ACE® Excel™
UHPLC Columns

ACE®
HPLC Columns

Exploiting Selectivity in HPLC and UHPLC With Rational Stationary Phase Design

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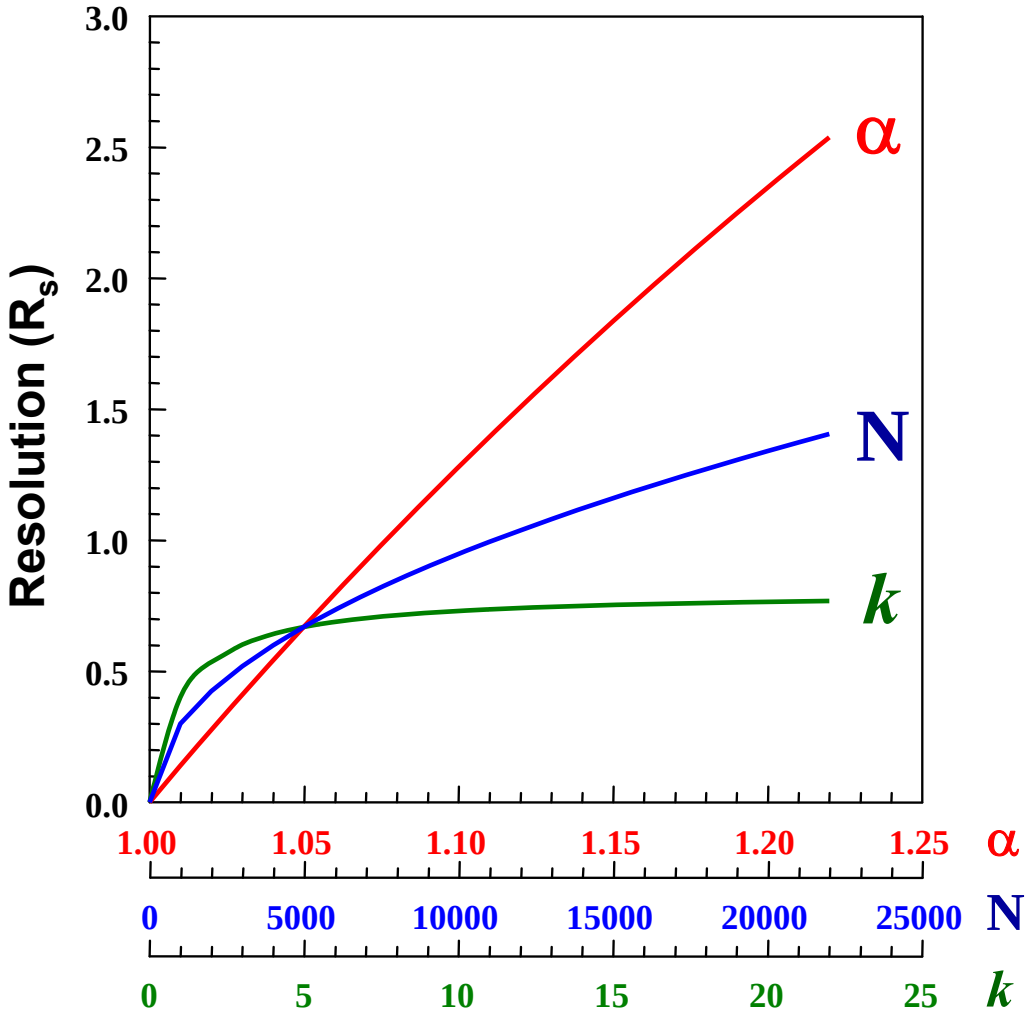
Outline

- 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 (e.g., 5 μm → 2.5 μm):

N = 10,000 → 20,000 plates

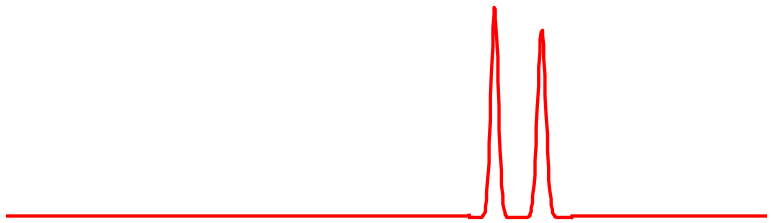
$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k}{1+k}$$

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

$$R_S = 2.6$$

$R_s = 1.8$

~40% Increase



$R_s = 2.6$

Opportunity to optimize further, e.g. reduce column length to speed up

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

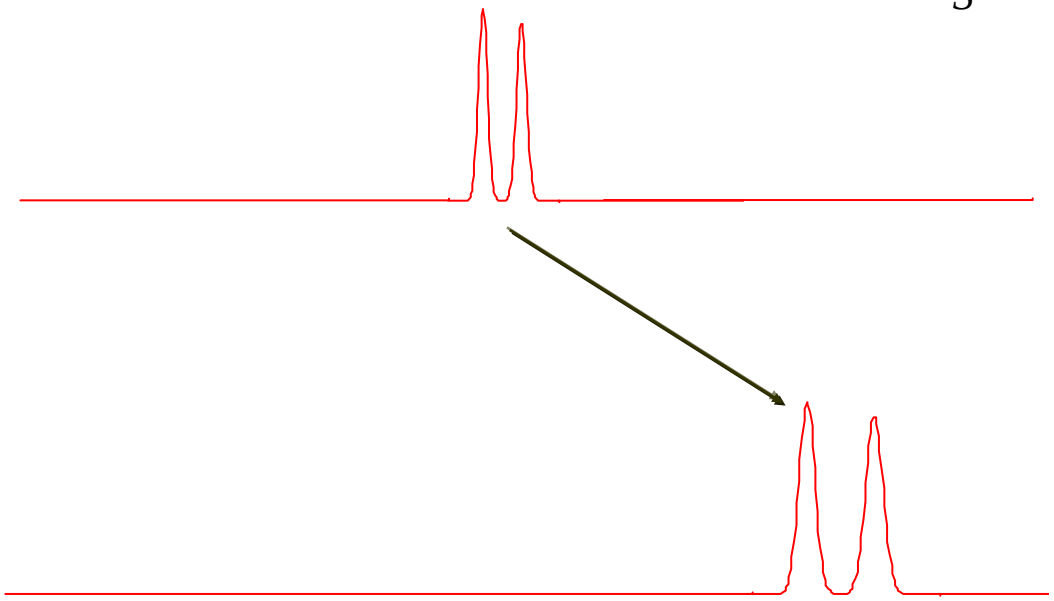
Double Retention Factor (e.g., decrease solvent strength):

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 (e.g., change column):

α = 1.1 → 1.2

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

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

$$R_S = 3.3$$

$R_s = 1.8$

Can now shorten run time with shorter column and/or faster flow rate

~80% Increase

$R_s = 3.3$

Significant opportunity to speed up for modest change in selectivity

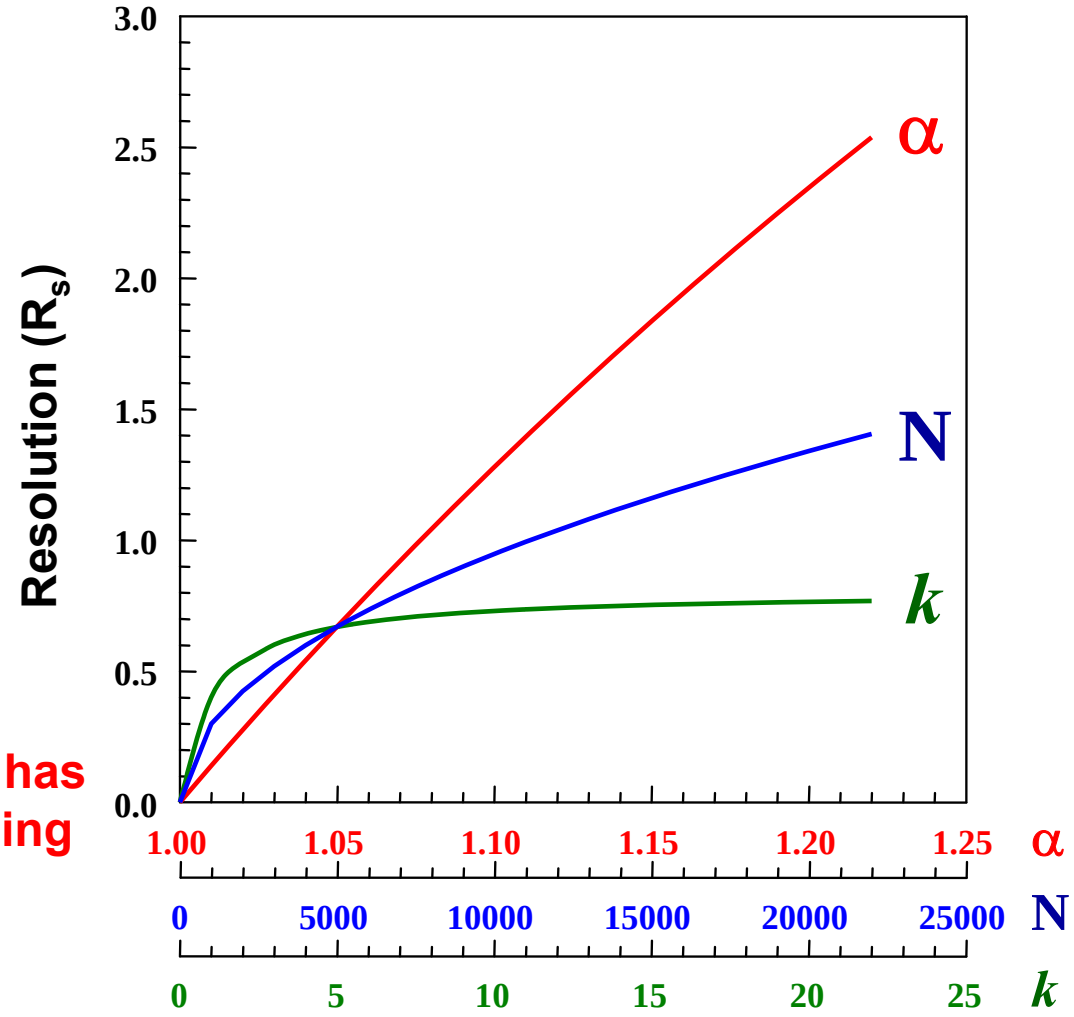
Selectivity: The Key to Chromatographic Peak Resolution

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?

MOST Influence

Isocratic Separations

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

LEAST Influence

Gradient Separations

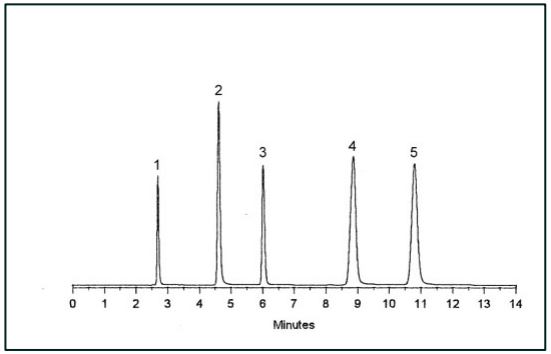
- All parameters for isocratic
- Gradient steepness
- ***k****
- Dwell volume
- Column dimensions

$$k^* = \frac{85 \times t_G \times F}{\Delta\Phi \times V_m \times S}$$

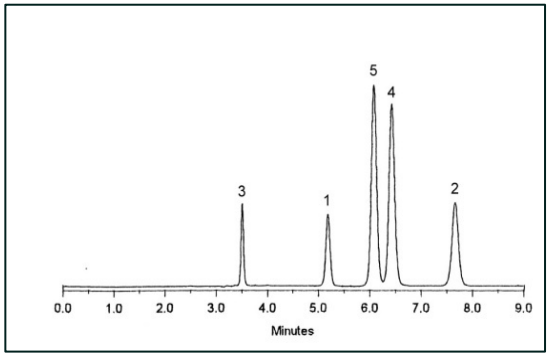
[a] Adapted from 'Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & Sons

Influencing Selectivity – Bonded Phase Effects / Basic Analytes

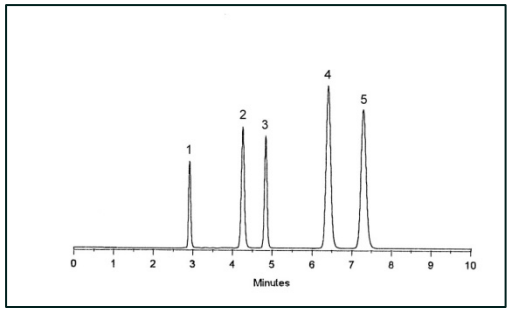
ACE C18 – Increase Retention



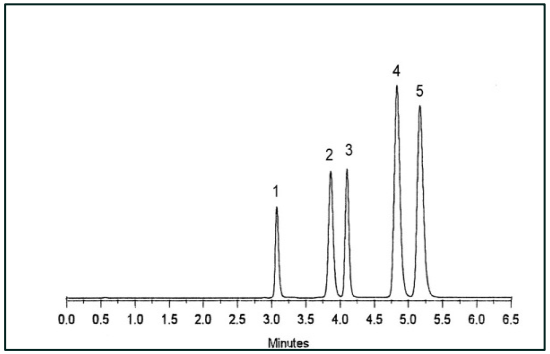
ACE CN – Elution Order



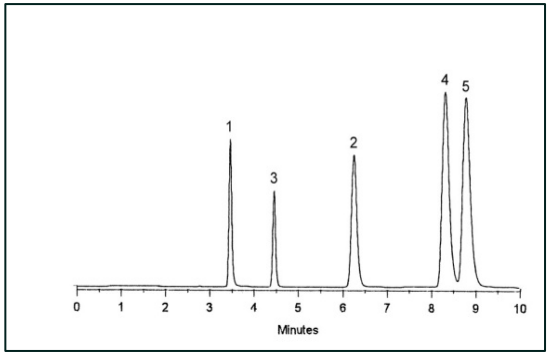
ACE C8 (start point)



