Exploiting Selectivity in HPLC and UHPLC With Rational Stationary Phase Design

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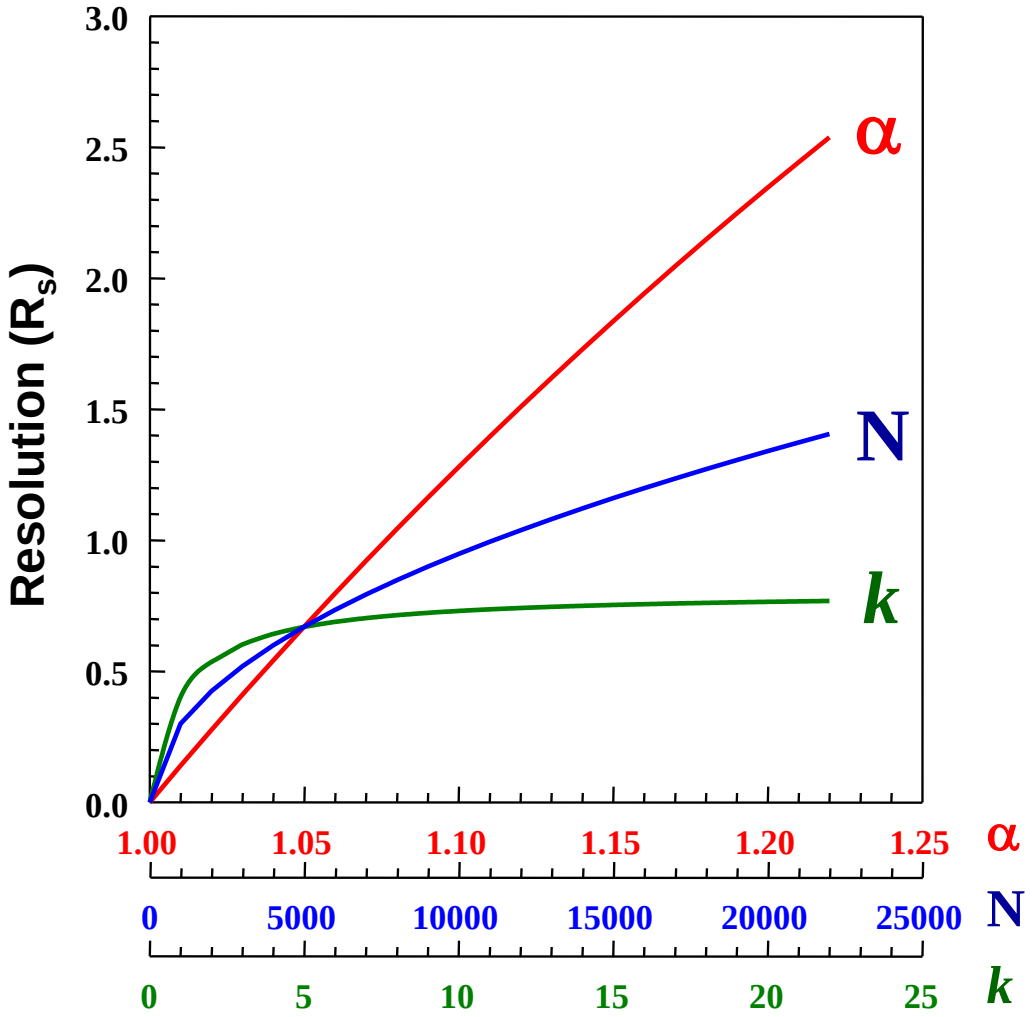
Outline

- Chromatographic selectivity
- Stationary phase design concepts
- The unique ACE® C18-AR and ACE® C18-PFP phases
- Introducing the NEW ACE® *Excel*™ UHPLC products
- Examples
- Conclusions

Chromatographic Peak Resolution

Efficiency Selectivity Retention

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



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

The Importance of *N*, *k* and α For Resolution

Typical separation:

N = 10,000 plates

k = 3.8 / 4.2 (4.0 mean)

α = 1.1

$$R_S = \frac{1}{4}\sqrt{10{,}000}\left(\frac{1.1-1}{1.1}\right)\left[\frac{4}{1+4}\right]$$

$$R_S = 1.8$$

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

Which looks like →

The Importance of *N*, *k* and α For Resolution

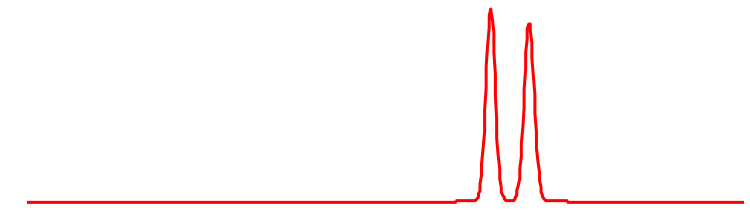
Double Efficiency (eg 5 μm → 2.5 μm):

N = 10,000 → 20,000 plates

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

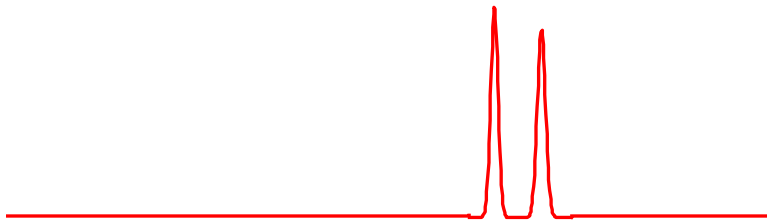
$$R_S = \frac{1}{4}\sqrt{20{,}000}\left(\frac{1.1-1}{1.1}\right)\left[\frac{4}{1+4}\right]$$

$$R_S = 2.6$$



$R_s = 1.8$

~40% Increase



$R_s = 2.6$

Opportunity to optimise further eg reduce column length to speed up

The Importance of *N*, *k* and α For Resolution

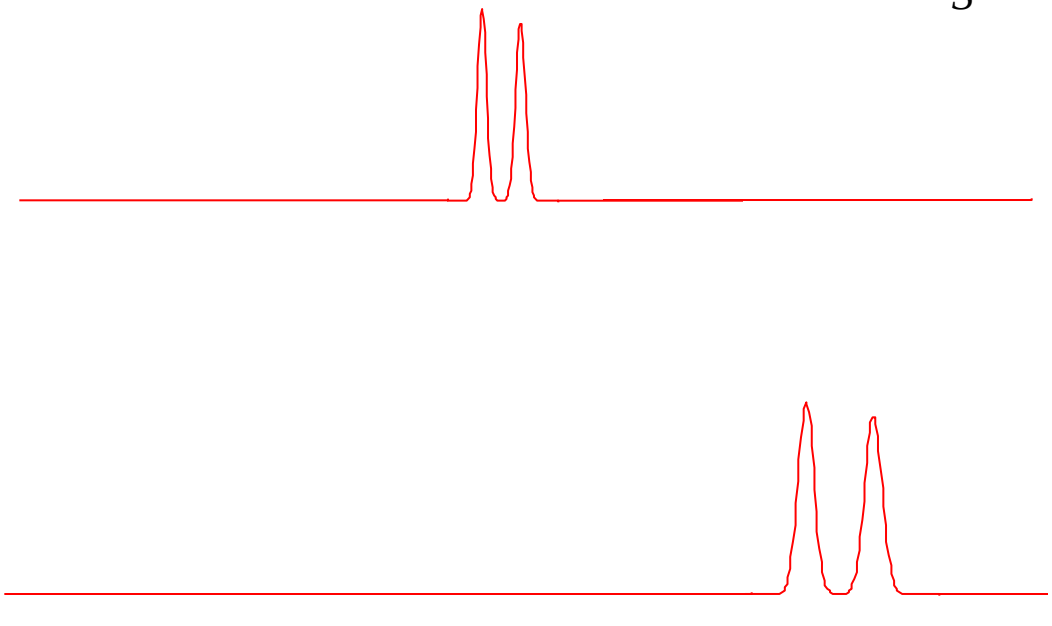
Double Retention Factor (eg decrease solvent strength):

k = 4 → 8

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

$$R_S = \frac{1}{4}\sqrt{10{,}000}\left(\frac{1.1-1}{1.1}\right)\left[\frac{8}{1+8}\right]$$

$$R_S = 2.0$$



$R_s = 1.8$

~10% Increase

$R_s = 2.0$

Slight improvement in resolution has led to increased analysis time

The Importance of *N*, *k* and α For Resolution

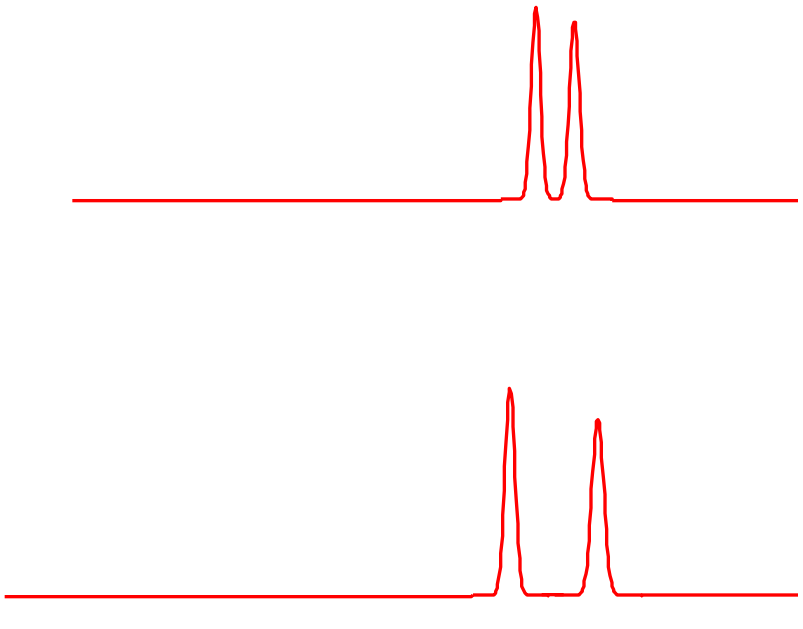
Increase Selectivity (eg change column):

