Accelerating HPLC / UHPLC Method Development Strategies by Leveraging Selectivity: Influence of Unique C18-Based Phases

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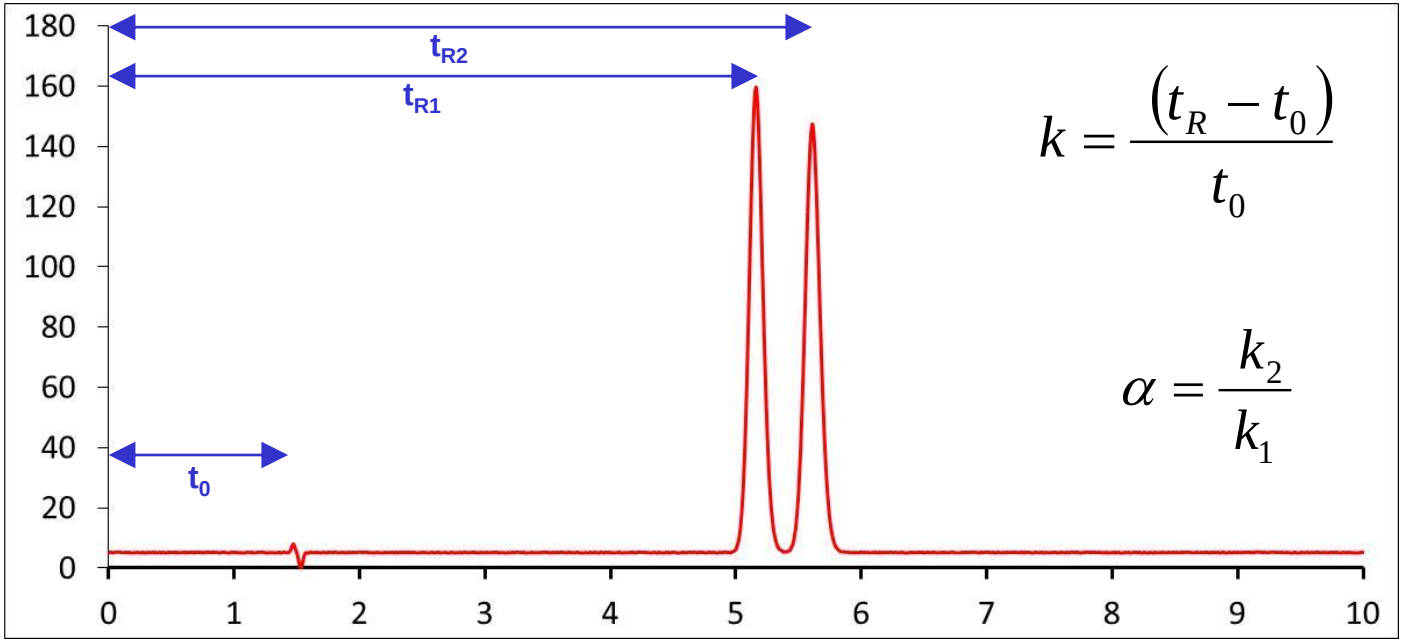
Outline

- What is selectivity?
- Method development workflows
- Maximizing selectivity through rational stationary phase design
- Selectivity diagrams and optimized method development workflows

What is Selectivity?

- Alpha (α) is the symbol used to denote the **separation factor** or **separation selectivity** between 2 adjacent peaks



- **Selectivity** can be thought of as '**peak spacing**'
- Selectivity values should be **> 1.0**

Resolution, Selectivity, Efficiency & Retention

Particle size, column length, dispersion etc

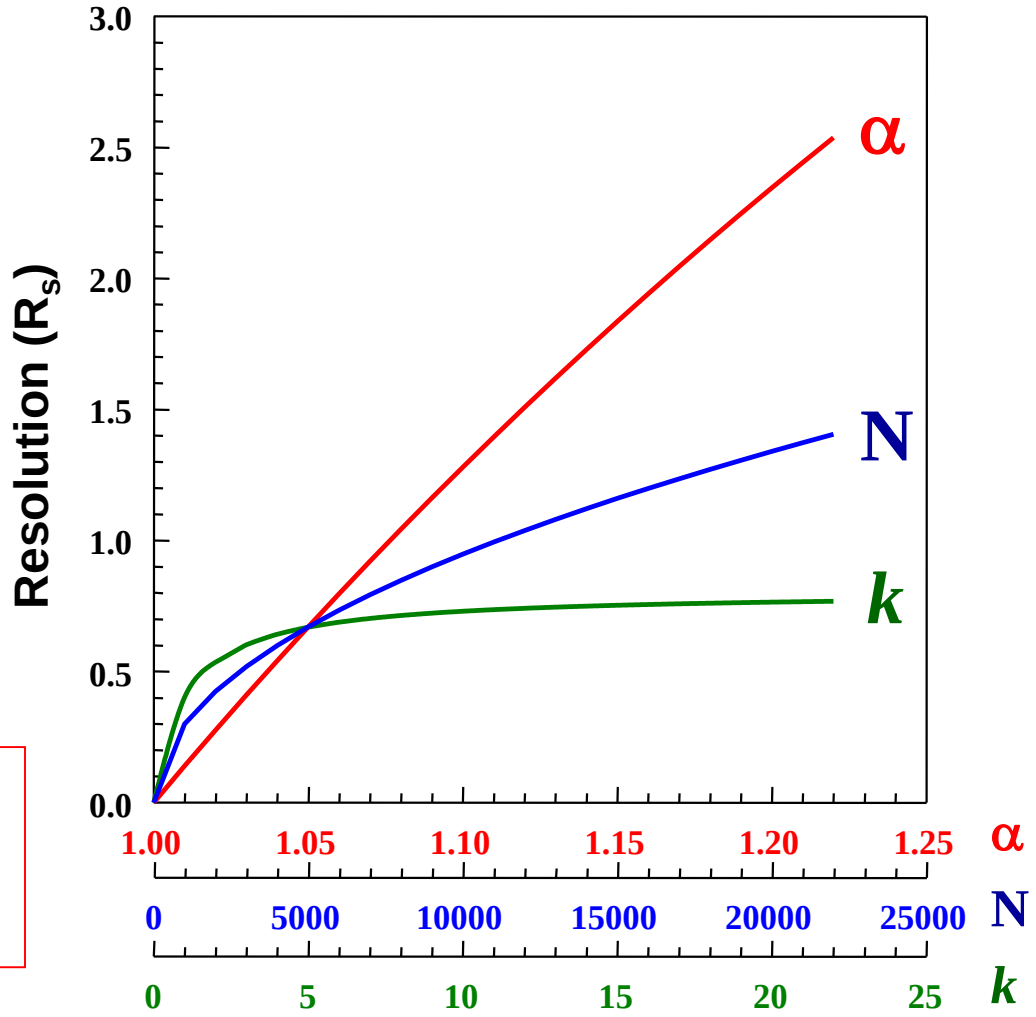
Phase design, eluent etc

Efficiency

Selectivity

Retention

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



Selectivity (α) is the key to resolution and efficiency (N) boosts performance

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

Which Factors[1] Affect Selectivity?

- **Strongly influenced by physicochemical properties of the analyte, stationary phase, eluent etc**
- **From a practical perspective:**

Isocratic Separations

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

MOST Influence

LEAST Influence

Gradient Separations

All parameters for isocratic **PLUS**

Gradient steepness,

k^* (t_G, F, V_m, $\Delta\Phi$, M),

$$k^* = \frac{t_G F}{\Delta\Phi V_m M}$$

Dwell volume,

Column dimensions.

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

Method Development / Screening Workflow: Overview

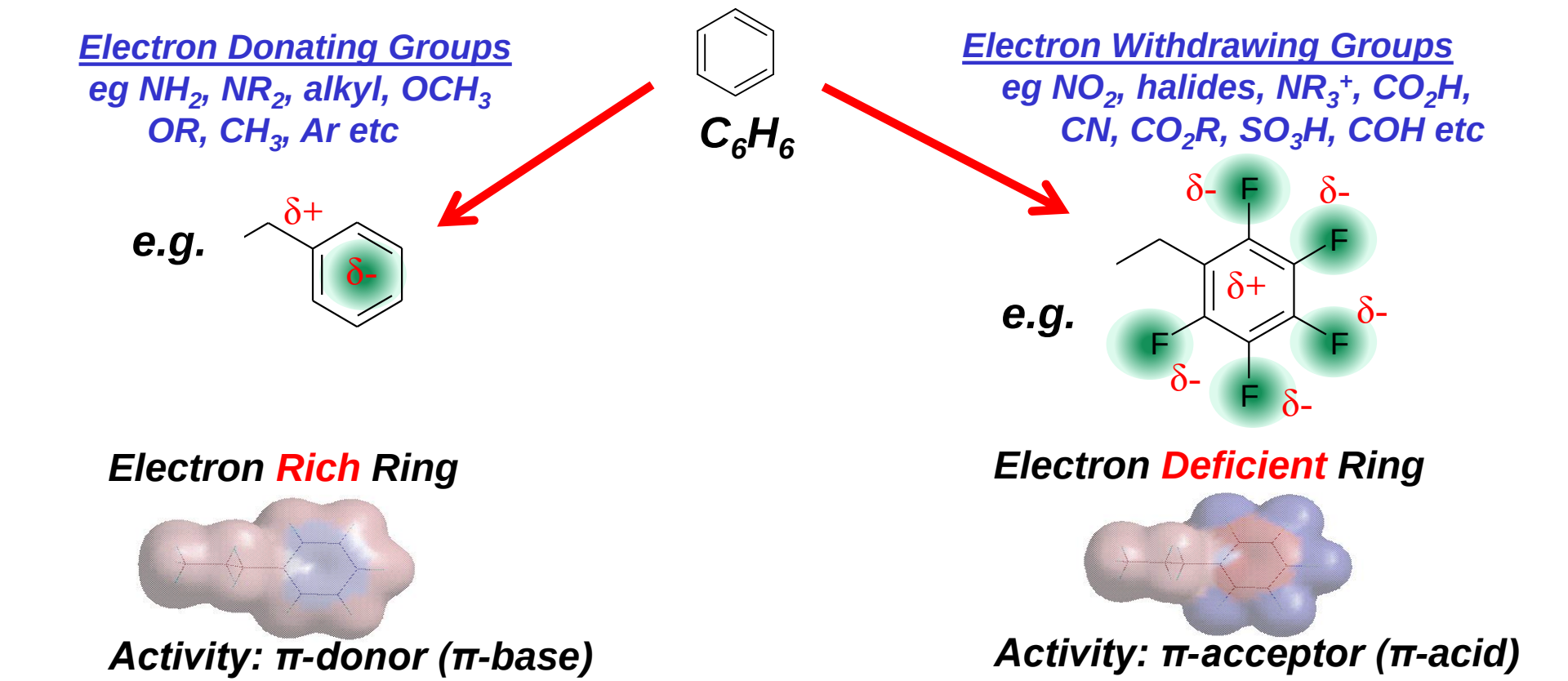
- **Typically multivariate**

- 1 column - 1 temperature - 1 pH - 1 organic modifier - 1 t_G	- 1 column - 2 temperatures - 1 pH - 1 organic modifier - 2 x t_G	- 1 column - 2 temperatures - 1 pH - 2 organic modifier - 2 x t_G	- ≥ 2 columns - 2 temperatures - 1 pH - 2 organic modifier - 2 x t_G	- ≥ 2 column - 2 temperatures - 2 or 3 pH - 2 organic modifier - 2 x t_G
2 x t_G	20C & 60C	MeOH & MeCN	Alkyl chains eg C18, C8 Aromatic eg Phenyl, C18-AR or C18-PFP Polar eg C18-PFP, C18-Amide	pH 2.5 pH 7 pH 10.7

INCREASING COMPLEXITY...BUT KNOWLEDGE RICH

- **Many potential runs to fully explore variables and their effects on retention and selectivity**
- **Would be helpful to reduce parameter options...**

Scientific Led Stationary Phase Design: Aromatic Phases



How do we exploit these properties for new stationary phases?

