Comparison of Phenyl and C18 bonded-phases to obtain separation selectivity of peptide mixtures

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Agenda

- Peptide separation selectivity options in RP versus standard alkyl bonded phase are often sought.
- Alternate selectivity could be obtained by use of phenyl bonded phases.
- Aim to explore selectivity differences of $\phi$-Hexyl, $\phi$-Butyl, $\phi$-Ethyl vs C18.
- Selectivity Differences of Identified Peptides using Test System under various conditions.
- Global vs specific selectivity differences.
- Examples of utility for selected peptides.
<table>
<thead>
<tr>
<th>Bonded Phase</th>
<th>Surface Coverage (µm/m²)</th>
<th>Endcapped</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18</td>
<td>1.7</td>
<td>No</td>
</tr>
<tr>
<td>ϕ-Hexyl</td>
<td>3.5</td>
<td>Yes</td>
</tr>
<tr>
<td>ϕ-Butyl</td>
<td>3.4</td>
<td>No</td>
</tr>
<tr>
<td>ϕ-Ethyl</td>
<td>2.1</td>
<td>No</td>
</tr>
</tbody>
</table>

¹Surface Coverage based on %Carbon
Original Halo Superficially Porous Particles Fused-Core®

- Low back pressure due to the particle design (solid core with a porous shell)
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)
Low pH Column Stability: φ-Hexyl
19,520 column volumes

Column: HALO φ-Hexyl
2.7µm, 2.1x100mm 160Å
A: Water/ 0.1% Trifluoroacetic Acid
B: 70-30 ACN/ Water/ 0.1% Trifluoroacetic Acid
Gradient: 9-55% B in 10 min.

Flow: 0.5 ml/min
Detection: 220 nm
Temperature: 60 ºC
Injection Volume: 2 µl

Standard Peptides/Proteins:
1. Gly-Tyr
2. Val-Tyr-Val
3. Methionine Enkephalin
4. Angiotensin II
5. Leucine Enkephalin
6. Bovine RNaseA
7. Bovine Insulin

Peak Shape Reproducible >>
Butyl & Ethyl low pH Stable
Mobile Phase Acid Modifier Selection for Bonded Phase Comparison:

Column: 2.1 x 150 mm Halo Peptide C18; Flow rate: 0.4 mL/min;
Gradient: 5 - 60% B in 20 min

Difluoroacetic acid (DFA)

Formic acid (FA)

Ammonium Formate (AF)

Standard Peptides
1. Asp-Phe
2. Angiotensin(1-7)amide
3. Tyr-Tyr-Tyr
4. Angiotensin(II)
5. Neurotensin
6. Angiotensin(1-2)human
7. β-endorphin
8. Sauvagine
9. Mellitin

DFA is LCMS compatible! (vs TFA)
WORK FLOW

Trastuzumab: (monoclonal antibody)
  Reduced/Alkylated
  Trypsin Digest
  Shimadzu Nexera-UV coupled to Orbitrap Velos Pro ETD
  Peptide Identification by MS$^2$ fragmentation spectra
  Extracted Ions Used to Measure Retention and Peptide Pair Selectivity Differences
Specific Comparison: Identified Peptides

UNRESOLVED PAIR 24 min

220nm

C18

φ-Hexyl

φ-Butyl

φ-Ethyl

EIC

Peak Widths
Specific Comparison: Identified Peptides

220nm

C18

φ-Hexyl

φ-Butyl

φ-Ethyl

EIC

UNRESOLVED PAIR 24 min
Specific Comparison: Identified Peptides

220nm

C18

φ-Hexyl

φ-Butyl

φ-Ethyl

EIC

LScAASGFNIKDTYIHWVR

HerDigest_ES-C18_BPL03.raw  #2062  RT: 25.6782 min

ITMS, 1120.0776@cid35.00, z=+2, Mono m/z=1119.57727 Da, MH+=2238.14726 Da, Match Tol.=1 Da
Specific Comparison: Identified Peptides

220nm

C18

φ-Hexyl

φ-Butyl

φ-Ethyl

EIC

FTISADTSKNTAYLQMNSLRAEDTAVYYcSR
Chromatographic Selectivity ($\alpha$)  

