# Discovery® BIO Wide Pore

*Solutions to Protein and Peptide Separation Challenges*

# Agenda:

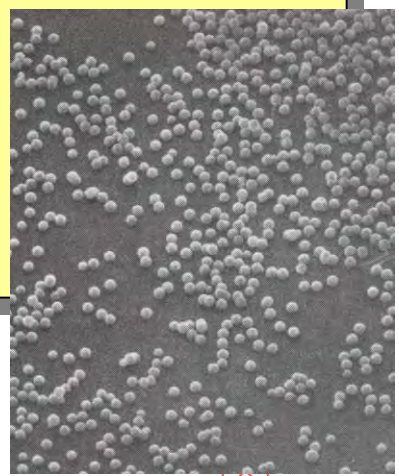
- What is Discovery BIO Wide Pore
- Physical characteristics
- Why we developed it and for whom
- Performance demonstrations
- Choosing a column

# What is Discovery BIO Wide Pore?

- Reversed-phase HPLC columns and capillaries
- 3, 5, and 10μm spherical silica particles
- 300Å pore diameter silica
- C5, C8, and C18 bonded phases
- Column IDs:  0.32mm to 21.2mm
- See handout for details on particle and bonded phase properties.

# Discovery BIO Wide Pore Silica

| | |
|---|---|
| **Shape:** | **spherical** |
| **Type:** | **B (sil-gel process)** |
| **Size:** | **3µm (2.8-3.2µm)** |
| | **5µm (4.5-5.1µm)** |
| | **10µm (9.0-11.0µm)** |
| **Distribution profile:** | **Single mode (particle and pore size)** |
| **Pore size:** | **260-340Å** |
| **Pore volume:** | **1mL/g** |
| **Surface area:** | **80-120m²/g** |
| **Metals analyzed:** | **Al, Ti, Fe, Zr** |
| **Metal content:** | **<10ppm, typically <2ppm** |



*Photomicrograph of Discovery BIO Wide Pore 5µm silica particles*

# Discovery BIO Wide Pore Phases

| | C5 | C8 | C18 |
|---|---|---|---|
| Silane: | pentyl | octyl | octadecyl |
| Endcap: | C1 | C1 | C1 |
| %C: | 3.2-3.8% | 4.8-5.3% | 9.0-9.5% |
| Coverage (µmole/m²): | 4.1-5.0 | 3.8-4.3 | 3.3-4.0 |
| Temp max: | 70°C | 70°C | 70°C |
| Pressure max (bar): | 400 | 400 | 400 |
| pH range (phosphate):* | 1 - 8 | 1 - 8 | 1 - 8 |