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 (pH 6.0) Flow: 1.00ml/min
Components; 1: Norephedrine, 2: Nortriptyline, 3: Toluene, 4: Imipramine, 5: Amitriptyline Wavelength: 215nm

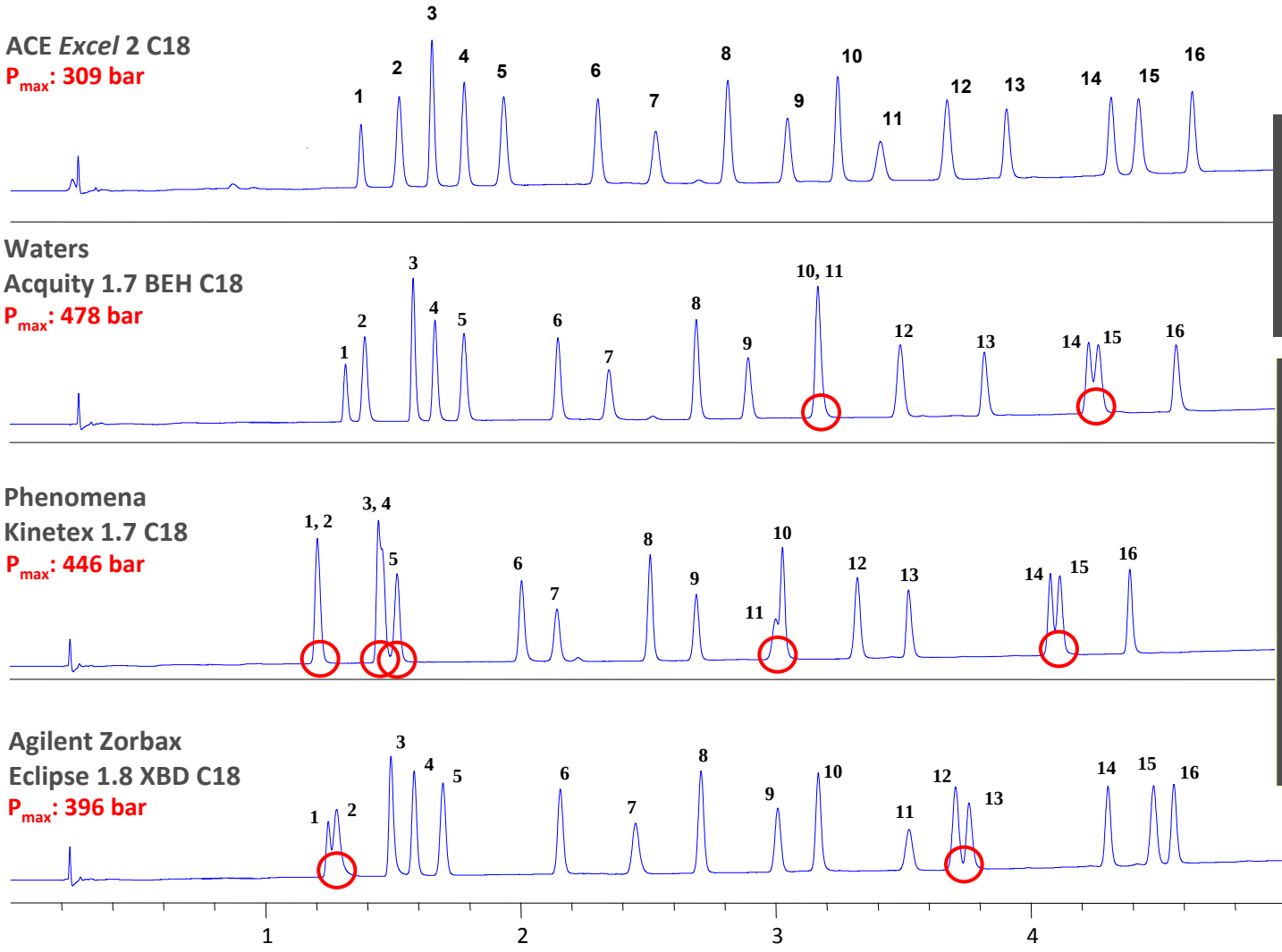
HPLC End User Surveys[a] ...Listening To The Analyst

- **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. Methyl benzoate
13. Remacemide
14. Nimesulide
15. Ethyl benzoate
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**

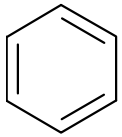
- **Advantages**
  - **Aromatic functionality potentially offer unique interactions with analytes (c.f. C18) giving alternative selectivity**
  - **Provides enhanced retention of polar compounds**
  - **Many aromatic functionality-based phases can be used in 100% aqueous eluents**

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

Aromatic Functionality: π – π Interactions

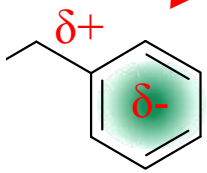
- A type of electron donor-acceptor interaction
- Originates from π systems in unsaturated functional groups on analytes and the stationary phase
- Types of π-π interaction can be manipulated for maximum effect (orthogonality) in phase design
  - eg phenyl: electron rich ring on the stationary phase 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



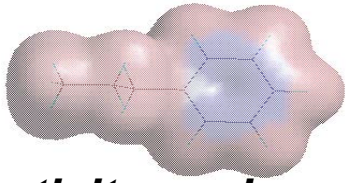
C_6H_6

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



e.g.

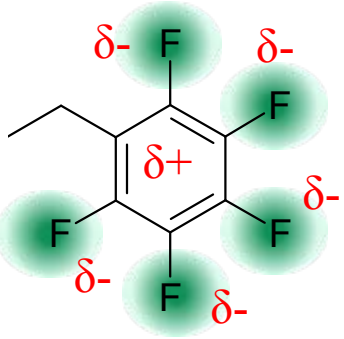
Electron Rich Ring



Activity: π-donor (π-base)

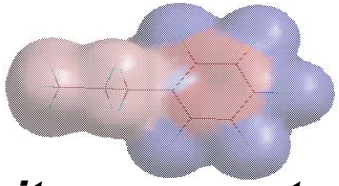
Classic π-π interaction

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



e.g.

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**

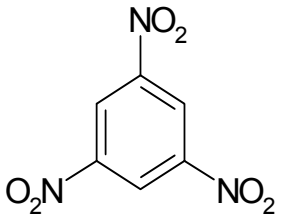
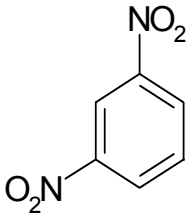
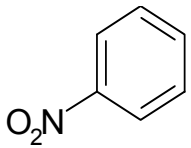
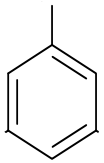
Separation mechanism	Typical C18	Typical Phenyl	ACE® C18-AR
Hydrophobicity	++++	+ / ++	++++
π-π Interaction	-	+++	+++
Dipole - Dipole	-	+	+
Hydrogen Bonding	-	++	++
Shape Selectivity	++	++	++ / +++

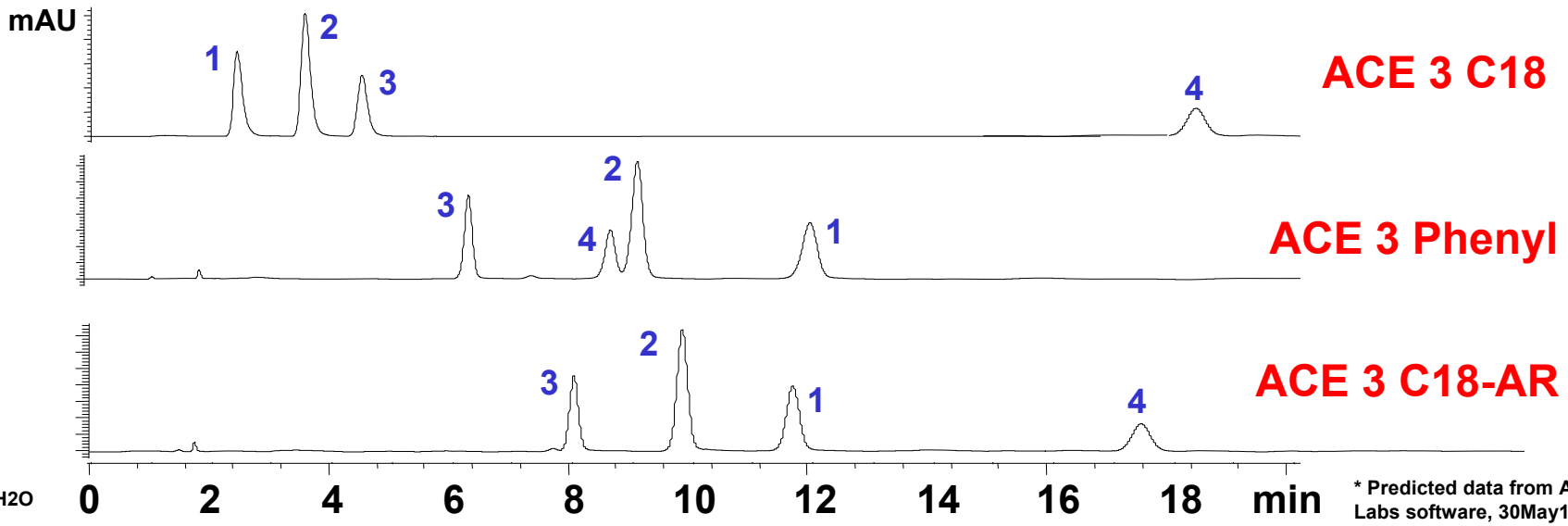
- **The predominance of each retention mechanism will be dictated by the analyte's physicochemical properties, its structure and the chromatographic conditions applied**

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

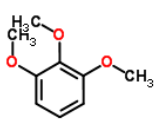
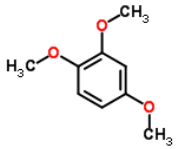
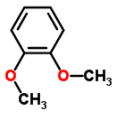
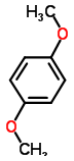
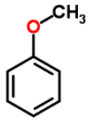
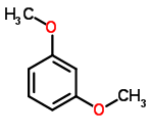
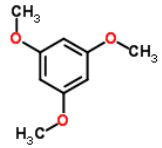
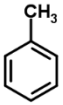
- Combining the character of C18+PFP into a single individual phase harnesses the best of both phases for unique selectivity

