High Resolution Polypeptide Separations on Ascentis Express Peptide ES-C18

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Outline

• Introduction and Background to Fused-Core® Particles
• Polypeptides and Pore Size
• Strategy for LC-MS of Basic Peptides
• Conclusions
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<sup>a</sup>

### Specifications

<table>
<thead>
<tr>
<th>Property</th>
<th>Specification</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>

<sup>a</sup>Advanced Materials Technology poster EAS2009; used with permission

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Uniform Fused-Core Particles
Narrow Particle Distribution

- Standard Fused-Core, 2.82 µm (SD 0.14 µm)
- Larger Pore Fused-Core, 2.85 µm (SD 0.14 µm)

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Comparison of Pore-Size Distribution

- Standard Fused-Core
- Fused-Core Peptide

90 Å
160 Å
Comparative Chromatograms
with 0.1% TFA, water/acetonitrile mobile phases; gradient elution

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

<table>
<thead>
<tr>
<th>Peak</th>
<th>Name</th>
<th>kDa</th>
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<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 anyhdrase</td>
<td>29.0</td>
</tr>
<tr>
<td>8</td>
<td>ovalbumin</td>
<td>44.3</td>
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</table>
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 $pK_a$ 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.
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.
Basic peptides: typical chromatograms

TFA, UV
- greater retention
- better peak shape

Formic acid, ESI(+)
- less retention
- poor peak shape for most basic peptides
Chromatographic Conditions for Basic Peptides

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

- **column**: 2(2.1) x 100 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**: 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)

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Column Performance: Formic acid vs TFA

David McCalley has published a series of peer-reviewed journal articles in which he details his line of investigation to uncover the 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

If, however, formic acid (0.1% v/v) is titrated to pH 3.5
- 36% ionized
- Formate anion, ~ 9.4 mM (>5x higher than at pH 2.6)
- Peak shape much improved

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Ascentis Express Peptide ES-C18, 160 Å, 2.7µm

chromatograms, equally scaled

pH 2.6

![Chromatogram pH 2.6]

pH 3.5

![Chromatogram pH 3.5]
Competitor Z C18, 300 Å, 3.5µm

pH 2.6

pH 3.5

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Competitor V C18, 120 Å, 1.5µm

pH 2.6

pH 3.5

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Competitor W C18, 130 Å, 1.7µm

pH 2.6

pH 3.5

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Some Quantitative Comparisons

<table>
<thead>
<tr>
<th>Column</th>
<th>Peak 1 (monobasic)</th>
<th>Peak 2 (dibasic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$w_{1/2}$</td>
<td>$h/w_{1/2}$</td>
</tr>
<tr>
<td>Ascentis Express Peptide ES-C18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 2.6</td>
<td>0.0258</td>
<td>2981</td>
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<tr>
<td>pH 3.5</td>
<td>0.0258</td>
<td>9725</td>
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<tr>
<td>Competitor Z C18, 3.5 µ</td>
<td></td>
<td></td>
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<tr>
<td>pH 2.6</td>
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<td>2267</td>
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<td>pH 3.5</td>
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<tr>
<td>Competitor V C18, 1.5 µ</td>
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<td>1493</td>
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<tr>
<td>pH 3.5</td>
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<tr>
<td>Competitor W C18, 1.7 µ</td>
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<td>pH 2.6</td>
<td>0.0198</td>
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<td>pH 3.5</td>
<td>0.0258</td>
<td>7568</td>
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</table>

- some cases of dramatic improvement in peak width and/or sharpness

* $w_{1/2}$ peak width at half height (min)
* $h/w_{1/2}$ peak height divided by width at half height; a measure of peak sharpness
* $TF_{0.1}$ tailing factor at 10% peak height

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Some Quantitative Comparisons

<table>
<thead>
<tr>
<th>Column</th>
<th>Peak 3</th>
<th>Peak 4</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$w_{\frac{1}{2}}$</td>
<td>$h/w_{\frac{1}{2}}$</td>
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<tr>
<td>Ascentis Express Peptide ES-C18</td>
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<tr>
<td>pH 2.6</td>
<td>0.0688</td>
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<td>pH 3.5</td>
<td>0.0344</td>
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<td>Competitor Z C18, 3.5 µ</td>
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<td>pH 2.6</td>
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<td>pH 3.5</td>
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<tr>
<td>Competitor V C18, 1.5 µ</td>
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<tr>
<td>pH 2.6</td>
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<td>pH 3.5</td>
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<td>Competitor W C18, 1.7 µ</td>
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<tr>
<td>pH 2.6</td>
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<tr>
<td>pH 3.5</td>
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</table>

- overall dramatic improvement in peak width, sharpness and tailing factor

* $w_{\frac{1}{2}}$ peak width at half height (min)
* $h/w_{\frac{1}{2}}$ peak height divided by width at half height; a measure of peak sharpness
* $T_{F0.1}$ tailing factor at 10% peak height

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pH 3.5 Peak Widths

![Bar chart showing peak widths for different peptides and columns at pH 3.5.](chart.png)
pH 3.5 Peak Sharpness

![Graph showing peak sharpness comparison between Asc. Express Peptide, Competitor Z, Competitor V, and Competitor W at pH 3.5. The x-axis represents peptide column and competitor, while the y-axis shows the H/w (peak height / width at half-height) values.]
pH 3.5 Peak Symmetry

Tailing Factor (10% peak height)

peptide column: Asc. Express Peptide Competitor z Competitor V Competitor W

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Results from Formic Acid pH Adjustment with Basic Peptides

The raised pH (3.5 vs 2.6) yields improvement in peak shape of basic peptides; the greater the basicity (high pI), the more dramatic the improvement.

This is consistent with McCalley’s explanation of the poor performance with formic acid at pH 2.6 being due to lack of ion-pairing between the formate anion and basic moieties of the peptide.

Compared to other high performance silica columns, Ascentis Express Peptide generally displays narrower, sharper peaks, without suffering from adverse peak tailing.

Increasing the pH further to 4.0, doesn’t result in improvement in peak shape (though the formic acid is 64% ionized), but can significantly affect selectivity (data not shown).
Conclusions

Improved peak shape of basic peptides with formic acid at pH 3.5 (vs formic acid with pH unadjusted – pH 2.6) fits with McCalley’s hypothesis of formate anion functioning to mitigate ionic repulsion via ion pairing with basic moieties of the peptides.

Ion-pairing of formate anion with peptide basic moieties may also mitigate ion-exchange interaction with silanols.

The pH 3.5 mobile phase is suggested for pharmaceutical peptides.

Further studies will attempt to:

- Elucidate the mechanism that permits better peak shape at pH 3.5 vs 2.6
- Differentiate the impact of higher pH on the peptide chromatography vs MS source effects.

Ascentis Express Peptide ES-C18 is a high-performance platform for peptide separations, up to 20 kDa.
References