Exploiting Selectivity in HPLC and UHPLC With Rational Stationary Phase Design

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Advanced Chromatography Technologies

www.ace-hplc.com
www.mac-mod.com
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

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

The Importance of $N$, $k$ and $\alpha$ For Resolution

Typical separation:

$N = 10,000$ plates

$k = 3.8 / 4.2$ (4.0 mean)

$\alpha = 1.1$

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

$$R_s = 1.8$$

Which looks like
The Importance of \( N, k \) and \( \alpha \) For Resolution

**Double Efficiency** (eg 5 \( \mu \)m \( \rightarrow \) 2.5 \( \mu \)m):

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

\( N = 10,000 \rightarrow 20,000 \) plates

\[ R_s = 2.6 \]

\( R_s = 1.8 \)

\( \sim 40\% \) Increase

Opportunity to optimise further eg reduce column length to speed up
Double Retention Factor (eg decrease solvent strength):

\[ k = 4 \rightarrow 8 \]

\[ R_s = \frac{\sqrt{N}}{4} \frac{\alpha-1}{\alpha} \frac{k}{1+k} \]

\[ R_s = 2.0 \]

\[ R_s = 1.8 \]

\[ \sim 10\% \text{ Increase} \]

Slight improvement in resolution has led to increased analysis time
The Importance of \( N, k \) and \( \alpha \) For Resolution

Increase Selectivity (eg change column):

\[ \alpha = 1.1 \rightarrow 1.2 \]

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

\[ R_s = 3.3 \]

Significant opportunity to speed up for modest change in selectivity
Selectivity: The Key to Chromatographic Peak Resolution

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

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

\[ \text{Resolution (Rs)} \]

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

\(\text{MOST Influence}\)

\(\text{LEAST Influence}\)

ACE C18 – Increase Retention

ACE C8 (start point)

ACE C4 – Decrease Retention

ACE CN – Elution Order

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 KH₂PO₄ (pH6.0)  Flow: 1.00ml/min
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
16 Pharmaceutically Relevant Analytes – C18 Columns

ACE Excel 2 C18
$P_{\text{max}}$: 309 bar

Waters
Acquity 1.7 BEH C18
$P_{\text{max}}$: 478 bar

Phenomenex
Kinetex 1.7 C18
$P_{\text{max}}$: 446 bar

Agilent Zorbax
Eclipse 1.8 XBD C18
$P_{\text{max}}$: 396 bar

C18 phases show ‘similar’ selectivity...

50x2.1mm
A: 20 mM $\text{KH}_2\text{PO}_4$, pH 2.7
B: 20 mM $\text{KH}_2\text{PO}_4$, pH 2.7 in MeOH/H$_2$O (65:35 v/v)
Gradient: 3 – 100 %B in 5 min
Flow rate: 0.6 ml/min
Temperature: 60°C
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

All trademarks are recognised...comparative separations may not be representative of all applications
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
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

Electron Donating Groups
eg NH₂, NR₂, alkyl, OCH₃
OR, CH₃, Ar etc

e.g. δ+ δ-

Electron Rich Ring
Activity: π-donor (π-base)

Classic π-π interaction

Electron Withdrawing Groups
eg NO₂, halides, NR₃⁺, CO₂H,
CN, CO₂R, SO₃H, 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
Combining the character of C18+phenyl into a single individual phase harnesses the best of both phases for unique selectivity

<table>
<thead>
<tr>
<th>Separation mechanism</th>
<th>Typical C18</th>
<th>Typical Phenyl</th>
<th>ACE® C18-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobicity</td>
<td>++++</td>
<td>+ / ++</td>
<td>++++</td>
</tr>
<tr>
<td>(\pi-\pi) Interaction</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Dipole - Dipole</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen Bonding</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Shape Selectivity</td>
<td>++</td>
<td>++</td>
<td>++ / +++</td>
</tr>
</tbody>
</table>

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*

π-acidity (order)

ACE 3 C18
ACE 3 Phenyl
ACE 3 C18-AR

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.

<table>
<thead>
<tr>
<th>Separation mechanism</th>
<th>Typical C18</th>
<th>Typical PFP</th>
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<tr>
<td>Hydrophobicity</td>
<td>++++</td>
<td>+ / ++</td>
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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*

<table>
<thead>
<tr>
<th>Peak Number</th>
<th>Structure</th>
<th>Log P</th>
<th>π-basicity (order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,2,3-TMB</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1,2,4-TMB</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1,2-DMB</td>
<td>1.7</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1,4-DMB</td>
<td>2.1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>MB</td>
<td>2.2</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>1,3-DMB</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>1,3,5-TMB</td>
<td>1.6</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Tol</td>
<td>2.7</td>
<td>-</td>
</tr>
</tbody>
</table>

♦ 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.