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

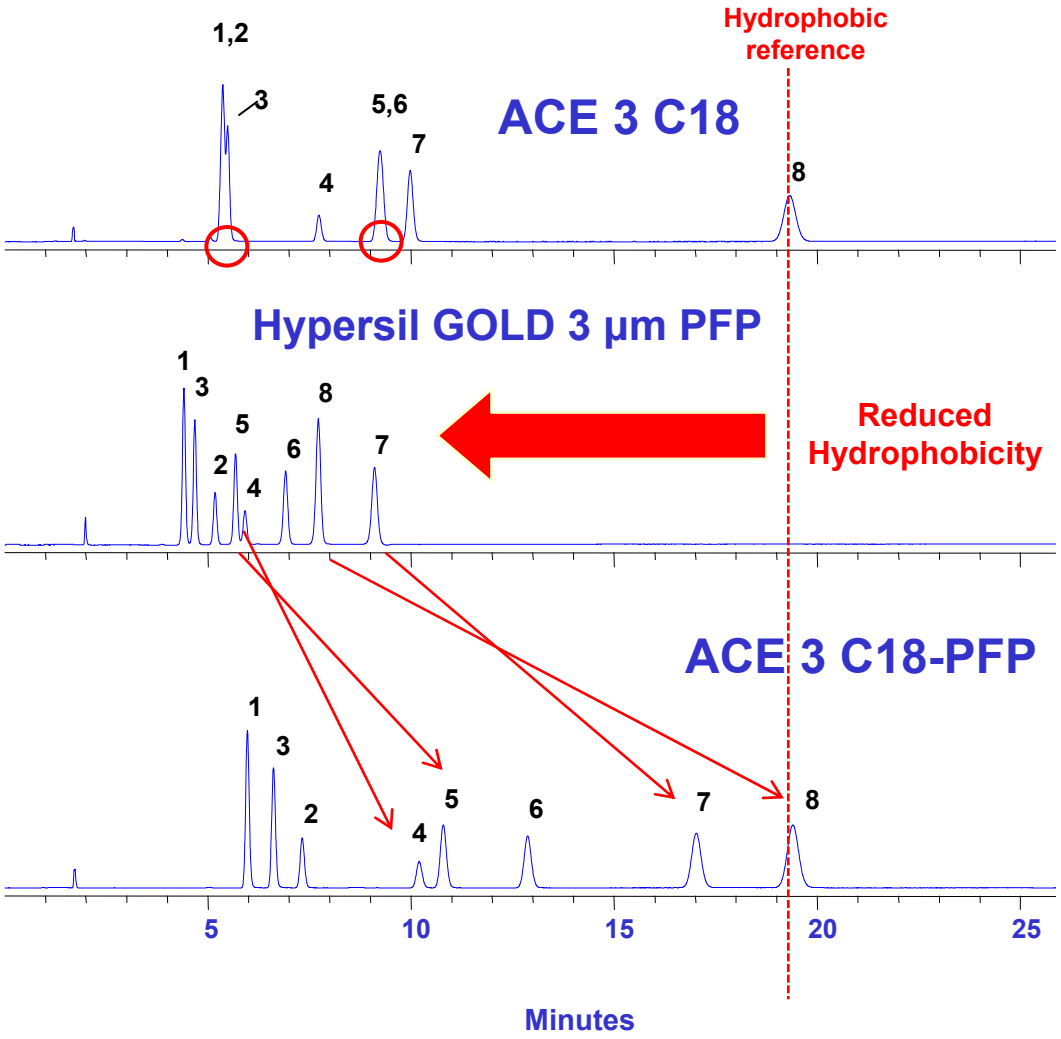
ACE® C18-PFP Selectivity*

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

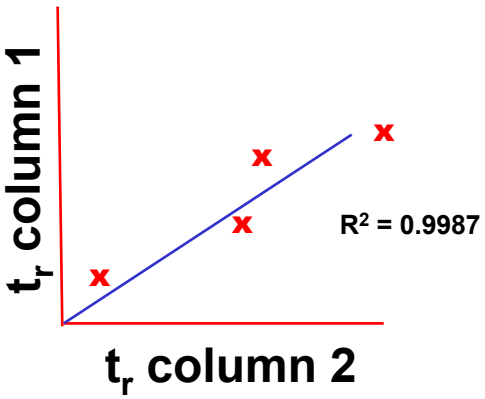


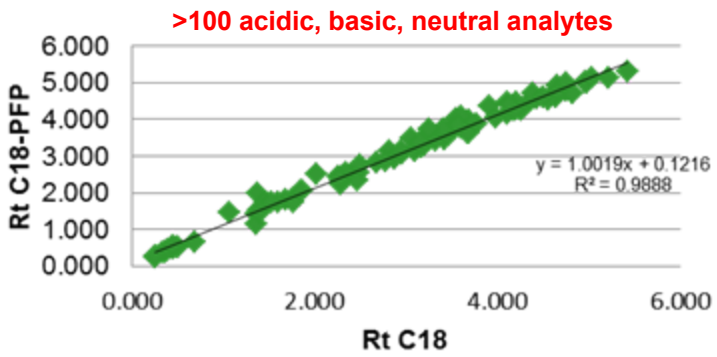
- C18 or PFP mechanisms alone not enough to fully resolve the methoxybenzene isomers
- ACE C18-PFP mechanism combines hydrophobicity, shape selectivity, dipole-dipole and π-π interactions
- Elution order, retention and selectivity all seen to differ
- Powerful positional isomer and shape selectivity

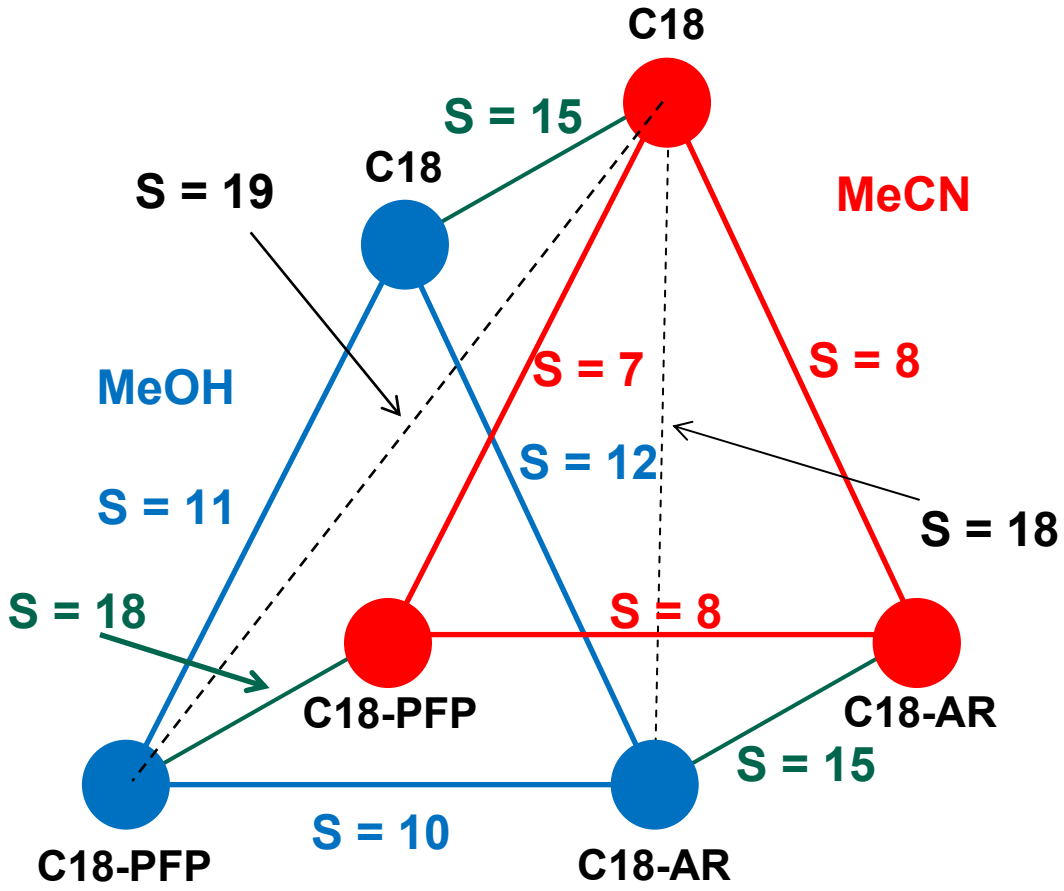
1) 1,2,3-trimethoxybenzene, 2) 1,2,4-trimethoxybenzene, 3) 1,2-dimethoxybenzene, 4) 1,4-dimethoxybenzene 5) methoxybenzene, 6) 1,3-dimethoxybenzene, 7) 1,3,5-trimethoxybenzene, 8) toluene (ref) Mobile phase 50:50 v/v MeOH / H_2O; Column= 150 x 4.6 mm id; 1.00 ml/min; 40C; 254 nm

ACE® Phase Comparisons With The Selectivity Descriptor*

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







Selectivity = 100 x $\sqrt{(1 - R^2)}$
= 100 x $\sqrt{(1 - 0.9888)}$
= 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

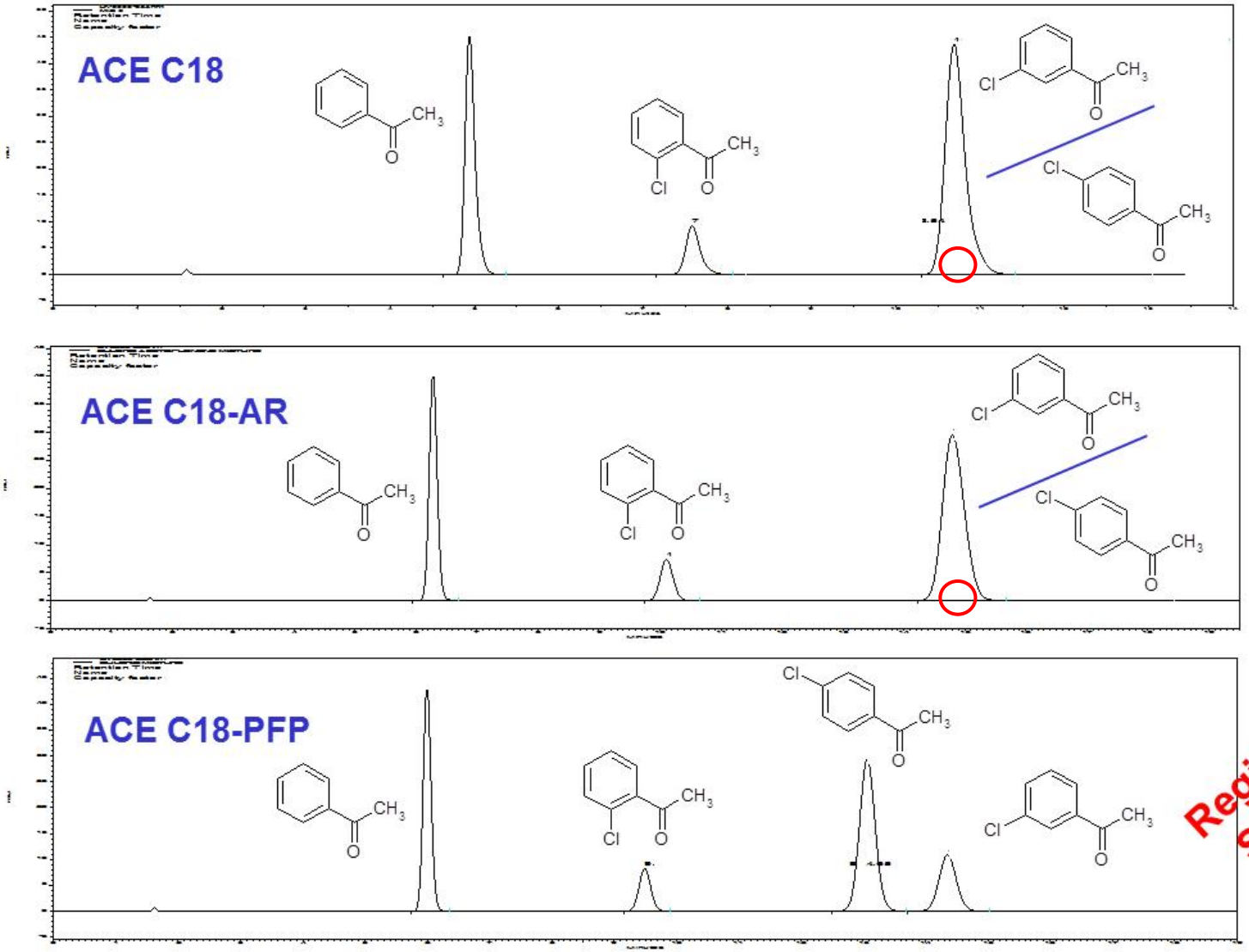
Shows value of using the 3 phases in a 2 solvent screen for method development work

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

- Useful for analytes that contain electron donating moieties 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**



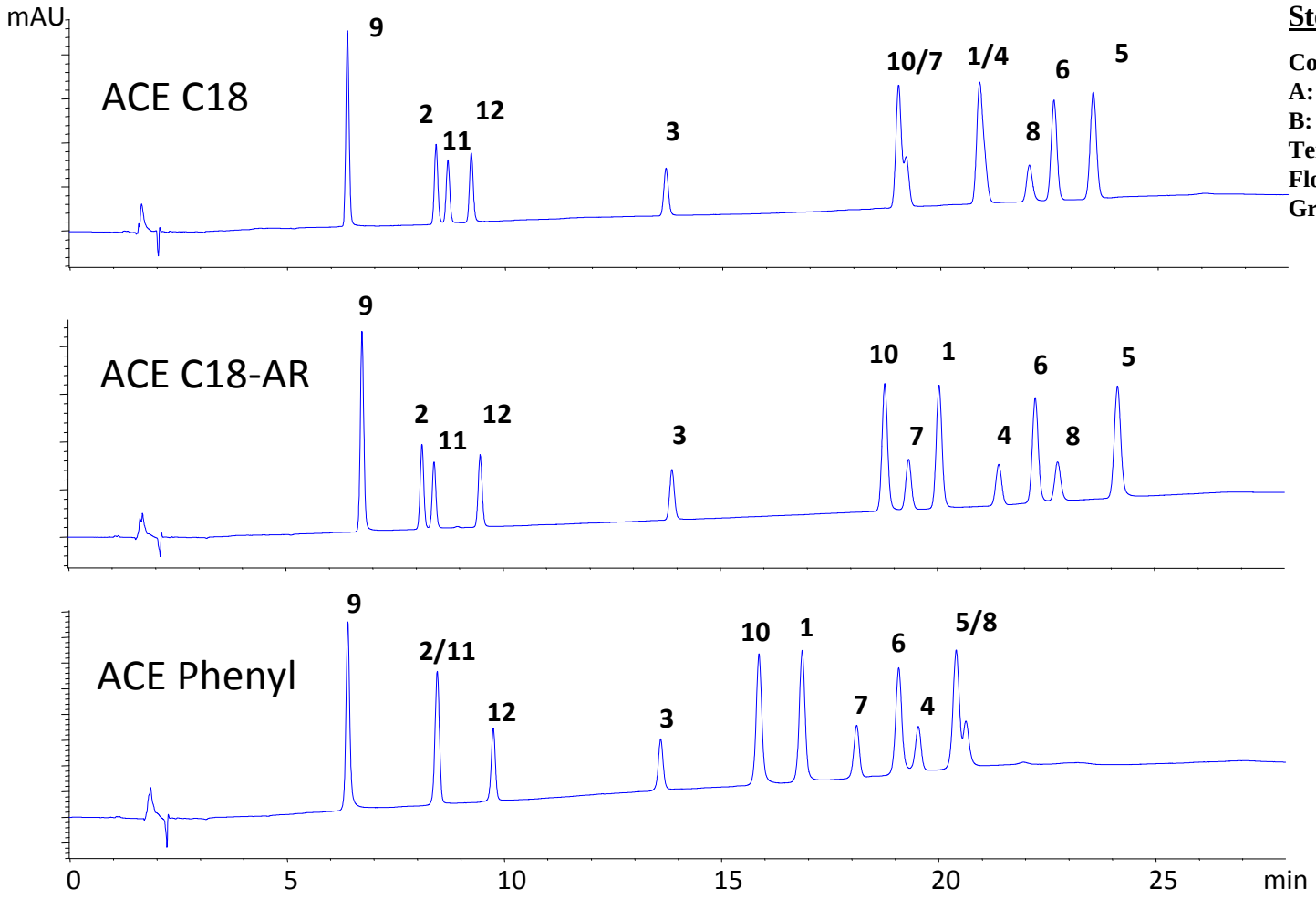
What Do I Use These Novel Phases For: ACE C18-AR?