α = 1.1 → 1.2

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

$$R_S = \frac{1}{4}\sqrt{10{,}000}\left(\frac{1.2-1}{1.2}\right)\left[\frac{4}{1+4}\right]$$

$$R_S = 3.3$$



R_s = **1.8**

~80% Increase

R_s = **3.3**

Significant opportunity to speed up for modest change in selectivity

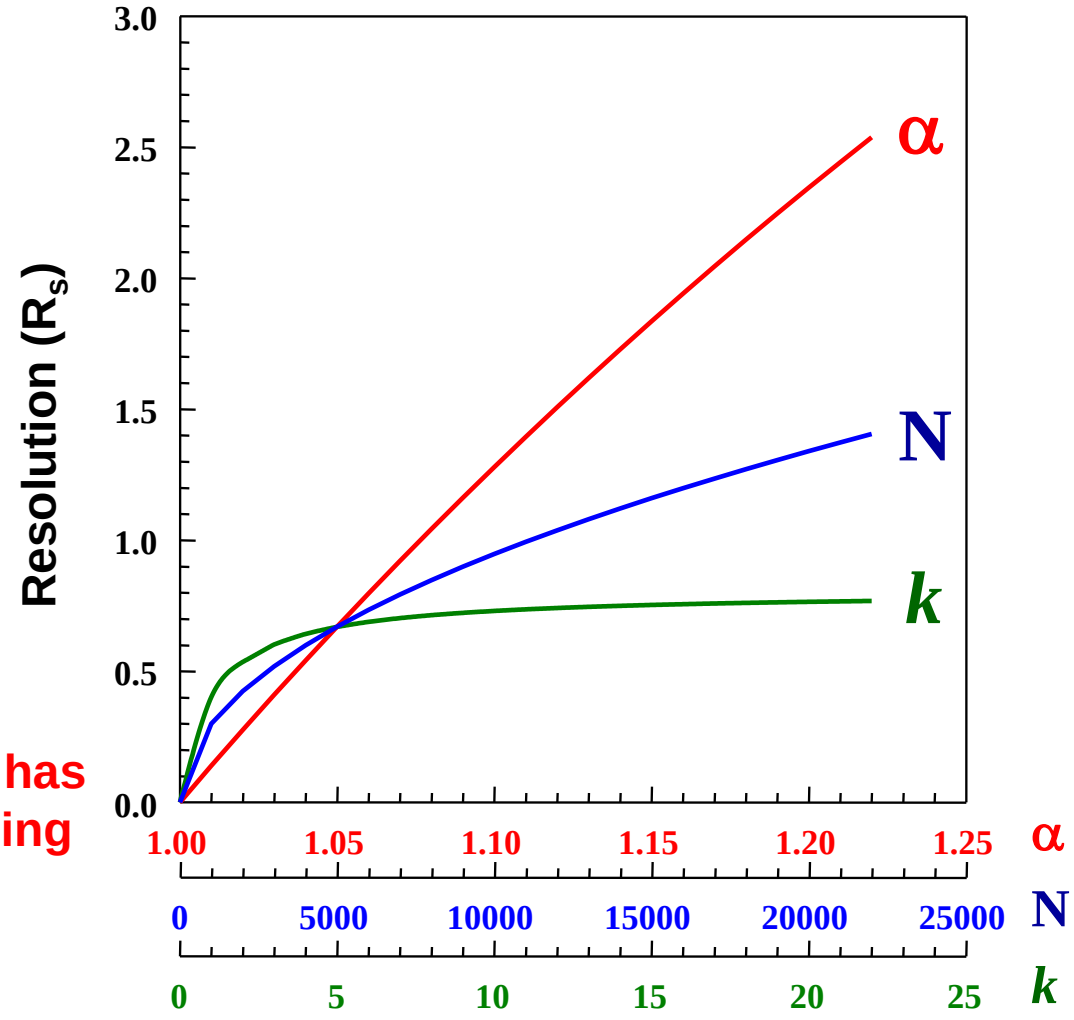
Selectivity: The Key to Chromatographic Peak Resolution

Efficiency | Selectivity | Retention

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

~40% ~80% ~10%

From the examples, selectivity has the greatest impact on increasing peak resolution

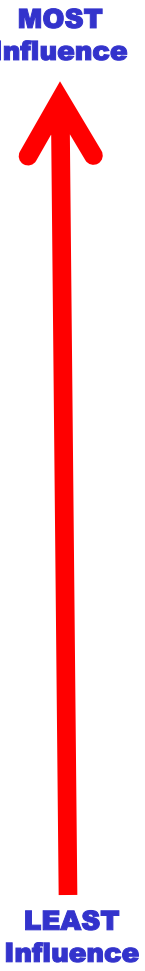


Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Which Factors[a] Affect Selectivity?

Isocratic Separations

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



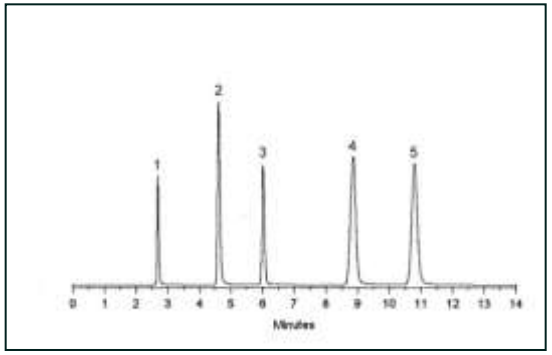
Gradient Separations

- All parameters for isocratic
- Gradient steepness
- *k**
- Dwell volume
- Column dimensions

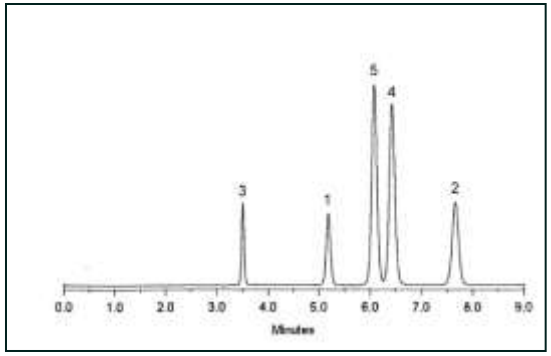
a Adapted from 'Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & sons

Influencing Selectivity – Bonded Phase Effects / Basic Analytes

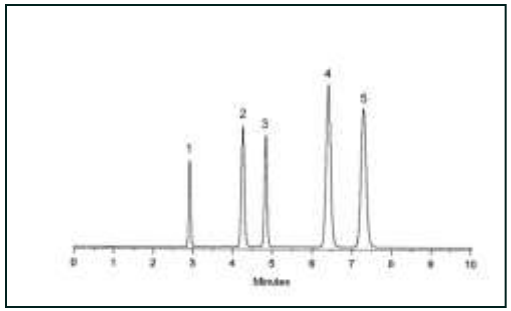
ACE C18 – Increase Retention



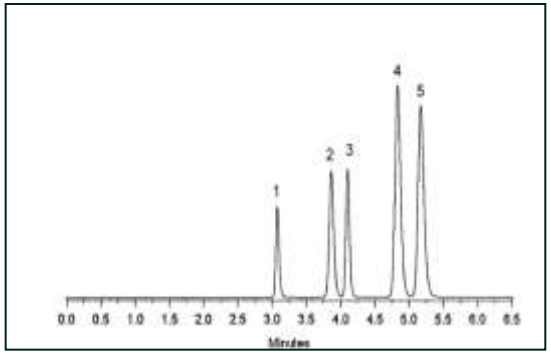
ACE CN – Elution Order



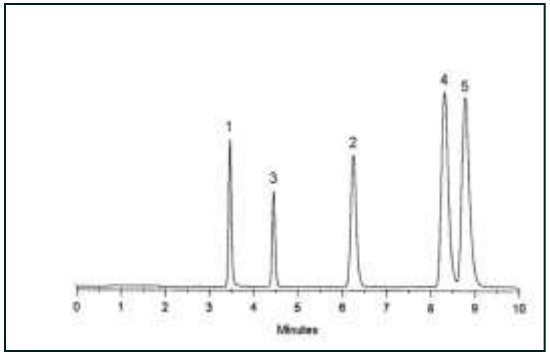
ACE C8 (start point)



ACE C4 – Decrease Retention



ACE Phenyl – Elution Order



Use ultra high purity silica for good chromatography and reproducibility

Column: 250 x 4.6mm 5µm Mobile phase: 80:20 MeOH/25mM KH_2PO_4 (pH6.0) Flow: 1.00ml/min
Components; 1: Norephedrine, 2: Nortriptyline, 3: Toluene, 4: Imipramine, 5: Amitriptyline Wavelength: 215nm

HPLC End User Surveys[a] ...Listening To The Analyst

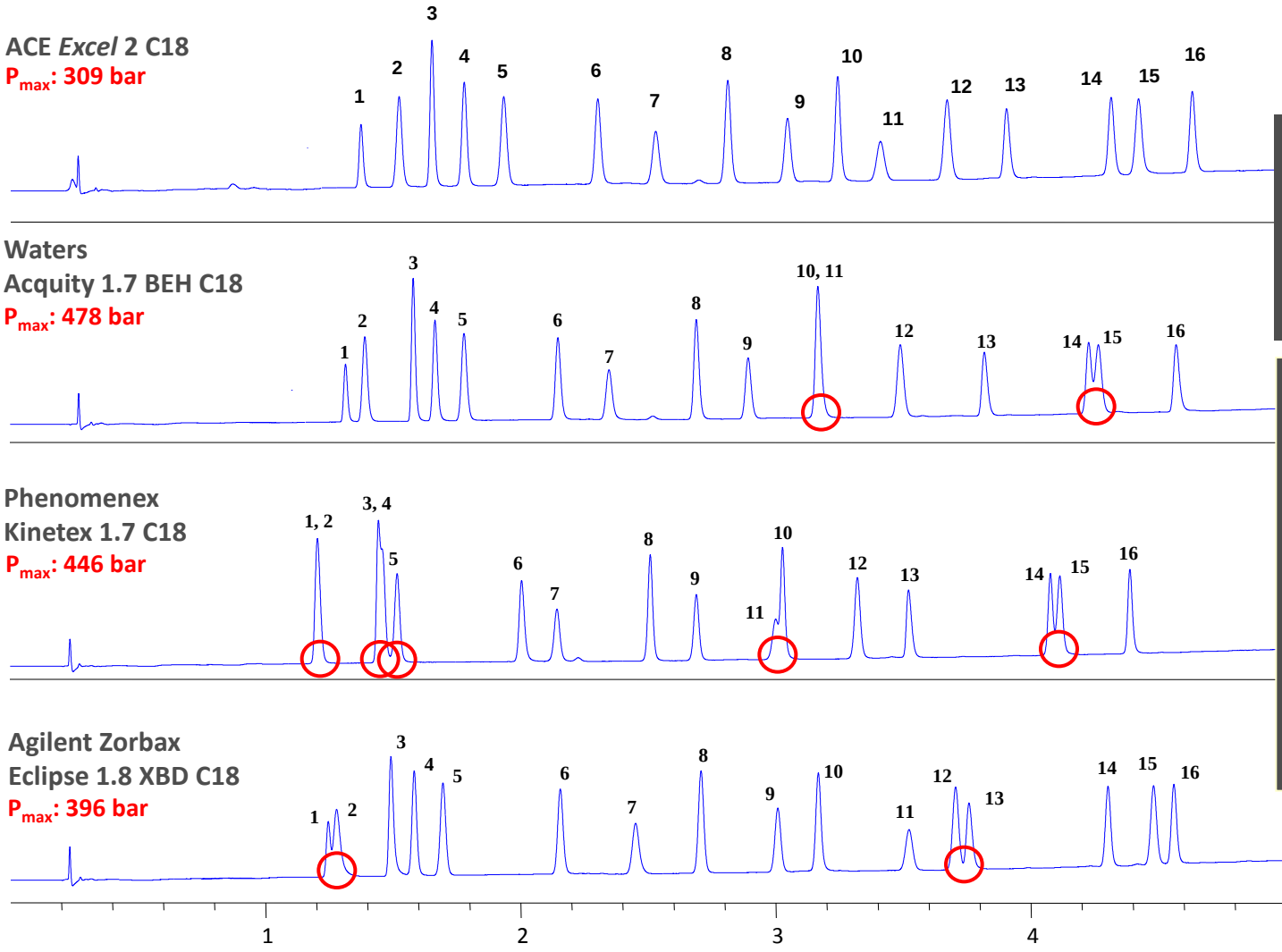
- **Column reproducibility and column lifetime are major factors for analysts**
  - Have been the top 2 feedback points since 2007
  - Critical in pharmaceutical and other major industries for method transfers / consistency and long term performance

