI, and pKa values were taken from www.chemicalize.org

Customer Example with Totally Porous ACE SuperC18, 2 µm

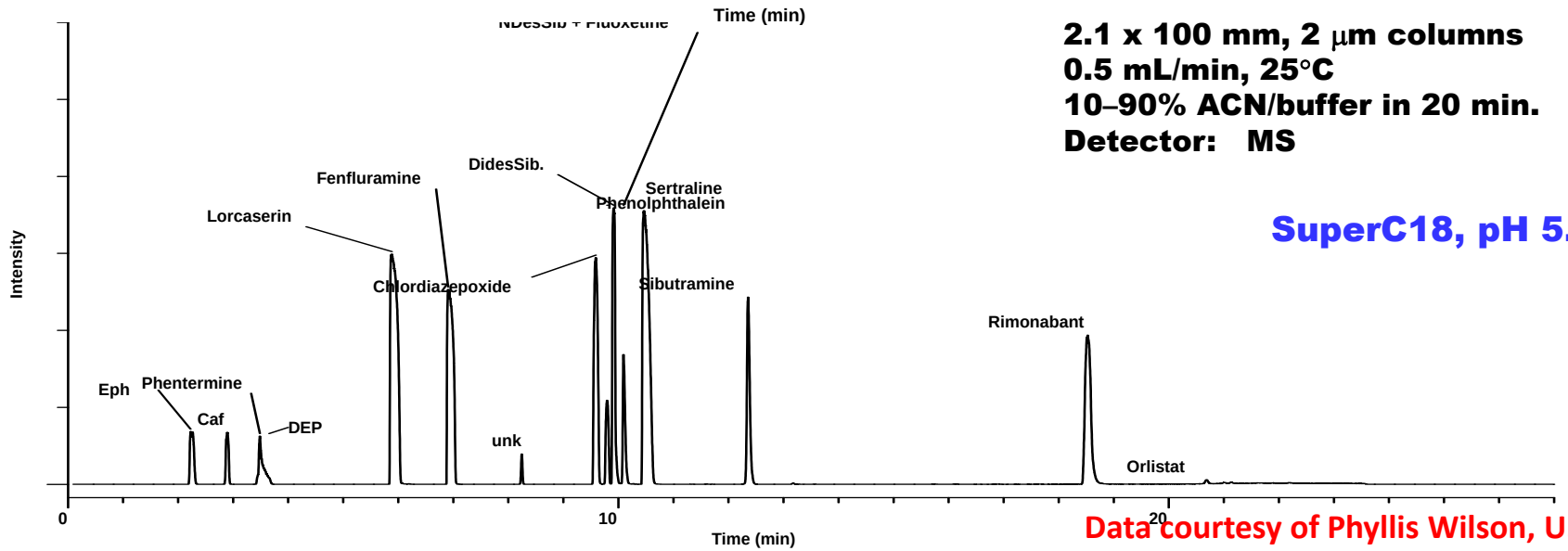
- **Had explored various TPP competitive phases at lower pH**
- **Found best results at pH 5.1**
- **Tried pH 5.1 with two TPP ACE phases**

C18-PFP, pH 5.1



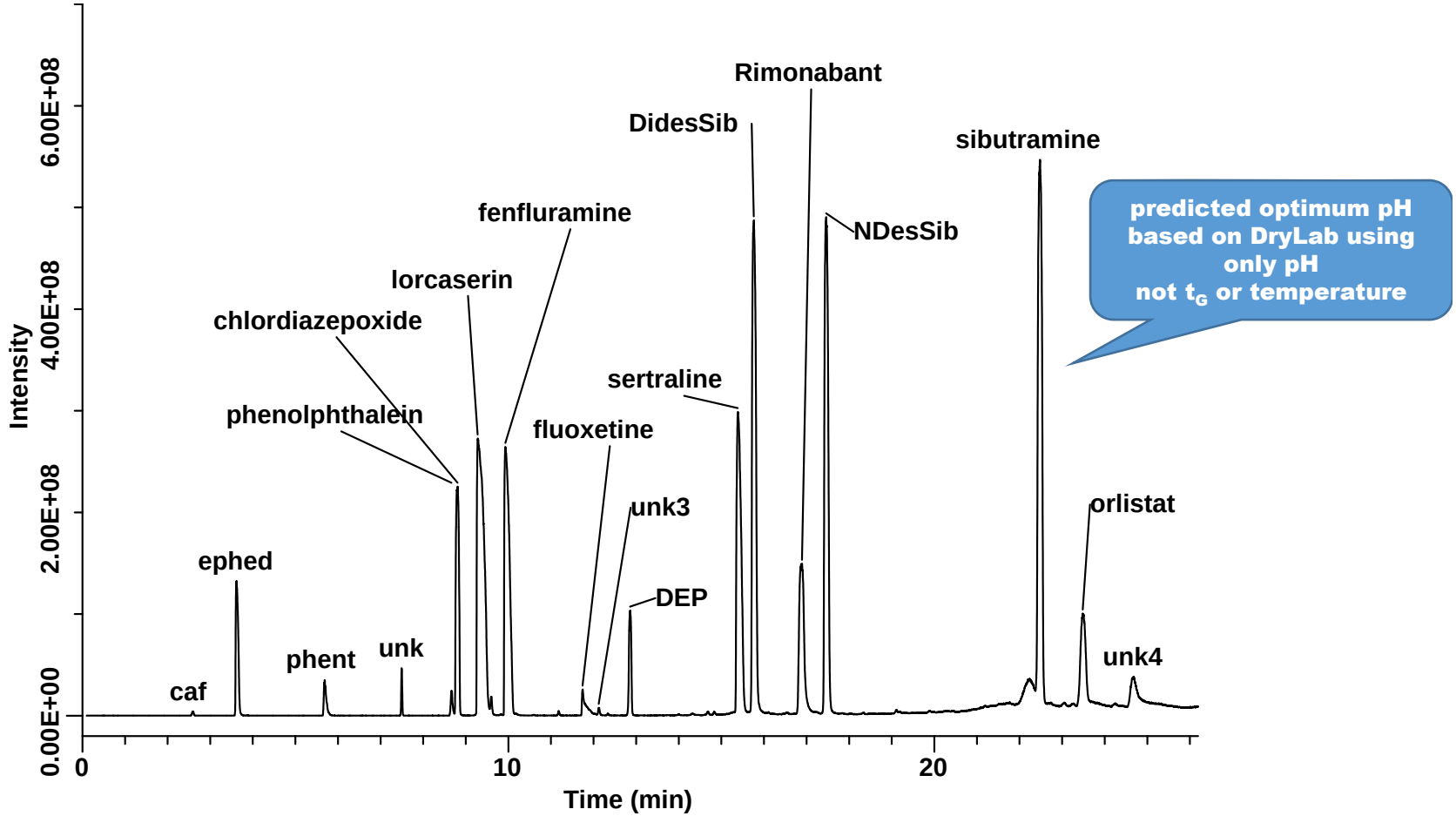
2.1 x 100 mm, 2 µm columns
0.5 mL/min, 25°C
10–90% ACN/buffer in 20 min.
Detector: MS