- 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?

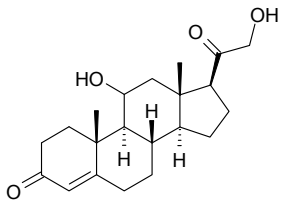


Steroid Mixture

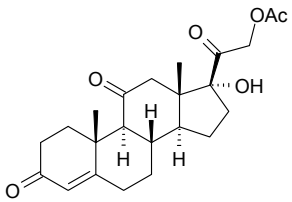
Columns:	**4.6 x 150 mm, 3 µm**
A:	**Water**
B:	**ACN**
Temp.	**20°C**
Flow Rate:	**1 mL/min**
Gradient:	**25 to 46%B, 24 min**

1) 17α Estradiol, 2)Prednisolone, 3) Corticosterone, 4) 19-Norethindrone, 5) Estrone, 6) 17α Ethynylestradiol, 7) Cortisone 21-acetate, 8) 21-Hydroxyprogesterone, 9) Estriol, 10) 17β Estradiol, 11) Hydrocortisone, 12) Cortisone

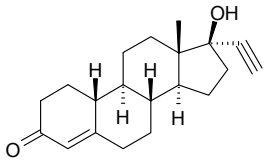
Steroid Sample



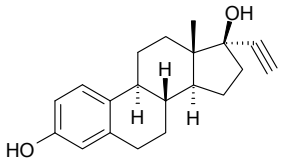
Corticosterone



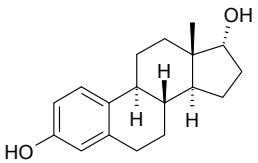
Cortisone 21-acetate



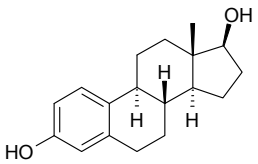
19-Norethindrone



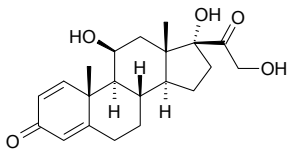
17 alpha-Ethynylestradiol



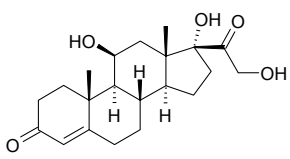
17 alpha-Estradiol



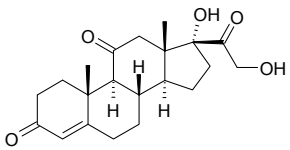
17 beta-Estradiol



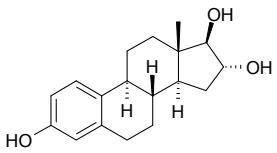
Prednisolone



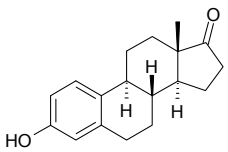
Hydrocortisone



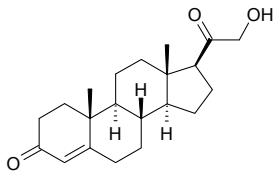
Cortisone



Estriol



Estrone



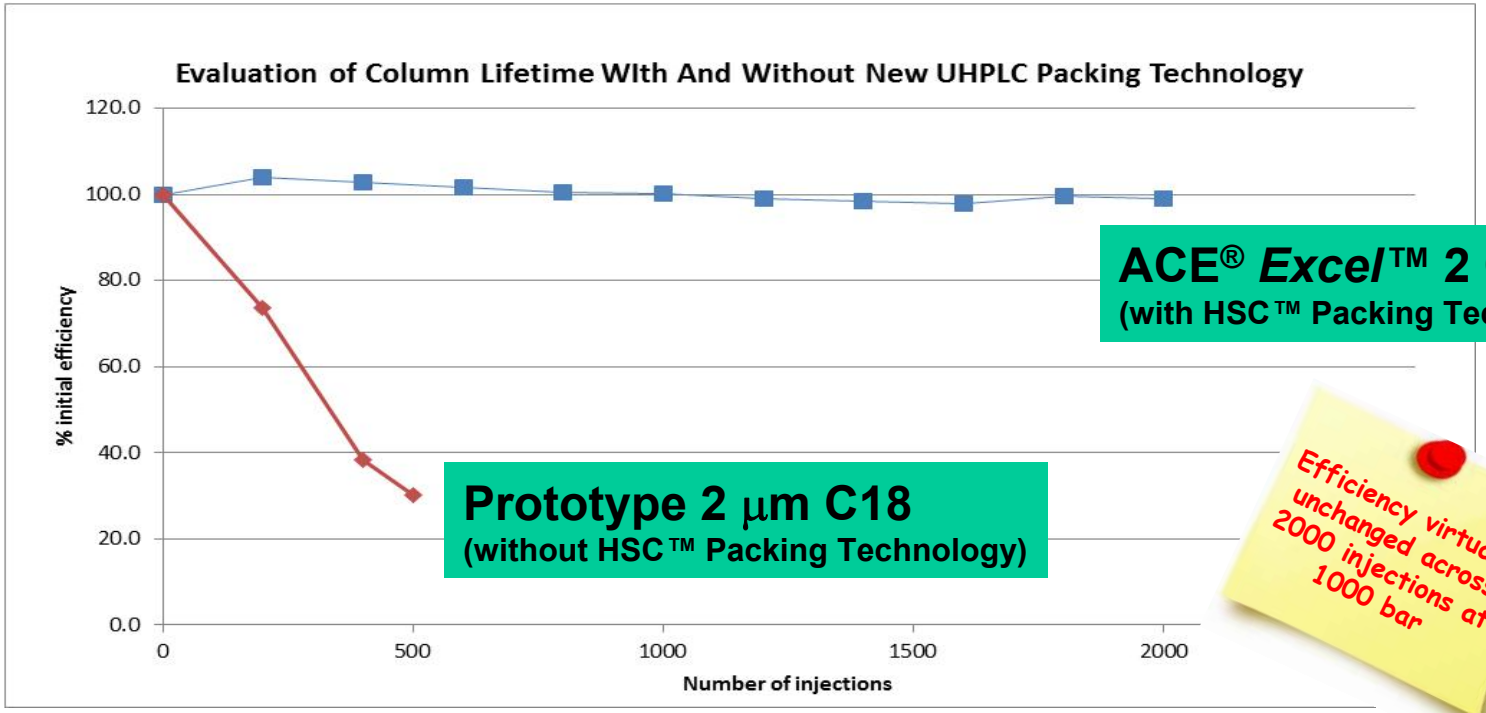
21-Hydroxyprogesterone

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

- NEW high efficiency, ultra-inert 2μm silica particles suitable for UHPLC at 1000bar (15,000psi)
- 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 (~30%) 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 HPLCs and UHPLCs

ACE® *Excel*™ UHPLC Column Robustness

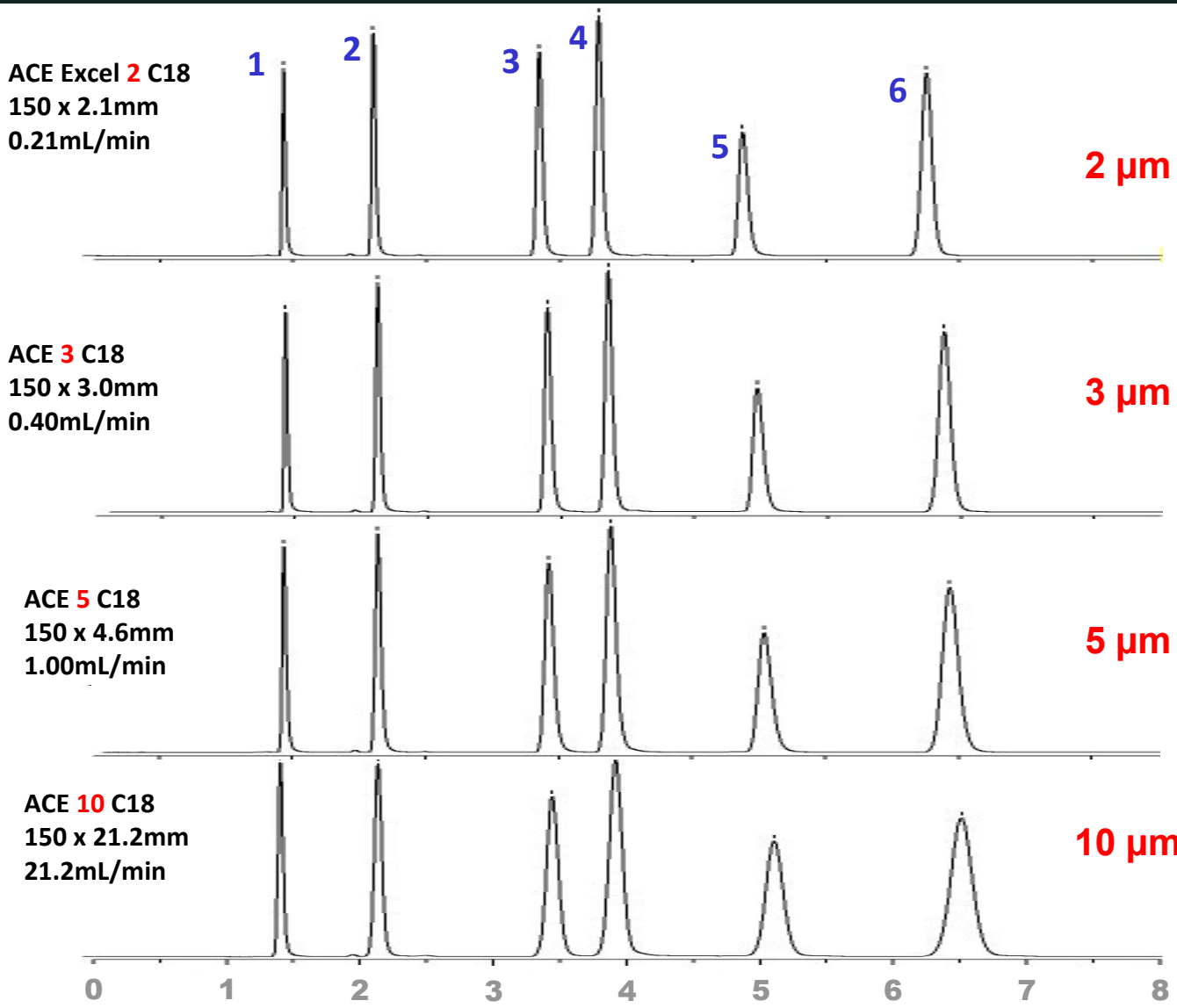
- **1000 bar for ~2000 gradient runs**
  - **Isocratic efficiency assessments every ~100 runs ←more demanding!**



