Affecting Reversed-Phase/MS Peptide Separations on High Performance Silica particles

Hillel K. Brandes, Craig R. Aurand, Richard A Henry, Dave S. Bell, and Wayne K. Way
Supelco, Div. of Sigma-Aldrich, Bellefonte, PA 16823 USA

sigma-aldrich.com
LC-MS Separation of Basic Peptides

Background

TFA (0.1% v/v) is typically used for RPC of peptides in conjunction with UV detection.
- Forms effective ion pair with basic moieties on the peptide.
- Keeps pH well below pKₐ of side-chain carboxyls to maximize retention.

TFA alone, is not suitable for RPC of peptides when MS detection employed.
- Surface tension of 0.1% (v/v) solutions precludes efficient nebulization in ESI source (1).
- TFA ions in gas phase form strong ion-pair with basic moieties on peptide, masking charge (1).
- Need to use alternative ion-pairing agent, that is MS-compatible.
- Formic acid is the usual selection; acetic acid less common.

Problem: generally much poorer peak shape and lower peak capacity with formic (or acetic) acid than when TFA is used, yet it remains the default substitute for RP LC-MS chromatography of peptides.
Reversed Phase Chromatography of Polypeptides: Hydrophobic Retention and Molecular Volume

A new Ascentis® Express particle with larger pores was needed for samples with larger solutes.

Fused-Core properties:

- 2.7 µm pure silica particle
- 1.7 µm solid core
- 0.5 µm porous shell
- 90 Å pores (2007)
- 160 Å pores (2010)
- Extremely tight particle size distribution
- Retains 75% of the sample capacity of porous particle (based on surface area)
## Properties of Ascentis® Express Peptide ES-C18

### Specifications

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>High Purity Type B</td>
</tr>
<tr>
<td>Phase</td>
<td>Sterically protected C18</td>
</tr>
<tr>
<td>pH range</td>
<td>1 – 9</td>
</tr>
<tr>
<td>Temperature</td>
<td>100 °C</td>
</tr>
<tr>
<td>Average pore diameter</td>
<td>160 Å</td>
</tr>
<tr>
<td>Surface area, nitrogen</td>
<td>80 sq.m/g</td>
</tr>
<tr>
<td>Pore volume</td>
<td>0.30 mL/g</td>
</tr>
<tr>
<td>Particle density</td>
<td>1.3 g/cc</td>
</tr>
</tbody>
</table>

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*Advanced Materials Technology poster EAS2009; used with permission*
Uniform Fused-Core™ Particles

[Two images of different types of particles]
Comparative Chromatograms
with 0.1% TFA, water/acetonitrile mobile phases; gradient elution, (2.1 x 150 mm)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Name</th>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ribonuclease A</td>
<td>13.7</td>
</tr>
<tr>
<td>2</td>
<td>adrenomedullin</td>
<td>5.73</td>
</tr>
<tr>
<td>3</td>
<td>cytochrome c</td>
<td>12.2</td>
</tr>
<tr>
<td>4</td>
<td>lysozyme</td>
<td>14.3</td>
</tr>
<tr>
<td>5</td>
<td>superoxide dismutase</td>
<td>32.5</td>
</tr>
<tr>
<td>6</td>
<td>soybean trypsin inhibitor</td>
<td>20.1</td>
</tr>
<tr>
<td>7</td>
<td>carbonic anyhydrase</td>
<td>29.0</td>
</tr>
<tr>
<td>8</td>
<td>ovalbumin</td>
<td>44.3</td>
</tr>
</tbody>
</table>

- polypeptides up to 20 kDa chromatograph with good peak shape
- above 20 kDa, exclusion from pores is evident
Peptide Structure

generic structure

\[
\begin{align*}
\text{NH}_2 & \quad \text{R}_1 \\
\text{O} & \quad \text{R}_2 \\
\text{NH} & \quad \text{R}_3 \\
\text{O} & \quad \text{R}_4 \\
\text{C} & \quad \text{OH}
\end{align*}
\]

\(pK_a = 9-10\) \quad \(pK_a = \sim 2\)

\(N\)-terminus \quad \text{C-terminus}

\(R\): amino acid side chain; neutral, polar, nonpolar, basic, or acidic

acetylated \(N\)-terminus

\[
\begin{align*}
\text{O} & \quad \text{R}_1 \\
\text{CH}_3 & \quad \text{R}_2 \\
\text{O} & \quad \text{R}_3 \\
\text{O} & \quad \text{R}_4 \\
\text{C} & \quad \text{OH}
\end{align*}
\]

neutral

\(pK_a = 10.8\) \quad \(pK_a = 12.5\)

tryptic peptide: \(C\)-terminal residue is lysine or arginine

\[
\begin{align*}
\text{NH}_2 \\
\text{HN} & \quad \text{NH}_2
\end{align*}
\]

Lysine side chain \quad Arginine side chain \quad Histidine side chain
Experimental Strategy

Compare and contrast column performance as a function of specific mobile phase modifications.