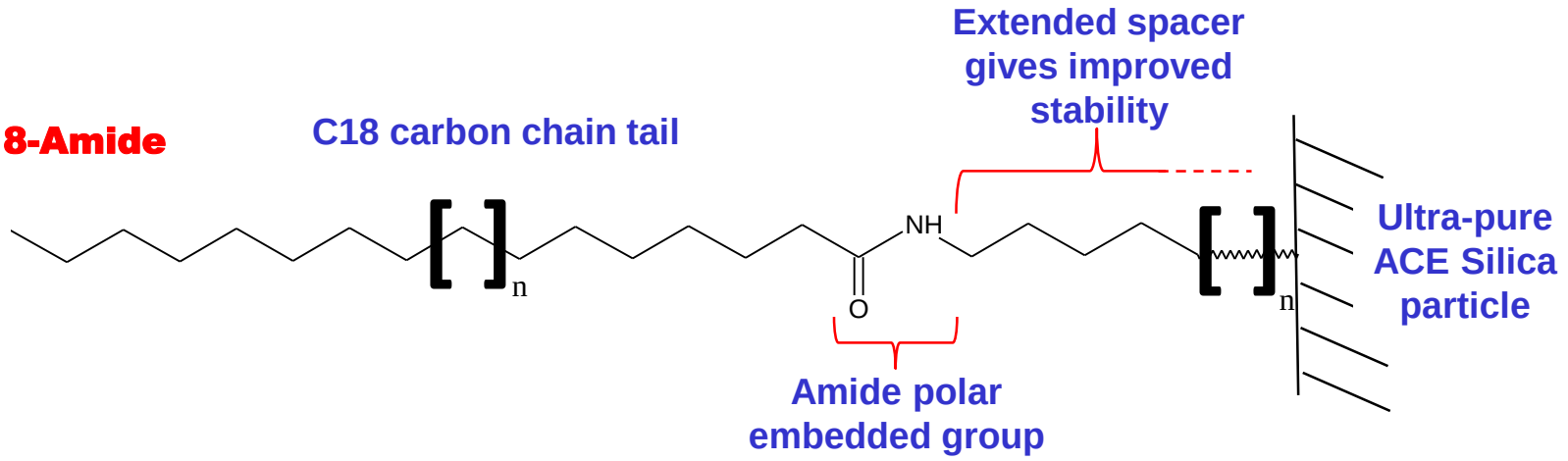
C18+Phenyl = ACE® C18-AR

C18+PFP = ACE® C18-PFP

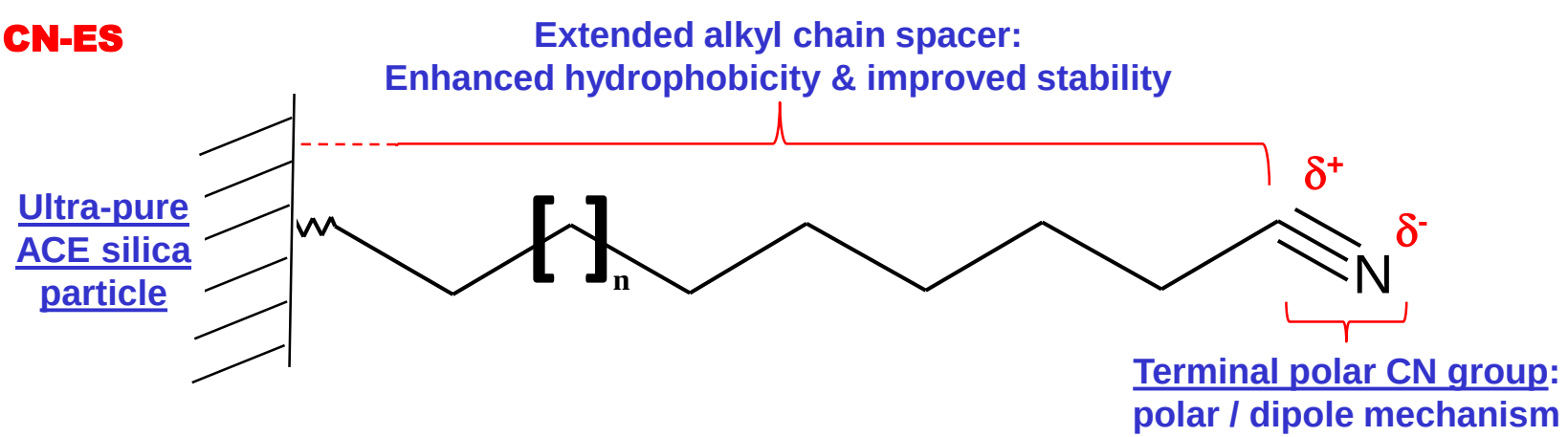
Both phases have multiple mechanisms of interaction, low bleed and are 100% wettable: i.e. maximize selectivity

Scientific Led Stationary Phase Design: Other Phases

ACE C18-Amide



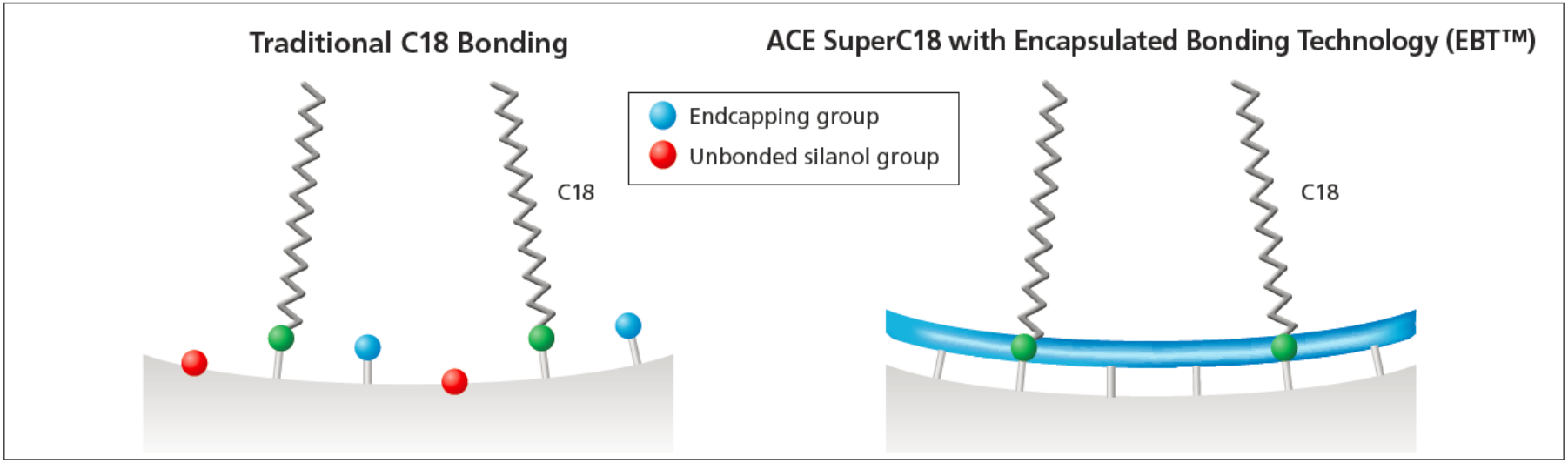
ACE CN-ES



Multiple mechanisms of interaction, low bleed and are 100% wettable: i.e. maximize selectivity

ACE® SuperC18™: Uniquely Designed To Exploit Eluent pH

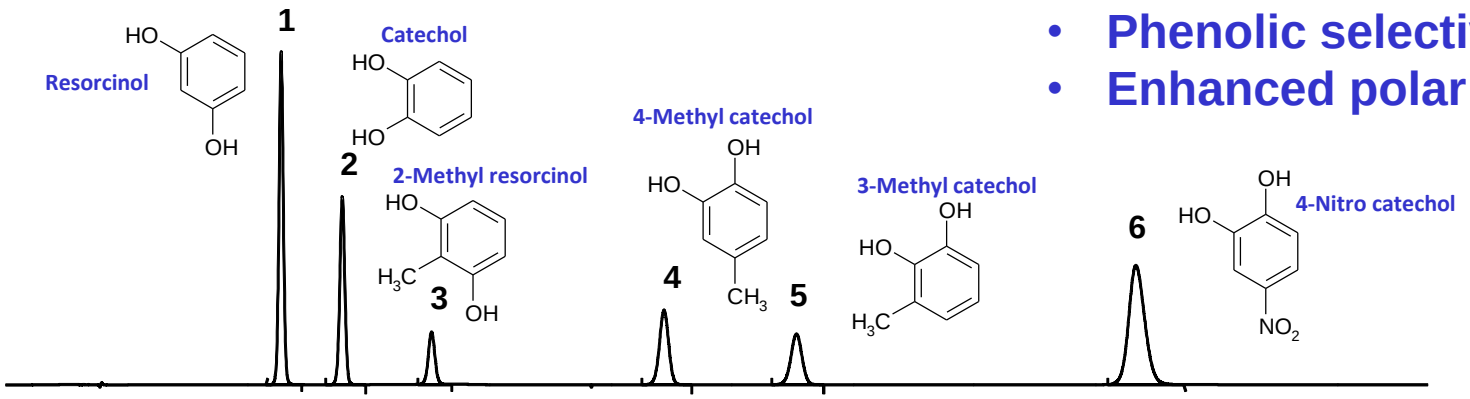
Encapsulated Bonding Technology (EBT™) for Improved Chromatography and Stability



- **Unique bonding technology** protects the silica surface
- **Extended pH range** compatible silica-based columns
- **Rational** stationary phase design

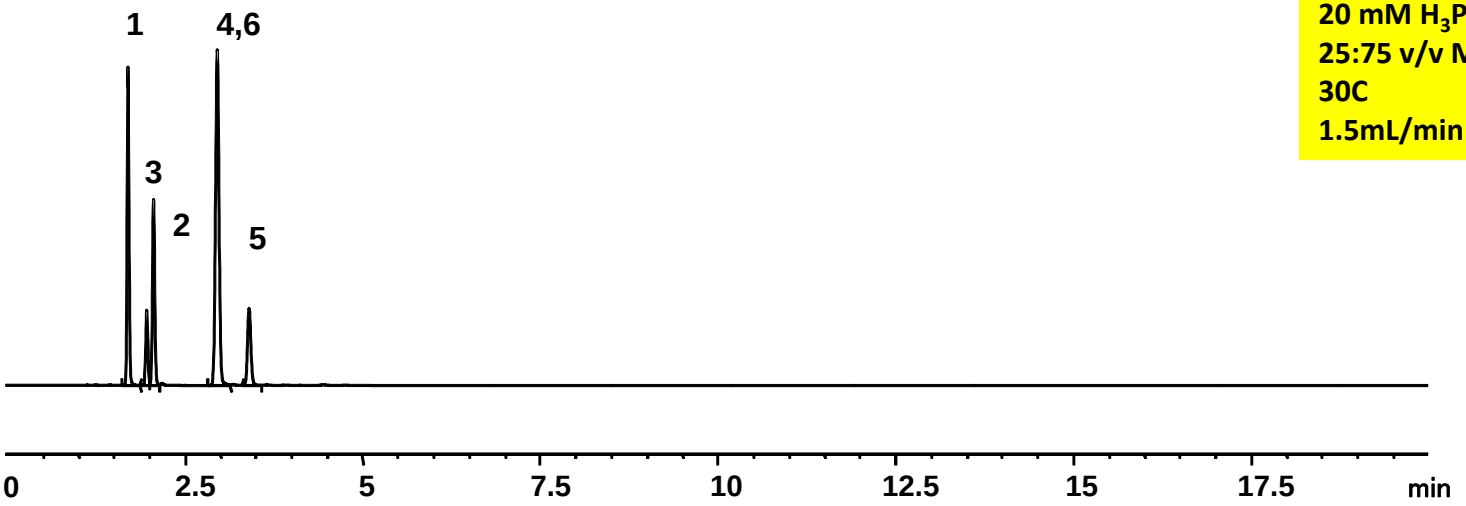
Catechols and Resorcinols Separations

ACE Excel C18-Amide



- **Phenolic selectivity**
- **Enhanced polar retention**

ACE C18

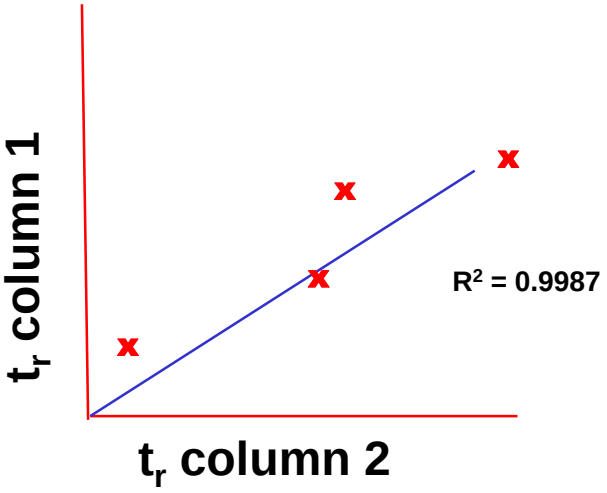


150Ax4.6mm, 3µm
Isocratic analysis

20 mM H_3PO_4 in
25:75 v/v MeCN/H_2O
30C
1.5mL/min

Selectivity Descriptor*

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

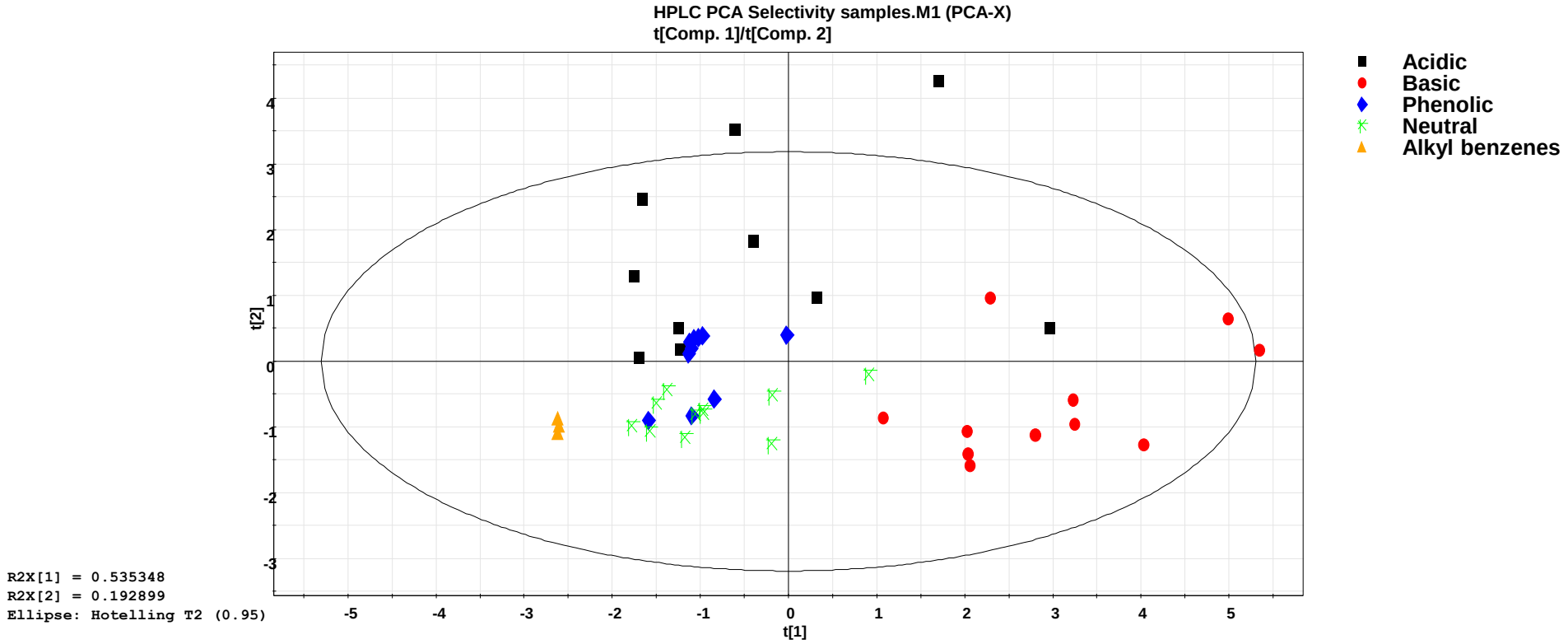


- Selectivity values from ~8 upwards indicate suitable changes in selectivity for method development.
- Large Selectivity values can be achieved with multiple parameter changes.