- **Reversed-phase is the dominant separation mode**
  - C18 & C8 = 60%; Phenyl = 16%; CN = 9.5%; Fluorinated = 5.9%
  - 92% analysts use C18 at some time in their work...they typically meet the above criteria

  - BUT limited selectivity

[a] *Current Trends in HPLC Column Usage*, R.E. Majors, 1Jan12, *Modern Medicine website*, accessed on 15Feb12: http://www.modernmedicine.com/modernmedicine/article/articleDetail.jsp?id=755108

16 Pharmaceutically Relevant Analytes – C18 Columns



50x2.1mm
A: 20 mM KH_2PO_4, pH 2.7
B: 20 mM KH_2PO_4, pH 2.7 in MeOH/H_2O (65:35 v/v)
Gradient: 3 – 100 %B in 5 min
Flow rate: 0.6 ml/min
Temperature: 60C
Detection: 214 nm

1. N-Acetylprocainamide
2. 3-Hydroxybenzoic acid
3. Pindolol
4. Methylphenylsulfoxide
5. Benzyl alcohol
6. Quinoxaline
7. 1,4-Dinitrobenzene
8. Phenacetin
9. 1,2-Dimethoxybenzene
10. Furosemide
11. Anisole
12. Methylbenzoate
13. Remacemide
14. Nimesulide
15. Ethylbenzoate
16. Diflunisal

C18 phases show 'similar' selectivity...

The Challenge…

- To engineer new phases with alternative selectivity but with the robust properties of the C18 ligand
  - Reproducible (column-to-column & batch-to-batch)
  - Excellent column lifetime
  - Superb efficiency provided by ultra-inert, ultra-pure silica particle
  - Low MS bleed
  - Usable in 100% aqueous eluents
- Available for HPLC & UHPLC separations
- Available as a 'Phase III Ready' product family
  - Globally available, supply chain, reproducible, multiple batches etc

Aromatic Functionality – Engineering New Stationary Phases

- **Phases with aromatic functionality include phenyl and pentafluorophenyl (PFP) based ligands**

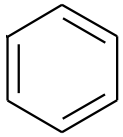
- **Advantages**
  - **Aromatic functionality potentially offer unique interactions with analytes (c.f. C18) giving alternative selectivity**
  - **Provides enhanced retention of polar compounds**
  - **Many aromatic functionality-based phases can be used in 100% aqueous eluents**

- **Disadvantages**
  - **Phenyl / PFP phases may suffer phase bleed**
  - **Batch-to-batch reproducibility & robustness may be weak**

Aromatic Functionality: π – π Interactions

- A type of electron donor-acceptor interaction
- Originates from π systems in unsaturated functional groups on analytes and the stationary phase
- Types of π-π interaction can be manipulated for maximum effect (orthogonality) in phase design
  - eg phenyl: electron rich ring on the stationary phase also acts as π-base and interacts well with electron poor analytes
  - eg PFP: electron poor ring on the stationary phase also acts as π-acid and interacts well with electron rich analytes

The Power of π...Scientific Led Stationary Phase Design

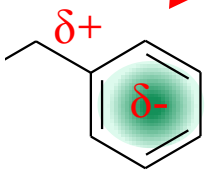


C_6H_6

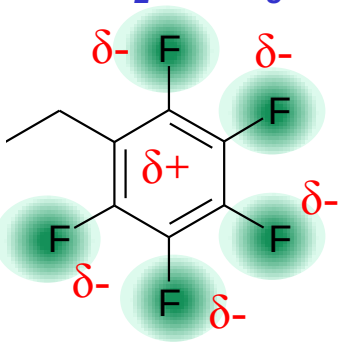
Electron Donating Groups
eg NH_2, NR_2, alkyl, OCH_3 OR, CH_3, Ar etc

Electron Withdrawing Groups
eg NO_2, halides, NR_3^+, CO_2H, CN, CO_2R, SO_3H, COH etc

e.g.

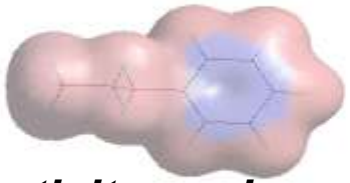


e.g.



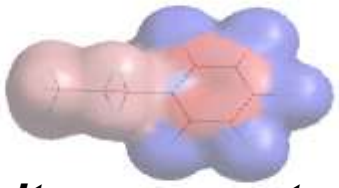
Classic π-π interaction

Electron Rich Ring



Activity: π-donor (π-base)

Electron Deficient Ring



Activity: π-acceptor (π-acid)

How do we exploit these properties for new stationary phases?

C18+Phenyl = ACE® C18-AR

C18+PFP = ACE® C18-PFP

Uniquely Designed Stationary Phases

- **ACE® C18-AR (USP L1)**
  - Ligand has C18 hydrophobic element PLUS phenyl character
- **ACE® C18-PFP (USP L1)**
  - Ligand has C18 hydrophobic element PLUS PFP character
- **Ultra-inert, ultra-pure silica particle technology as used in all ACE® products for high peak efficiency**
- **Available in 3, 5 & 10µm, (ACE®) and 2µm (ACE® *Excel*™)**

Multi-mode interaction mechanisms result in enhanced chromatographic selectivity giving the analyst new options for method development

ACE® C18-AR: Multi-Mode Separation Mechanisms

- **Combining the character of C18+phenyl into a single individual phase harnesses the best of both phases for unique selectivity**

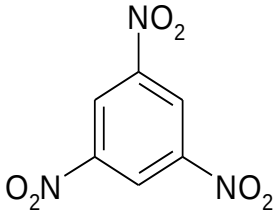
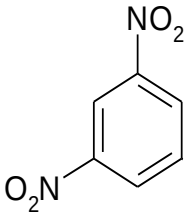
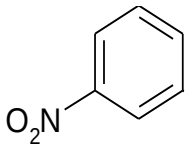
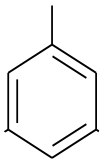
Separation mechanism	Typical C18	Typical Phenyl	ACE® C18-AR
Hydrophobicity	++++	+ / ++	++++
π-π Interaction	-	+++	+++
Dipole - Dipole	-	+	+
Hydrogen Bonding	-	++	++
Shape Selectivity	++	++	++ / +++

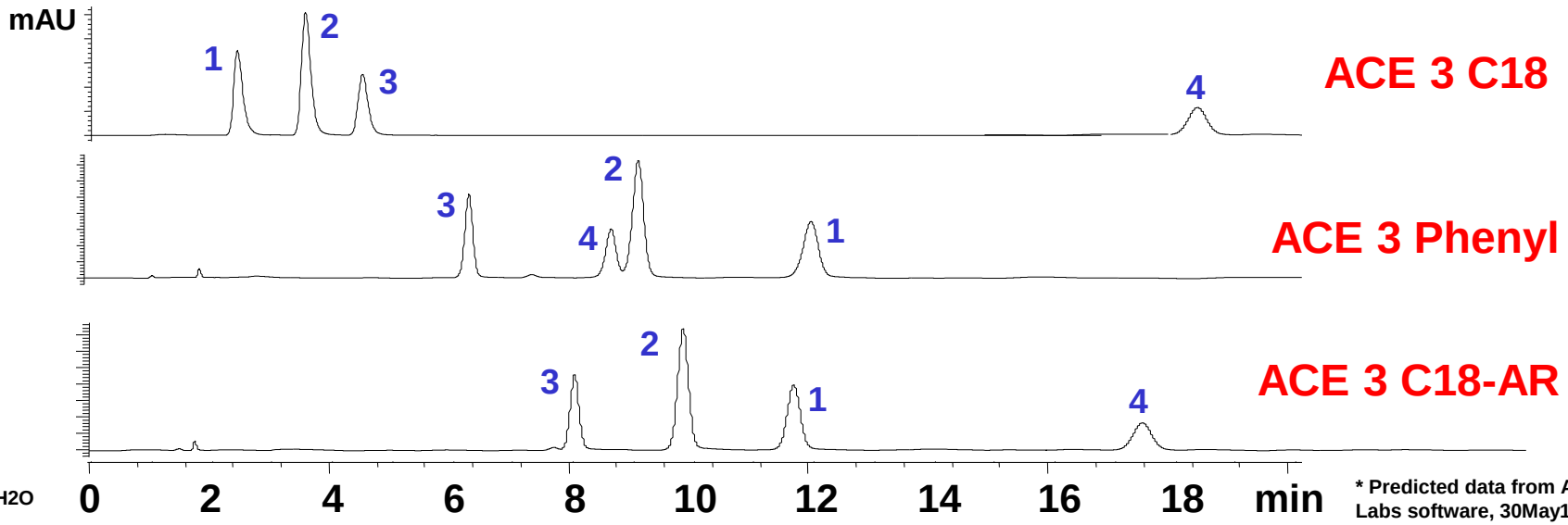
- **The predominance of each retention mechanism will be dictated by the analyte's physicochemical properties, its structure and the chromatographic conditions applied**

Multi-Mode Interactions Offer the Chromatographer More

ACE® C18-AR Aromatic Selectivity

- Illustrating hydrophobicity and π-base character / aromatic selectivity with a simple example using substituted aromatics

	1. TNB	2. DNB	3. NB	4. Tol
				
Log P*	1.2	1.6	1.9	2.7
π-acidity (order)	1	>	2	>	3	-



150x4.6 mm id
1 mL/min
40C, 210nm
1:1 v/v MeOH:H2O

* Predicted data from ACD Labs software, 30May12

ACE® C18-PFP: Multi-Mode Separation Mechanism

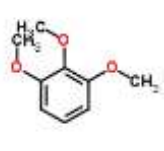
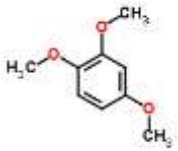
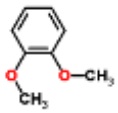
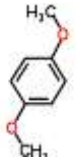
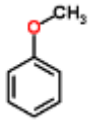
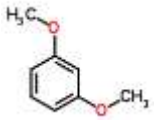
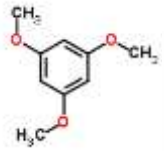
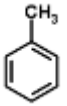
- **Combining the character of C18+PFP into a single individual phase harnesses the best of both phases for unique selectivity**

Separation mechanism	Typical C18	Typical PFP	ACE® C18-PFP
Hydrophobicity	++++	+ / ++	++++
π-π Interaction	-	+++	+++
Dipole - Dipole	-	++++	++++
Hydrogen Bonding	-	+++	+++
Shape Selectivity	++	+++	++++