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

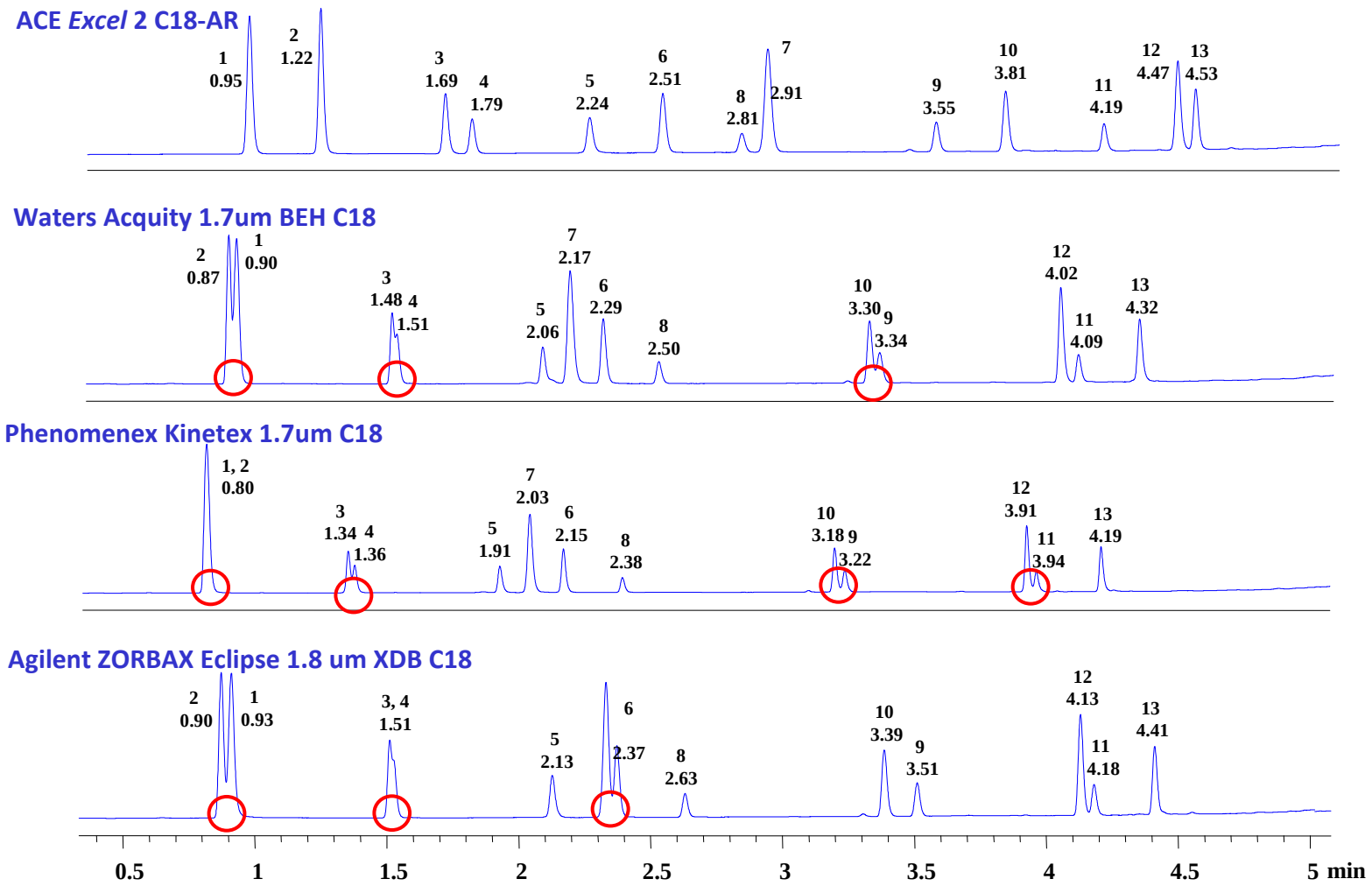
HPLC

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

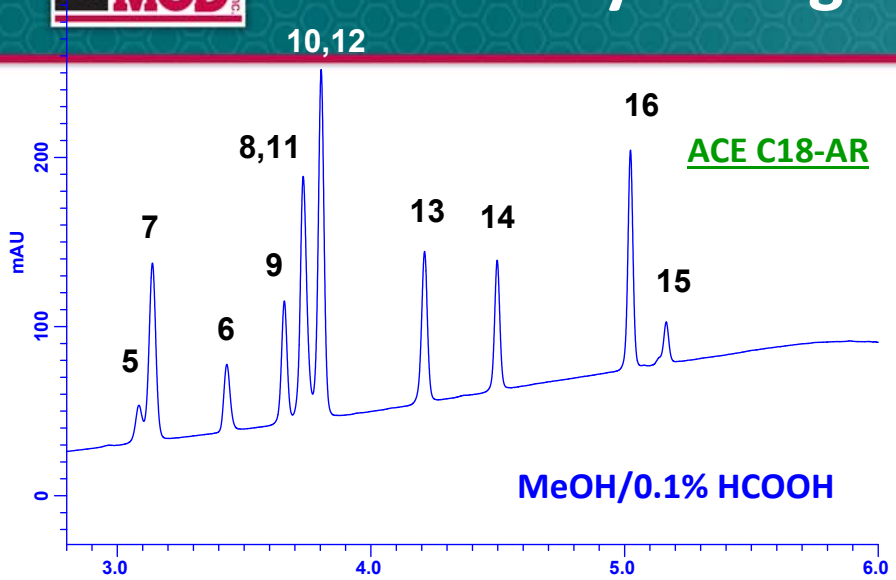
P_{max}: 540 bar

P_{max}: 540 bar

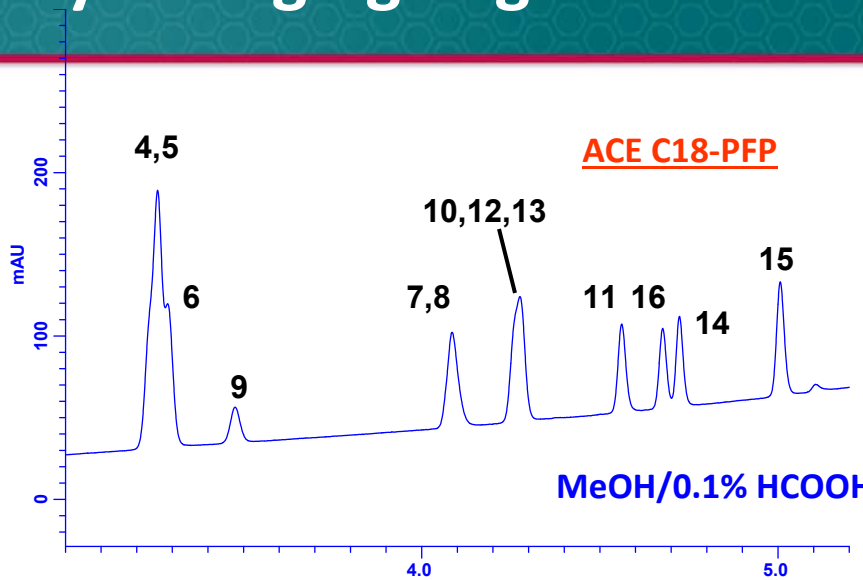
Conditions: A = 5mM formic acid (aq); B = 5mM formic acid in MeOH; tg= 3 to 100%B in 5 min; 0.6 ml/min; 40C; 254nm

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

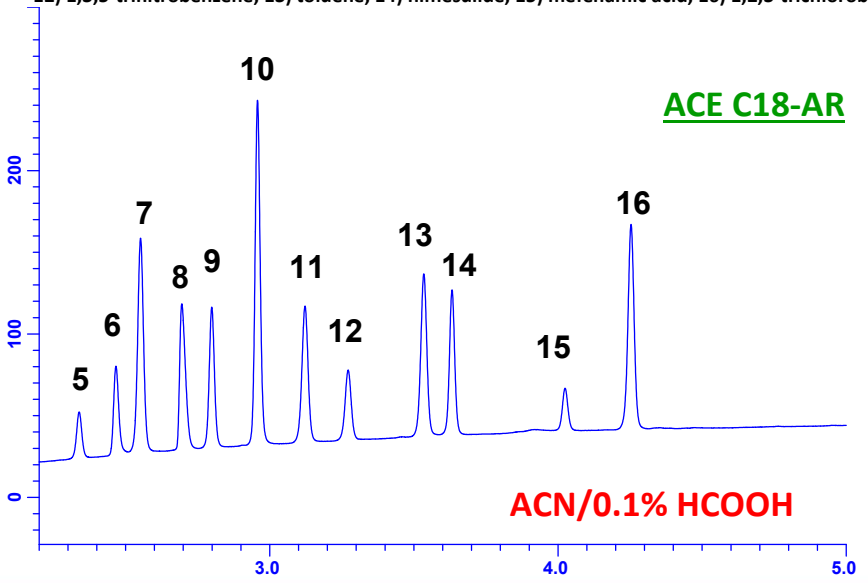
Selectivity Changes by Changing Organic Modifier

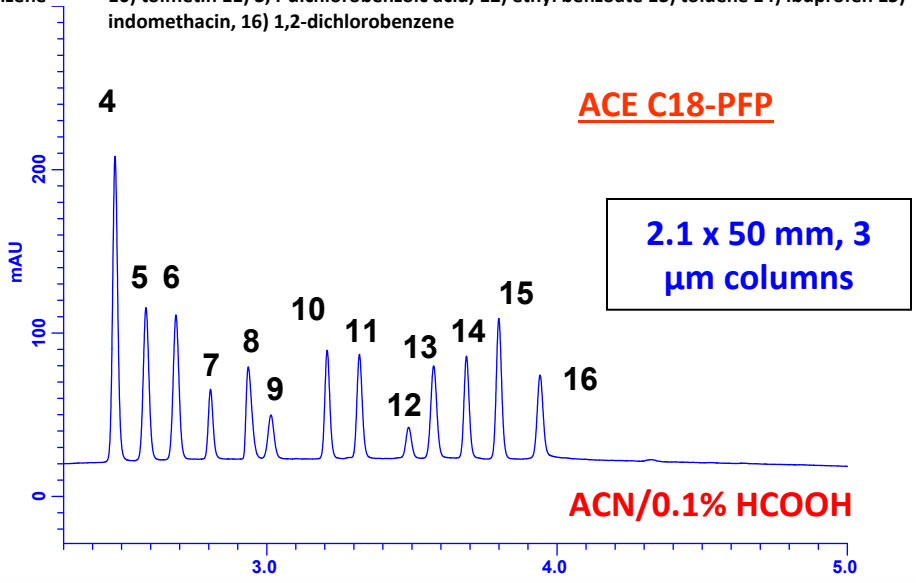


5) benzoic acid, 6) myrecetin, 7) p-cresol, 8) propranolol, 9) ethylparaben, 10) furosemide, 11) anisole, 12) 1,3,5-trinitrobenzene, 13) toluene, 14) nimesulide, 15) mefenamic acid, 16) 1,2,3-trichlorobenzene



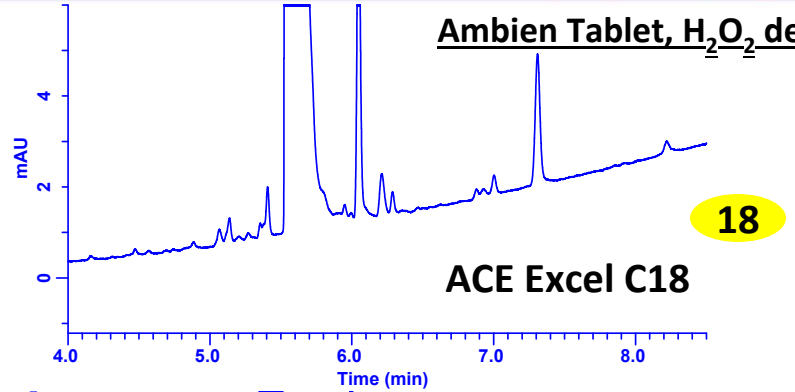
4) phenacetin, 5) p-cresol, 6) acetophenone, 7) quercetin, 8) carvedilol, 9) 1,3-dinitrobenzene, 10) tolmetin 11) 3,4-dichlorobenzoic acid, 12) ethyl benzoate 13) toluene 14) ibuprofen 15) indomethacin, 16) 1,2-dichlorobenzene





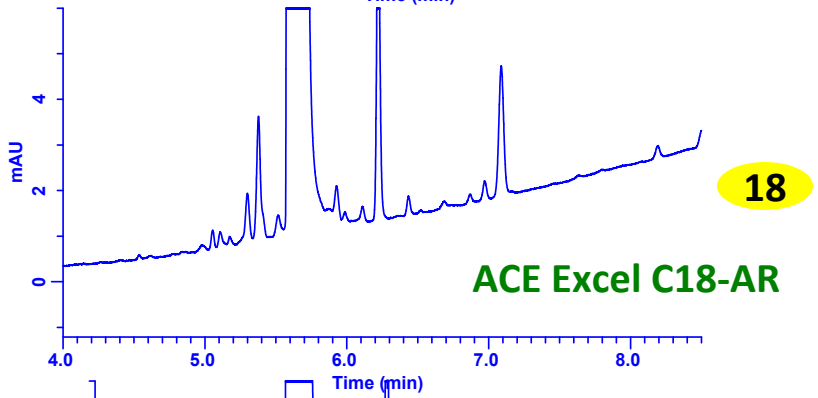
Gradient Column Phase Screening:
Optimum selectivity + UHPLC efficiency