* Neue, O'Gara, Méndez "Selectivity in Reversed-Phase Separations: Influence of the Stationary Phase", J. Chromatogr. A 1127 (2006), 161-174.

Diverse Analytes / Probes

- A mixture of 45 acidic, basic & neutral small molecule analytes were used that represent a broad range of physico-chemical properties.

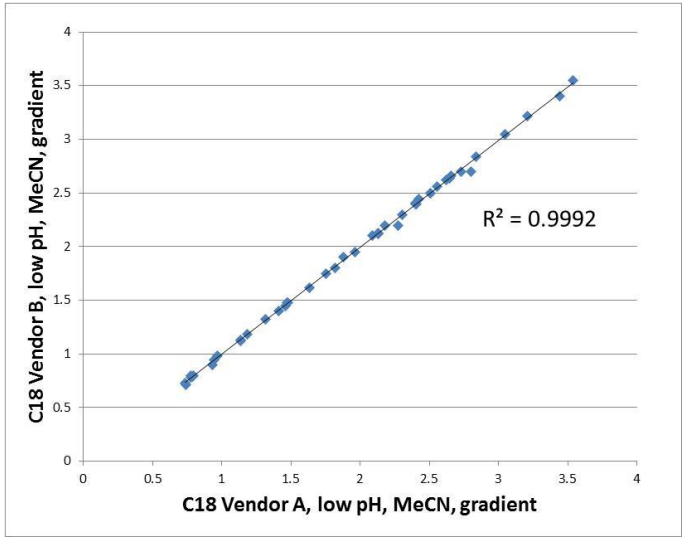


- Challenging stationary phases with these probes gives an indication of chromatographic selectivity for each phase and between phases.

Low Selectivity Differences

- **Selectivity values 0 to < ~8.**

- **Little difference in analyte retention on the 2 phases therefore little scatter seen. High correlation.**



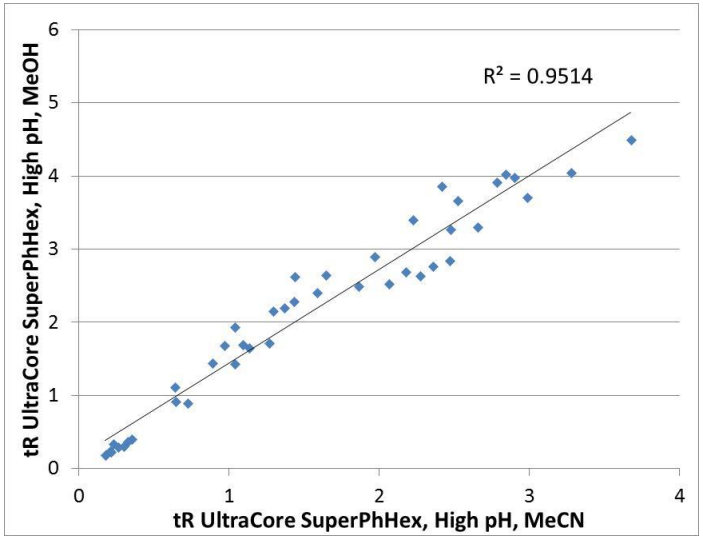
Selectivity = 3

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

Moderate Selectivity Differences

- Selectivity values > ~8 and < ~30.

- Changes in analyte retention between the 2 phases observed. May include elution order changes. Scatter observed and correlation reduces.

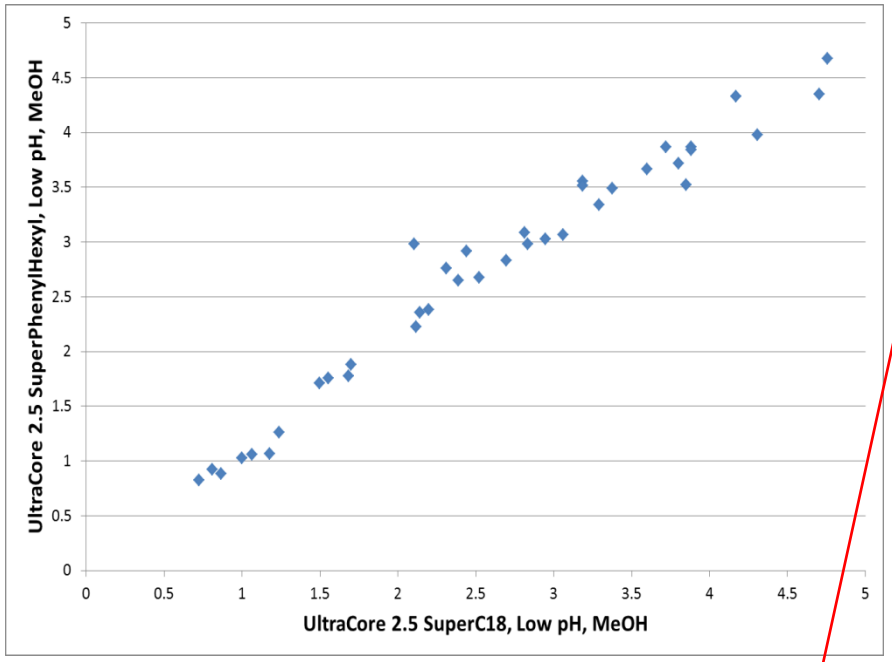


Selectivity = 22

- Typically observed when changing a variable eg comparing different phases OR changing solvent.

Selectivity: Exploring Solid Core Bonded Phase Effects

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

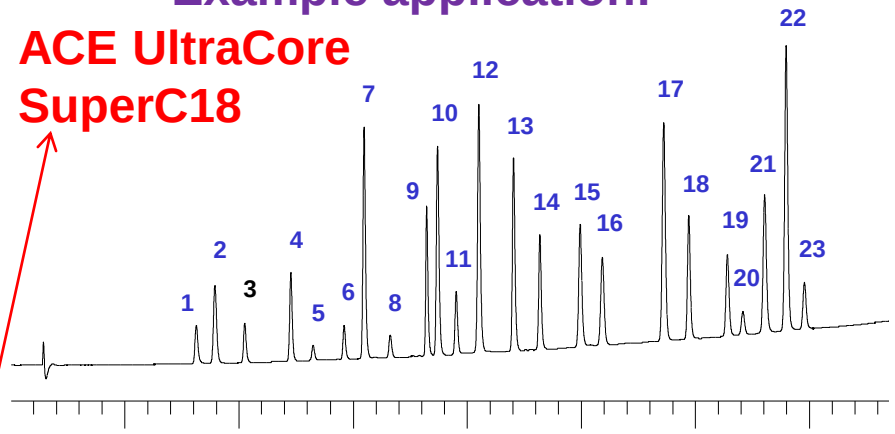


Selectivity = 19

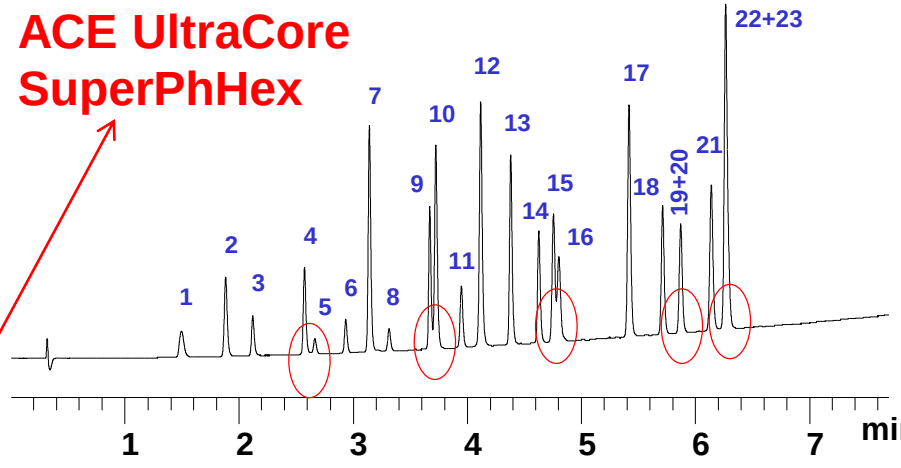
Changes in peak spacing and elution order noted

Example application:

ACE UltraCore SuperC18



ACE UltraCore SuperPhHex



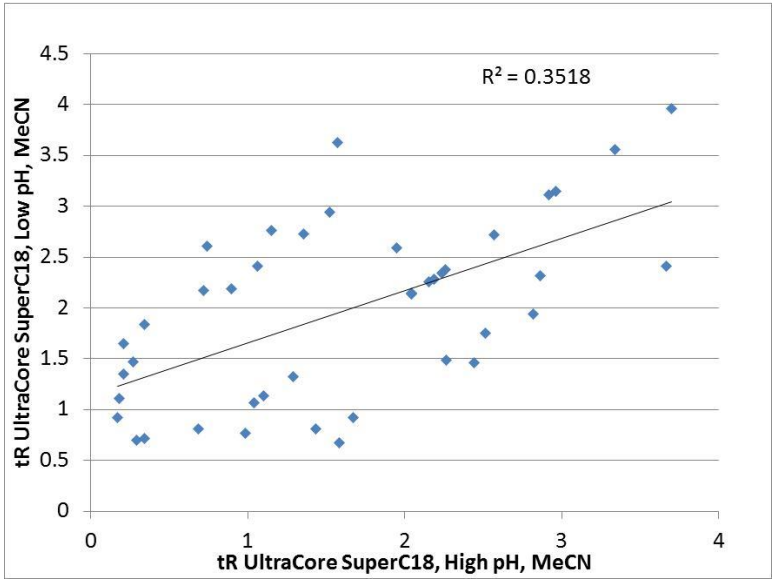
50x2.1mm, 2.5µm, gradient analysis, A= 20mM $HCOONH_4$, pH3 (aq), B= 20mM $HCOONH_4$, pH 3 in MeCN/water 9:1 v/v, 3-100%B in 7.5 mins, hold 100%B for 1.5 mins, 40°C, 0.40 mL/min, 254 nm.

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 dimethylpthalate, 12 piroxicam, 13 hydro cortisone-21-acetate, 14 ketoprofen, 15 ethylbenzoate, 16 toluene, 17 valerophenone, 18 mefenamic acid 19 hexanophenone, 20 propylbenzene, 21 phenanthrene, 22 heptaphenone, 23 butylbenzene

High Selectivity Differences

- **Selectivity values > ~30.**

- **Significant changes in analyte retention and elution order between the 2 phases. Scatter observed.**

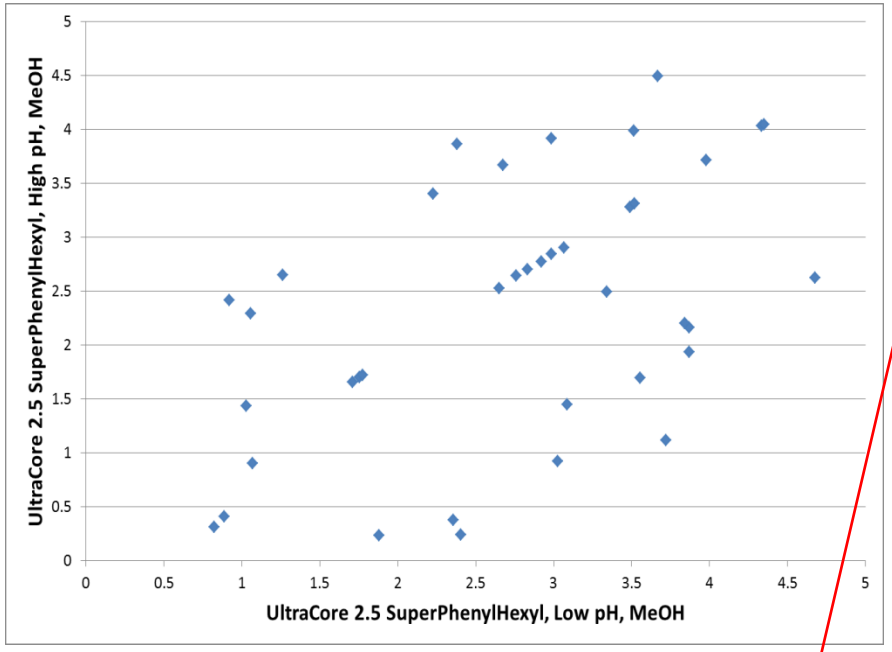


Selectivity = 81

- **Typically observed when changing multiple variables. Or changing eluent pH (eg low vs high) with ionisable analytes in the sample. ← Column pH range limitations.**

Selectivity Plot: Exploring Eluent pH With SuperPhenylHexyl

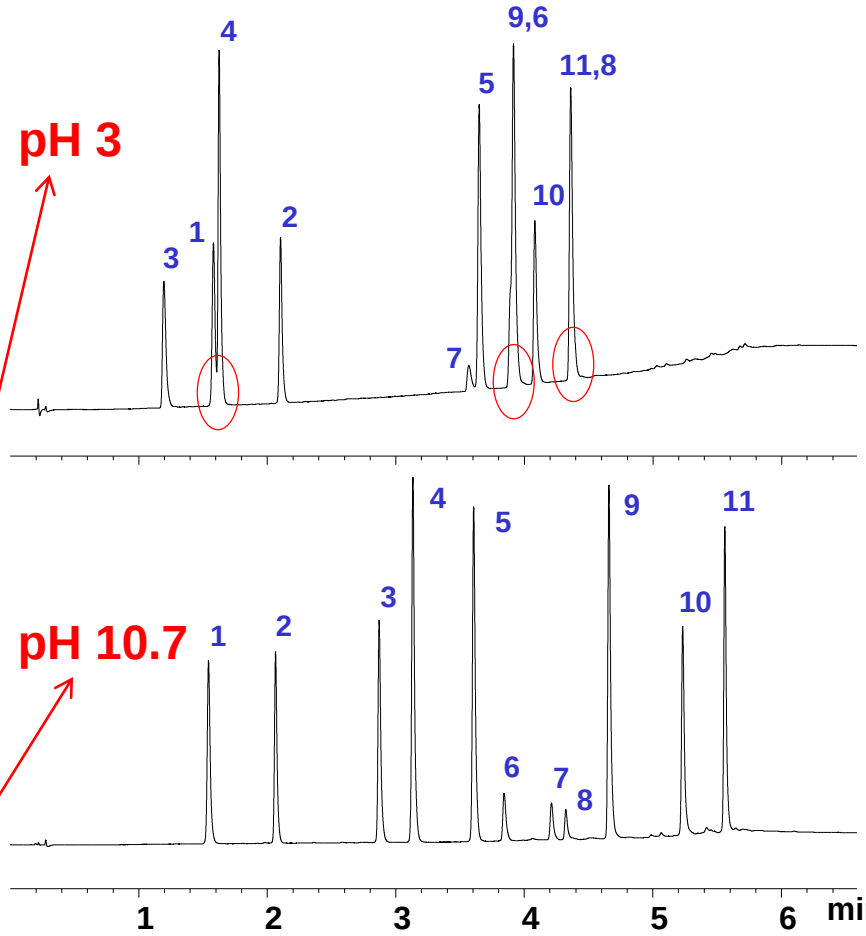
SuperPhenylHexyl, low pH, MeOH vs SuperPhenylHexyl, high pH, MeOH



Selectivity = 83

Significant changes in elution order noted

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 carvdilol, 10 nortriptyline, 11 clomipramine.