SuperC18, pH 5.1



Data courtesy of Phyllis Wilson, U.S. FDA

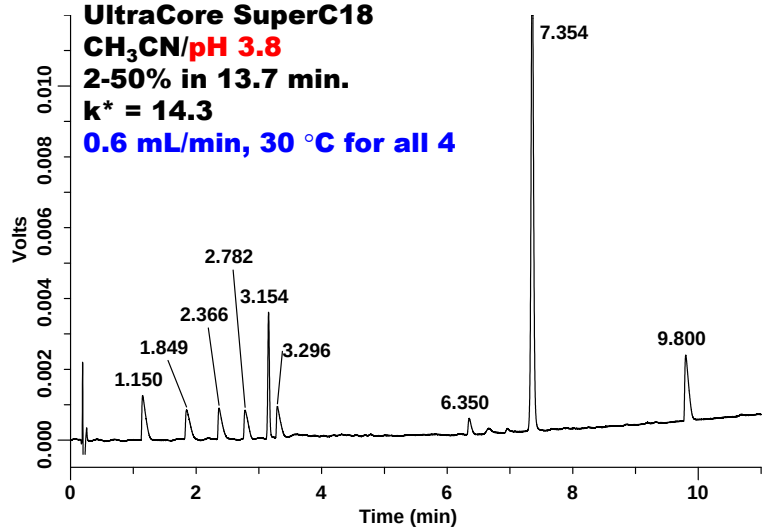
More Complex Appetite Suppressant Mixture: optimum pH from DryLab®

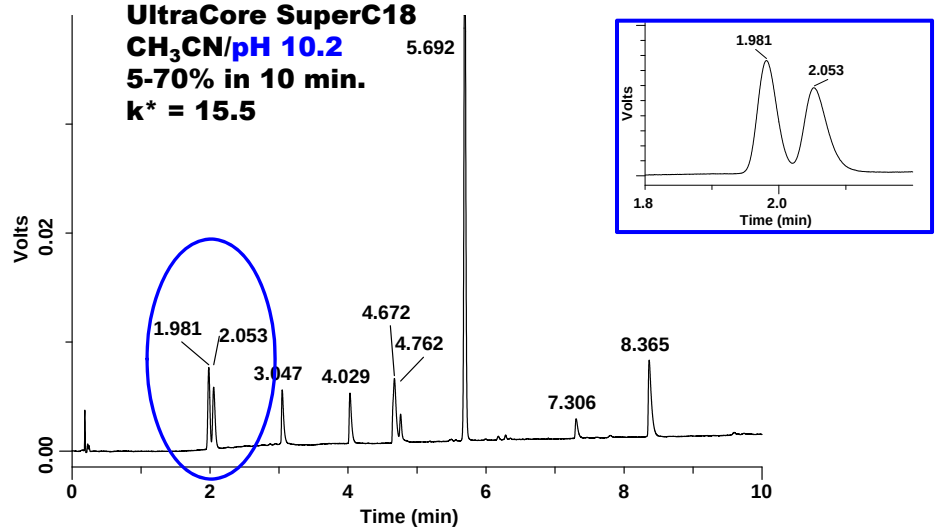


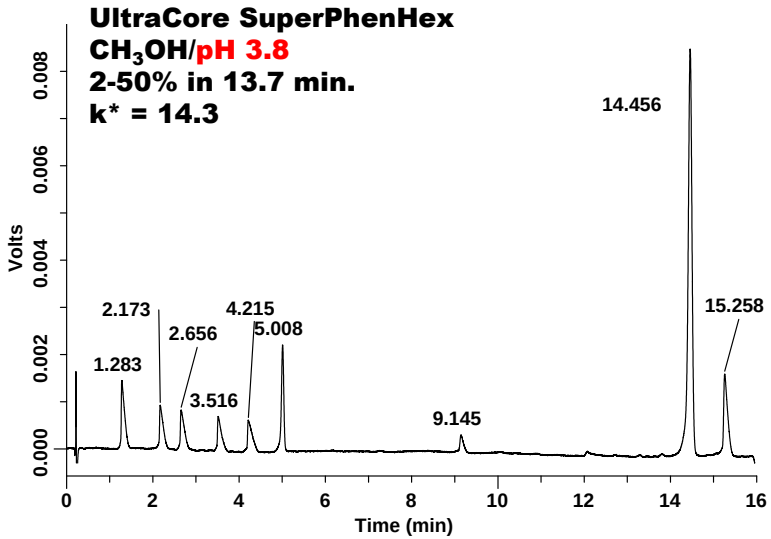
2.1 x 100 mm, 2 µm ACE Excel SuperC18
0.5 mL/min, 25°C, 10–90% CH3CN/20 mM pH 9.3 buffer in 20 min.
MS Detection