- **The predominance of each retention mechanism will be dictated by the analyte's physicochemical properties, its structure and the chromatographic conditions applied**

Multi-Mode Interactions Offer the Chromatographer More

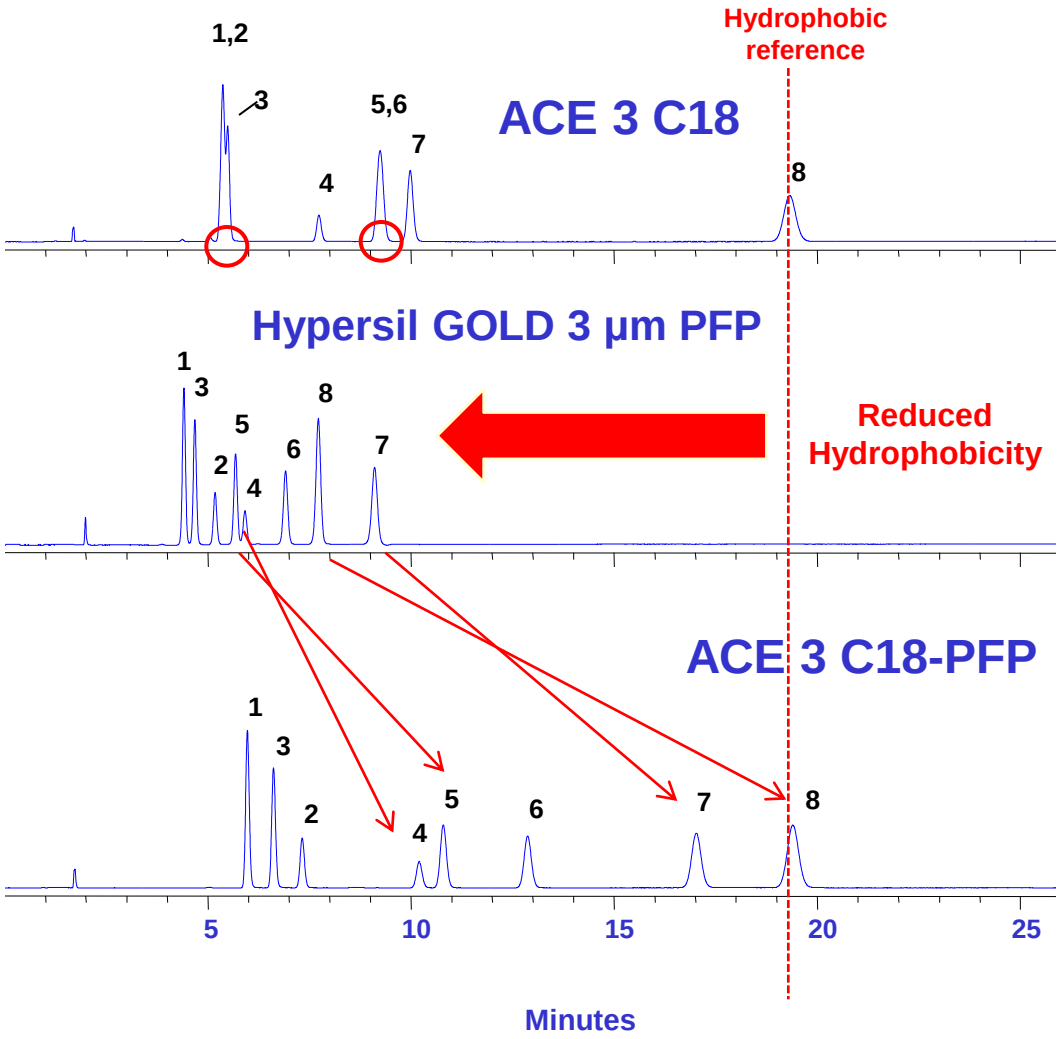
ACE® C18-PFP Selectivity*

Peak Number:	1	2	3	4	5	6	7	8
								
	1,2,3-TMB	1,2,4-TMB	1,2-DMB	1,4-DMB	MB	1,3-DMB	1,3,5 TMB	Tol
Log P:	1.7	1.6	1.7	2.1	2.2	2.2	1.6	2.7
π-basicity (order):	1	1	2	2	3	2	1	-

- Elution / retention **not simply** a function of **π-basicity** and **Log P**
- Retention mechanism for C18-PFP **multi-modal**

*Structures from www.chemspider.com
Predicted data from ACD Labs software, 30May12

ACE® C18-PFP Selectivity

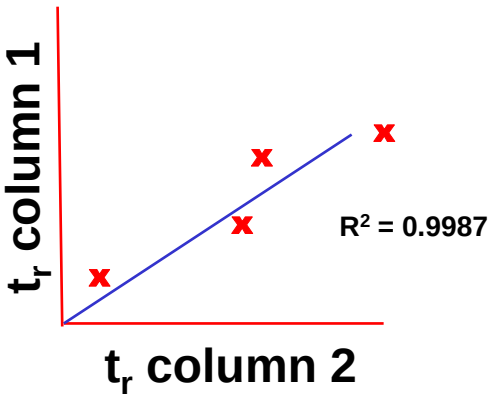


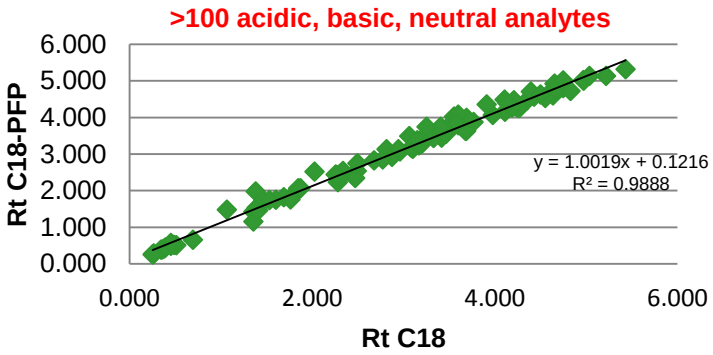
- C18 or PFP mechanisms alone not enough to fully resolve the methoxybenzene isomers
- ACE C18-PFP mechanism combines hydrophobicity, shape selectivity, dipole-dipole and π-π interactions
- Elution order, retention and selectivity all seen to differ
- Powerful positional isomer and shape selectivity

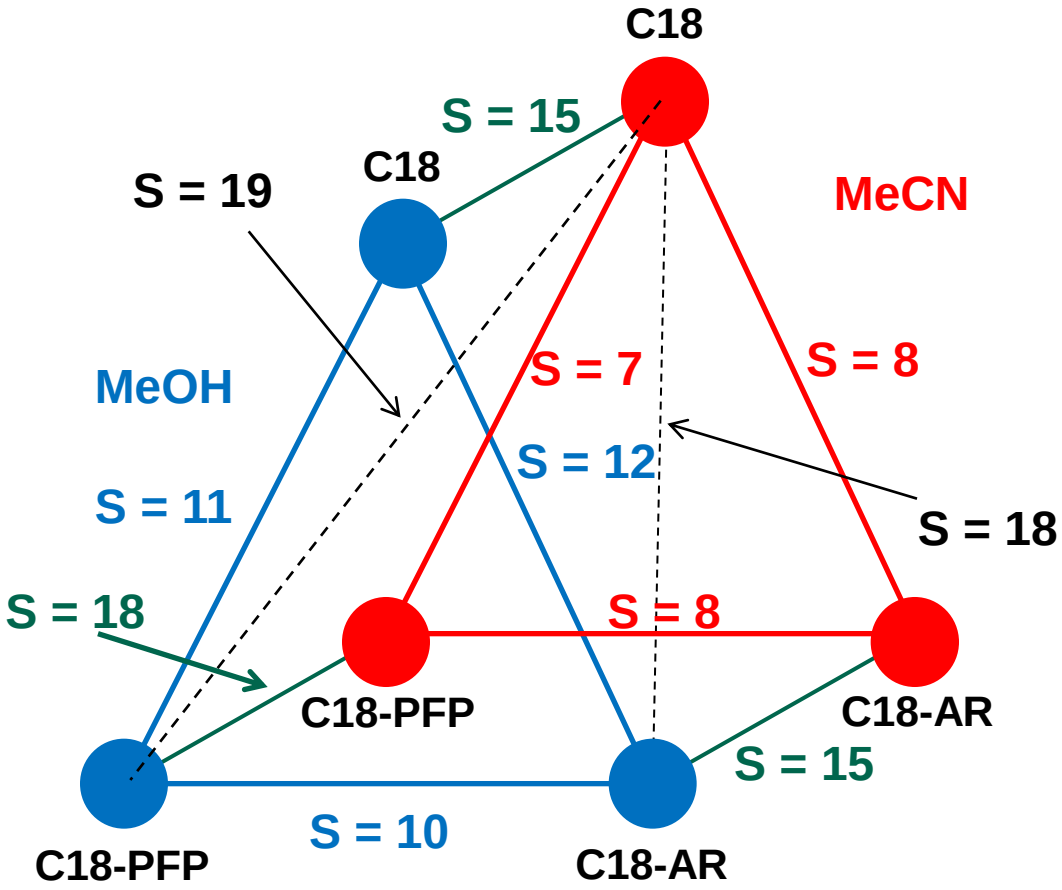
1) 1,2,3-trimethoxybenzene, 2) 1,2,4-trimethoxybenzene, 3) 1,2-dimethoxybenzene, 4) 1,4-dimethoxybenzene 5) methoxybenzene, 6) 1,3-dimethoxybenzene, 7) 1,3,5-trimethoxybenzene, 8) toluene (ref) Mobile phase 50:50 v/v MeOH / H_2O; Column= 150 x 4.6 mm id; 1.00 ml/min; 40C; 254 nm

ACE® Phase Comparisons With The Selectivity Descriptor*

Selectivity = 100 x √ (1 – R^2)







Selectivity = 100 x √ (1 – R^2)
= 100 x √ (1 – 0.9888)
= 10.6

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

Ranking ACE® Phase Orthogonality With MeOH and MeCN

- **For the 102 acidic, basic and neutral analytes assessed**

MeOH

Column 1	Column 2	Selectivity 'S'
C18	C18-AR	12
C18	C18-PFP	11
C18-AR	C18-PFP	10

MeCN

Column 1	Column 2	Selectivity 'S'
C18	C18-AR	8
C18-AR	C18-PFP	8
C18	C18-PFP	7

MeOH	MeCN	Selectivity Value
C18-PFP	C18	19
C18-AR	C18	18
C18-AR	C18-PFP	18
C18-PFP	C18-AR	18
C18-PFP	C18-PFP	18
C18	C18-AR	17
C18	C18-PFP	17
C18	C18	15
C18-AR	C18-AR	15