Degraded Sample With Unknown Number of Peaks

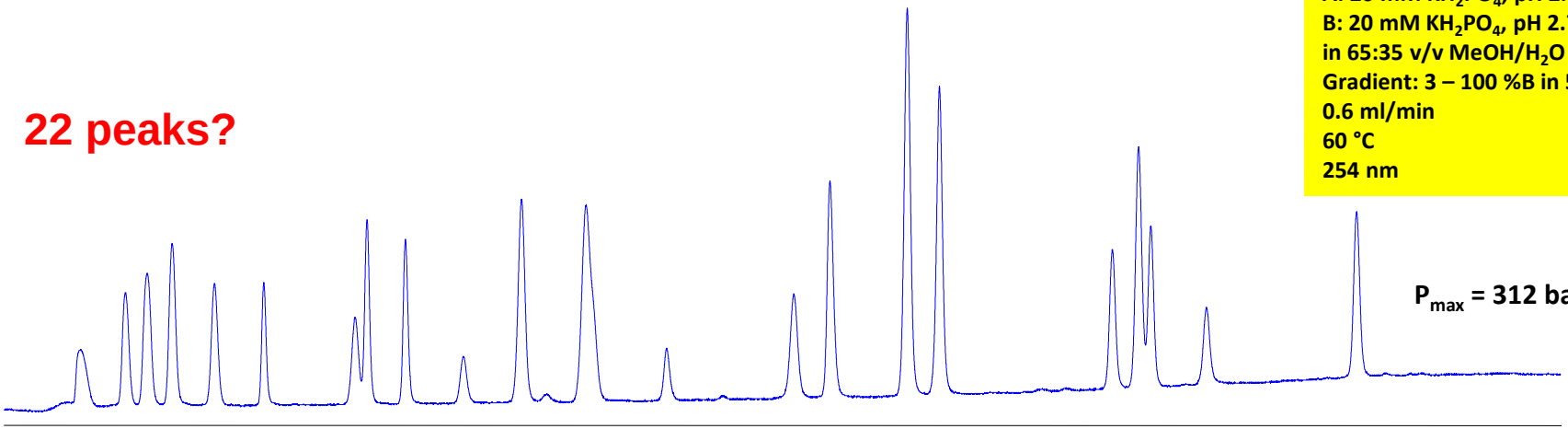
50x2.1mm, 2um
Gradient analysis

A: 20 mM KH_2PO_4, pH 2.7 (aq)
B: 20 mM KH_2PO_4, pH 2.7
in 65:35 v/v MeOH/H_2O
Gradient: 3 – 100 %B in 5 mins
0.6 ml/min
60 °C
254 nm

ACE Excel 2 C18

Mechanism = Hydrophobicity

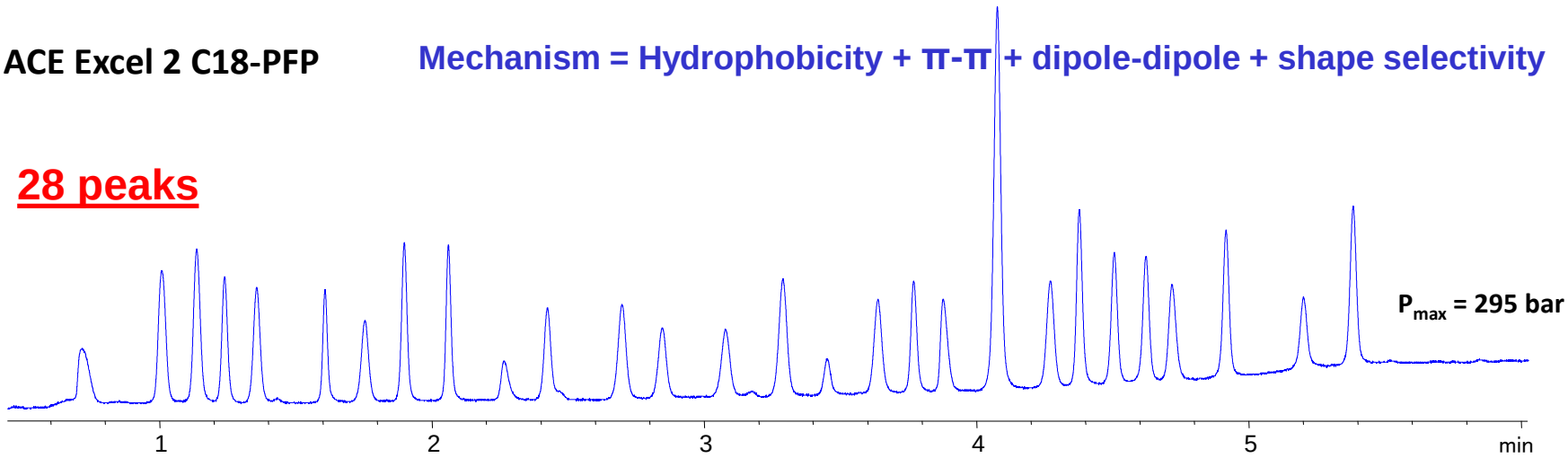
22 peaks?



ACE Excel 2 C18-PFP

Mechanism = Hydrophobicity + π-π + dipole-dipole + shape selectivity

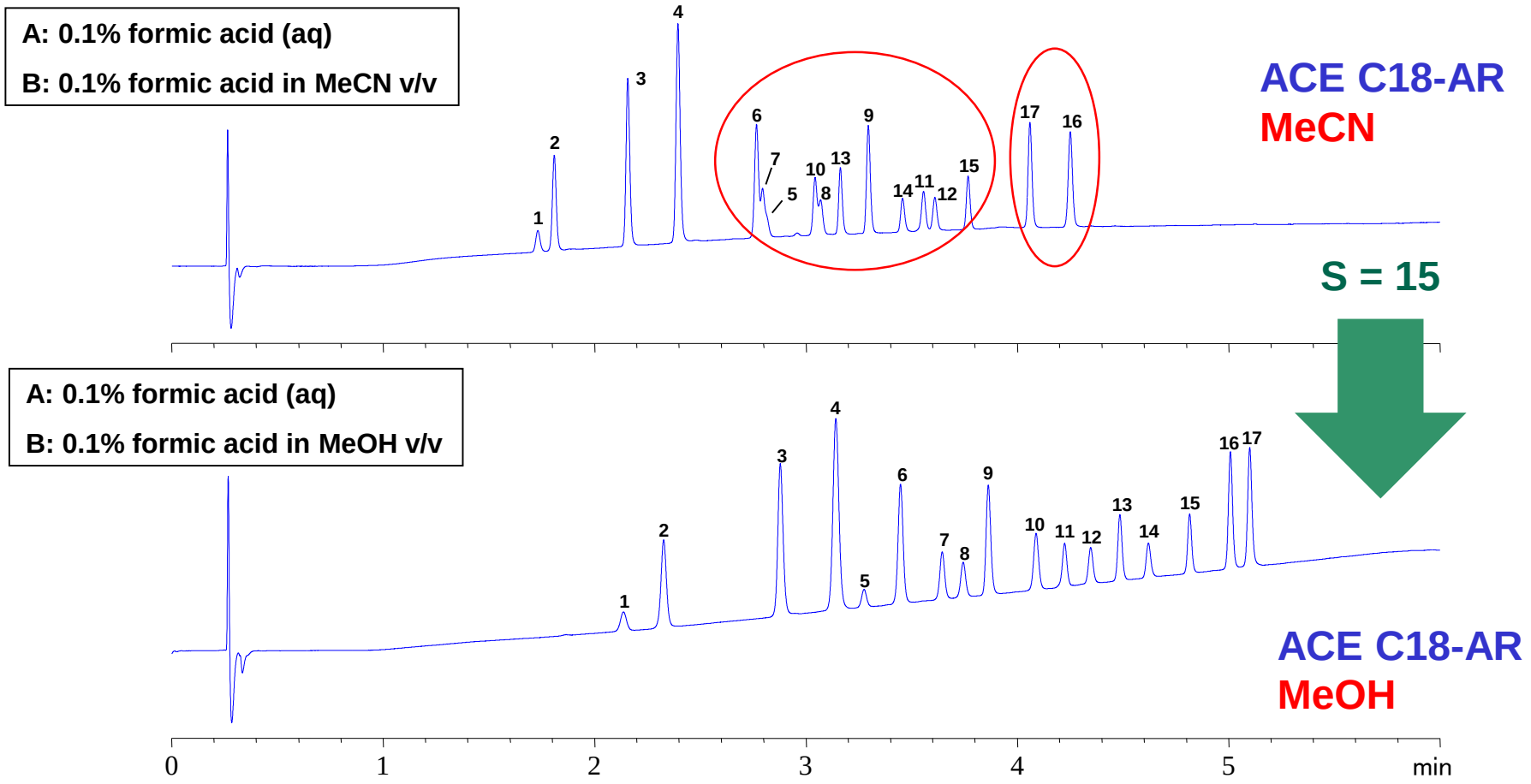
28 peaks



Stationary Phase Selectivity Is Powerful

Selectivity: Changing Organic Solvent Type

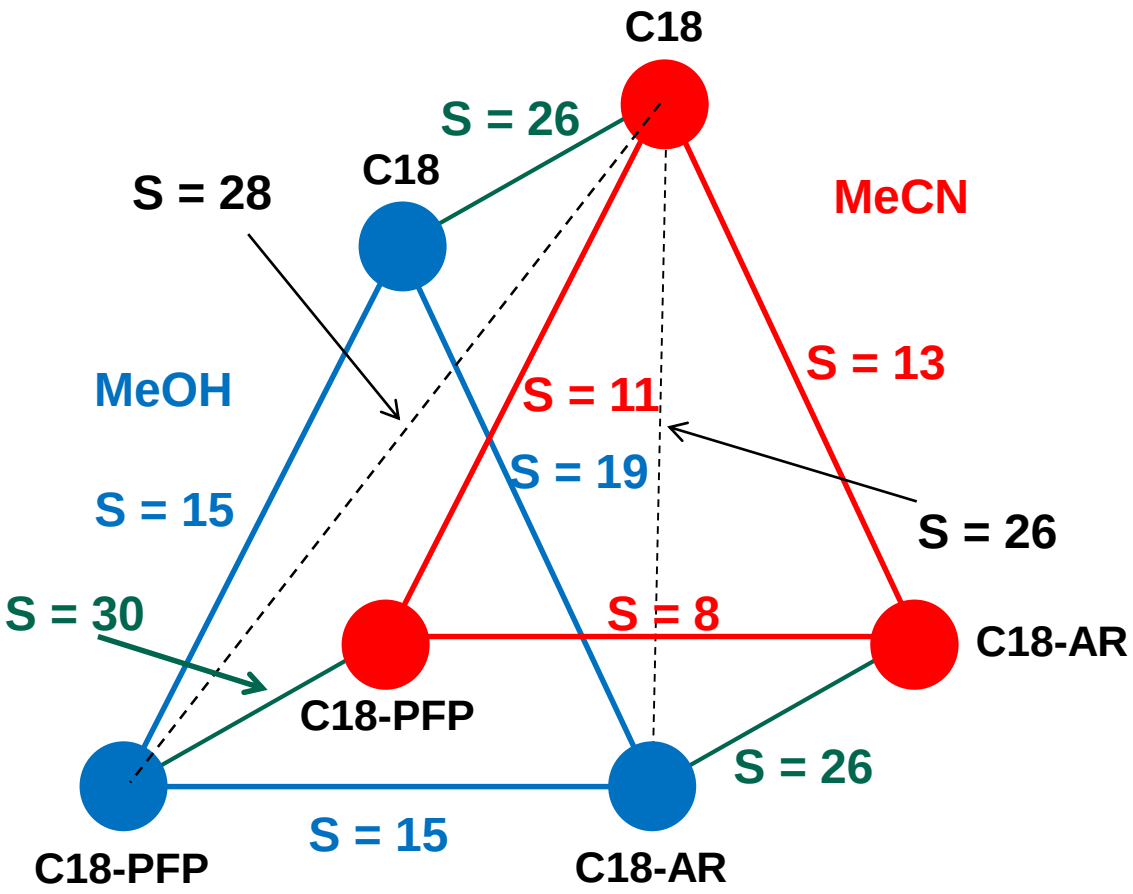
- **Exploring selectivity with solvent type:**