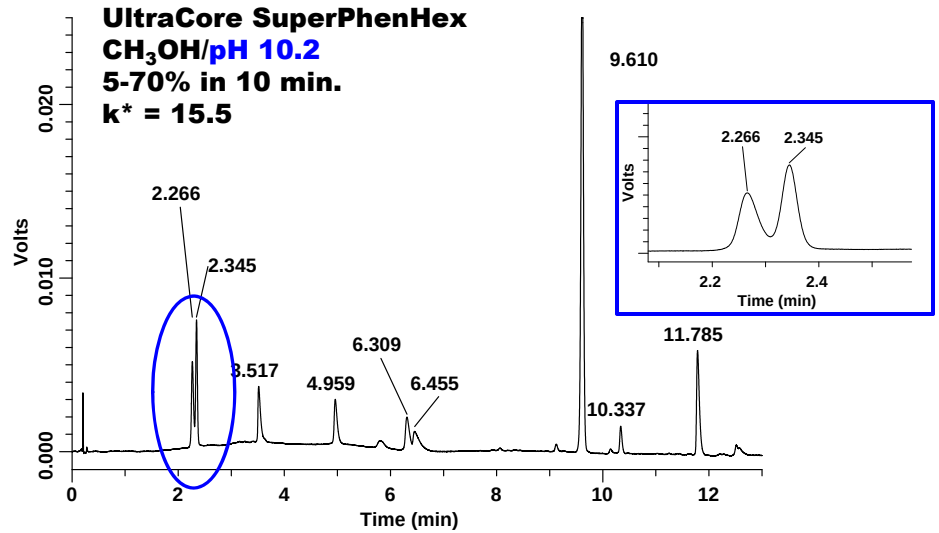
Data courtesy of Phyllis Wilson, U.S. FDA

