Protein/Peptide Separation: 
A Comparison of Stability and Selectivity 
of C5 and C4 Bonded Phases 

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Introduction

Short-chain alkyl bonded phases, such as C3 and C4, are preferred for reversed-phase HPLC separations of large biomolecules, such as proteins, or very hydrophobic peptides. However, they are not stable at low pH due to the hydrolysis of the siloxane bond of the bonded phases.

In this presentation, we compare a newly developed C5 phase with leading commercial C4 phases in terms of stability, selectivity and resolution of proteins/peptides. The results show that this C5 phase exhibits similar selectivity to C4 phases, but with higher stability and better resolution.
**Discovery BIO Wide Pore C5 Columns**

**Silica:**
- 3 μm, 5 μm, 10 μm spherical particles
- 300 Å pore size
- 1 mL/g pore volume
- Minimal metal content
  (Al, Fe, Ti, Zr < 10 ppm ea.)

**Bonded phase:**
- Dimeric C5 bonding
- 3.5% carbon content
- Endcapped
- High reproducibility
## Protein Recovery on Discovery BIO Wide Pore C5

<table>
<thead>
<tr>
<th>Protein</th>
<th>M.W.</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>14,400</td>
<td>97.7%</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,800</td>
<td>98.5%</td>
</tr>
<tr>
<td>Insulin (bovine)</td>
<td>5,800</td>
<td>98.9%</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>13,700</td>
<td>98.0%</td>
</tr>
<tr>
<td>BSA</td>
<td>66,000</td>
<td>99.3%</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,400</td>
<td>97.7%</td>
</tr>
</tbody>
</table>
Reproducibility of Discovery BIO Wide Pore C5
Tryptic Digest of Cytochrome C on Different Bonding Lots

Column: Discovery BIO Wide Pore C5, 15cm x 4.6mm, 5µm;
Mobile Phase: (A) 0.07% TFA in H₂O, (B) 0.1% TFA in MeCN;
Flow Rate: 1ml/min;
Temp: 35 °C;
Detection: 220nm;
Injection: 50µL;
Sample: Cytochrome c (Bovine) Digest
Gradient: 0-35% B in 60 min
High Stability at 0.1% TFA: Discovery BIO Wide Pore C5

Mobile phase A: 0.1% TFA in 95:5 water : MeCN
Mobile phase B: 0.1% TFA in 25:75 water : MeCN
Column: 4.6 x 50 mm, 5μ
Flow: 2.0 mL/min
Temperature: 35 °C
Detection: UV 220 nm
Injection: 5 μL
Sample: HPLC Peptide Standard (Sigma Cat. No. H2016)
Gradient: 0 to 30% B in 22 min
Elution order: 1. Gly-Tyr
2. Val-Tyr-Val
3. Met enkephalin
4. Leu enkephalin
5. Angiotensin II
High Stability at pH 8.0: DiscoveryBIO Wide Pore C5

Mobile phase: 5:95 methanol : 25mM pH8.0 phosphate buffer
Column: 4.6 x 50 mm, 5μ
Flow: 2.0 mL/min
Temperature: 35 °C
Detection: UV 254 nm

Injection: 5 μL
Elution order: 1. Sorbic acid
2. Pyridine
3. Procainamide
4. Caffeine

Retention Time (min)

Column Volumes
Stability at 0.1% TFA: DiscoveryBIO C5 vs Vendor A C4

Mobile phase A: 0.1% TFA in 95:5 water:MeCN
Mobile phase B: 0.1% TFA in 25:75 water:MeCN
Column: 4.6 x 50 mm, 5μm
Flow: 2.0 mL/min
Temperature: 35 °C
Detection: UV 220 nm
Injection: 5 μL
Sample: HPLC Peptide Standard (Sigma Cat. No. H2016)
Gradient: 0 to 30% B in 22 min
Comparison: %Change after 560 gradients
Elution order: 1. Gly-Tyr
2. Val-Tyr-Val
3. Met enkephalin,
4. Leu enkephalin
5. Angiotensin II
Stability at 0.1% TFA: Discovery BIO C5 vs Vendor B C4

Mobile phase A: 0.1% TFA in 95:5 water : MeCN
Mobile phase B: 0.1% TFA in 25:75 water : MeCN
Column: 4.6 x 50 mm, 5 μm
Flow: 2.0 mL/min
Temperature: 35 °C
Detection: UV 220 nm
Injection: 5 μL
Sample: HPLC Peptide Standard (Sigma Cat. No. H2016)
Gradient: 0 to 30% B in 22 min
Comparison: %Change after 230 gradients
Elution order: 1. Gly-Tyr
2. Val-Tyr-Val
3. Met enkephalin
4. Leu enkephalin
5. Angiotensin II
Hydrophobicity on Short-Chain Phases