Ambien Tablet, H_2O_2 degraded



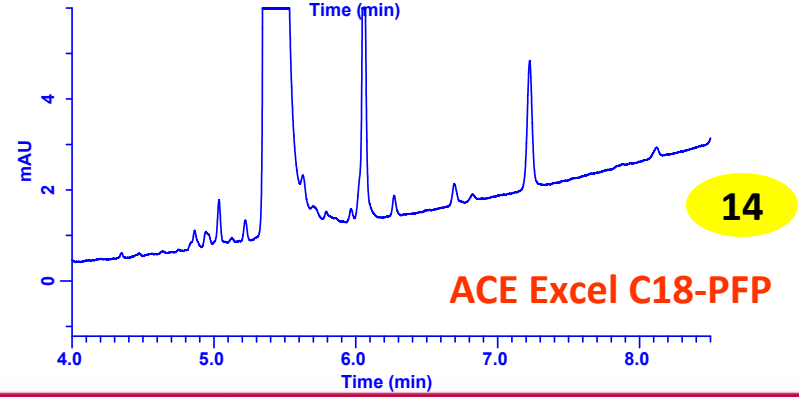
18

ACE Excel C18



18

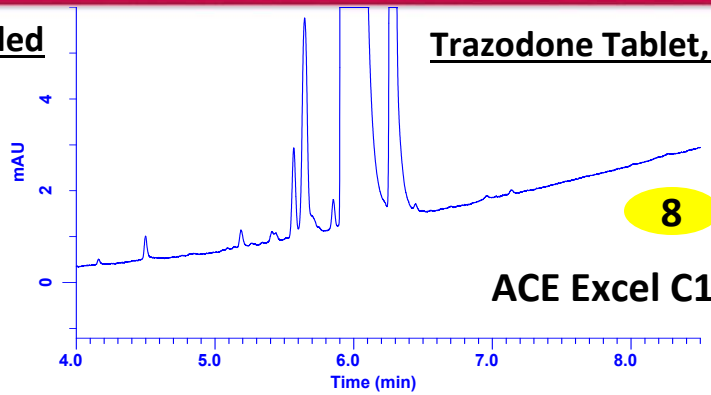
ACE Excel C18-AR



14

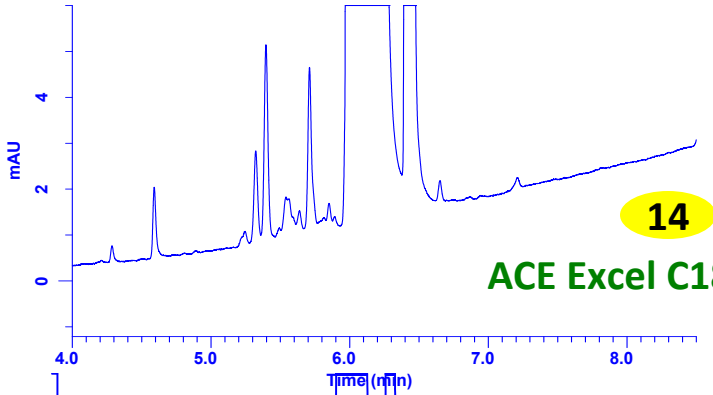
ACE Excel C18-PFP

Trazodone Tablet, H_2O_2 degraded



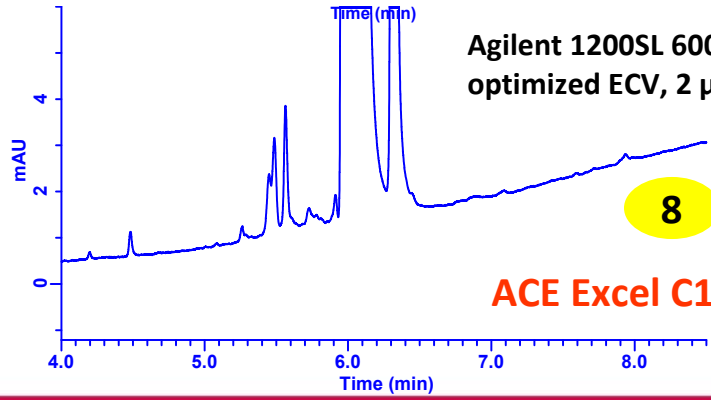
8 # Peaks

ACE Excel C18



14

ACE Excel C18-AR



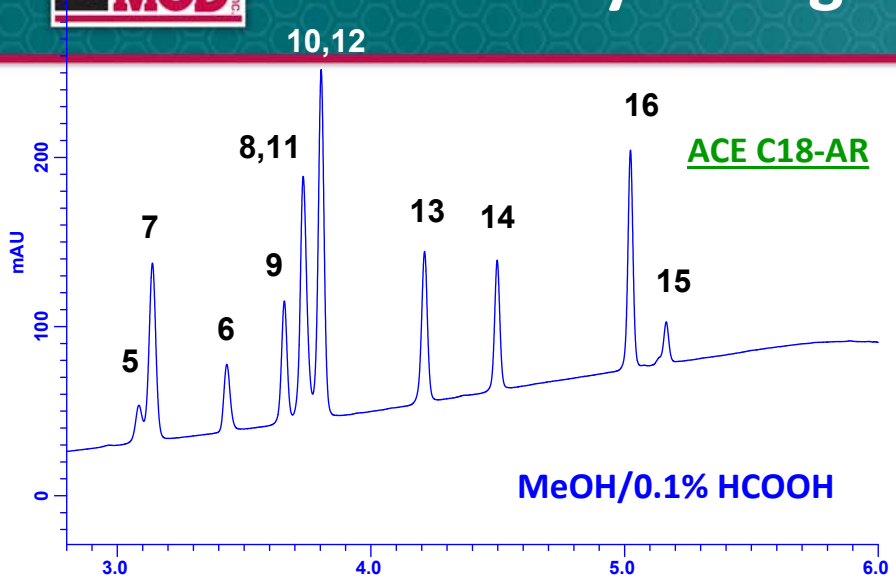
Agilent 1200SL 600 bar system
optimized ECV, 2 µL flow cell

8

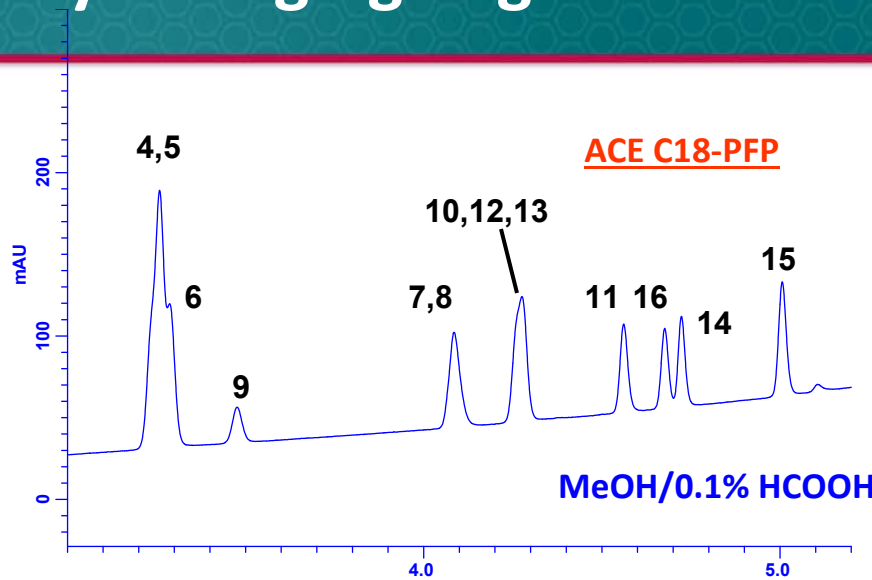
ACE Excel C18-PFP

0–80% ACN/0.1% HCOOH, 10 mM NH_4COO, 12-minutes

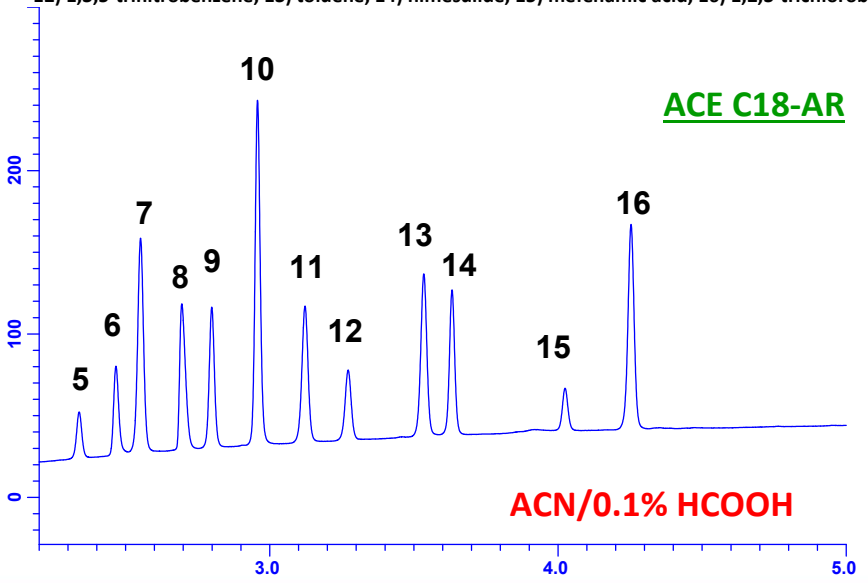
Selectivity Changes by Changing Organic Modifier

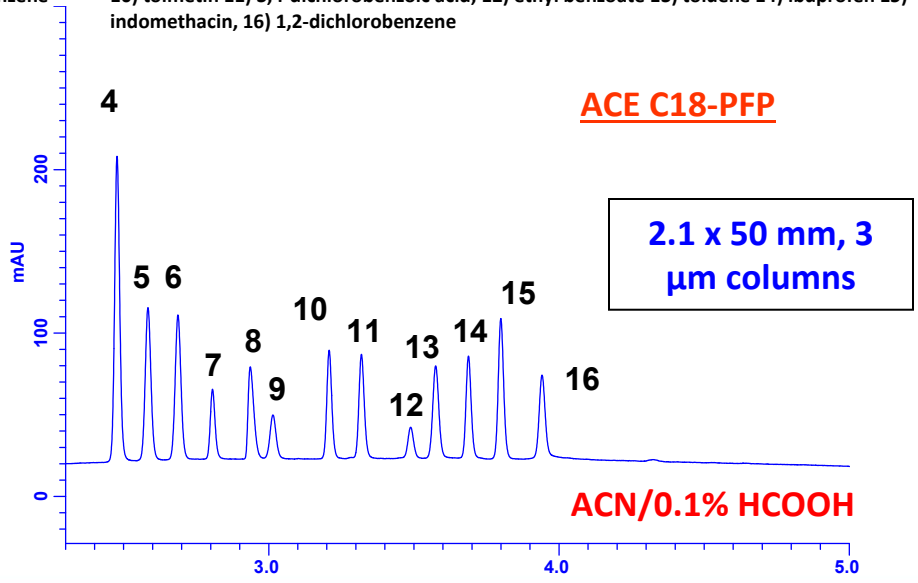


5) benzoic acid, 6) myrecetin, 7) p-cresol, 8) propranolol, 9) ethylparaben, 10) furosemide, 11) anisole, 12) 1,3,5-trinitrobenzene, 13) toluene, 14) nimesulide, 15) mefenamic acid, 16) 1,2,3-trichlorobenzene



4) phenacetin, 5) p-cresol, 6) acetophenone, 7) quercetin, 8) carvedilol, 9) 1,3-dinitrobenzene, 10) tolmetin 11) 3,4-dichlorobenzoic acid, 12) ethyl benzoate 13) toluene 14) ibuprofen 15) indomethacin, 16) 1,2-dichlorobenzene





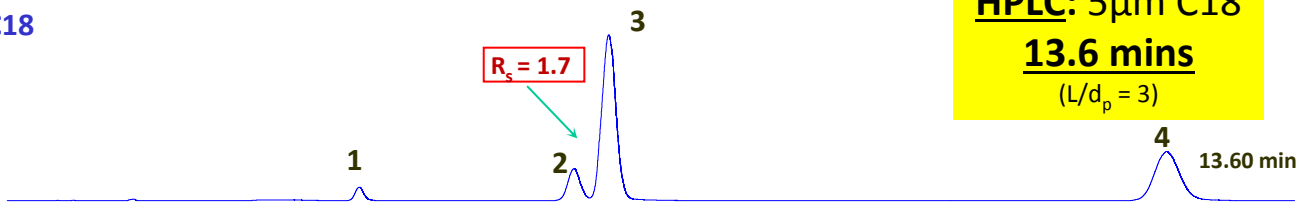
Selectivity, Speed & Scaling

Isocratic & Gradient HPLC → UHPLC

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

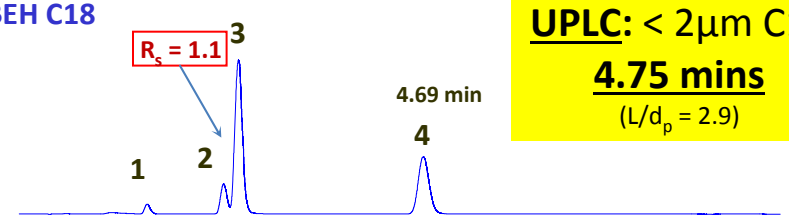
Aim: obtain $R_s \geq 1.7$ in shortest possible time for mixture

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



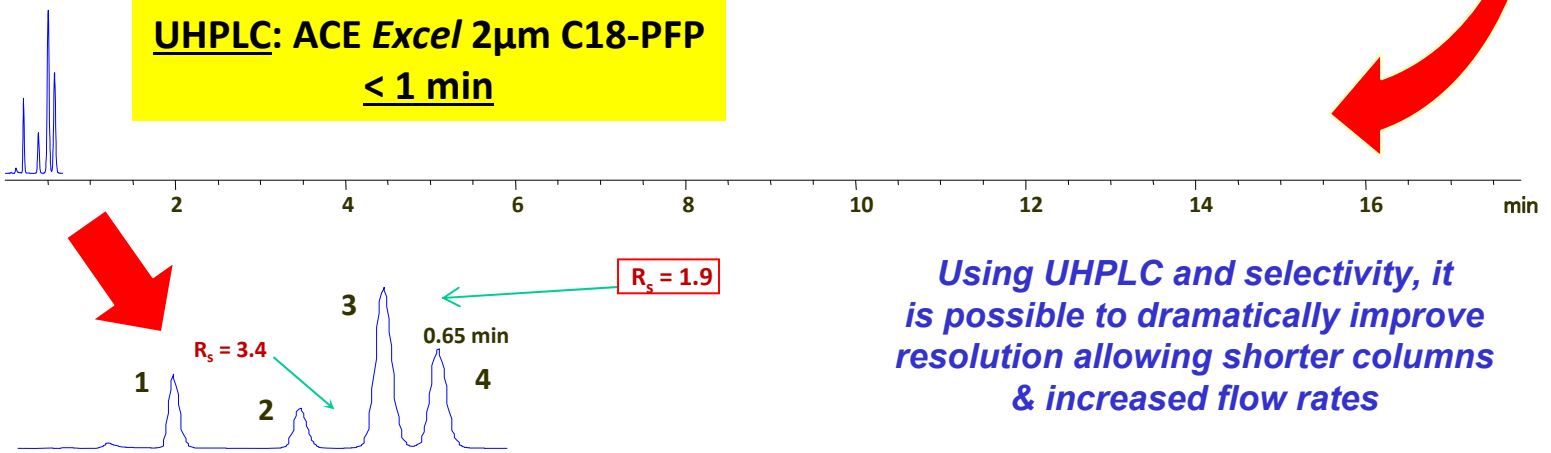
UPLC: < 2µm C18
4.75 mins
($L/d_p = 2.9$)