Examples with CH_3CN and CH_3OH at pH 3.8 and pH 10.2 on both phases

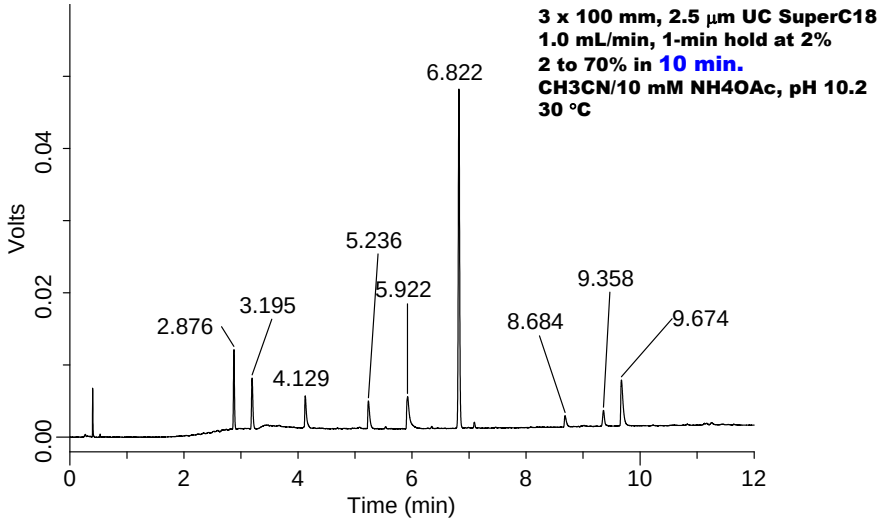


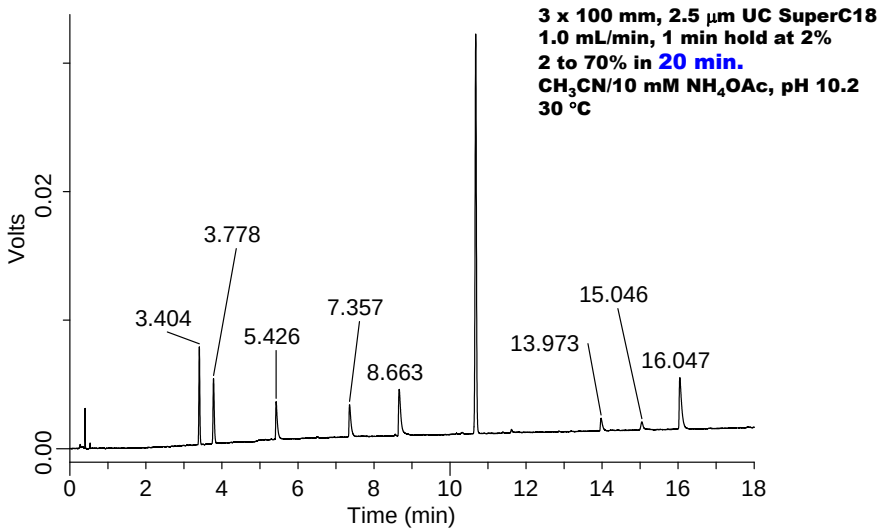


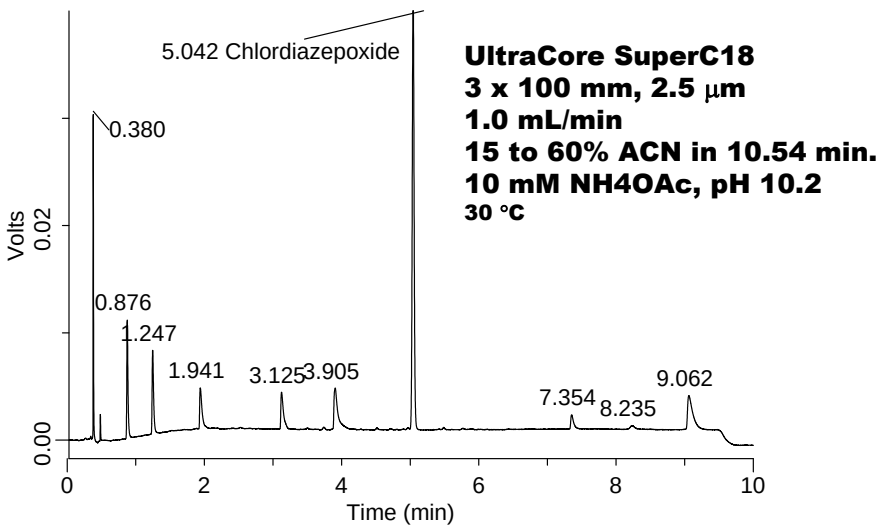




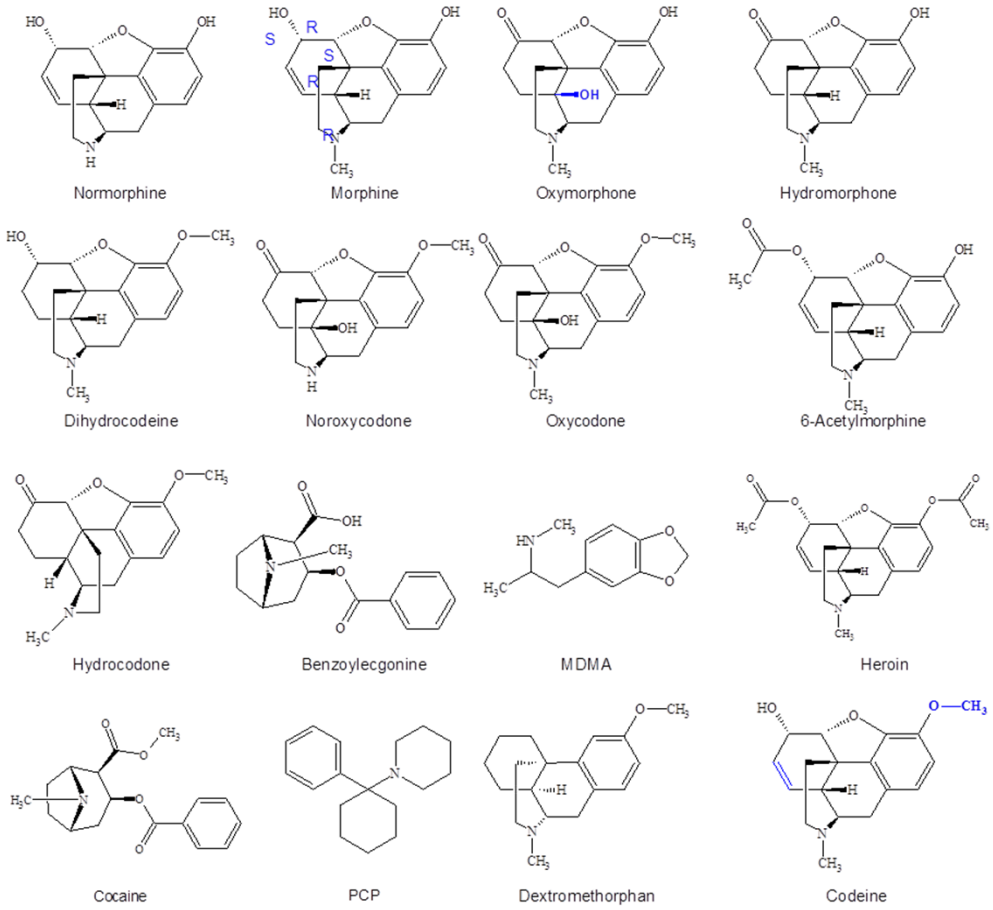
9 Appetite Suppressants: UltraCore SuperC18 DryLab Optimized Linear Gradient