**ACE® C18-PFP Selectivity**

- **ACE 3 C18**
- **Hypersil GOLD 3 μm PFP**
- **Reduced Hydrophobicity**
- **ACE 3 C18-PFP**

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 / H₂O; Column= 150 x 4.6 mm id; 1.00 ml/min; 40°C; 254 nm

All trademarks are recognised...comparative separations may not be representative of all applications.
Selectivity = 100 \times \sqrt{(1 - R^2)}

Selectivity = 100 \times \sqrt{(1 - 0.9888)}

= 10.6

For the 102 acidic, basic and neutral analytes assessed

<table>
<thead>
<tr>
<th>MeOH</th>
<th></th>
<th>Selectivity ‘S’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1</td>
<td>Column 2</td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>C18-AR</td>
<td>12</td>
</tr>
<tr>
<td>C18</td>
<td>C18-PFP</td>
<td>11</td>
</tr>
<tr>
<td>C18-AR</td>
<td>C18-PFP</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MeCN</th>
<th></th>
<th>Selectivity ‘S’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1</td>
<td>Column 2</td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>C18-AR</td>
<td>8</td>
</tr>
<tr>
<td>C18-AR</td>
<td>C18-PFP</td>
<td>8</td>
</tr>
<tr>
<td>C18</td>
<td>C18-PFP</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MeOH</th>
<th>MeCN</th>
<th>Selectivity Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18-PFP</td>
<td>C18</td>
<td>19</td>
</tr>
<tr>
<td>C18-AR</td>
<td>C18</td>
<td>18</td>
</tr>
<tr>
<td>C18-AR</td>
<td>C18-PFP</td>
<td>18</td>
</tr>
<tr>
<td>C18-PFP</td>
<td>C18-AR</td>
<td>18</td>
</tr>
<tr>
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<td>C18-PFP</td>
<td>18</td>
</tr>
<tr>
<td>C18</td>
<td>C18-AR</td>
<td>17</td>
</tr>
<tr>
<td>C18</td>
<td>C18-PFP</td>
<td>17</td>
</tr>
<tr>
<td>C18</td>
<td>C18</td>
<td>15</td>
</tr>
<tr>
<td>C18-AR</td>
<td>C18-AR</td>
<td>15</td>
</tr>
</tbody>
</table>

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 -NH₂, -NR₂, -OCH₃, -OH, -alkyl, -Ar etc
- eg nucleotides, nucleosides, nucleobases, halogenated aryl / aromatics, catecholamines, tetracyclines, beta blockers, structural isomers, coumarins etc
- **Excellent** shape and **positionnal 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 -NO₂, -halides, -NR₃⁺, -SO₂, -CO₂H, -SO₃H, -CO₂R, -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

Separation of 12 Steroid Standards

1. Estriol
2. Prednisolone
3. Hydrocortisone
4. Cortisone
5. Corticosterone
6. 17β-Estradiol
7. Cortisone-21-acetate
8. 17α-Estradiol
9. 19-Norethindrone
10. 17α-Ethynylestradiol
11. 21-Hydroxyprogesterone
12. Estrone
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
NEW High Stability Column (HSC™) Packing Technology Significantly Improves 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.
ACE® Excel™ UHPLC Columns – Scalability & Reproducibility

ACE Excel 2 C18
150 x 2.1mm
0.21mL/min

ACE 3 C18
150 x 3.0mm
0.40mL/min

ACE 5 C18
150 x 4.6mm
1.00mL/min

ACE 10 C18
150 x 21.2mm
21.2mL/min

UHPLC

HPLC

Prep LC

MP: 35:65 v/v MeCN:0.1% TFA (aq); 22C; 254nm; 1. uracil; 2. 4-hydroxybenzoic acid; 3. acetylsalicylic 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

ACE Excel 2 C18-AR

Waters Acquity 1.7um BEH C18

Phenomenex Kinetex 1.7um C18

Agilent Zorbax Eclipse 1.8 um XDB C18

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

All trademarks are recognised...comparative separations may not be representative of all applications
Selectivity, Speed & Scaling
Isocratic & Gradient HPLC → UHPLC
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

$R_s = 1.7$

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

$R_s = 1.1$

$4.75$ mins
($L/d_p = 2.9$)

UPLC: <2µm C18

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

$R_s = 1.9$

< 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$_2$O; Temperature 40°C; 254 nm

All trademarks are recognised...comparative separations may not be representative of all applications
Aim: obtain \( R_s \geq 1.7 \) in shortest possible time for mixture

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

**ACE 5µm C18**
- 100 x 4.6 mm
- 1 ml/min, \( t_c = 29 \) min
- max pressure: 92 bar
- 40 min cycle time

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

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

HPLC: 5µm C18
- 22.49 mins

UHPLC: 2µm C18
- 4.17 mins
- \( \sim x25 \) Quicker

UHPLC: 2µm C18-PFP
- 0.61 mins

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

HPLC ↔ UHPLC
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.

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.

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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

Summary: Unwanted Selectivity Changes

- Pressure induced $k$ and $\alpha$ 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, 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 $\alpha$ observed.

- Successful HPLC ↔ UHPLC possible...the analyst just needs to be vigilant.

Connections : Losses in $N$ and $A_s$

Peak Dispersion
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.
Correctly fitted columns make the most of your column and system.

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

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!
Unique Selectivities

Free Guides

MACMOD Analytical = http://www.mac-mod.com/
ACT = http://www.ace-hplc.com
Thank You For Your Attention

amckeown@ace-hplc.com

www.ace-hplc.com
www.mac-mod.com