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



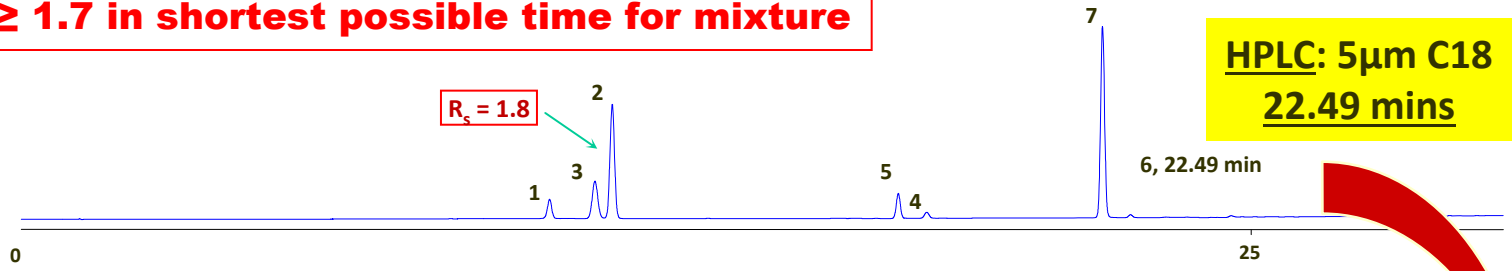
Using UHPLC and selectivity, it is possible to dramatically improve resolution allowing shorter columns & increased flow rates

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)

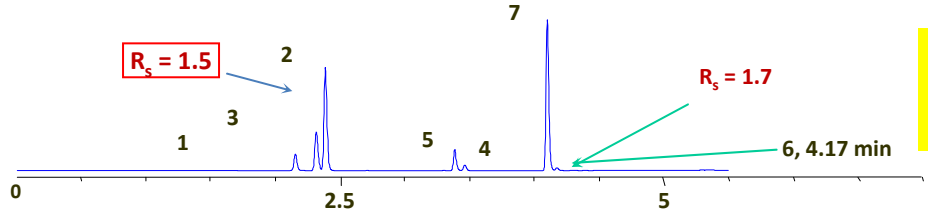
Aim: obtain $R_s \geq 1.7$ in shortest possible time for mixture

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

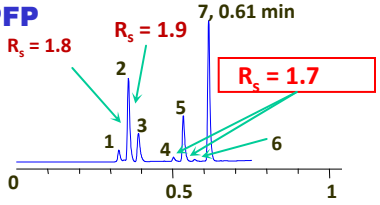
ACE *Excel* 2µm C18
50 x 2.1 mm
0.6 ml/min, t_G = 5 min
max pressure: 367 bar
9 min cycle time



UHPLC: 2µm C18
4.17 mins

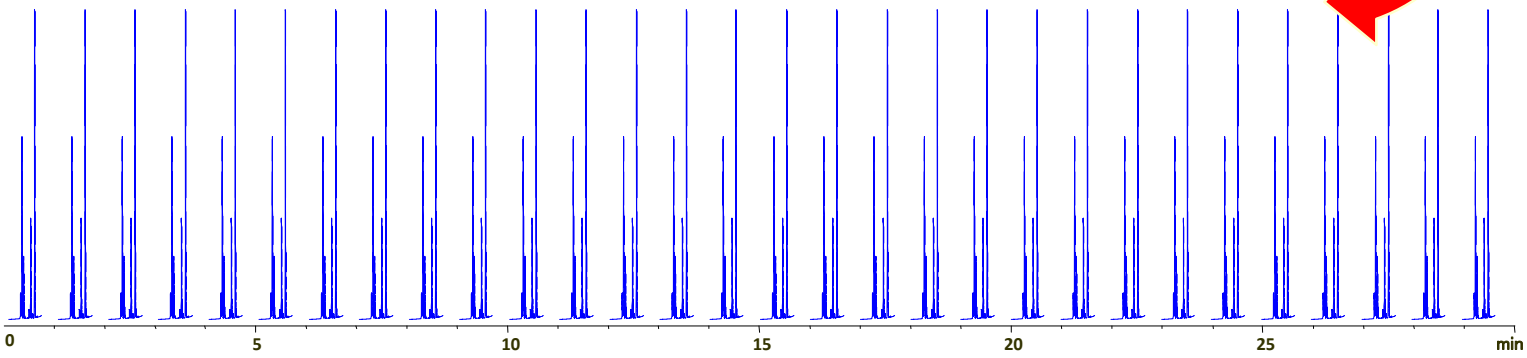
~ x25 Quicker

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, ethyl benzoate; 5, nimesulide; 6, ibuprofen; 7, indomethacin.

Pressure Effects

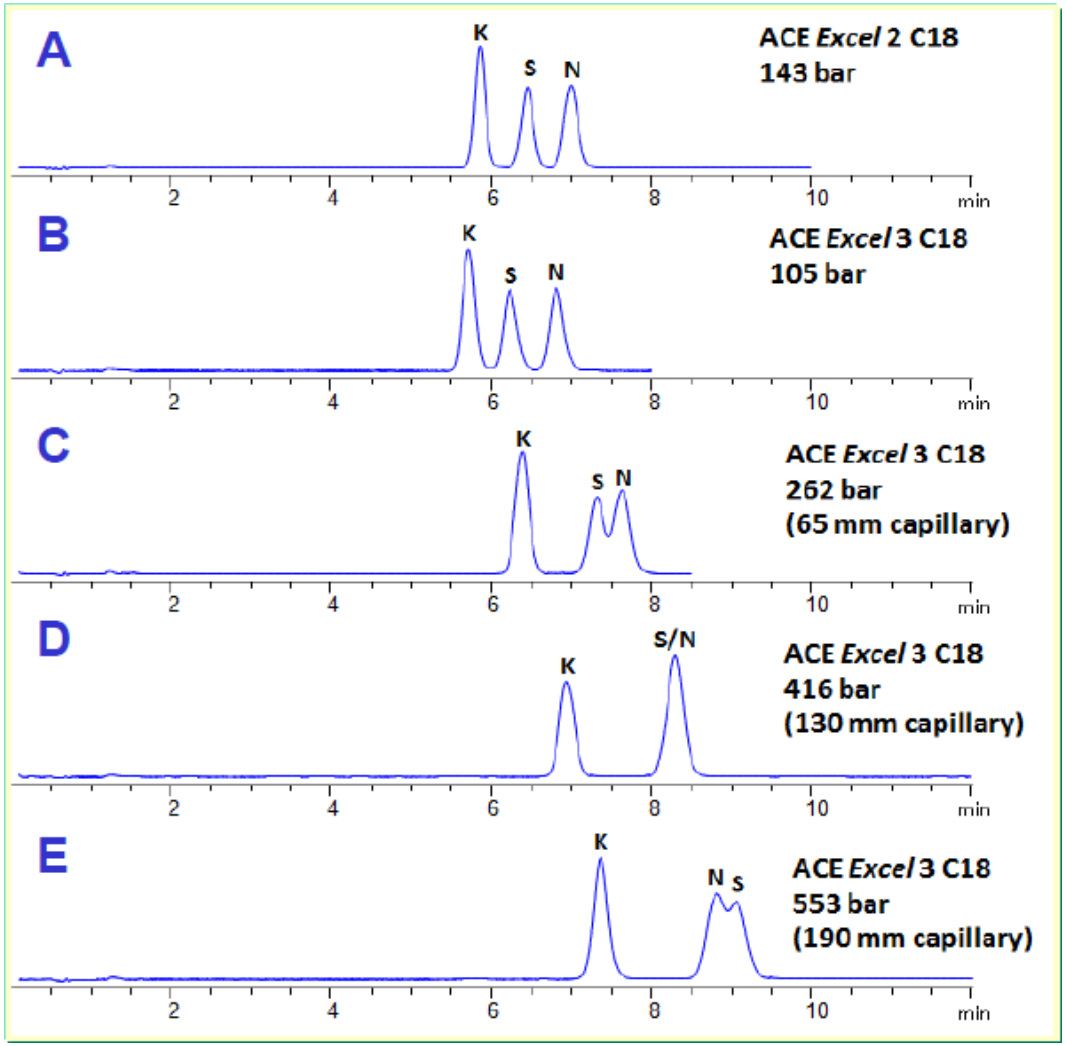
HPLC ↔ UHPLC

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



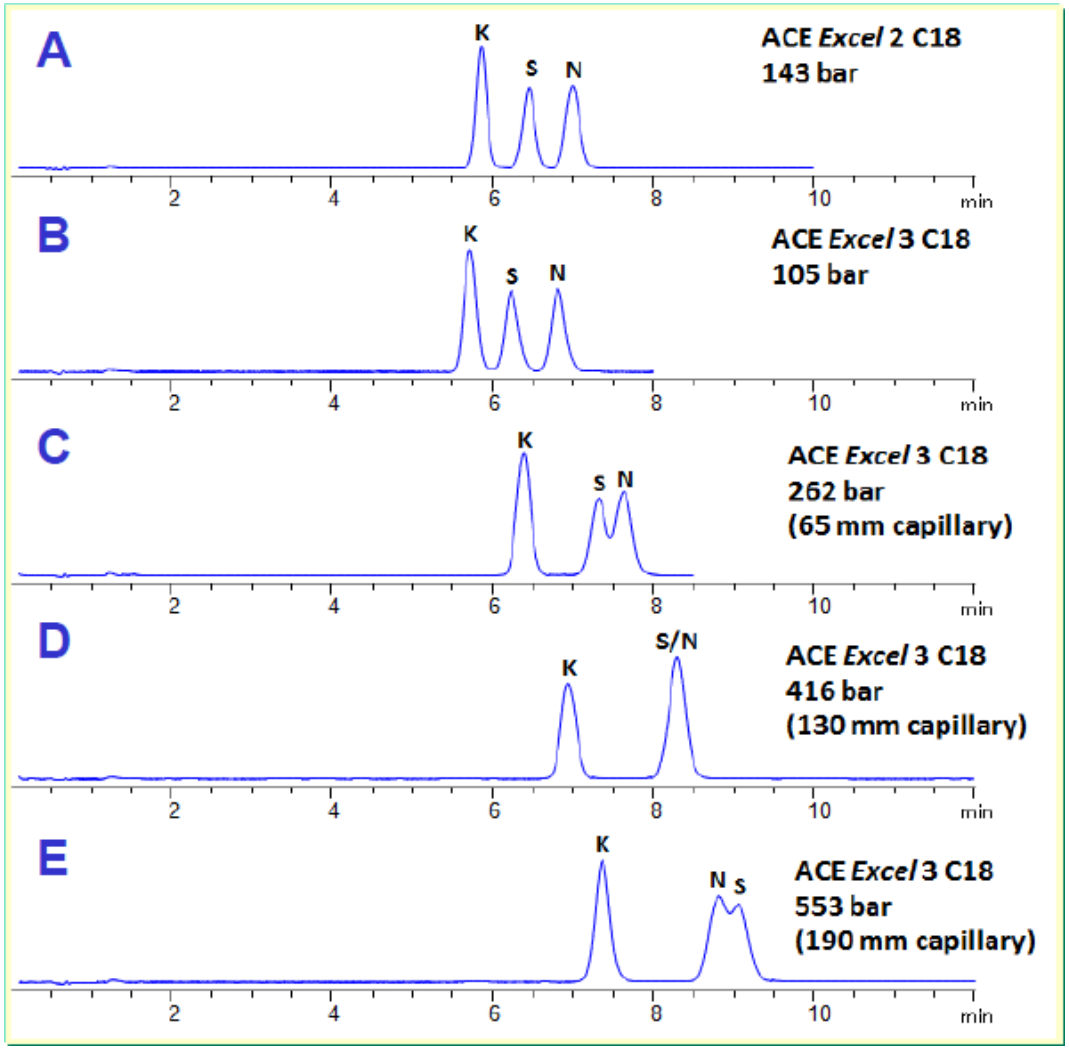
Agilent 1290, 50 x 2.1 mm (constant flow and restrictor capillary used)
Mobile phase: A=0.1% FA in water: B=0.1% FA in MeOH (51:49 v/v)
Flow Rate: 0.21 ml/min, Temperature: 40 °C
K= Ketoprofen; S= Sulindac; N=Naproxen

Initial 2µm and 3µm data are similar (A, B)

- **Scalability looks good**

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

Effect of Pressure on Selectivity and Retention Factor



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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

Initial 2µm and 3µm data are similar (A, B)

- Scalability looks good

Retention and selectivity seen to change with pressure (B→E)