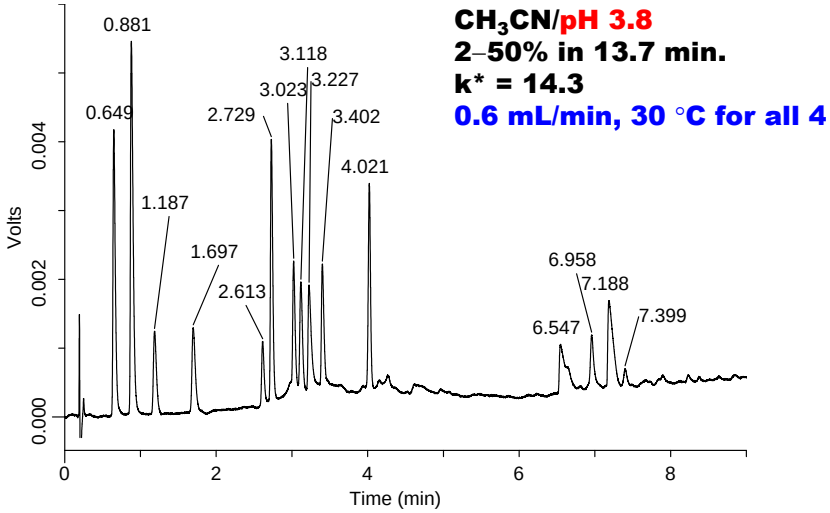
Sample: 16 Drugs of Abuse

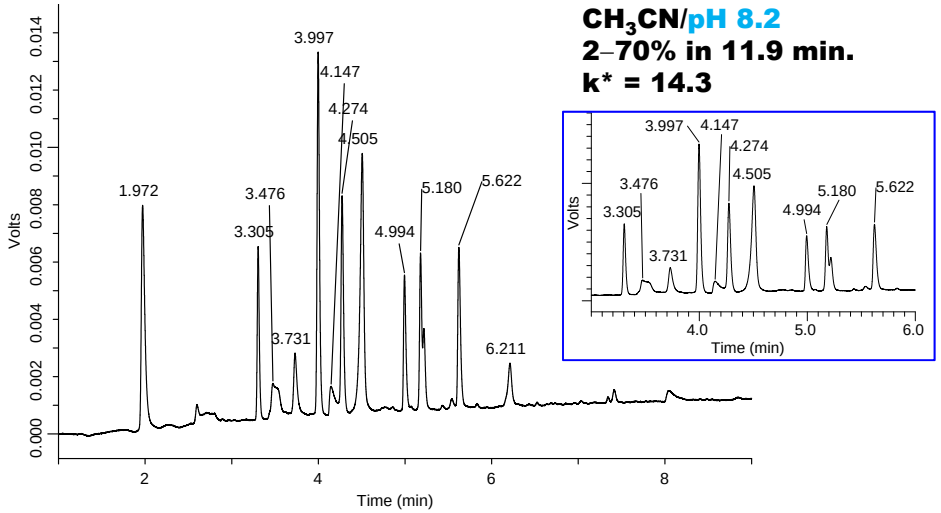


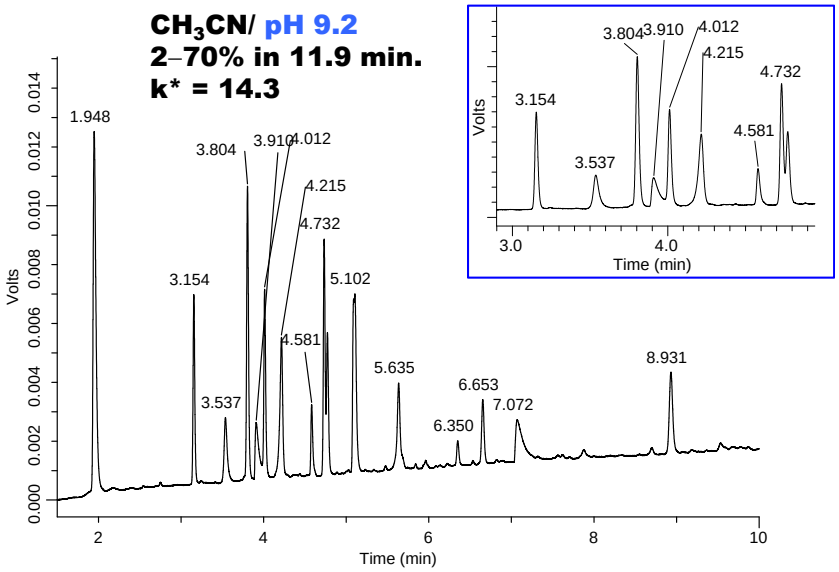
MW	Compound Name	pKa	log P	pI	RT SPH 2-50% pH 2.8
271.31	Normorphine	10.12	0.26	10.12	0.707
285.34	Morphine	9.66	0.90	9.66	1.051
301.34	Oxymorphone	9.14	0.78	9.14	1.405
285.34	Hydromorphone	9.34	1.62	9.34	1.789
301.38	Dihydrocodeine	9.33	1.55	11.74	2.432
299.36	Codeine	9.19	1.34	11.49	2.519
301.34	Noroxycodone	9.14	0.78	9.14	2.663
315.36	Oxycodone	8.21	1.03	10.89	2.807
327.37	6-Acetylmorphine	8.42	1.09	9.25	2.874
299.36	Hydrocodone	8.61	1.96	13.30	2.989
289.33	Benzoylecgonine	9.54	-0.59	6.49	3.268
193.24	MDMA	10.14	1.86	NA	3.518
369.41	Heroin	9.10	1.55	NA	3.966
303.35	Cocaine	8.85	2.28	NA	4.051
243.39	Phencyclidine (PCP)	10.64	4.49	NA	4.616
271.40	Dextromethorphan	9.85	3.49	NA	4.793

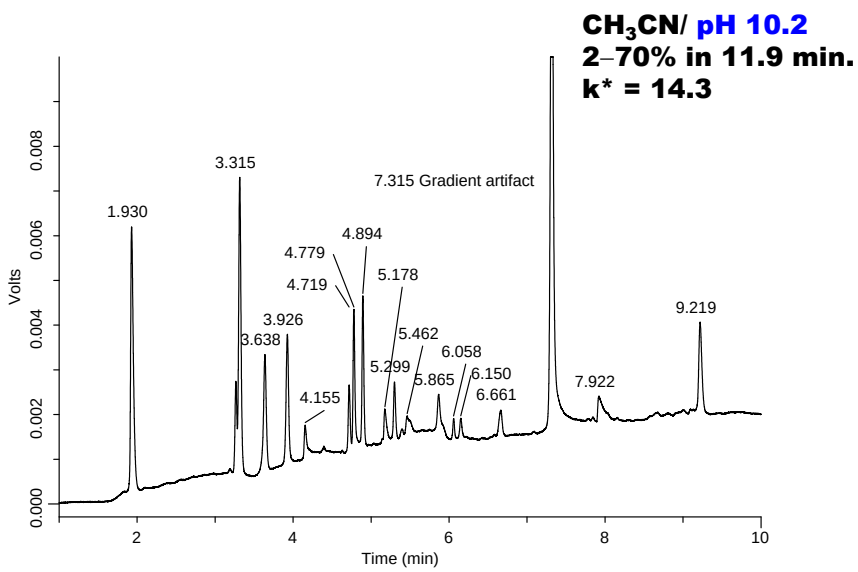
Isobaric compounds highlighted in same color
RTs for SuperPhenylHexyl 2-50% gradient