$\phi$-Butyl C18

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Extracted Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.50</td>
<td>C18</td>
</tr>
<tr>
<td>22.5'</td>
<td></td>
</tr>
<tr>
<td>22.9'</td>
<td></td>
</tr>
<tr>
<td>26.50</td>
<td></td>
</tr>
</tbody>
</table>

$\varphi$-Butyl

$\alpha = k_2/k_1$

$\alpha = k_2/k_1$

MS Selectivity ($\alpha$)  

EIC

C18

1148 m/z

948 m/z

$\varphi$-Butyl

$k_1 = (t_1 - t_0)/t_0$  
$t_0 =$ void time

$\alpha = k_2/k_1$
Measuring Differences in Selectivity for Peptide Pairs

\[ k = \frac{(t_1 - t_0)}{t_0} \]

\[ \alpha = \frac{k_2}{k_1} \]

\[ \Delta \alpha = \alpha_{\text{phenyl}} - \alpha_{\text{c18}} \]

\[ |\Delta \alpha| = |\alpha_{\text{phenyl}} - \alpha_{\text{c18}}| \]

Average (\(\Delta \alpha\)):

\[ \frac{1}{(n-1)} \sum_{n-1} \Delta \alpha \]

Average Absolute Value (\(|\Delta \alpha|\)):

\[ \frac{1}{(n-1)} \sum_{n-1} |\Delta \alpha| \]

\(n = \) number of peptides; \(n-1 = \) number of peptide pairs
Selectivity Differences for 42 Peptide Pairs

\[ \Delta \alpha = \alpha_{\text{phenyl}} - \alpha_{c_{18}} \]
# Average Selectivity Differences for Peptide Pairs

<table>
<thead>
<tr>
<th>Varied Bonded Phase (Same Mobile Phase)</th>
<th>φ-Hexyl:C18</th>
<th>φ-Butyl:C18</th>
<th>φ-Ethyl:C18</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1/(n-1) \sum_{n-1} \Delta\alpha$</td>
<td>0.017</td>
<td>0.025</td>
<td>0.028</td>
</tr>
<tr>
<td>$1/(n-1) \sum_{n-1}</td>
<td>\Delta\alpha</td>
<td>$</td>
<td>0.044</td>
</tr>
</tbody>
</table>
Pair-wise Comparison of Bonded Phase Orthogonality

Varied Bonded Phases (Same Mobile Phase)

<table>
<thead>
<tr>
<th></th>
<th>Dim</th>
<th>R.H.A.</th>
<th>1-r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18/φ-Hexyl</td>
<td>1.10</td>
<td>0.048</td>
<td>0.0045</td>
</tr>
<tr>
<td>C18/φ-Butyl</td>
<td>1.23</td>
<td>0.073</td>
<td>0.0123</td>
</tr>
<tr>
<td>C18/φ-Ethyl</td>
<td>1.19</td>
<td>0.057</td>
<td>0.0079</td>
</tr>
<tr>
<td>φ-Hexyl/φ-Butyl</td>
<td>1.21</td>
<td>0.040</td>
<td>0.0035</td>
</tr>
<tr>
<td>φ-Hexyl/φ-Ethyl</td>
<td>1.15</td>
<td>0.030</td>
<td>0.0024</td>
</tr>
<tr>
<td>φ-Butyl/φ-Ethyl</td>
<td>1.16</td>
<td>0.034</td>
<td>0.0026</td>
</tr>
</tbody>
</table>

“perfect Dim” 2

Dim = Dimensionality
R.H.A. = Relative Hull Area
1-r² = Pearson Correlation Coefficient

Could You Observe Larger Selectivity Differences by Changing the Mobile Phase?
## Average Selectivity Differences for Peptide Pairs

### Varied Mobile Phases (Same Bonded Phase)

<table>
<thead>
<tr>
<th>Phase Combination</th>
<th>Absolute Difference</th>
<th>0.076</th>
<th>0.103</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:C18</td>
<td>φ-Hexyl:φ-Hexyl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Varied Bonded Phases (Same Mobile Phase)