Utilize peptide probes of varying basicity and hydrophobicity as probes:

1. ac-GGGLGGAGGLKG  
   monoisotopic mass: 941.5
2. ac-KYGLGGAGGLKG  
   monoisotopic mass: 1118.6
3. ac-GGAVKALKGLKG  
   monoisotopic mass: 1139.7
4. ac-KYALKALKGLKG  
   monoisotopic mass: 1330.8

Peptide probes shown in order of increasing basicity and hydrophobicity.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Name</th>
<th>Side-chain Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>glycine</td>
<td>neutral</td>
</tr>
<tr>
<td>A</td>
<td>alanine</td>
<td>neutral, hydrophobic</td>
</tr>
<tr>
<td>L</td>
<td>leucine</td>
<td>neutral, very hydrophobic</td>
</tr>
<tr>
<td>V</td>
<td>valine</td>
<td>neutral, very hydrophobic</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
<td>neutral, polar, hydrophobic</td>
</tr>
<tr>
<td>K</td>
<td>lysine</td>
<td>basic, hydrophilic</td>
</tr>
</tbody>
</table>
Chromatographic Conditions for Basic Peptides

Columns are run under equivalent conditions of gradient slope (Δ %MeCN per column volume).

- **column**: 2(2.1) x 150 mm; C18
- **mobile phase A**: 0.1 % (v/v) additive
- **mobile phase B**: 25:75, (0.4 % additive) : acetonitrile
- **gradient**: initial = 15% B, slope = 2% MeCN / column volume
- **flow rate**: 0.3 mL/min
- **temp.**: 35 °C
- **det.**: UV 210 nm OR ESI(+) TOF
- **injection**: 1 µL
- **sample**: basic peptide mix (shown previously)
  - 5 mg/L peptide 1 & 3, 1 mg/L peptide 2, 15 mg/L peptide 4

**additive**: TFA, pH 2.0 @ 0.1% (pH unadjusted) OR
formic acid, pH 2.6 @ 0.1% (pH unadjusted) OR
formic acid, pH 3.5 (pH adjusted with ammonium hydroxide)
Column Performance: Formic acid vs TFA

David McCalley has published a series of peer-reviewed journal articles in which he offers a mechanistic explanation as to why peak shape and peak capacity of peptides are so negatively impacted by the use of formic acid as the additive, compared to TFA [2 –5]. This is outlined as follows:

TFA 0.1% (v/v) aqueous solution (~ 13 mM)
  pH = 2.0
  98% ionized (pKa = 0.3)
  TFA anion, ~ 12.7 mM

Formic acid, 0.1% (v/v) aqueous solution (~ 26 mM)
  pH = 2.6
  7% ionized (pKa = 3.7)
  Formate anion, ~ 1.8 mM

Formic acid (0.1% v/v) titrated to pH 3.5
  36% ionized
  Formate anion, ~ 9.4 mM (>5x higher than at pH 2.6)
  Peak shape much improved
Basic peptides: typical chromatograms

TFA, UV
- greater retention
- better peak shape

Formic acid, ESI(+)
- less retention
- poor peak shape for most basic peptides
Basic Peptides: MS Sensitivity

Ascentis Express Peptide ES-C18

0.1% formic acid (pH 2.6)

0.1% formic acid (pH 3.5)

0.01% TFA

0.1% TFA (pH 2)
Ascentis Express Peptide ES-C18, 160 Å, 2.7 µ

chromatograms, equally scaled

pH 2.6

![Chromatogram pH 2.6](image)

pH 3.5

![Chromatogram pH 3.5](image)
Competitor Z C18, 300 Å, 3.5 µ

pH 2.6

pH 3.5
Competitor W C18, 130 Å, 1.7 μ

pH 2.6

pH 3.5
Discussion

The results from these comparisons of the basic peptides with 0.1% formic acid, pH 2.6 versus pH 3.5 (ammonium hydroxide as titrant) demonstrate a general phenomenon of improved peak shape at the higher pH. But why?

As indicated previously, one possibility is simply the availability of formate as a counter-anion to ion-pair with analyte basic moieties to reduce charge-charge repulsion.

Another possibility is mitigation of ionic interaction with silanols. In the case of pH 3.5, a counter-cation is introduced that may mitigate ionic interactions. Therefore experiment should be repeated, with ammonium formate, pH 2.6.

– In titrating formic acid to pH 3.5, about 8 mM NH$_4^+$ cation was introduced.
– Prepare 8 mM ammonium formate, and titrate down to pH 2.6.
– With ammonium formate pH 2.6 (formic acid as titrant), do we see a similar affect on peak shape as with formic acid pH 3.5 (ammonium hydroxide as titrant)?
Ascentis Express Peptide ES-C18

0.1% formic acid, pH 2.6

8 mM ammonium formate, pH 2.6

0.1% formic acid, pH 3.5
Summary of pH study

TFA remains an ion-pair reagent of choice for reversed-phase peptide chromatography coupled to UV detection.