UltraCore SuperC18, 2.1 x 50 mm, 2.5 µm
4 pHs with CH_3CN

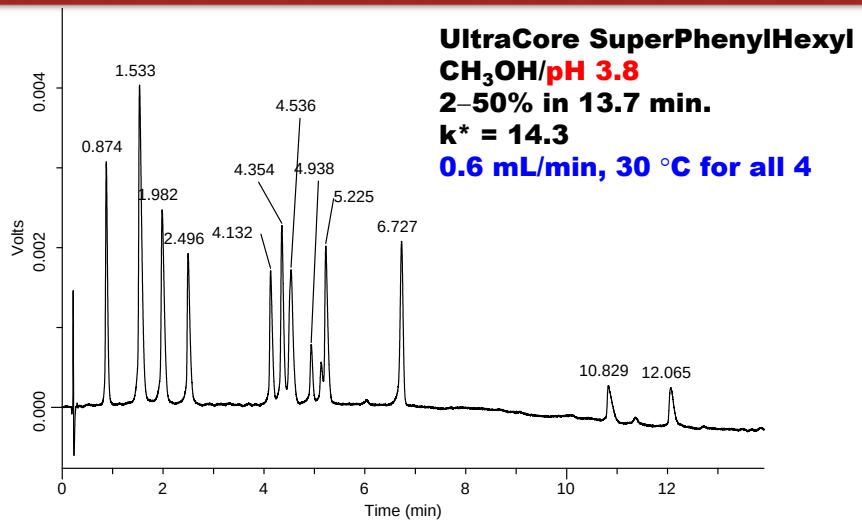


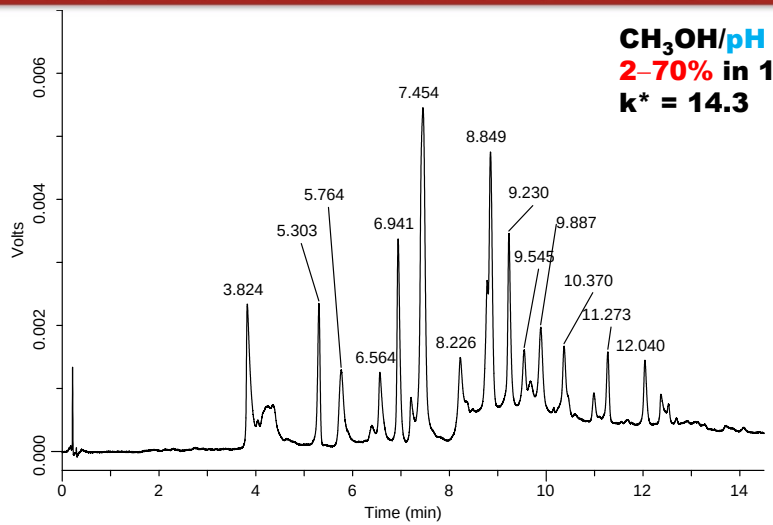


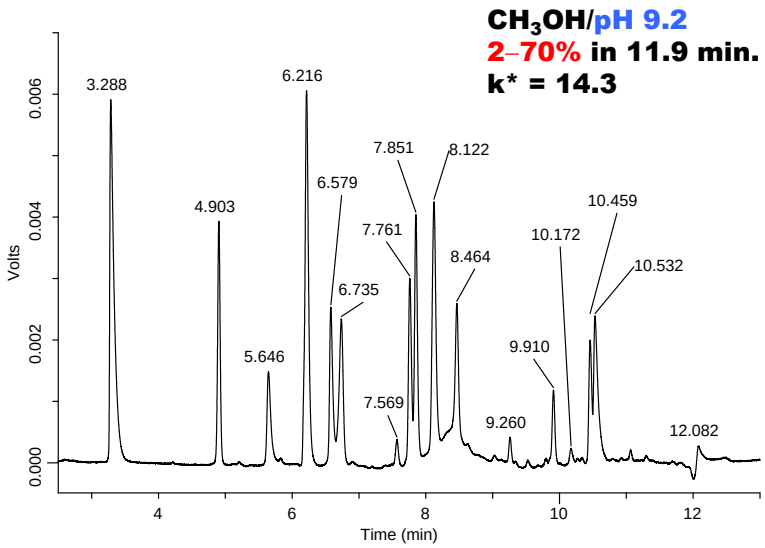


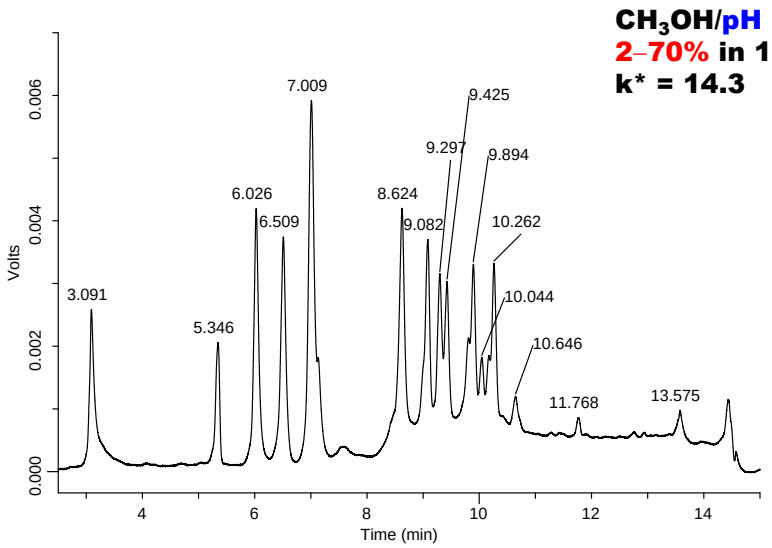


UltraCore SuperPhenylHexyl, 2.1 x 50 mm: 4 pHs with CH_3OH

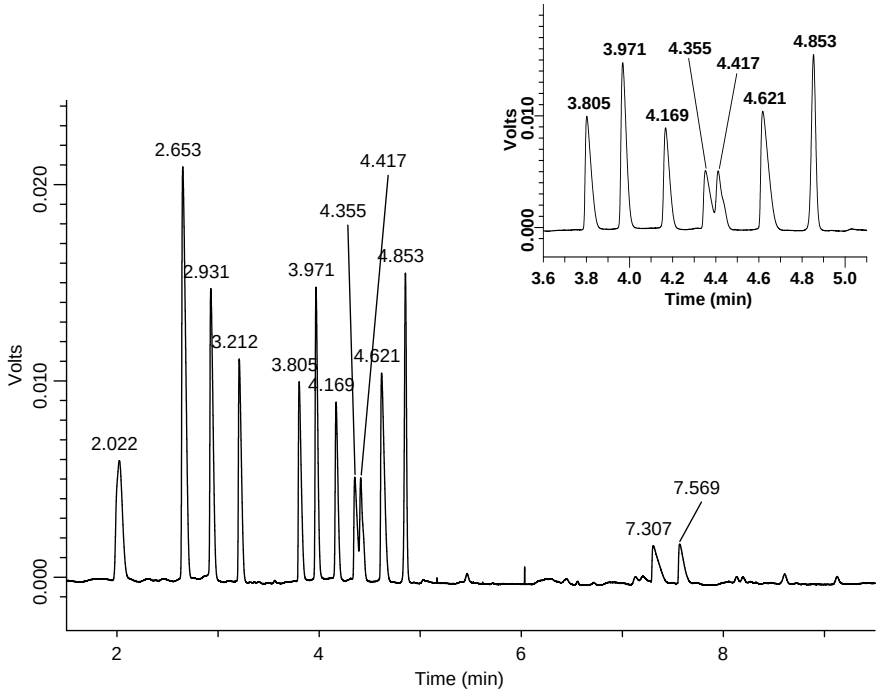




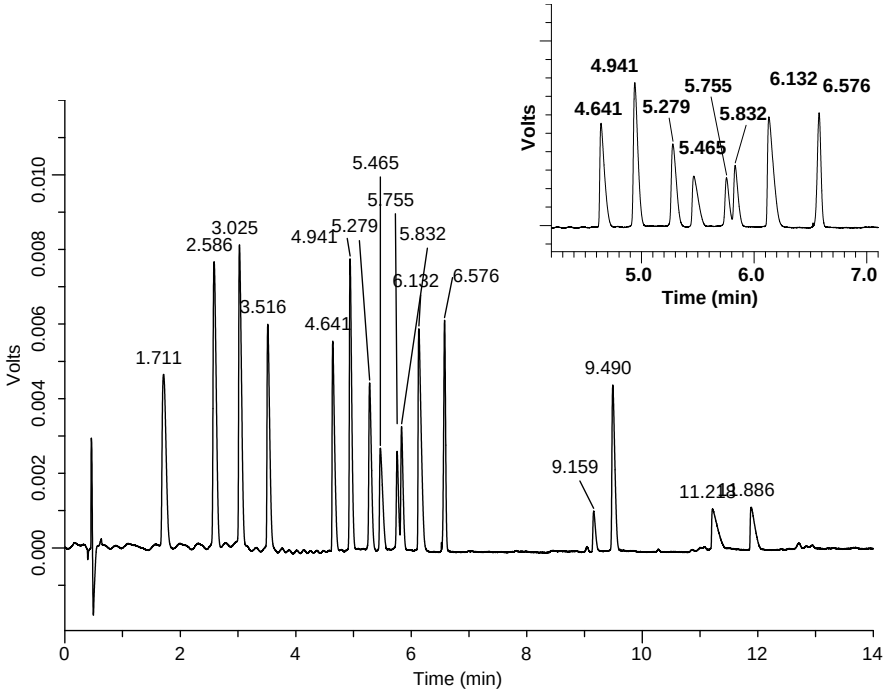




Separation on 3 x 100 SuperPhenylHexyl: All 16 drugs of abuse separated