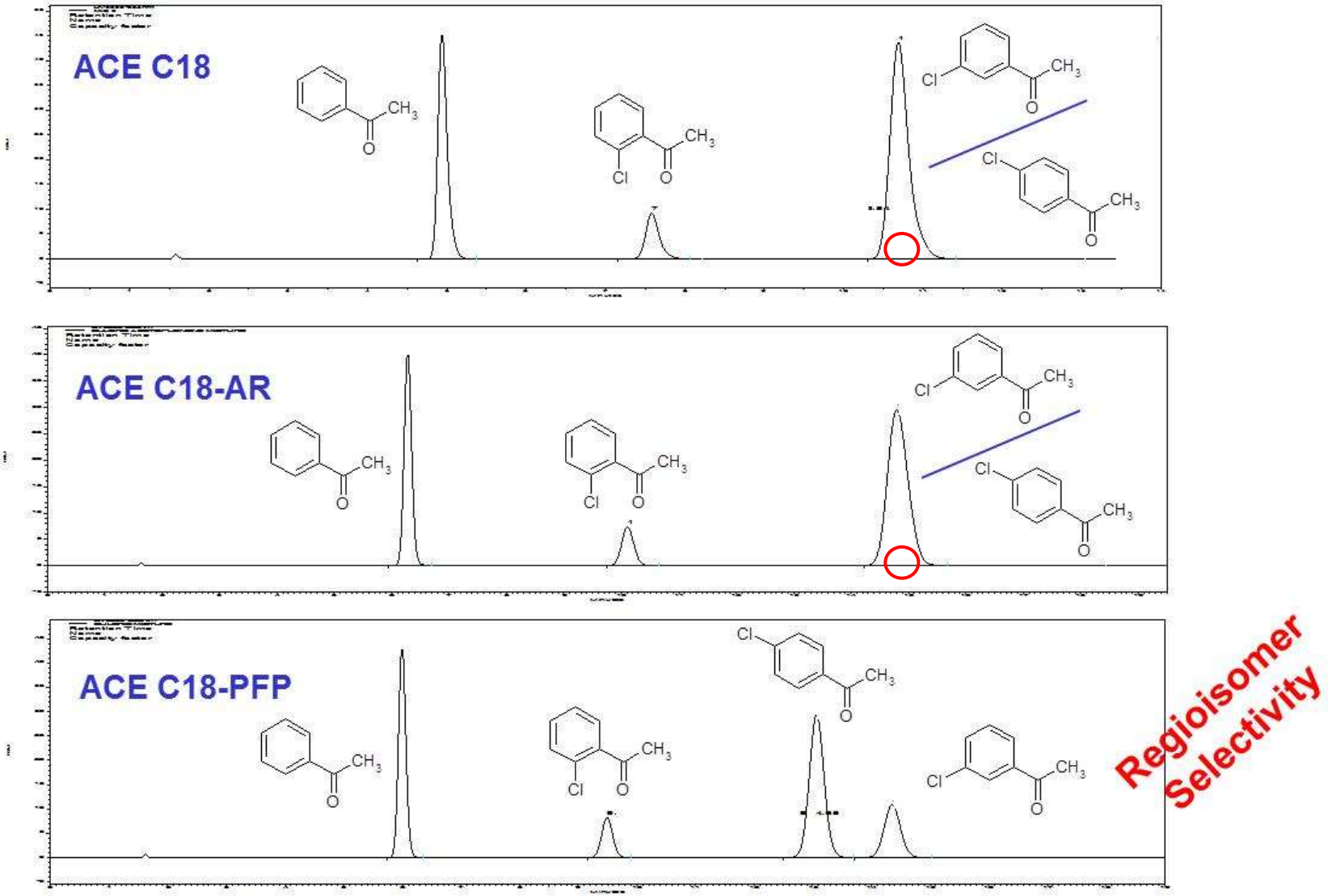
Shows value of using the 3 phases in a 2 solvent screen for method development work

What Do I Use These Novel Phases For: ACE C18-PFP?

- Useful for analytes that contain electron donating moieites eg -NH_2, -NR_2, -OCH3, -OH, -alkyl, -Ar etc
- eg nucleotides, nucleosides, nucleobases, halogenated aryl / aromatics, catecholamines, tetracyclines, beta blockers, structural isomers, coumarins etc
- Excellent shape and positional isomer selectivity

What Do I Use These Novel Phases For: ACE C18-PFP?

- **C18-PFP: chloroacetophenone halogenated isomers separation**



What Do I Use These Novel Phases For: ACE C18-AR?

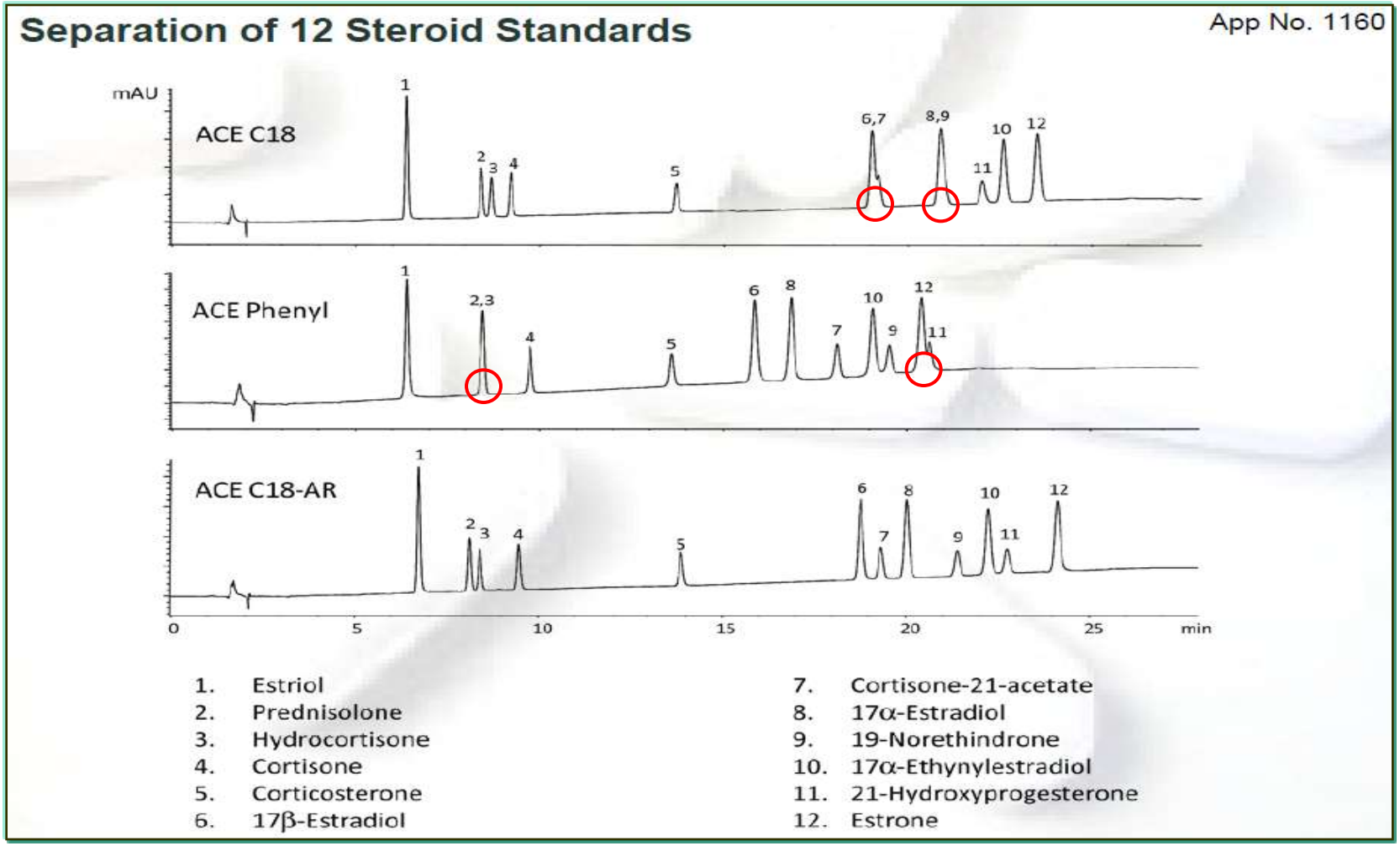
- Useful for analytes that contain electron withdrawing moieties eg -NO_2, -halides, -NR_3^+, -SO_2, -CO_2H, -SO_3H, -CO_2R, -CHO etc

- eg aromatic compounds, anthocyanins, steroids, analgesics, phenolics, water soluble vitamins, sulphur containing compounds, quinolones, positional isomers etc

- Moderate shape selectivity

What Do I Use These Novel Phases For: ACE C18-AR?

- **C18-AR: steroids separation**

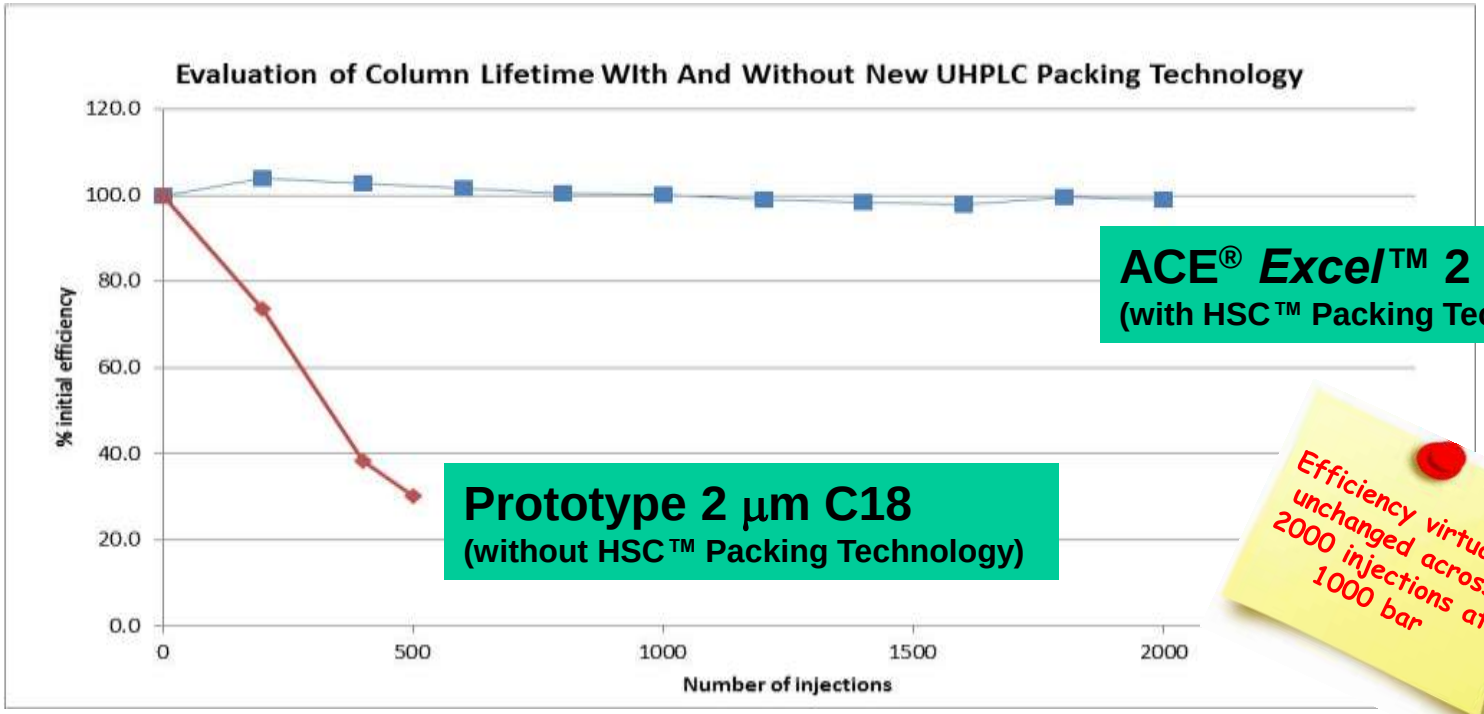


Combining Selectivity With The NEW ACE® *Excel* ™ Format

- **NEW high efficiency, ultra-inert 2μm silica particles suitable for UHPLC at 1000bar (15000psi)**
- **Nine selectivities – including the unique C18-AR and C18-PFP**
- **High reproducibility: column-to-column and batch-to-batch**
- **Ultra-robust phases: NEW low dispersion column hardware and NEW High Stability Column (HSC™) packing technology**
- **Engineered with lower back pressures compared to other <2μm phases due to 2μm particle size and frit technology**
- **Fully scalable to ACE® 3μm, 5μm and 10μm phases**
- **Fully compatible with all commercial HPLC and UHPLC kit**