[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

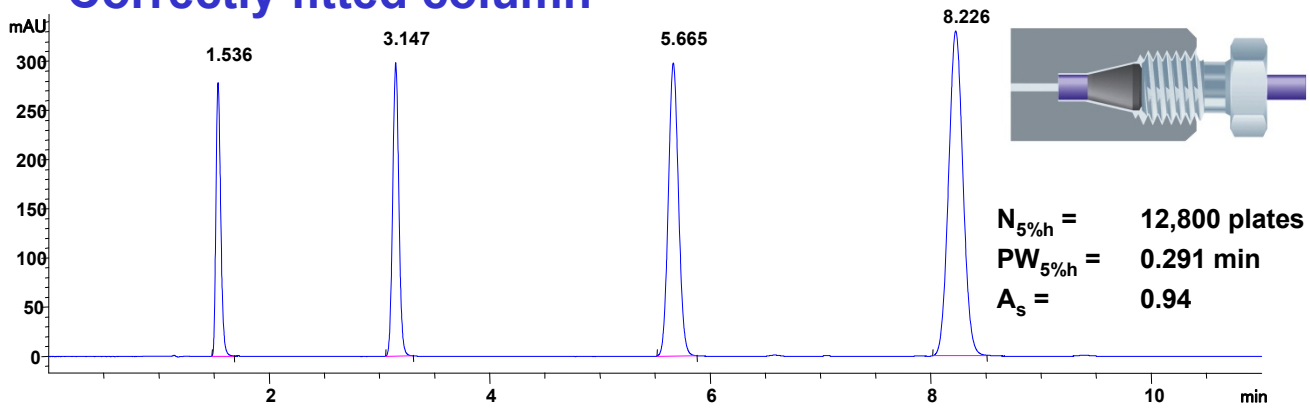
Peak Dispersion

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

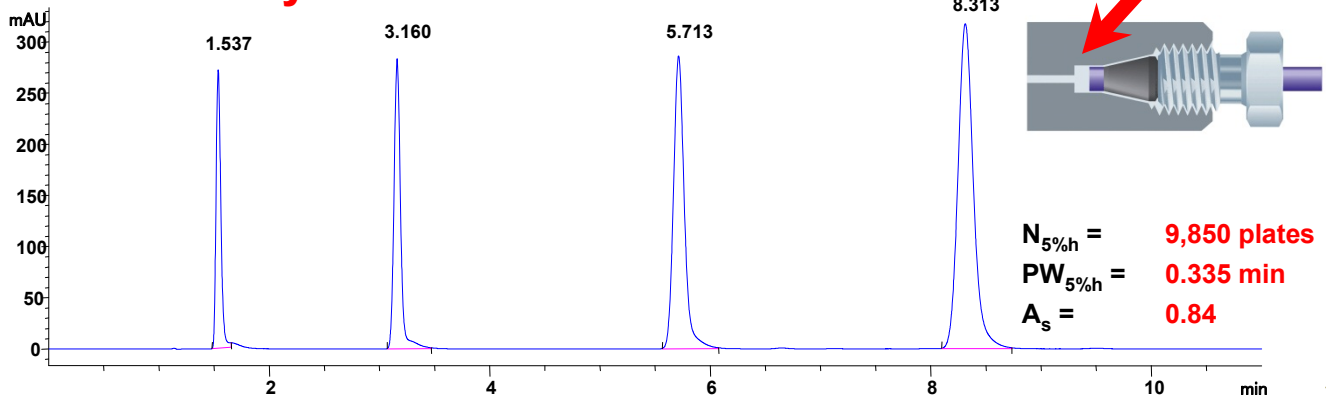
Correctly fitted column



$N_{5\%h}$ = 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



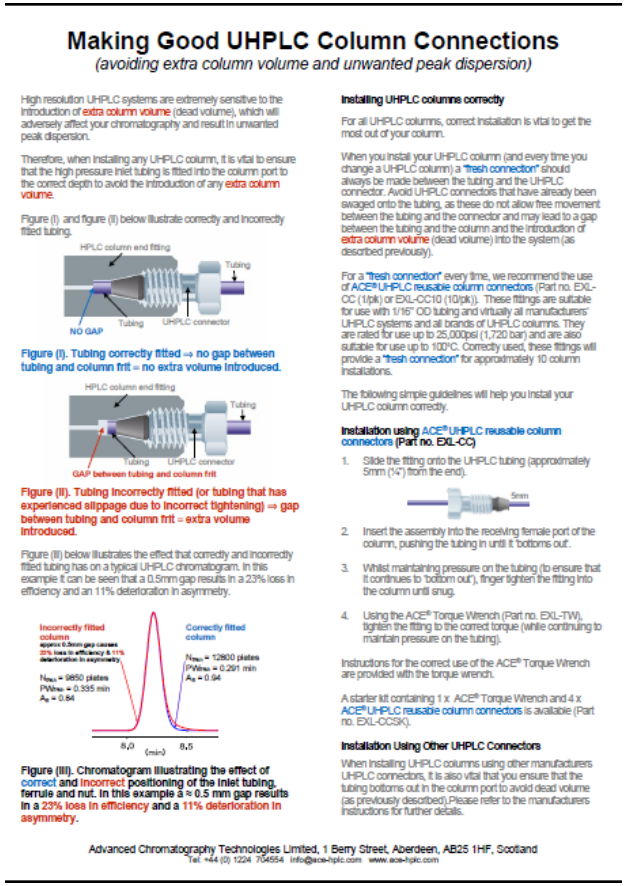
$N_{5\%h}$ = 9,850 plates
$PW_{5\%h}$ = 0.335 min
A_s = 0.84

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

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

Summary: Column Connections

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



Making Good UHPLC Column Connections

(avoiding extra column volume and unwanted peak dispersion)

High resolution UHPLC systems are extremely sensitive to the introduction of extra column volume (dead volume), which will adversely affect your chromatography and result in unwanted peak dispersion.

Therefore, when installing any UHPLC column, it is vital to ensure that the high pressure inlet tubing is fitted into the column port to the correct depth to avoid the introduction of any extra column volume.

Figure (I) and figure (II) below illustrate correctly and incorrectly fitted tubing.

HPLC column end fitting — Tubing — NO GAP — Tubing — UHPLC connector

Figure (I). Tubing correctly fitted ⇒ no gap between tubing and column frit ⇒ no extra volume introduced.

HPLC column end fitting — Tubing — Tubing — UHPLC connector — GAP between tubing and column frit

Figure (II). Tubing incorrectly fitted (or tubing that has experienced slippage due to incorrect tightening) ⇒ gap between tubing and column frit ⇒ extra volume introduced.

Figure (III) below illustrates the effect that correctly and incorrectly fitted tubing has on a typical UHPLC chromatogram. In this example it can be seen that a 0.5mm gap results in a 23% loss in efficiency and an 11% deterioration in asymmetry.

Incorrectly fitted column — approx 0.5mm gap causes 23% loss in efficiency & 11% deterioration in asymmetry — N_{theo} = 9850 plates, PW_{50} = 0.335 min, A_s = 0.84 — Correctly fitted column — N_{theo} = 12800 plates, PW_{50} = 0.291 min, A_s = 0.94 — 8.0 (min) 8.5

Figure (III). Chromatogram illustrating the effect of correct and incorrect positioning of the inlet tubing, ferrule and nut. In this example a ≈ 0.5 mm gap results in a 23% loss in efficiency and a 11% deterioration in asymmetry.

Installing UHPLC columns correctly

For all UHPLC columns, correct installation is vital to get the most out of your column.

When you install your UHPLC column (and every time you change a UHPLC column) a “fresh connection” should always be made between the tubing and the UHPLC connector. Avoid UHPLC connectors that have already been swaged onto the tubing, as these do not allow free movement between the tubing and the connector and may lead to a gap between the tubing and the column and the introduction of extra column volume (dead volume) into the system (as described previously).

For a “fresh connection” every time, we recommend the use of ACE® UHPLC reusable column connectors (Part no. EXL-CC (1/pk) or EXL-CC10 (10/pk)). These fittings are suitable for use with 1/16” OD tubing and virtually all manufacturers’ UHPLC systems and all brands of UHPLC columns. They are rated for use up to 25,000psi (1,720 bar) and are also suitable for use up to 100°C. Correctly used, these fittings will provide a “fresh connection” for approximately 10 column installations.

The following simple guidelines will help you install your UHPLC column correctly.

Installation using ACE® UHPLC reusable column connectors (Part no. EXL-CC)

1. Slide the fitting onto the UHPLC tubing (approximately 5mm (¼”) from the end).

2. Insert the assembly into the receiving female port of the column, pushing the tubing in until it ‘bottoms out’.
3. Whilst maintaining pressure on the tubing (to ensure that it continues to ‘bottom out’), finger tighten the fitting into the column until snug.
4. Using the ACE® Torque Wrench (Part no. EXL-TW), tighten the fitting to the correct torque (while continuing to maintain pressure on the tubing).

Instructions for the correct use of the ACE® Torque Wrench are provided with the torque wrench.

A starter kit containing 1 x ACE® Torque Wrench and 4 x ACE® UHPLC reusable column connectors is available (Part no. EXL-CCSK).

Installation Using Other UHPLC Connectors

When installing UHPLC columns using other manufacturers UHPLC connectors, it is also vital that you ensure that the tubing bottoms out in the column port to avoid dead volume (as previously described).Please refer to the manufacturers instructions for further details.

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Making Good UHPLC Column Connections

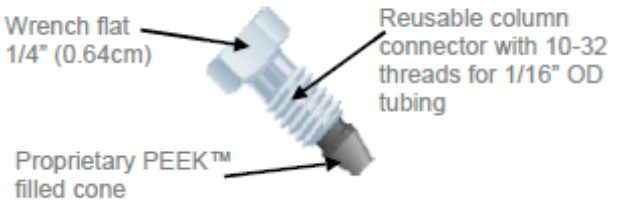
UHPLC column outlet end connections

ACE® UHPLC reusable column connectors (EXL-CC) are suitable for use at both the column inlet and the column outlet, to make good UHPLC connections. Alternatively, at the column outlet end an ACE® reusable PEEK finger-tight column connector (ACE-FT, suitable for use up to 5,000psi, 345 bar) can be used to make a good connection.

To avoid extra column volume and unwanted peak dispersion, it is additionally important to make good connections at the outlet end of your UHPLC column. Similar principles as previously described for the inlet end connection should be employed.

ACE® UHPLC Reusable Column Connectors

- **Compact, one-piece, easy to use connector**
- **Pressure rated to 25,000psi (1,720 bar)**
- **Usable at elevated temperatures (up to 100°C)**
- **Fits virtually all manufacturers' UHPLC systems**
- **Fits all brands of UHPLC columns**
- **Reusable, non-permanent swaging design**
- **10 make and break cycles***



Part Numbers

Description UHPLC column inlet / outlet connectors	Part No.
ACE® UHPLC reusable column connectors (1/pk) suitable for use up to 25,000psi (1,720 bar)	EXL-CC
ACE® UHPLC reusable column connectors (10/pk) suitable for use up to 25,000psi (1,720 bar)	EXL-CC10
ACE® UHPLC reusable column connectors starter kit (contains 1 x Part no. EXL-TW and 4 x Part no. EXL-CC)	EXL-CCSK
ACE® Torque Wrench	EXL-TW

Description UHPLC column outlet connectors	Part No.
ACE® reusable PEEK finger-tight column connector (1/pk) (suitable for use up to 5,000psi, 345 bar	ACE-FT
ACE® reusable PEEK finger-tight column connector (10/pk) (suitable for use up to 5,000psi, 345 bar	ACE-FT10

Also downloadable from the ACE website:

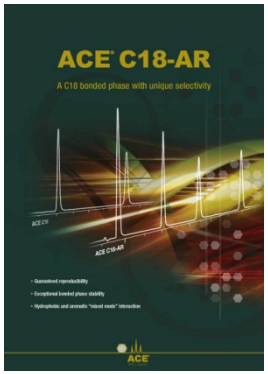
www.ace-hplc.com

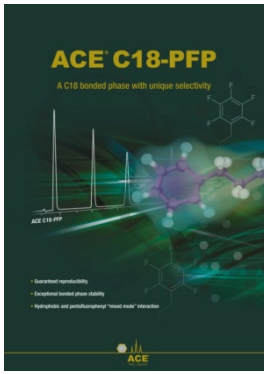
Overall Summary & Conclusions

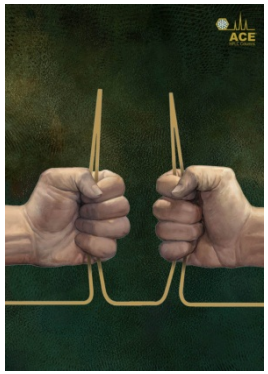
- Understanding the properties of building blocks in stationary phase design led to these unique ACE® products
- ACE® C18-AR and ACE® C18-PFP are powerful tools for method development due to unique but complementary selectivities
- These unique phases are available for HPLC as the ACE® range and also for 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

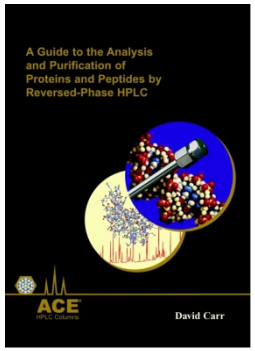
Unique Selectivities

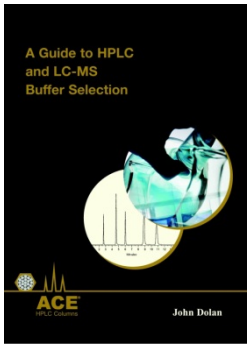


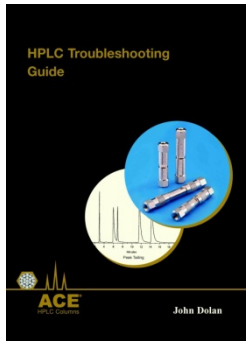




Free Guides







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

Thank You For Your Attention

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