<table>
<thead>
<tr>
<th>Phase Combination</th>
<th>Absolute Difference</th>
<th>0.044</th>
<th>0.072</th>
<th>0.064</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ-Hexyl:C18</td>
<td>φ-Butyl:C18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ-Hexyl:C18</td>
<td>φ-Ethyl:C18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<< Improved!
φ-Hexyl:φ-Hexyl vs φ-Butyl:C18
## Pair-wise Comparison of Mobile Phase Orthogonality

<table>
<thead>
<tr>
<th>Varied Mobile Phase (Same Bonded Phase)</th>
<th>Dim</th>
<th>R.H.A.</th>
<th>1-r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFA:AFFA (C18)</td>
<td>1.206</td>
<td>0.070</td>
<td>0.012</td>
</tr>
<tr>
<td>DFA:AFFA (φ-Hexyl)</td>
<td>1.187</td>
<td>0.068</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Dim = Dimensionality  
R.H.A. = Relative Hull Area  
1-r² = Pearson Correlation Coefficient  

Conclusions

• For identified peptides, the order of increasing selectivity difference relative to C18 was: φ-Hexyl < φ-Ethyl < φ-Butyl

• When the bonded phase was varied (same mobile phase), φ-Butyl demonstrated the greatest average selectivity difference relative to C18

• When the mobile phase was varied (same bonded phase), φ-Hexyl demonstrated the greater average selectivity difference than C18

• Models for measuring selectivity differences and for measuring orthogonality were in agreement; varied mobile phase comparison ongoing.

• A wider range of useful operating conditions (pH, temperature, etc.) could take advantage of improvements in bonded phases for HPLC and LCMS applications.
Thanks To:

Joe DeStefano      Tim Langlois
Bob Moran          Will Miles
Brian Wagner       Ron Orlando
Matt Jackson       Jason Lawhorn

Supported by NIH Grant GM116224 (Boyes).
Conditions and Procedure: Column Comparison

Trypsin Digest Sample: Reduced and alkylated trastuzumab (monoclonal antibody) was digested at 1:30 protein to enzyme for 4hrs in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by formic acid acidification and direct injection.

<table>
<thead>
<tr>
<th>Instrument: Nexera/Orbitrap Velos Pro ETD</th>
</tr>
</thead>
<tbody>
<tr>
<td>{Particle Size} µm: 2.7</td>
</tr>
<tr>
<td>{Pore Size} Å: 160</td>
</tr>
<tr>
<td>{Sample Conc.} mg/mL: 0.1mg/mL</td>
</tr>
<tr>
<td>{Temperature} °C: 60</td>
</tr>
<tr>
<td>Bonded Phases: C18, Phenyl Hexyl, Phenyl Butyl, Phenyl Ethyl</td>
</tr>
<tr>
<td>{Column Size} mm: 2.1x100mm</td>
</tr>
<tr>
<td>{Digest Injection V.} µL: 10 µL</td>
</tr>
<tr>
<td>{Flow Rate} mL/min: 0.3</td>
</tr>
</tbody>
</table>

| Mobile Phase 1 (A): 10mM Difluoroacetic Acid (DFA) |
| Mobile Phase 1 (B): 10mM DFA in ACN |
| MP1 Gradient: 2-50% B |
| Mobile Phase 2 (A): 10mM Ammonium Formate (AF)/10mM Formic Acid (FA) |
| Mobile Phase 2 (B): 10mM Ammonium Formate (AF)/10mM Formic Acid (FA) in 90% ACN |
| MP2 Gradient: 2.2-56% B |
| {Gradient Time} min: 60 |
| % ACN/min: 0.8 |
| MS Scan: 300-2000 m/z |
| ESI Source: 3.5 kV |
| {Sampling Rate} Hz: 10 |
| {Response} s: 0.1 |
| Wavelength: 220 nm |
Global Comparison: Identified Peptides

Extracted Ion Current

- **C18**
- **Phenyl Hexyl**
- **Phenyl Butyl**
- **Phenyl Ethyl**

**10mM DFA**

**High Confidence Peptide Spectral Match (above)**

MS^2 used for identification of peptides

Compared ions that were identified using extracted ion current