ACE® *Excel*™ UHPLC Column Robustness

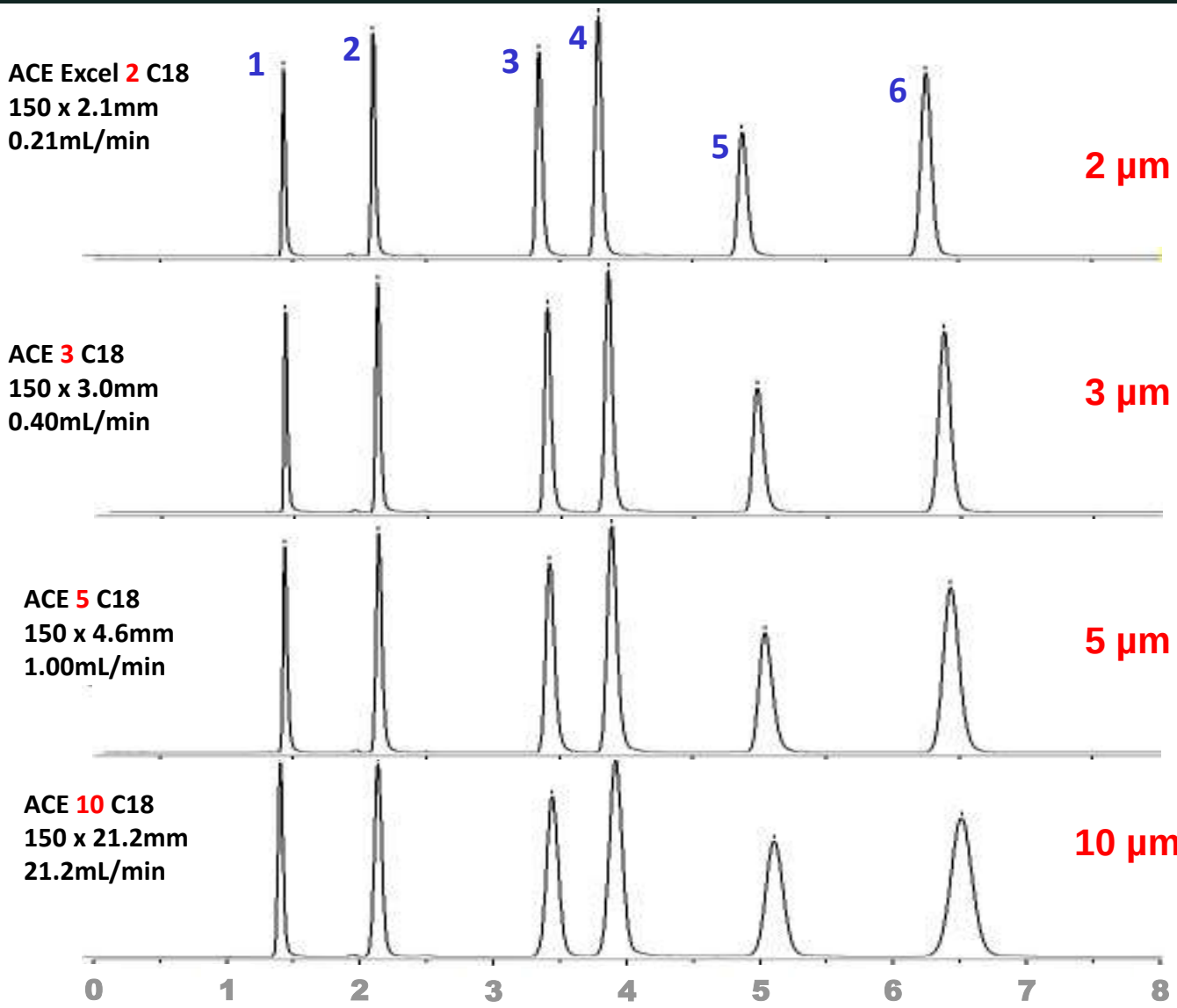
- **1000 bar for ~2000 gradient runs**
  - **Isocratic efficiency assessments every ~100 runs ←more demanding!**



100x2.1mm; MPA 0.1% FA (aq); MPB: 0.1% FA in MeOH; 0.73mL/min; gradient: 20-90%B in 6 mins.

NEW High Stability Column (HSC™) Packing Technology Significantly Improves UHPLC Column Robustness

ACE® *Excel*™ UHPLC Columns – Scalability & Reproducibility



UHPLC

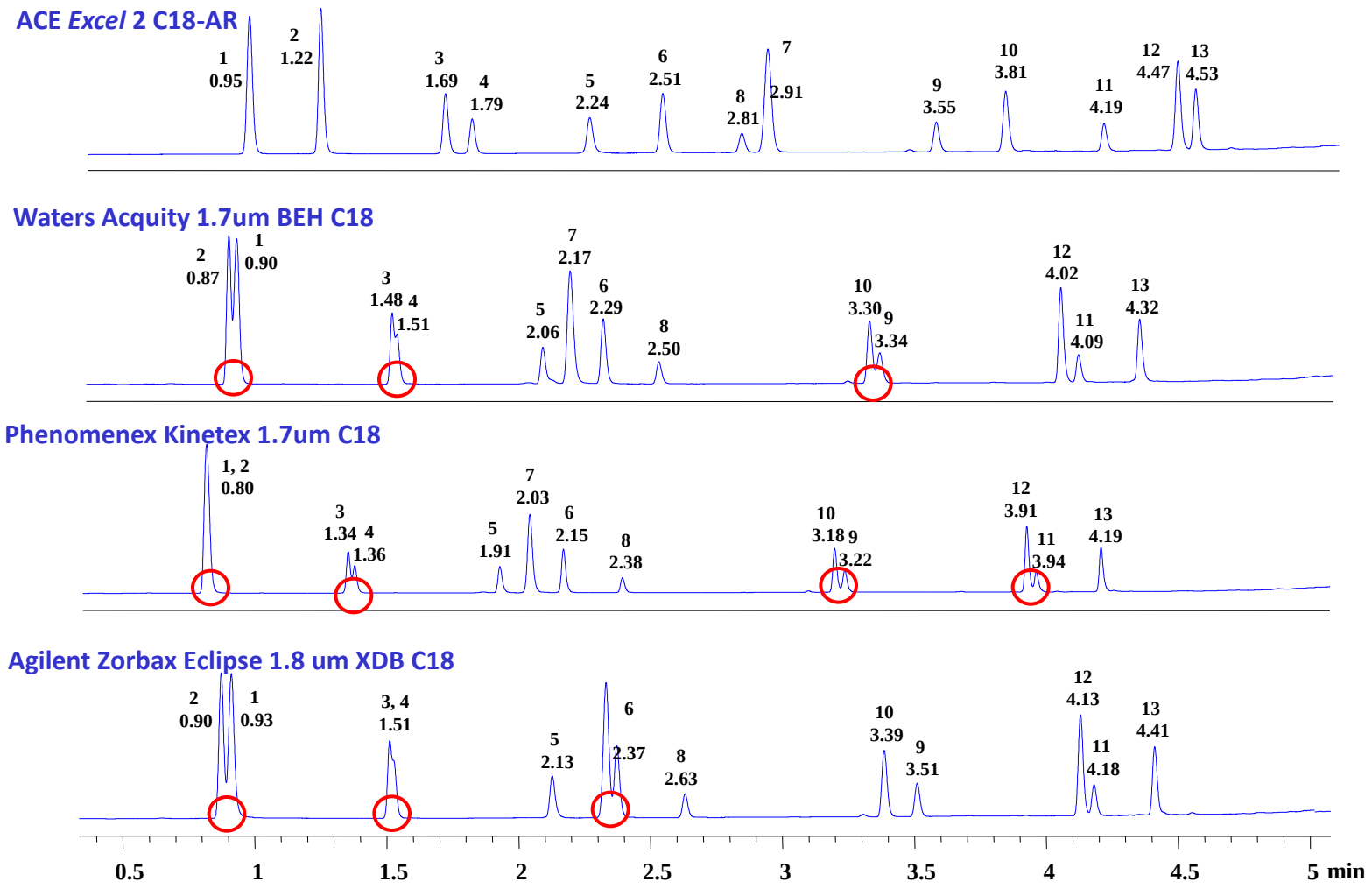
HPLC

Prep LC

MP: 35:65 v/v MeCN:0.1% TFA (aq); 22C; 254nm; 1. uracil; 2. 4-hydroxybenzoic acid; 3. acetylsalicyclic acid; 4. benzoic acid; 5. 2-hydroxybenzoic acid 6. ethyl paraben

ACE® *Excel*™ Has Typically Lower Back Pressure For UHPLC

- **Specifically engineered for lower UHPLC backpressures**



P_{max}: 364 bar

P_{max}: 581 bar

P_{max}: 540 bar

P_{max}: 540 bar

Conditions: A=5mM formic acid (aq); B=5mM formic acid in MeOH; tg= 3 to 100%B in 5 min; 0.6 ml/min; 40C; 254nm

1. Paracetamol 2. Hydrochlorothiazide 3. Methylphenylsulphoxide 4. Methylphenylsulphone 5. Aspirin 6. Phenacetin 7. 1,3-dinitrobenzene 8. 1,2,4-trimethoxybenzene 9. Ethylbenzoate 10. Nimesulide 11. Ibuprofen 12. Indomethacin 13. Mefenamic acid