50x2.1mm, 3 µm, gradient analysis, 3-100%B in 5.0 mins, hold 100%B for 1.0 mins, 40°C, 0.60 mL/min, 254 nm.
1 3-hydroxybenzoic acid, 2 methylphenylsulfoxide, 3 quinoxaline, 4 salicylic acid, 5 benzylcyanide,
6 1,2-dimethoxybenzene, 7 ethyl paraben, 8 1,4-dimethoxybenzene, 9 bendroflumethiazide, 10 piroxicam,
11 benzylchloride, 12 thioanisole, 13 sulindac, 14 chrysin, 15 ibuprofen, 16 1,2,3-trichlorobenzene,
17 meclofenamic acid

Advanced Selectivity Method Development Platform #1

- 45 analytes, 3 stationary phases, 2 solvents



ACE C18

- Hydrophobic

ACE C18-AR

- Hydrophobic
- π-π interactions

ACE C18-PFP

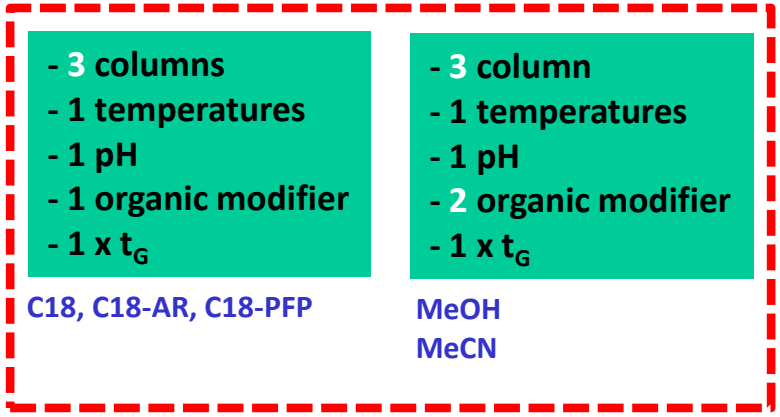
- Hydrophobic
- π-π interactions
- Dipole-dipole
- Shape selectivity

Complementary Mechanisms of Interaction

Data from Euerby et al., submitted to LC GC, July 2015

Advanced Selectivity Method Development Platform #1

Workflow Schematic



3 columns, 2 solvents method development / screening approach based on selectivity data

Initial Acetaminophen UHPLC Screening Chromatograms

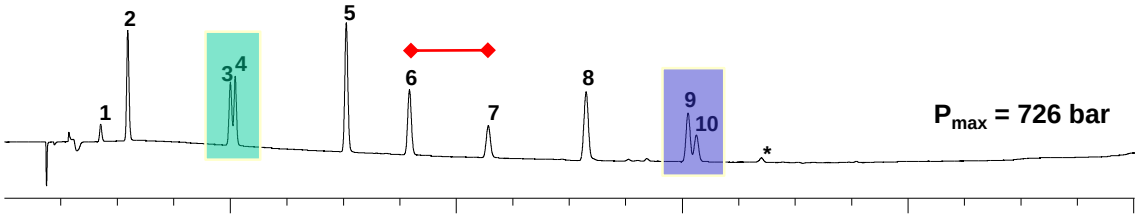
Conditions

A: 20 mM ammonium acetate pH 6.0
B: 20 mM ammonium acetate pH 6.0 in MeCN:water 9:1 *v/v*
Gradient: 0-95% B in 10 mins., hold 2.5 mins, ramp down 0.5 mins.
Post time: 5 mins
Inj. Vol.: 2 µL
Temp: 40 °C
Flow rate: 1.2 mL/min

ACE Excel 2µm C18 100 x 3.0mm

C18

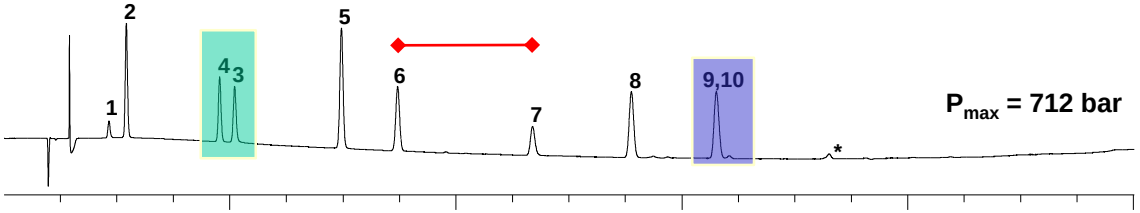
Mechanism = Hydrophobicity



ACE Excel 2µm C18-AR 100 x 3.0mm

C18-AR

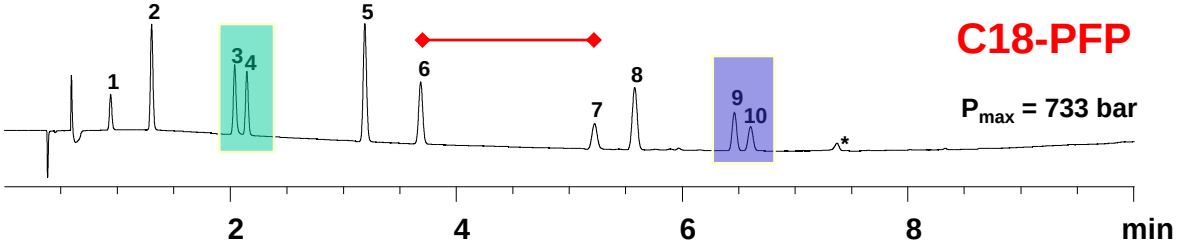
Mechanism = Hydrophobicity + π-π



ACE Excel 2µm C18-PFP 100 x 3.0mm

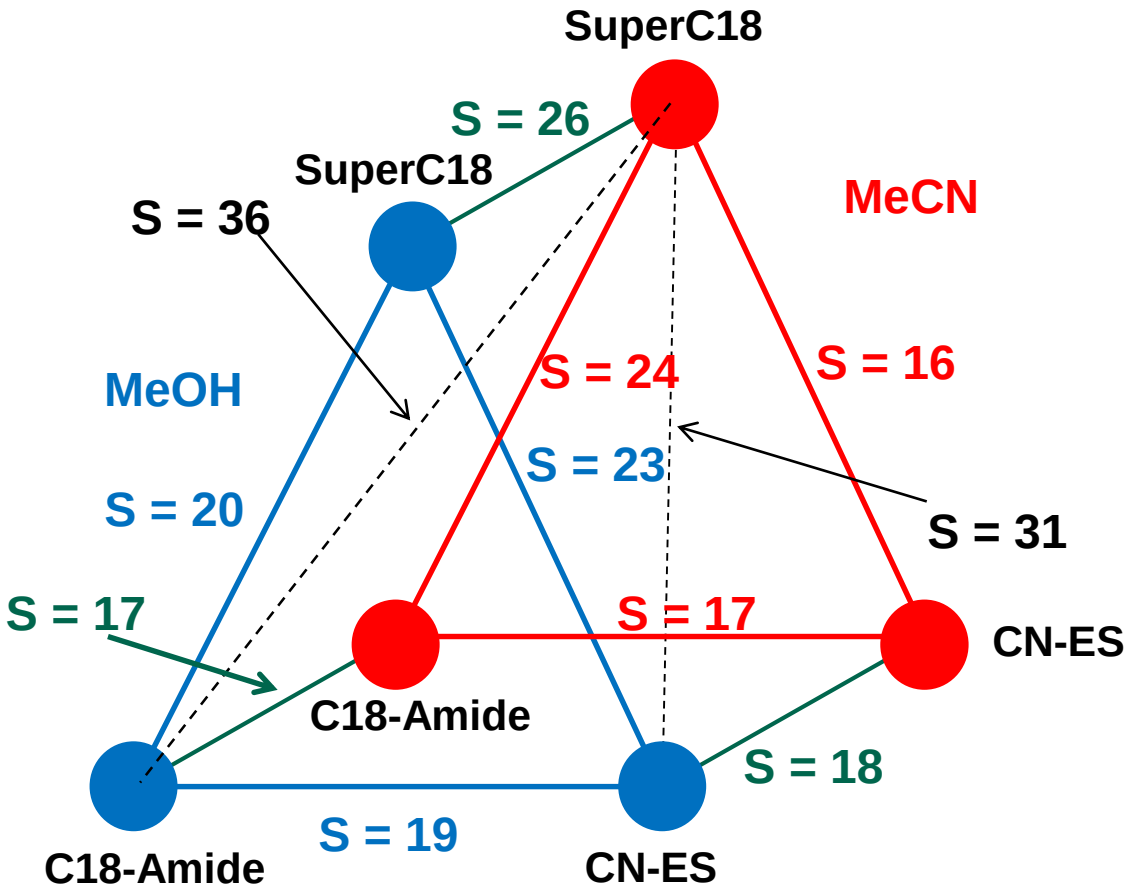
Mechanism = Hydrophobicity + π-π + dipole-dipole + shape selectivity

C18-PFP



Extended Selectivity Method Development Platform #2

- 45 analytes, 3 stationary phases, 2 solvents



ACE SuperC18

- Hydrophobic
- High pH stable

ACE C18-Amide

- Hydrophobic
- Phenolic selectivity
- Polar retention

ACE CN-ES

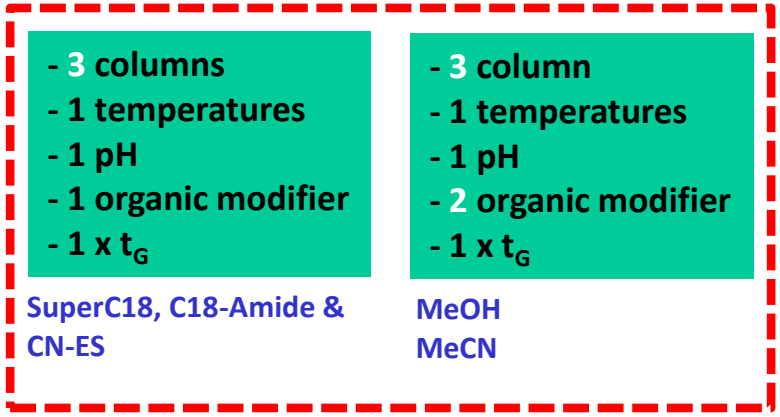
- Hydrophobic
- Dipole-dipole
- Polar retention

Complementary Mechanisms of Interaction

Data from Euerby et al., submitted to LC GC, July 2015

Extended Selectivity Method Development Platform #2

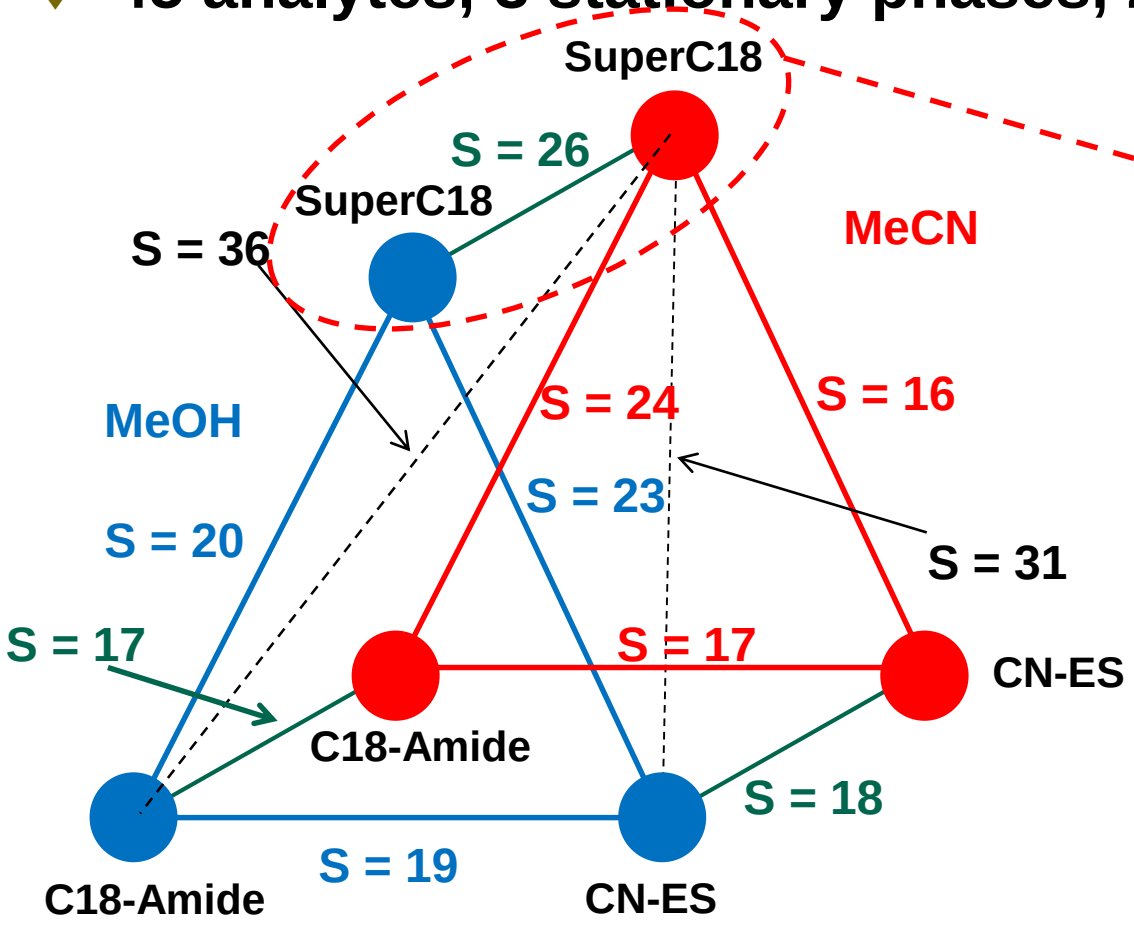
Workflow Schematic



3 columns, 2 solvents method development / screening approach based on selectivity data