For MS-compatible conditions, the two previous examples demonstrate the similar results between the two conditions in which the ammonium cation concentration is the same. The formate anion concentration is quite different in the two conditions.

If particularly basic peptides are to be chromatographed, probably the best case is to apply the pH 3.5 condition in which both cation and anion counterions are present which may each function by different mechanisms to improve peak shape.

   a suggested buffer may be 10 mM ammonium formate, pH 3.5.

These results as well as variability in results with 0.1% formic acid (pH 2.6, unadjusted) depending on the specific column, would suggest further investigations into sorption isotherms of the basic peptides that may reveal different classes of sites on the silica surfaces – both on a single column as well as between columns.

For typical LC-MS of tryptic digests, 0.1% formic acid (pH 2.6, unadjusted) remains a generally recommended protocol.
Complex Tryptic Peptide Mixes: Investigation of Peak Capacity

Peak capacity is a measure of how many peaks can fit into a chromatogram and is thus a measure of separation power.

Experimental Setup

- **columns**: 2.1 x 150 mm; C18
- **mobile phase A**: 0.1 % formic acid
- **mobile phase B**: 25:75, (0.4 % formic acid) : acetonitrile
- **gradient**: as indicated (in terms of column volumes)
- **flow rate**: as indicated
- **temp**: 35 °C
- **det**: ESI(+) TOF
- **injection**: 2 µL
- **sample**: mixture of several tryptic digests; 10 pmol / µL
Tryptic Digests: MS Sensitivity

Ascentis Express Peptide ES-C18

0.1% formic acid (pH 2.6)

0.1% formic acid (pH 3.5)

0.01% TFA

0.1% TFA (pH 2)
Ascentis Express Peptide ES-C18 vs standard column (*expanded scale*)

Ascentis Express Peptide ES-C18
- 0.3 mL/min
- 246 bar (3570 psi) initial

Gradient

<table>
<thead>
<tr>
<th>CV</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>46</td>
<td>65</td>
</tr>
</tbody>
</table>

C18, 5µ
- 0.3 mL/min
- 102 bar (1480 psi) initial

46 column volumes
Peak Capacity

\[ P_c \text{ (gradient)} = \text{gradient time} / \text{average peak width} \]

About 20 peaks from across the chromatogram were extracted from the MS data to serve as a sampling. Peak widths (baseline) were compiled for this sampling of peptides.

<table>
<thead>
<tr>
<th>Column</th>
<th>(t_g^*)</th>
<th>(w_{ave})</th>
<th>(P_c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascentis Express Peptide ES-C18</td>
<td>41.3</td>
<td>0.1213</td>
<td>340</td>
</tr>
<tr>
<td>Standard 5µ C18</td>
<td>47.2</td>
<td>0.1951</td>
<td>242</td>
</tr>
</tbody>
</table>

* recall that the gradient volume is scaled to the same number of column volumes.

\(t_g\) and \(w\) are units of minutes.

Average peak width of Ascentis Express Peptide is 40% less than that of a standard 5µ C18.
Ascentis Express Also Suited for Proteolytic Digests

BSA tryptic digest
LC-MS TIC
4.6 x 150 mm Ascentis Express C18 (90Å)
Flow split to TOF-MS

Luigi Mondello, et. al., Department of Pharmaceutical Chemistry, University of Messina, Messina, Italy
Surface Deactivation Can Improve Chromatography

New column; basic peptide probes, 0.1% formic acid, pH 2.6

After chromatographing single injection of tryptic digests: basic peptide probes, 0.1% formic acid, pH 2.6
Conclusions

When weaker and less hydrophobic acids than TFA are used, basic peptides can show evidence of nonspecific retention by different modes (tailing).

Improved peak shape of basic peptides with formic acid is achieved in the presence of a counter cation; an ammonium formate buffer, pH 3.5, may also have advantages in making formate anion available for ion pairing with basic peptide moieties.

Utilizing an ammonium formate buffer at pH 3.5 has application with basic peptides, such as pharmaceuticals or proteolytic digests other than with trypsin (multiple basic residues per peptide).

Peak shape of basic peptides is highly variable with dilute solutions of formic acid when comparing one column to another. Further studies will attempt to correlate this with differences in surface activity and perhaps subclasses thereof.

Surface deactivation is not an uncommon phenomenon with modern silica-based particles and reversed-phase chromatography of polypeptides. Consider a column pretreatment protocol.

Ascentis Express Peptide ES-C18 is a high-performance platform and suitable for separations of polypeptide up to a molecular weight of 20,000.
References


Acknowledgements

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