*Discovery BIO Wide Pore C5 is similar to C4, but with higher efficiency*

<table>
<thead>
<tr>
<th>Column</th>
<th>t(min)</th>
<th>t(min)</th>
<th>k'</th>
<th>USP tailing</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uracil</td>
<td>toluene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vendor A C4</td>
<td>1.761</td>
<td>5.057</td>
<td>1.87</td>
<td>0.86</td>
<td>12148</td>
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<tr>
<td>Vendor B C4</td>
<td>1.898</td>
<td>5.078</td>
<td>1.68</td>
<td>0.94</td>
<td>11719</td>
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<tr>
<td>DSC BIO WP C5</td>
<td>1.946</td>
<td>5.841</td>
<td>2.00</td>
<td>1.06</td>
<td>14531</td>
</tr>
</tbody>
</table>

Mobile phase: 45/55 Methanol/Water
Column: 4.6 x 150 mm, 5µm, 300Å
Flow: 1.0 mL/min
Temperature: 35 °C
Detection: UV 254 nm
Injection: 10 µL of testing mix
Elution order: uracil, acetophenone, benzene, toluene
Less Silanol Activity /Better Peak Shape on Discovery BIO Wide Pore C5

Mobile phase: 15:85 Methanol: 10mM pH6.8 acetate buffer
Column: 4.6 x 150 mm, 5µm
Flow: 1.0 mL/min
Temperature: 35°C
Detection: UV 254 nm
Injection: 5 µL
Elution order: 1. Uracil
2. Procainamide
3. Sorbic acid
4. Pyridine
5. Caffeine
6. Phenol
Similar Selectivity between Discovery BIO Wide Pore C5 and C4 Phases

Separation of Sigma Peptide Mix

Mobile phase A: 80:20 (water/0.1%TFA): (MeCN/0.1%TFA)
Mobile phase B: 66:34 (water/0.1%TFA): (MeCN/0.1%TFA)
Column: 4.6 x 150 mm, 5μm
Flow: 1.0 mL/min
Temperature: 30°C
Detection: UV 220 nm
Injection: 10 μL, ~0.25μg each peptide
Sample: Sigma Peptide Mix (Cat. No. P2693)
Gradient: 0 to 100% B in 14 min after a 1 min delay
Elution order: 1. Arg⁸-Vassopressin
2. Bradykinin, fragment 1-5
3. Oxytocin
4. Met-Enkephalin
5. Luteinizing hormone releasing hormone
6. Leu-Enkephalin
7. Bradykinin
8. Bombesin
9. Substance P (RPKPQQFFGLM-amide)
Similar Selectivity between Discovery BIO Wide Pore C5 and C4 phases

Separation of Insulin Variants

Vendor A C4

Vendor B C4

Discovery BIO C5

Column: 4.6 x 150 mm, 5μm
Mobile phase A: 71:29 (water/0.1%TFA): (MeCN/0.1%TFA)
Mobile phase B: 68:32 (water/0.1%TFA): (MeCN/0.1%TFA)
Flow: 1.0mL/min
Temperature: 30°C
Detection: UV 215nm
Injection: 5μL
Sample: Insulin variants Mix
Gradient: 0 to 100% B in 30 min
Elution order: 1. Bovine
2. Human
3. Porcine
Higher Resolution on Discovery BIO Wide Pore C5

Separation of Cytochrome c from Different Sources

Vendor A C4

Vendor B C4

Discovery BIO C5

Column: 4.6 x 150 mm, 5μm
Mobile phase A: 70:30 (water/0.1%TFA): (MeCN/0.1%TFA)
Mobile phase B: 64:36 (water/0.1%TFA): (MeCN/0.1%TFA)
Flow: 1.0 mL/min
Temperature: Room temperature
Detection: UV 220 nm
Injection: 12μL
Sample: Cytochrome c (different sources), 10 μg each.
Gradient: 0 to 100% B in 30 min
Elution order: 1. Horse
2. Rabbit
3. Cow
4. Pigeon
5. Chicken
6. Dog
Better Peak Shape on Discovery BIO Wide Pore C5

Protein Separation

Column: 4.6 x 150 mm, 5μm
Mobile phase A: 75:25 (water/0.1%TFA): (MeCN/0.1%TFA)
Mobile phase B: 25:75 (water/0.1%TFA): (MeCN/0.1%TFA)
Flow: 1.0 mL/min
Temperature: Room temperature
Detection: UV 220 nm
Injection: 12 μL
Sample: Protein test mix
Gradient: 0 to 100% B in 25 min
Elution order: 1. RNAse
2. Cytochrome c
3. Lysozyme
4. BSA
5. Myoglobin
6. Ovalbumin
Summary

*Discovery BIO Wide Pore C5 Phase:*

- More stable and longer life time than C4 phases
- Similar selectivity to C4 phases
- Less non-specific interaction between analytes and the silica surface, resulting in better peak shape.
- High protein recovery
- High reproducibility