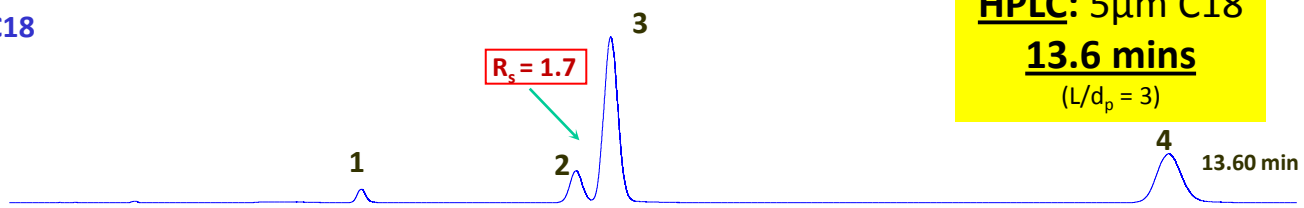
Selectivity, Speed & Scaling

Isocratic & Gradient HPLC → UHPLC

ACE® *Excel*™ C18-PFP Selectivity & Throughput (Isocratic)

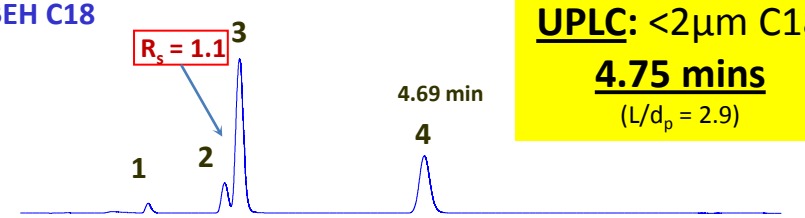
Aim: obtain $R_s \geq 1.7$ in shortest possible time for mixture

Waters XBridge 5µm C18
150 x 4.6 mm
1.00 ml/min
163 bar



HPLC: 5µm C18
13.6 mins
(L/d_p = 3)

Waters Acquity 1.7µm BEH C18
50 x 2.1 mm
0.21 ml/min
246 bar



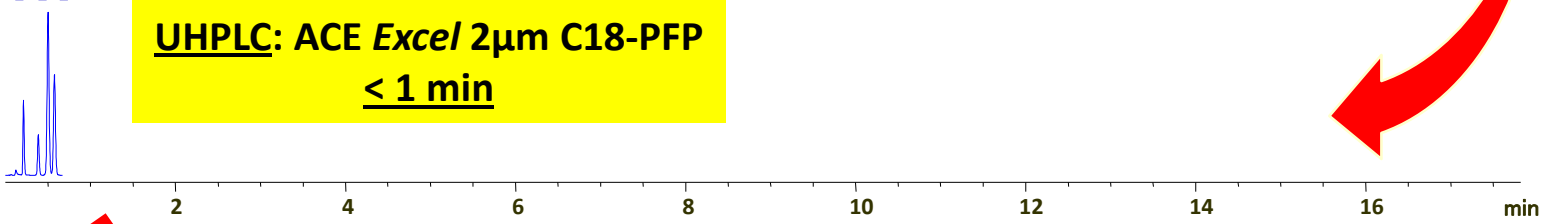
UPLC: <2µm C18
4.75 mins
(L/d_p = 2.9)

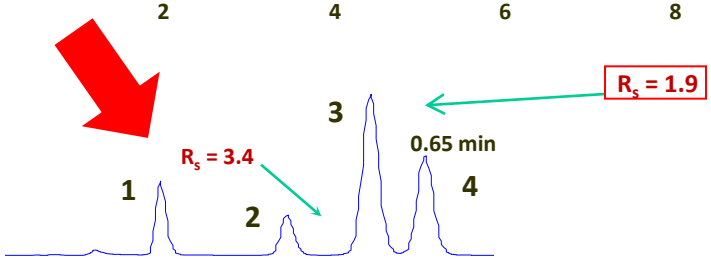
To maintain R_s and reduce run time, keep L / dp ratio constant

~ x23 Quicker

ACE *Excel* 2µm C18-PFP
30 x 2.1 mm
1.30 ml/min
492 bar

UHPLC: ACE *Excel* 2µm C18-PFP
< 1 min





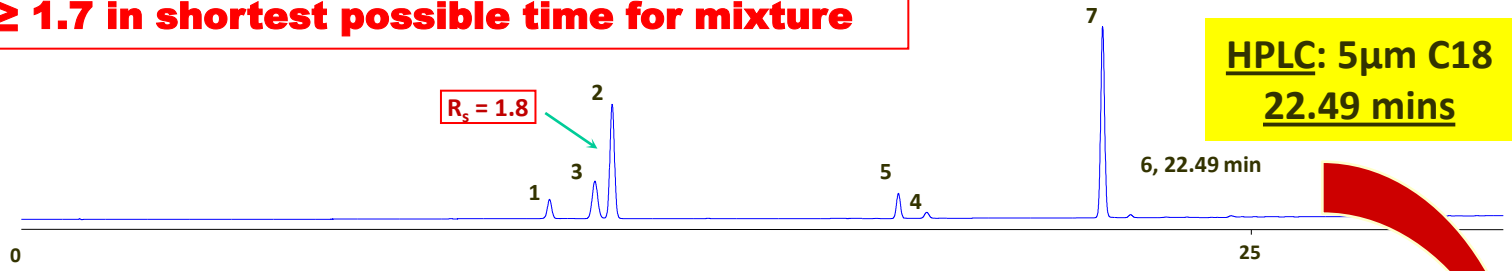
Using UHPLC and selectivity, it is possible to dramatically improve resolution allowing shorter columns & increased flow rates

Sample: 1) 1,2,dimethoxybenzene, 2) 1,3-dimethoxybenzene, 3) 1,3,5-trimethoxybenzene, 4) toluene (reference).
Mobile phase 50:50 MeOH / H_2O; Temperature 40°C; 254 nm

ACE® *Excel*™ C18-PFP Selectivity & Throughput (Gradient)

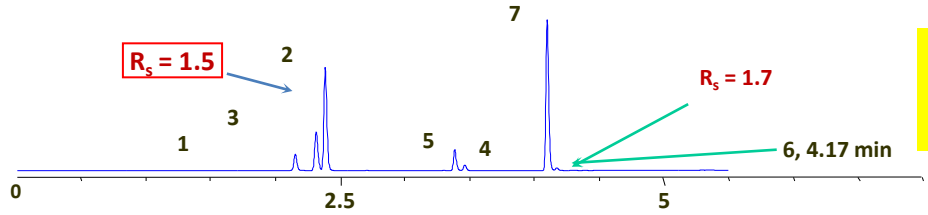
Aim: obtain $R_s \geq 1.7$ in shortest possible time for mixture

ACE 5µm C18
100 x 4.6 mm
1 ml/min, t_G = 29 min
max pressure: 92 bar
40 min cycle time



HPLC: 5µm C18
22.49 mins

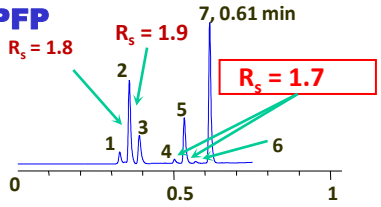
ACE *Excel* 2µm C18
50 x 2.1 mm
0.6 ml/min, t_G = 5 min
max pressure: 367 bar
9 min cycle time



UHPLC: 2µm C18
4.17 mins

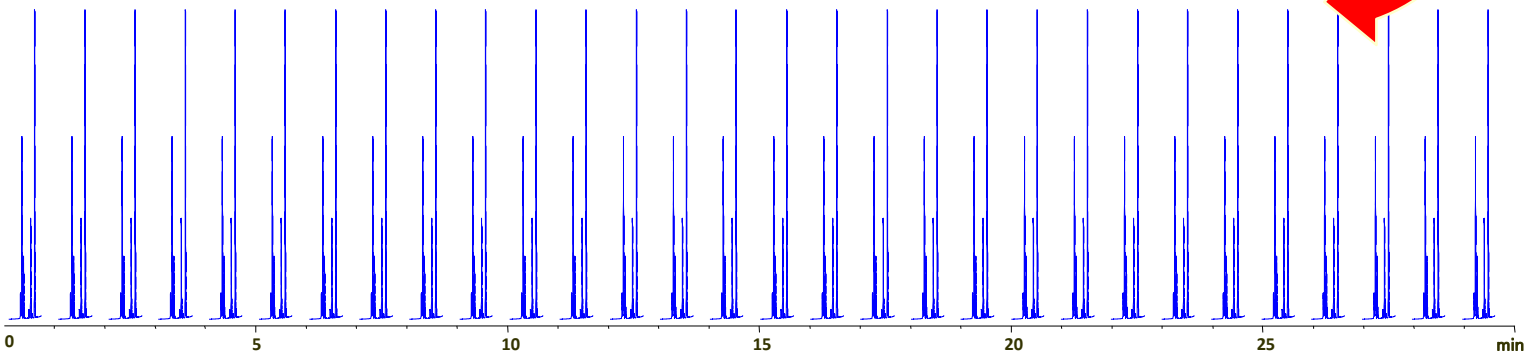
~ x25 Quicker

ACE *Excel* 2 µm C18-PFP
30 x 2.1 mm
2.5 ml/min, tG = 0.7 min
max pressure: 914 bar
1 min cycle time



UHPLC: 2µm C18-PFP
0.61 mins

$R_s \geq 1.7$



1, aspirin; 2, phenacetin; 3, 1,3-dinitrobenzene ; 4, ethylbenzoate; 5, nimesulide; 6, ibuprofen; 7, indomethacin.