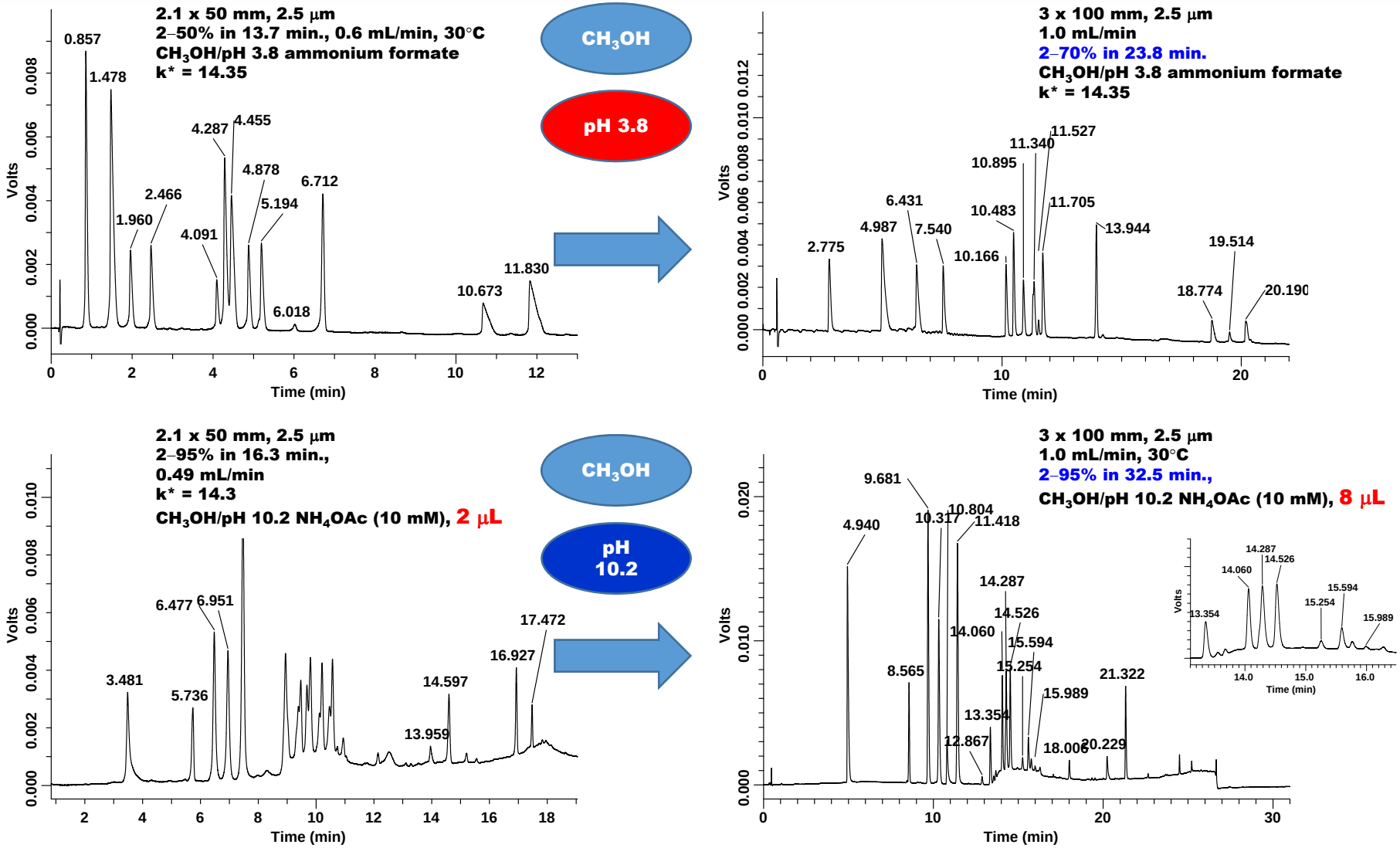


2-70% CH_3CN/pH 3.8 in 15 min.
1.0 mL/min



2-70% CH_3CN/pH 3.8 in 30 min.
1.0 mL/min

Scaling Separations to 3 x 100 mm UltraCore SuperPhenylHexyl with CH_3OH



High resolution example

ACE HPLC / UHPLC Columns

9

Impurity Profile of a Technical Grade Herbicide

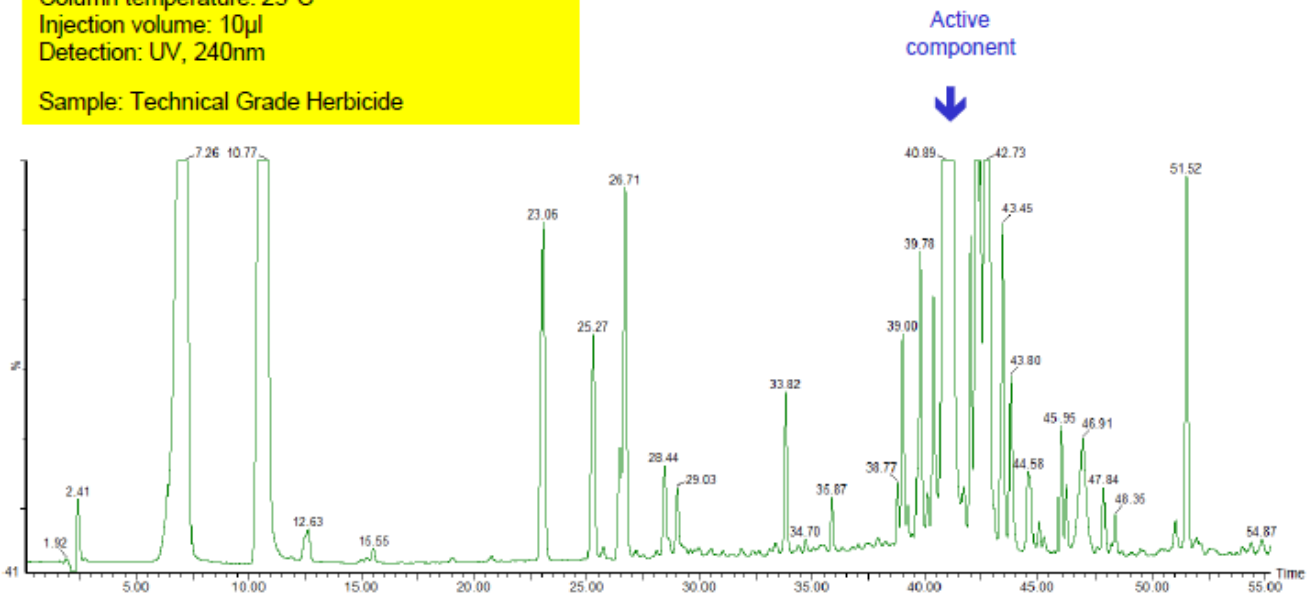
ACE UltraCore SuperC18, 2.5µm, 150 x 4.6mm
Gradient analysis
A = CH_3CN – H_2O – TFA (5:95:0.05 v/v/v)
B = CH_3CN – TFA (99.9:0.05 v/v/v)