Extended Selectivity Method Development Platform #2

- 45 analytes, 3 stationary phases, 2 solvents



ACE SuperC18 — High pH

- Hydrophobic
- High pH stable

ACE C18-Amide

- Hydrophobic
- Phenolic selectivity
- Polar retention

ACE CN-ES

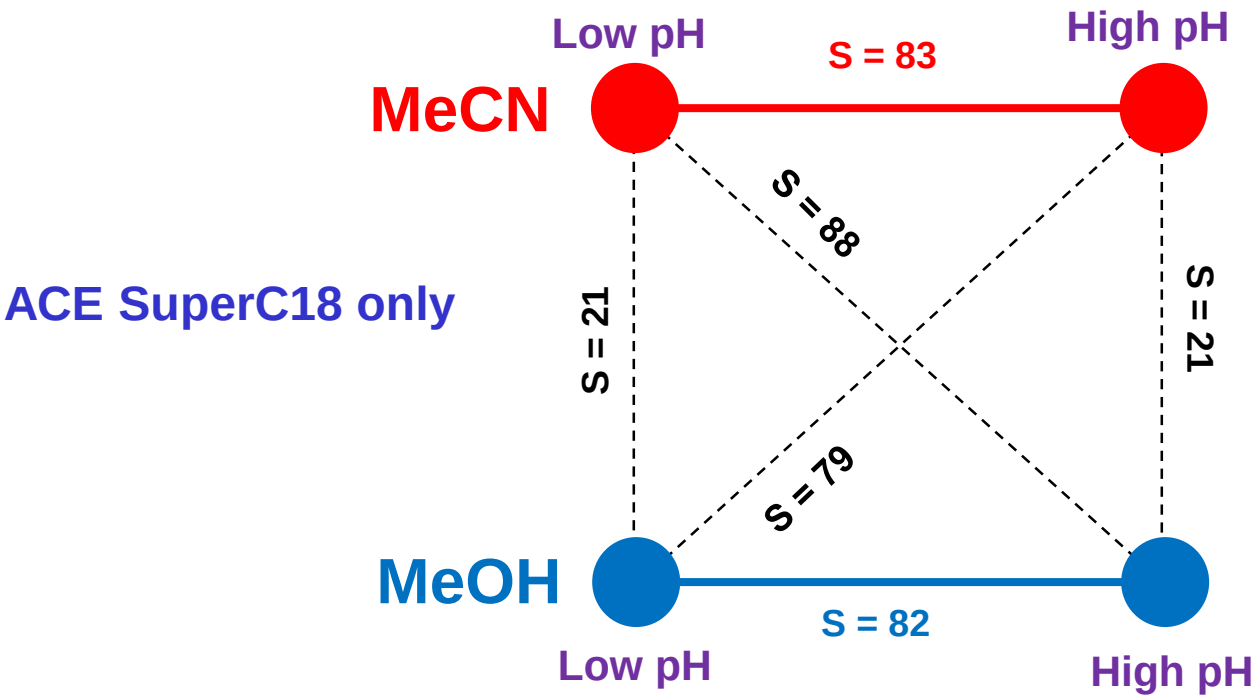
- Hydrophobic
- Dipole-dipole
- Polar retention

Complementary Mechanisms of Interaction

Data from Euerby et al., submitted to LC GC, July 2015

Extended Selectivity Method Development Platform #3

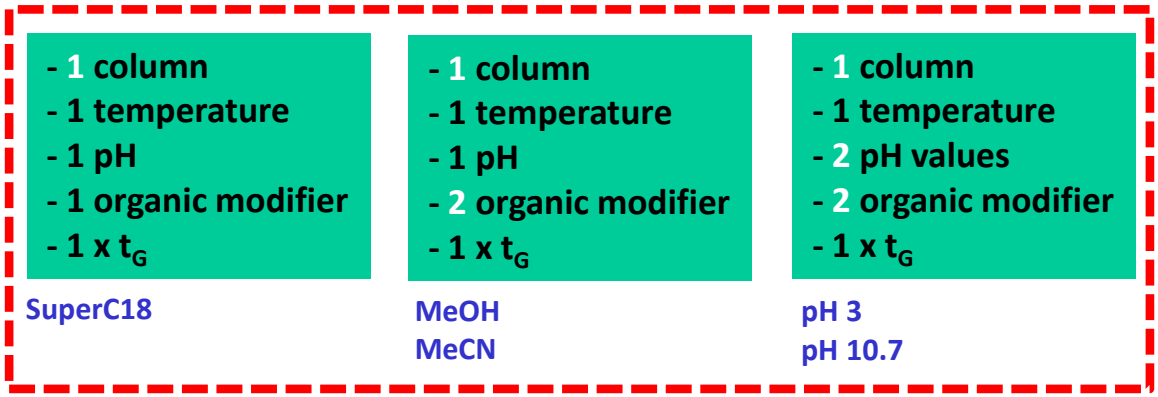
- ACE SuperC18: 45 analytes, 2 solvents, pH 2.5 & pH 10.7



SuperC18 Gives the Option to Explore a Wide pH Range to Tune Selectivity

Extended Selectivity Method Development Platform #3

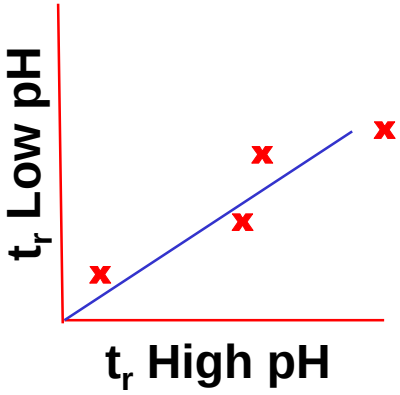
Workflow Schematic

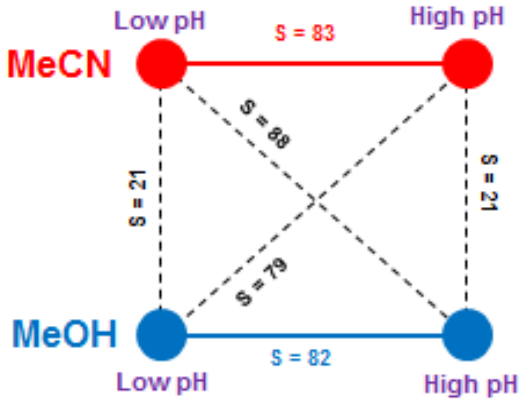


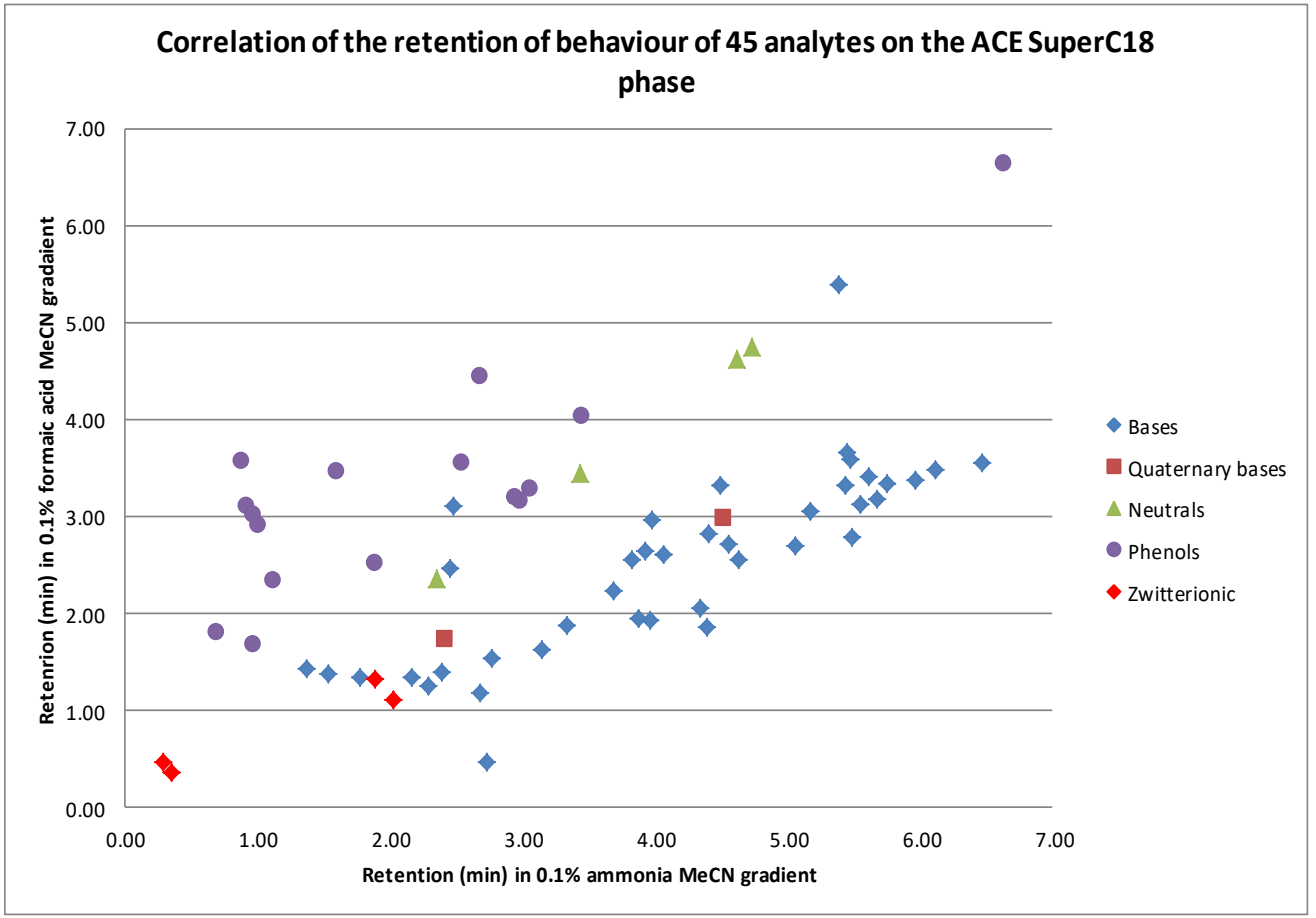
1 column, 2 solvent, 2 pH method development / screening approach based on selectivity data

ACE® SuperC18™: Exploring Selectivity with High / Low pH

Selectivity = 100 x √ (1 – R^2)





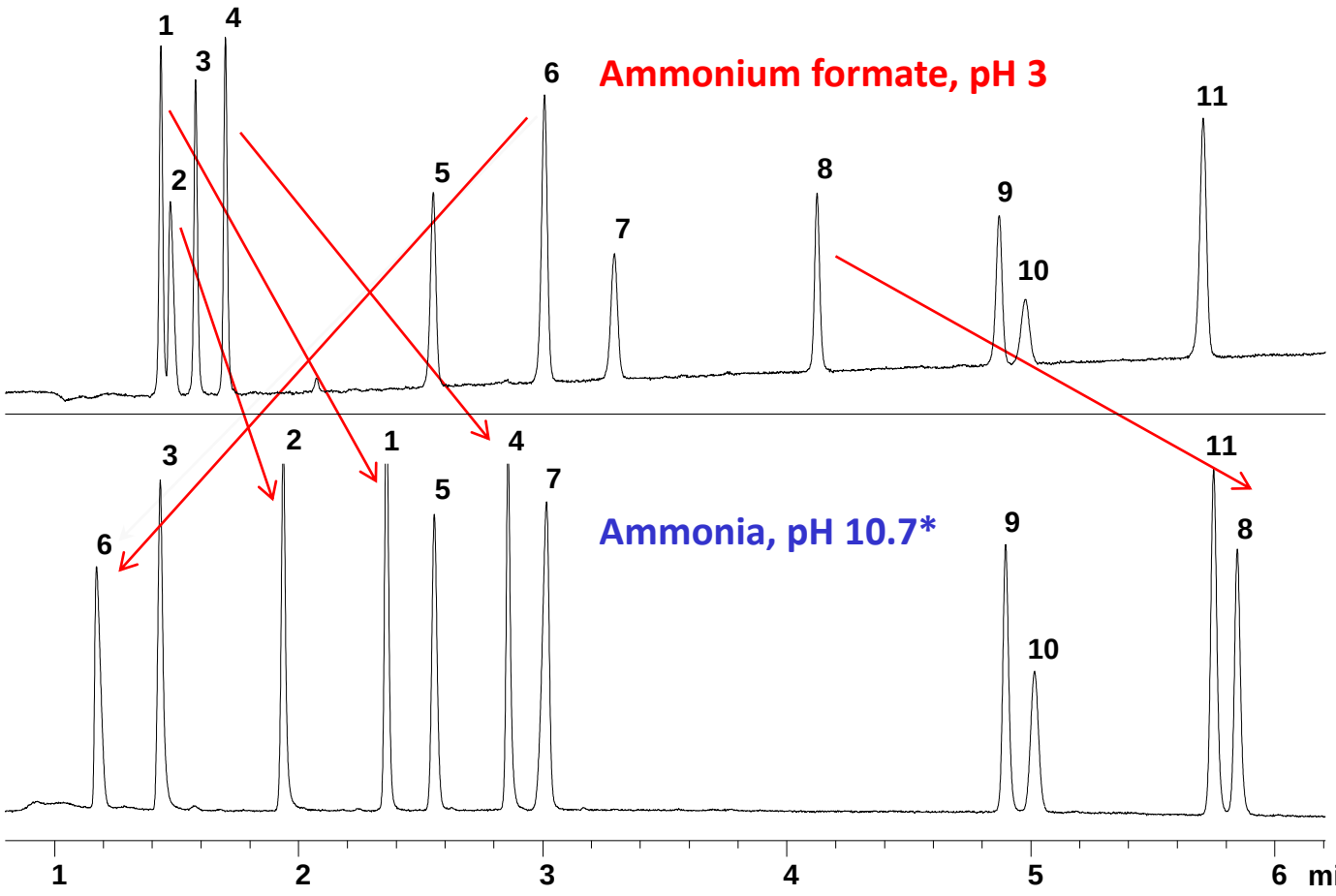


Selectivity = 83 → Powerful For Method Development

Low / High pH Switching...