Pressure Effects

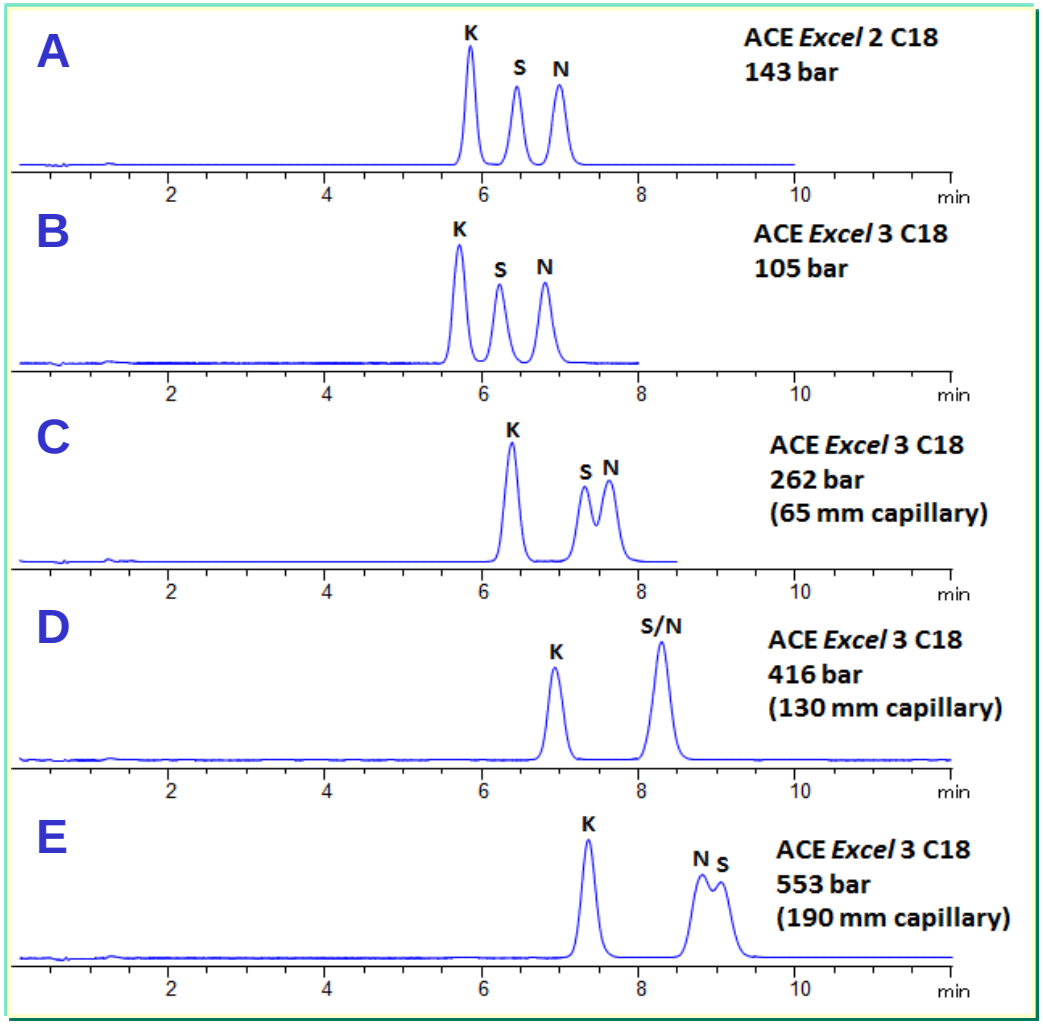
HPLC ↔ UHPLC

Background

- Pressure is a complex physical parameter that affects many elements of a chromatography system
- Chromatographic selectivity and retention changes at elevated pressures have been investigated and reported[a]
- Observations are highly dependent upon the analytes and may be seen with any manufacturer phases operated under UHPLC conditions
- Changes are typically not helpful for HPLC ↔ UHPLC activities

[a] Fallas et al. / J. Chromatogr. A 1209 (2008) 195–205

Effect of Pressure on Selectivity and Retention Factor



- Initial 2µm and 3µm data are similar (A, B)
  - Scalability looks good
- Retention and selectivity seen to change with pressure (B→E)

Agilent 1290, 50 x 2.1 mm (constant flow and restrictor capillary used)
Mobile phase: A=0.1% FA in water: B=0.1% FA in MeOH (51:49 v/v)
Flow Rate: 0.21 ml/min, Temperature: 40 °C
K= Ketoprofen; S= Sulindac; N=Naproxen

[a] Fallas et al. / J. Chromatogr. A 1209 (2008) 195–205

Summary: Unwanted Selectivity Changes

- Pressure induced *k* and α changes may be seen for any manufacturer phases under UHPLC conditions
- Changes in selectivity and retention may be significant with ionised analytes and large MW analytes[a], but the impact on neutral molecules is typically smaller
- Current discussions / theory focus on changes in analyte molar volume as the principle cause for changes in *k* and α observed
- Successful HPLC ↔ UHPLC possible...the analyst just needs to be vigilant

[a] Fallas et al. / J. Chromatogr. A 1209 (2008) 195–205

Connections : Losses in *N* and A_s

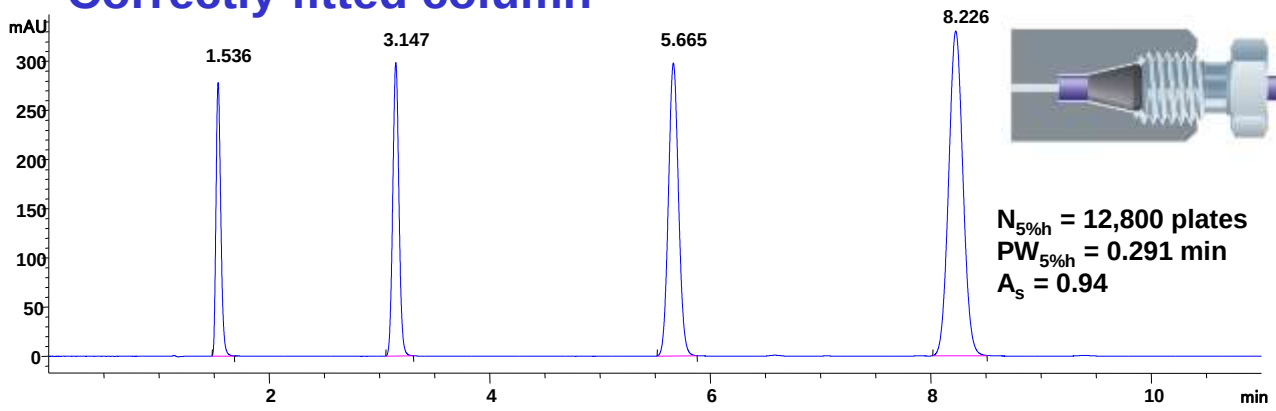
Peak Dispersion

Background

- UHPLC / optimised HPLC instruments are very sensitive to the introduction of extra column volume
- Any time you install a column (from any manufacturer) it is vital to ensure good connections
- Aim for a 'fresh connection' every time to ensure a snug fit between tubing and column and reduce the likelihood of an unwanted gap and / or tubing slippage
- Free movement of the ferrule and nut when installing the column gives you a fresh connection

Losses in Performance Due to Incorrect Column Fitting

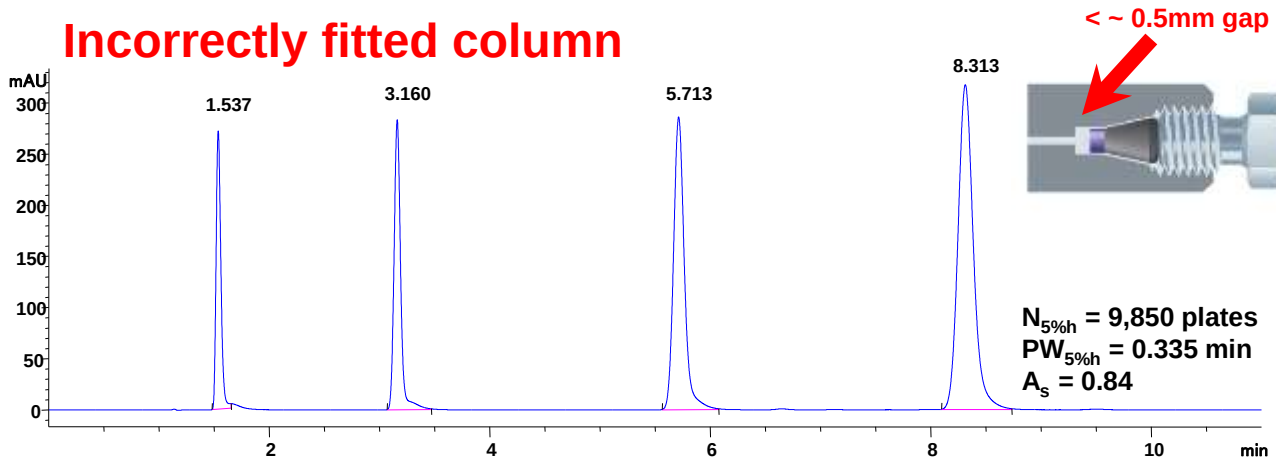
Correctly fitted column



$N_{5\%h}$ = 12,800 plates
$PW_{5\%h}$ = 0.291 min
A_s = 0.94

- **Correctly** fitted columns **make the most** of your column and system

Incorrectly fitted column



< ~ 0.5mm gap

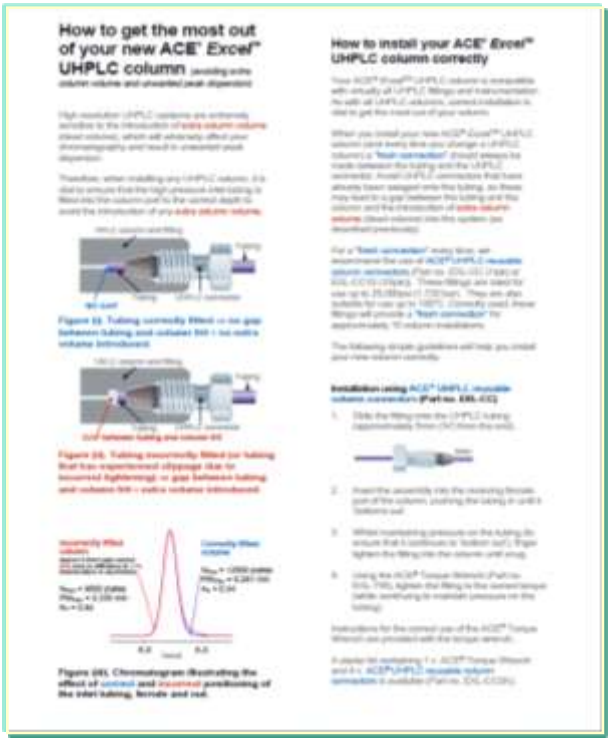
$N_{5\%h}$ = 9,850 plates
$PW_{5\%h}$ = 0.335 min
A_s = 0.84

- **Incorrectly** connected columns lead to **reduced** efficiency, **reduced** asymmetry and possibly **leaks**

- **Loss** of **~23%** for ***N***
  Loss of **~11%** for A_s

Summary: Column Connections

- Extra column volume **reduces** peak efficiency and asymmetry
- Make a **fresh connection every time** you install **any** column
- ACE recommend **reusable fittings** for a **fresh connection** every time
- All ACE® *Excel*™ columns have a **FREE** ‘**Making Great UHPLC Connections**’ leaflet in **every** box


How to get the most out of your new ACE® *Excel*™ UHPLC column

How to install your ACE® *Excel*™ UHPLC column correctly

Also downloadable from the ACE website:

www.ace-hplc.com

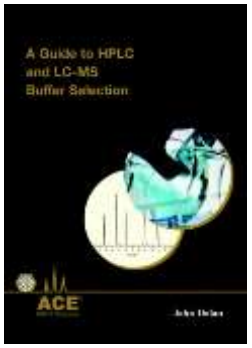
Overall Summary & Conclusions

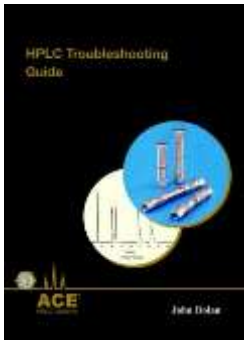
- Understanding the properties of building blocks in stationary phase design led to these unique ACE® products
- ACE® C18-AR and ACE® C18-PFP are powerful tools for method development due to unique but complementary selectivities
- These unique phases are available for HPLC as the ACE® range and also UHPLC as the NEW ACE® *Excel*™ 2 µm format
- These phases meet analyst demands of reproducibility, robustness & low phase bleed with excellent peak efficiency
- Operating at high pressures can deliver excellent results but remain vigilant - selectivity and retention may be affected...and even column connections become critical!

Full Information On All ACE Products Available

Unique Selectivities

Free Guides





MACMOD Analytical = http://www.mac-mod.com/
ACT = http://www.ace-hplc.com

Thank You For Your Attention

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