Time (mins)	%B	Time (mins)	%B
0	10	55	100
3	10	56	10
35	100	60	10

Flow rate: 0.60ml/min
Column temperature: 25°C
Injection volume: 10µl
Detection: UV, 240nm

Sample: Technical Grade Herbicide

Solid-Core columns are excellent for high-resolution separations in longer dimensions



Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

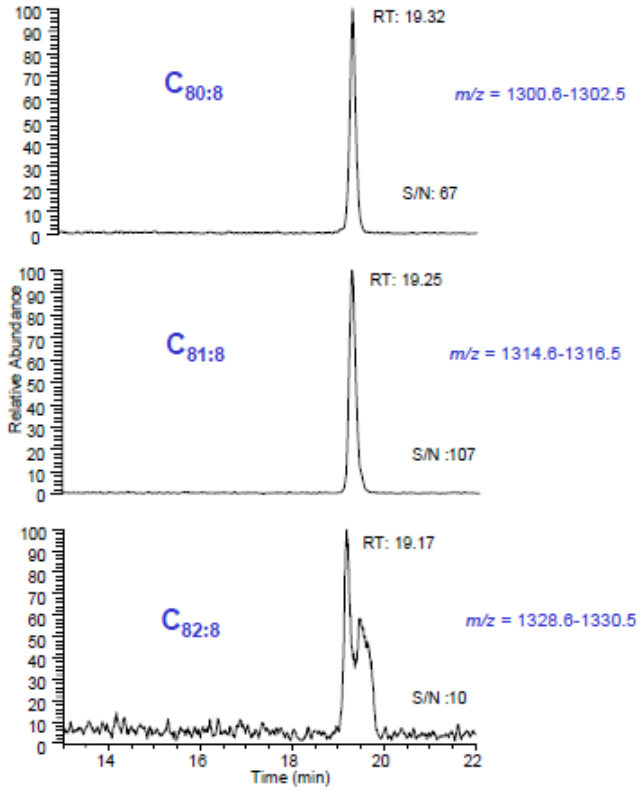
UltraCore: ideal for LC-MS

Very hydrophobic tetracarboxylic acids with gradient from mostly methanol to mostly IPA (2-propanol)

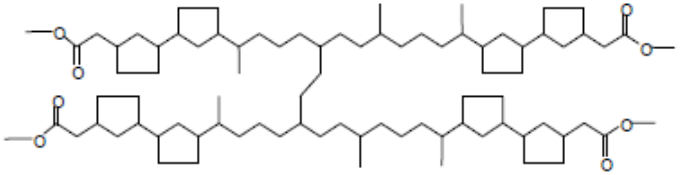
ACE HPLC / UHPLC Columns

8

Polycyclic Tetracarboxylic Acids



C80-82 polycyclic tetracarboxylic acids isolated from oilfield deposits



Tetramethyl ester of $C_{80:8}$ ring acid

ACE UltraCore SuperPhenylHexyl 2.5µm, 100 x 2.1mm
Gradient analysis
A: CH_3OH – H_2O (98:2) containing 10mM ammonium acetate
B: IPA-H_2O (98:2) containing 10mM ammonium acetate

T (mins)	%B	T (mins)	%B
0	0	15	100
1	0	25	100

Flow rate: 0.15ml/min
Column temperature: Ambient
Injection volume: 5µl
LCQ Ion trap MS
LC-ESI-MS extracted ion chromatograms
Compounds detected as ammoniated quasimolecular ions $[M+NH_4]+$
Detection limit ~ 0.1ppm

Reproduced with permission of Dr Paul Sutton, Petroleum & Environmental Geochemistry Group, Plymouth University, UK

Data courtesy of Dr. Alan McKeown,
Advanced Chromatography Technologies

Summary

- Discover the features and advantages of the ACE solid-core columns with their broad pH range for UHPLC/UPLC and HPLC separations
- Learn how to use pH, the most powerful parameter for ionizable analytes, and other parameters as part of an effective RPLC method development strategy
- A discussion of how to incorporate the various parameters into a method development strategy and an opportunity for questions will follow.

✓ Revisited the importance of selectivity on resolution

✓ Described the factors that affect selectivity most and how they can be used in your method development strategy

✓ Introduced ACE UltraCore solid-core UHPLC and HPLC columns and presented data that shows how they can provide orthogonal selectivity

✓ Discussed how ACE UltraCore columns can be used in a systematic approach for method development using different organic modifiers, stationary phases, and mobile phase pH.

Thank you for attending!

MAC-MOD Analytical, Inc.
103 Commons Court
Chadds Ford, PA 19317
800-441-7508
Email us: info@mac-mod.com
Web site: www.mac-mod.com

- **Established 1986**
- **Was exclusive global distributor of ZORBAX columns prior to HP's (Agilent) purchase from Rockland Technologies**
- **Exclusive distributor of ACE columns in USA since 1999**
- **Exclusive distributor of HALO columns in USA since 2006**
- **Main US Distributor of Prontosil columns since 1999**