- **Screening platforms / method development systems**
  - **Peak tracking**

ACE SuperC18



A1= 10mM $HCOONH_4$, pH3 (aq)
B1= 10mM $HCOONH_4$, pH 3 in 90% MeCN
A2= 0.1% NH_3, pH 10.7 (aq)
B2= 0.1% NH_3, pH10.7 in 90% MeCN

ACE Excel 3 SuperC18
50x2.1mm
Gradient: 3 – 100 %B in 7 mins
Flow rate: 0.42 mL/min
Temperature: 40C
Detection: 254 nm
Injection: 2uL

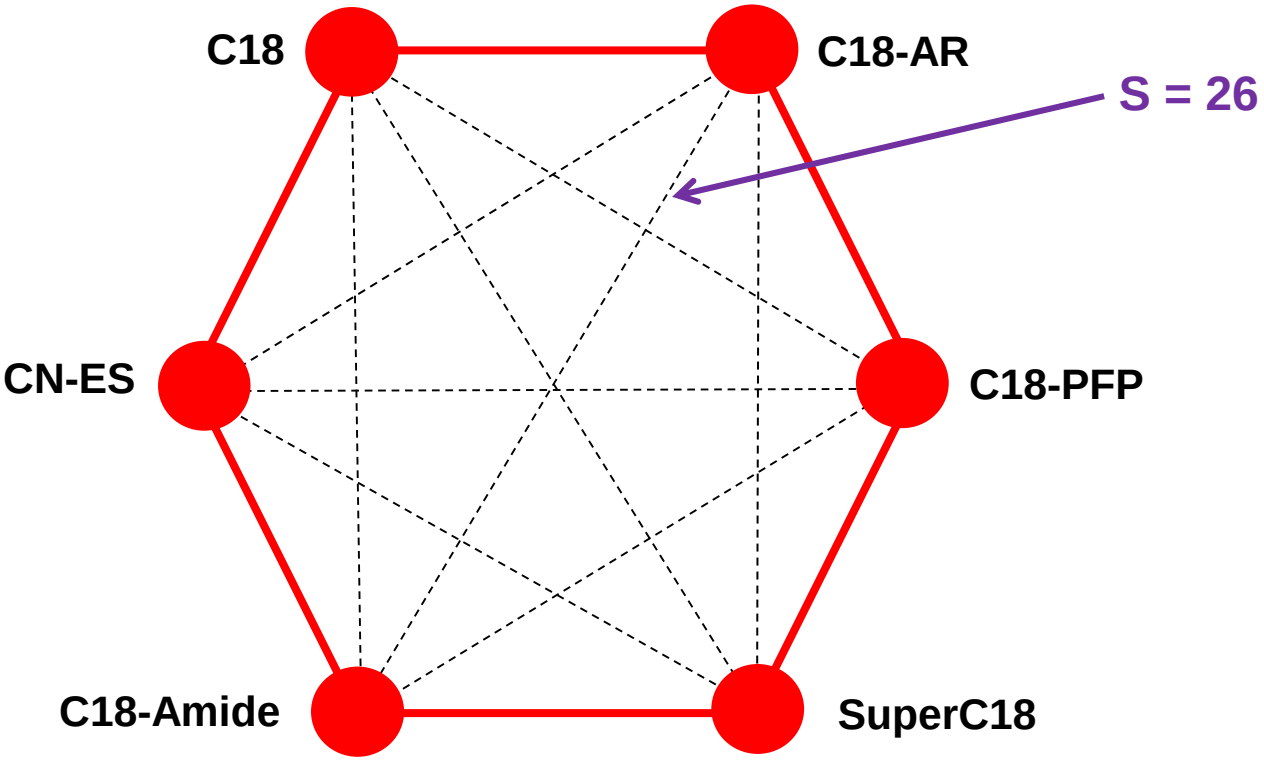
1. Nizatidine 2. Salbutamol 3. Amiloride 4. N-Acetylprocainamide 5. Quinoxaline 6. Methyl paraben 7. p-Cresol 8. Reserpine 9. Piperine 10. Toluene 11. Felodipine

*Equivalent to 18mM

Total Selectivity, Method Development: 6 Column Switcher

- 45 analytes, 6 stationary phases, 1 solvent, 1 low pH

MeCN

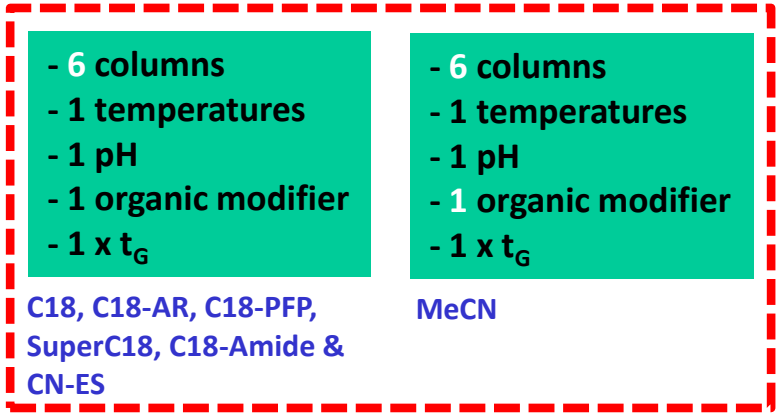


Total 6 Column Method Development Platform Based Upon The Power of Phase Selectivity

Data from Euerby et al., submitted to LC GC, July 2015

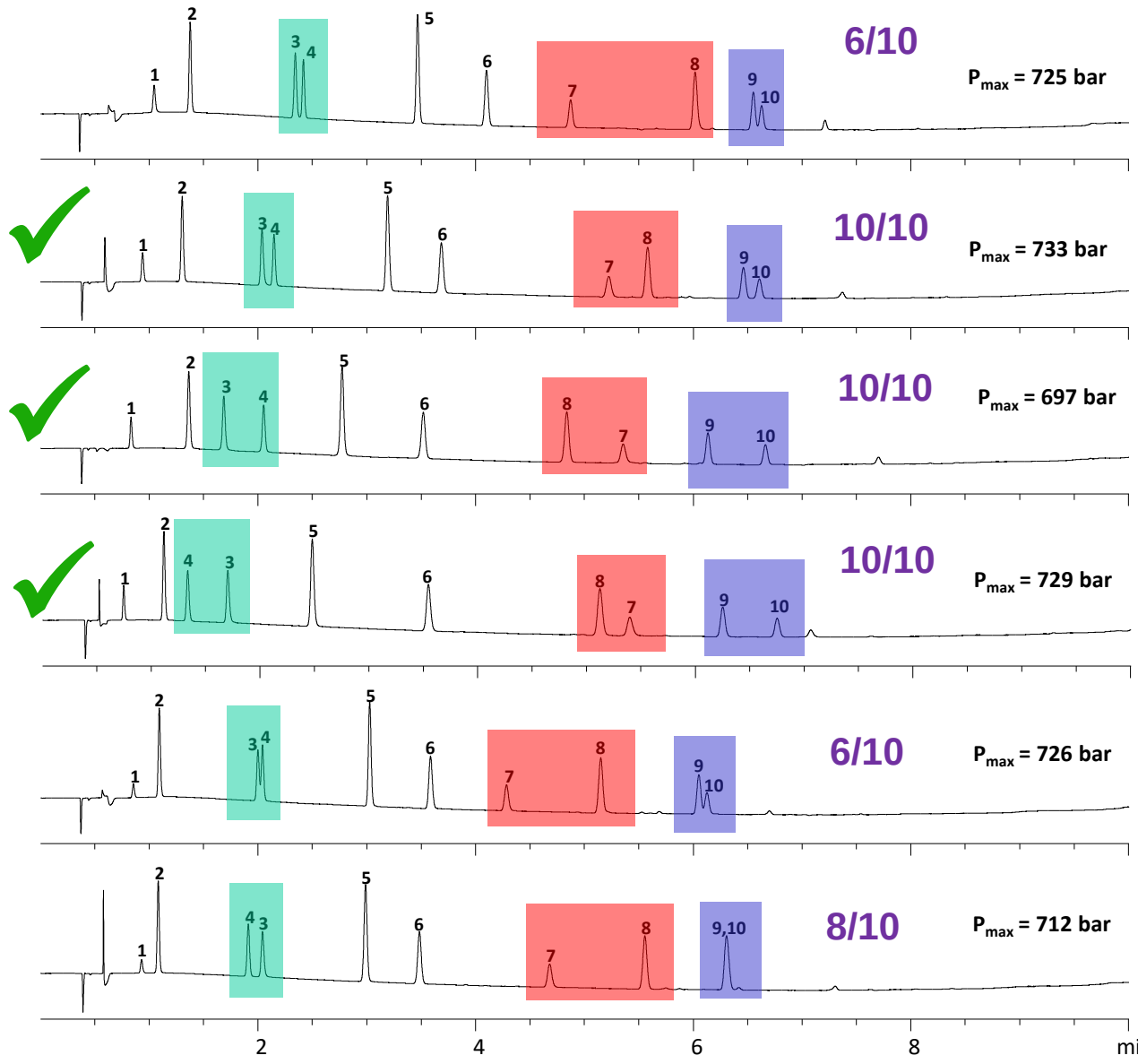
Total Selectivity, Method Development: Screening Platform

Workflow Schematic



6 columns, 1 solvents method development / screening approach based on selectivity data

Total Selectivity, Method Development: Screening Platform



Acetaminophen and related analytes

ACE Excel 2 Super C18 100 x 3.0 mm

ACE Excel 2 C18-PFP 100 x 3.0 mm

ACE Excel 2 C18-Amide 100 x 3.0 mm

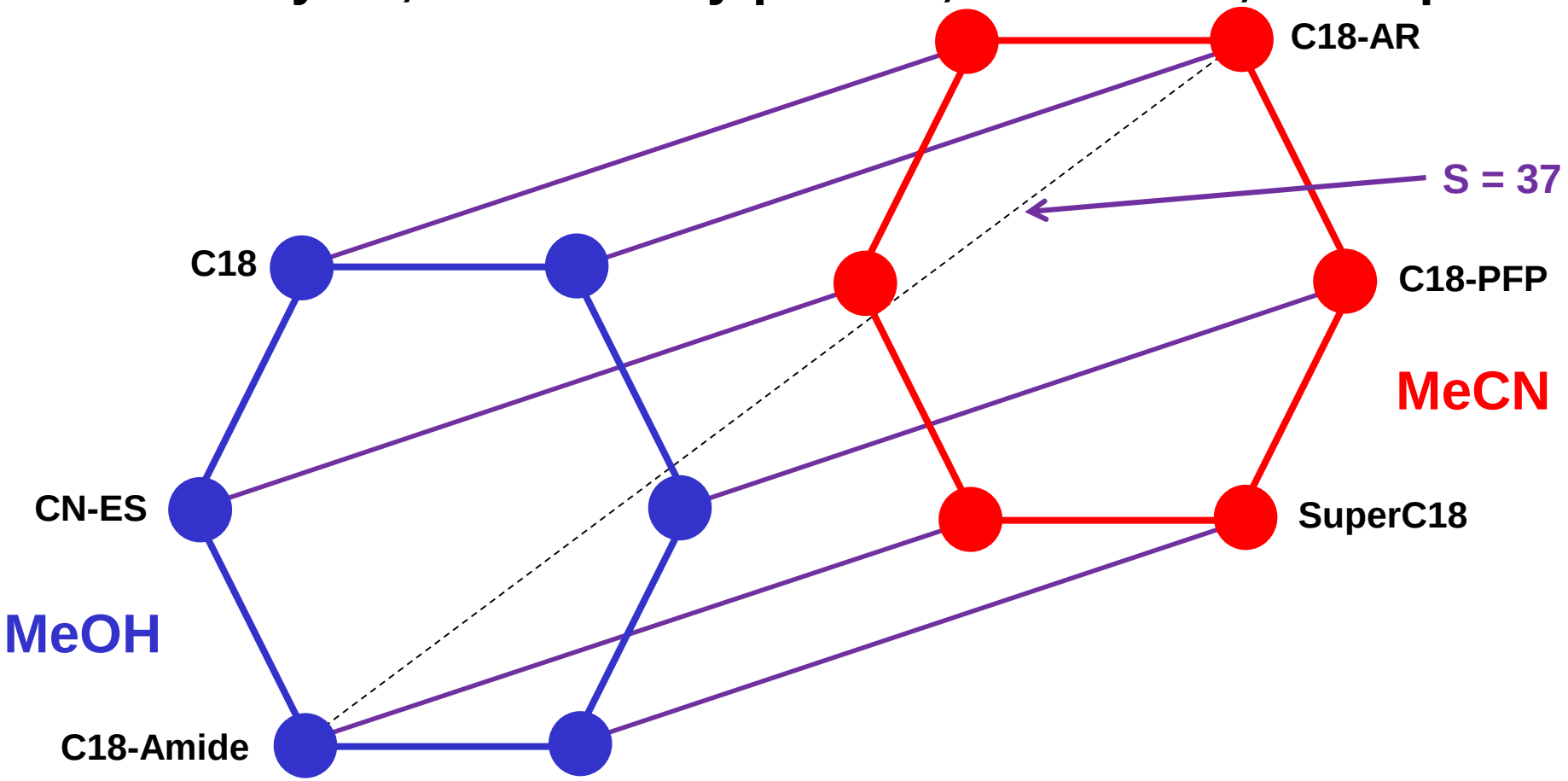
ACE Excel 2 CN-ES 100 x 3.0 mm

ACE Excel 2 C18 100 x 3.0 mm

ACE Excel 2 C18-AR 100 x 3.0 mm

Total Selectivity, Method Development: 6 Column Switcher

- 45 analytes, 6 stationary phases, 2 solvents, 1 low pH

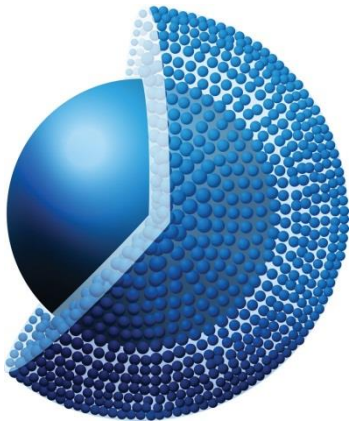


Total 6 Column Method Development Platform Based Upon The Power of Selectivity

Data from Euerby et al., submitted to LC GC, July 2015

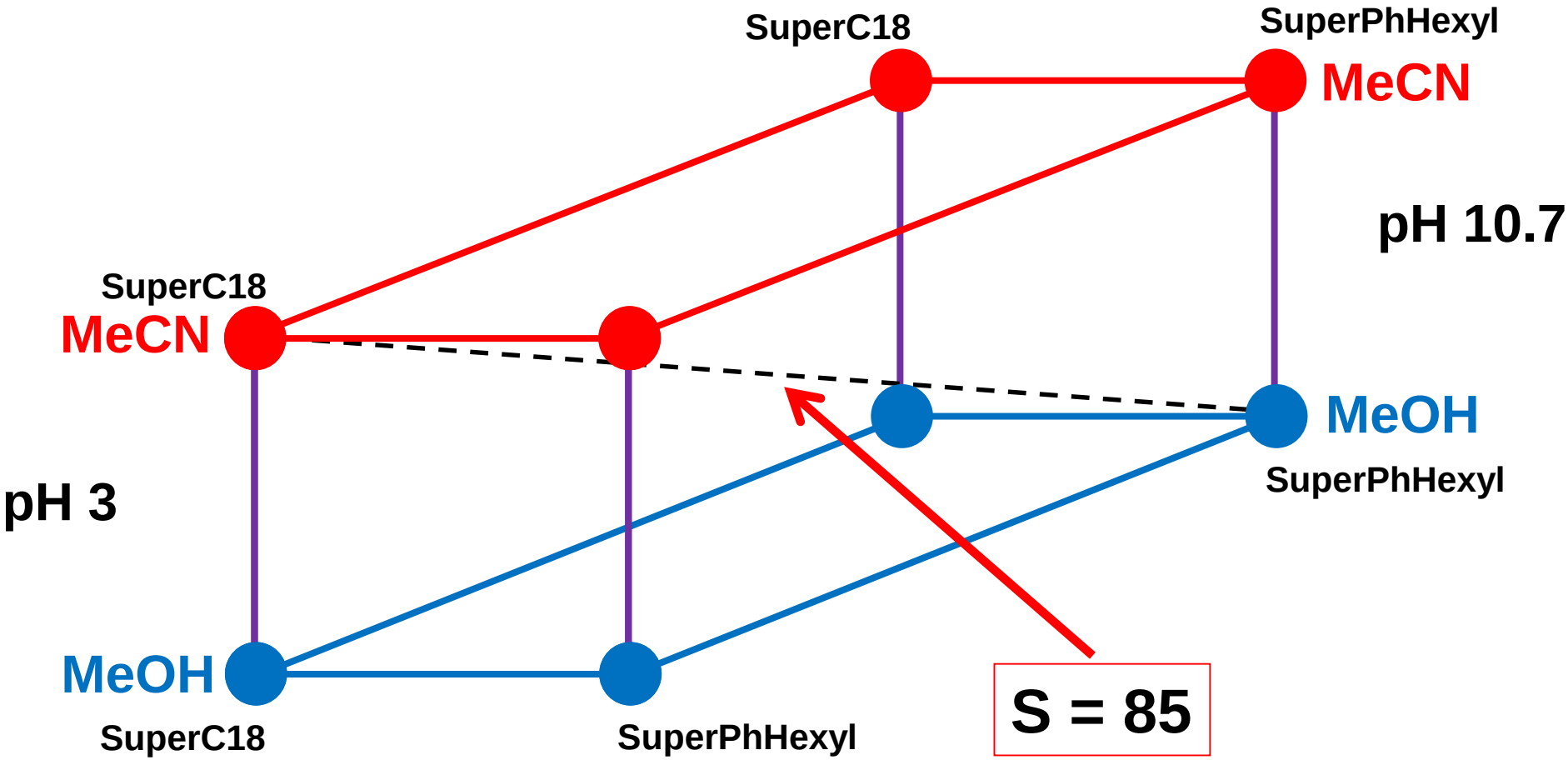
Solid Core Particles: UltraCore Method Development Platform

- **Silica** based solid core particles
- **SuperC18** and **SuperPhenylHexyl** bonded phases for alternative selectivity: **hydrophobic / aromatic** interactions.
- **Encapsulated Bonding Technology** provides **inertness** (sharp peaks) & **protects** the silica surface from eluent **pH 1.5 – 11.0**.



ACE UltraCore

UltraCore Method Development Platform #4

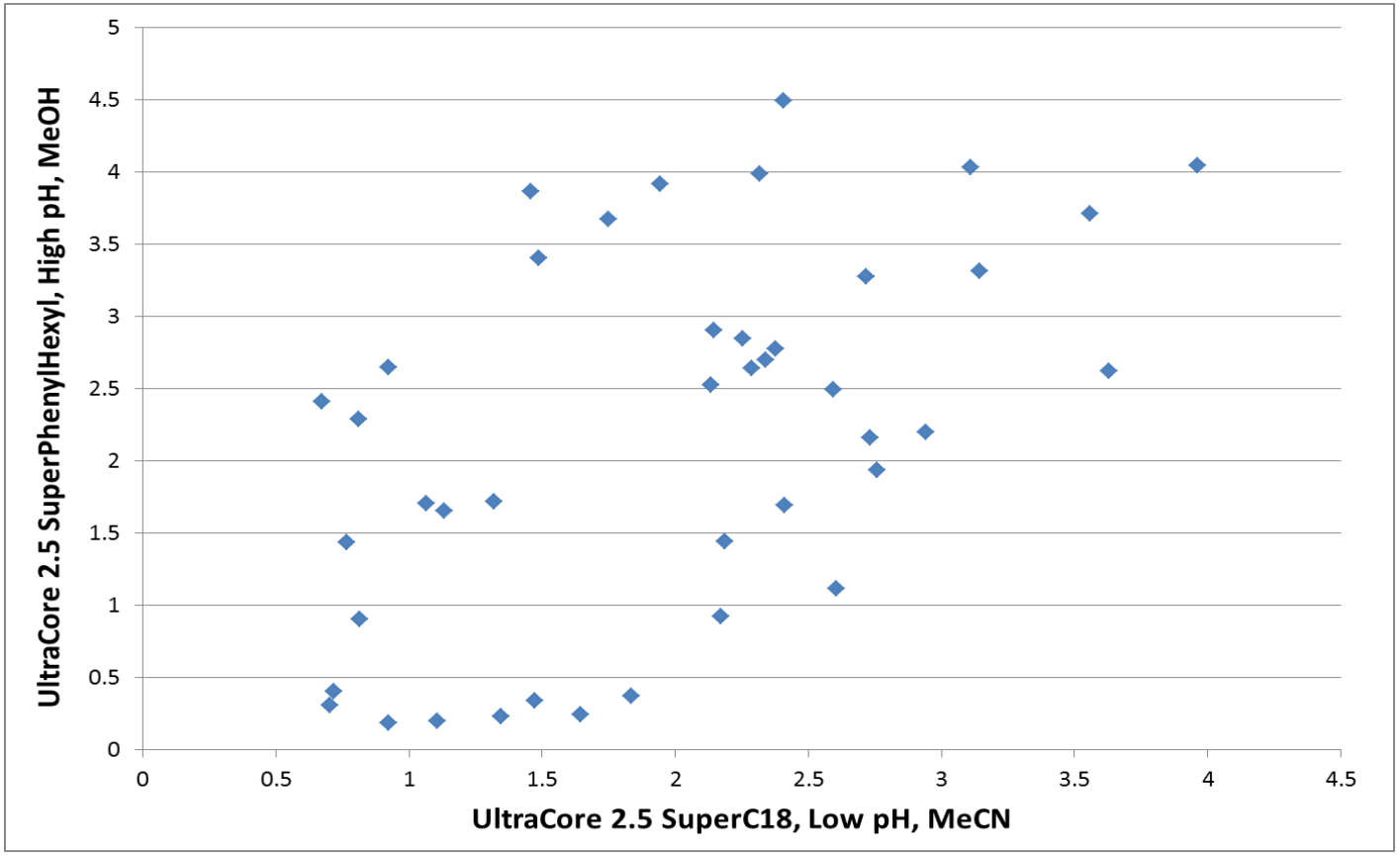


ACE® UltraCore™ Method Development Platform

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

UltraCore: Exploring Phase, Solvent & pH Selectivity

ACE UltraCore SuperC18, low pH, MeCN vs SuperPhenylHexyl, high pH, MeOH



Range of 50 Analytes To Describe Selectivity

Selectivity = 85 → Fully Explore The Selectivity 'Space'

UltraCore Method Development Platform #4

Workflow Schematic

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

Information Rich Data Based on Selectivity

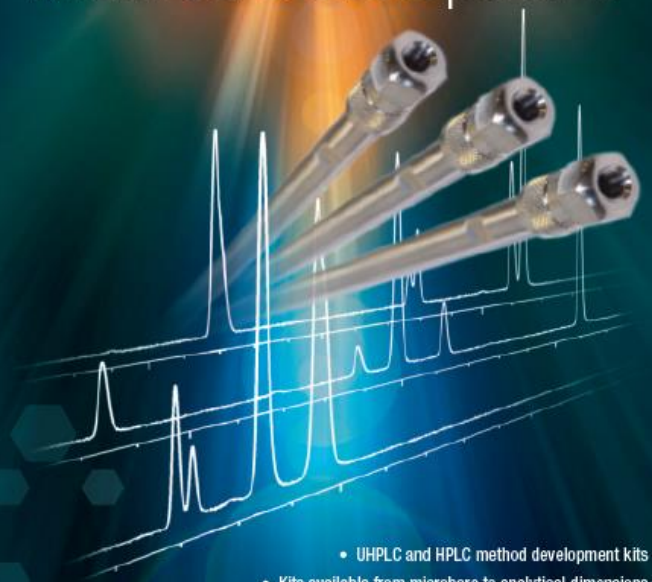
2 UltraCore columns, 2 solvents, 2 pH method development / screening approach based on selectivity data

ACE® Method Development Kit Brochure

Selectivity Offer:
2 and 3 column kits available for the same price as a single column

ACE®
Method Development Kits

Intelligent Solutions for Method Development



- UHPLC and HPLC method development kits
- Kits available from microbore to analytical dimensions
- Porous, solid-core and biomolecule options
- Wide range of particle sizes and complementary phases available
- Excellent peak shape, efficiency, reproducibility and lifetime
- Highly cost effective

ACE
UHPLC and HPLC Columns

ACE Method Development Kits

Intelligent Solutions for Method Development

- **Highly cost effective -** ACE Method Development Kits are available for the same price as a single column!
- 4 different ACE Method Development Kits available from microbore (0.5mm id) through to analytical (4.6mm id) dimensions for rapid, systematic method development.
- Each kit contains carefully selected ACE phases which enables the power of selectivity to be fully exploited.
- Each ACE phase provides different selectivity due to differing interactions.

		Bonded Phase	Separation Mechanism and Relative Strength[1]				
			Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
1	ACE Advanced Method Development Kit (see pages 4-7)	ACE C18	****	-	-	*	**
		ACE C18-AR	****	*** (donor)	*	**	***
		ACE C18-PFP	****	*** (acceptor)	****	***	****
2	ACE Extended Method Development Kit (see pages 8-11)	ACE SuperC18	****	-	-	-	**
		ACE C18-Amide	****	-	**	****	**/***
		ACE CN-ES	***	*	***	**	*
3	ACE UltraCore Method Development Kit (see pages 12-14)	ACE UltraCore SuperC18	***	-	-	-	**
		ACE UltraCore SuperPhenylHexyl	**	*** (donor)	*	**	***
4	ACE Bioanalytical 300Å Method Development Kit (see pages 15-17)	ACE C18-300	**	-	-	*	*
		ACE C4-300	*	-	-	-	-
		ACE Phenyl-300	*	** (donor)	*	**	**

[1] Approximate value – determined by semi-quantitative mechanism weightings and/or by reference to other ACE phases using >100 characterising analytes.

FREE Method Development Support!

- Not sure which ACE phase or kit will work best for your application?
- FREE Application Support and FREE Method Development Service
- Trust your method development to our experts and free up time for your other projects!
- Contact our expert method development team via **info@ace-hplc.com** or contact your local distributor

Learn More: www.ace-hplc.com

ACE® Method Development Kit Brochure (II)

		Bonded Phase	Separation Mechanism and Relative Strength[1]				
			Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
1	ACE Advanced Method Development Kit (see pages 4-7)	ACE C18	****	-	-	*	**
		ACE C18-AR	****	*** (donor)	*	**	***
		ACE C18-PFP	****	*** (acceptor)	****	***	****
2	ACE Extended Method Development Kit (see pages 8-11)	ACE SuperC18	****	-	-	-	**
		ACE C18-Amide	****	-	**	****	**/***
		ACE CN-ES	***	*	***	**	*
3	ACE UltraCore Method Development Kit (see pages 12-14)	ACE UltraCore SuperC18	***	-	-	-	**
		ACE UltraCore SuperPhenylHexyl	**	*** (donor)	*	**	***
4	ACE Bioanalytical 300Å Method Development Kit (see pages 15-17)	ACE C18-300	**	-	-	*	*
		ACE C4-300	*	-	-	-	-
		ACE Phenyl-300	*	** (donor)	*	**	**

[1] Approximate value – determined by semi-quantitative mechanism weightings and/or by reference to other ACE phases using >100 characterising analytes.

Overall Conclusions

- **Selectivity** is helpful in chromatography
- **Understanding selectivity** aids method development by focussing efforts on **high impact** variables
- It is possible to **design** stationary phases to **maximize selectivity**
- **Screening columns** with **differing retention mechanisms** is useful for method development
- Various **optimized** method development platforms **based on selectivity** have been described

Thank You For Your Attention

All ACE products are available globally

USA: http://www.mac-mod.com/