*using organic buffers, pH max is higher

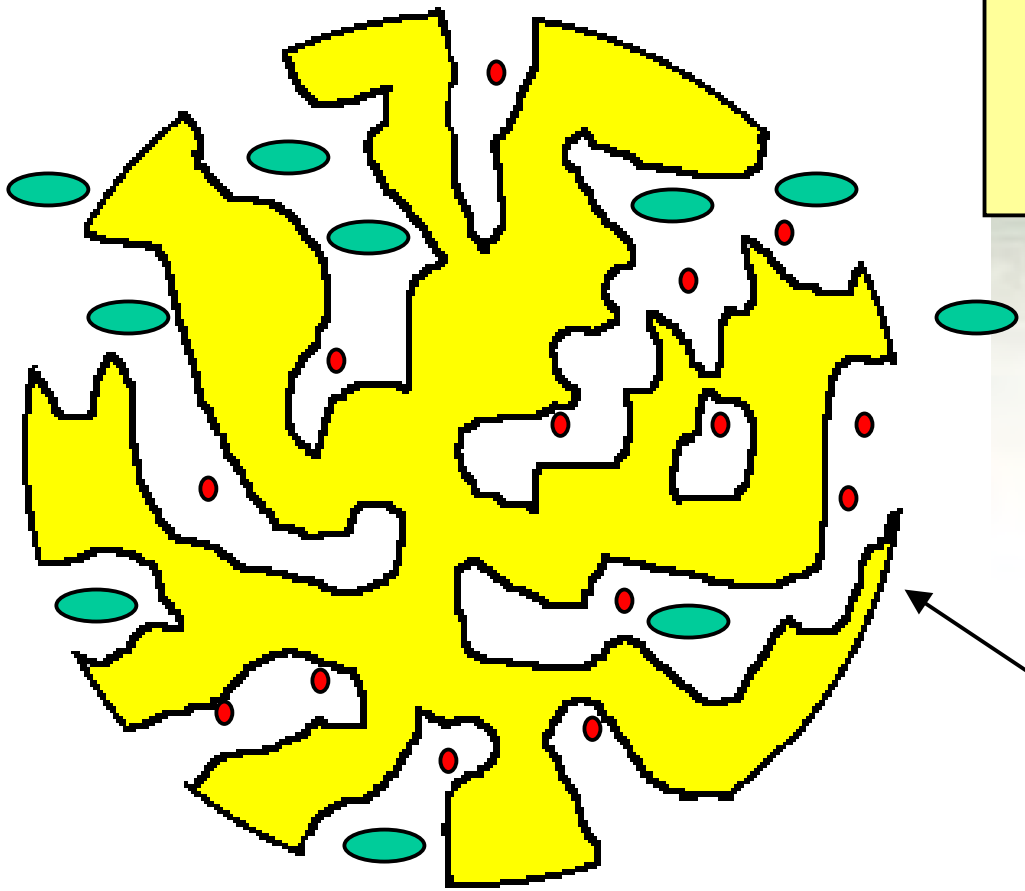
# Discovery BIO Wide Pore Dimensions

- Capillary (0.32, 0.5mm ID)*
- Microbore (1mm ID)
- Narrowbore (2.1mm ID)
- Standard Analytical (4.0, 4.6mm ID)
- Semi-Prep (10mm ID)
- Prep (21.2mm ID)*

*look for <0.32mm and >21.2mm ID

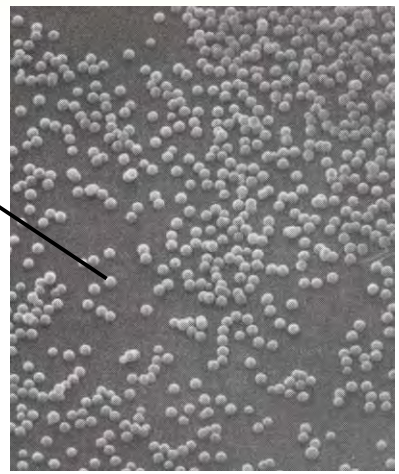
# Why "Wide Pore?"

>80% of the surface area is inside the particle - where separation occurs.



*Cross-section of Discovery BIO Wide Pore silica particle*

*Photomicrograph of Discovery BIO Wide Pore silica particles*



# What are its key features?

- Improves Resolution by providing:
  - > Choices in selectivity
  - > High efficiency
- Stable at high and low pH
- Reproducible
- Scalable from analytical to preparative
- LC-MS compatible (no-bleed, low TFA)

# For whom was it developed?

**Biochemists and researchers in proteomics or biopharmaceuticals who are:**

- **Separating native or recombinant proteins or peptides**
- **Working with synthetic peptides**
- **Using peptide maps to sequence proteins**
- **Employing LC-MS or conventional detectors**

# Why was it developed?

***To meet the challenges of protein and peptide HPLC separations.***

**What are those challenges?**

- **Complex protein and/or peptide mixtures**
- **Small sample volumes and proteins at low concentrations or low copy numbers**
- **Need for detailed characterization**
- **Maintaining the separation (trouble-free operation)**

# #1 Complex Protein and/or Peptide Mixtures

***"The selectivity and efficiency offered by Discovery BIO Wide Pore gives maximum power for resolving complex mixtures of proteins, natural or synthetic peptides, and peptide maps. Exceptional pH stability allows full use of mobile phase pH to adjust the separation."***

*Demonstrations:*

- *BIO Wide Pore C5 has greater efficiency and resolution than competitive phases.*
- *Choices in selectivity of BIO Wide Pore C5, C8, C18 phases.*
- *Harness the power of mobile phase pH to alter selectivity.*

# Competitive RP-HPLC Columns

**Waters Symmetry®300 C18**
**Vydac 214TP, 218TP, 238TP**
**Zorbax® SB300-C18**
**Phenomenex Jupiter C18**

# Demonstrating Efficiency: Proteins on C5

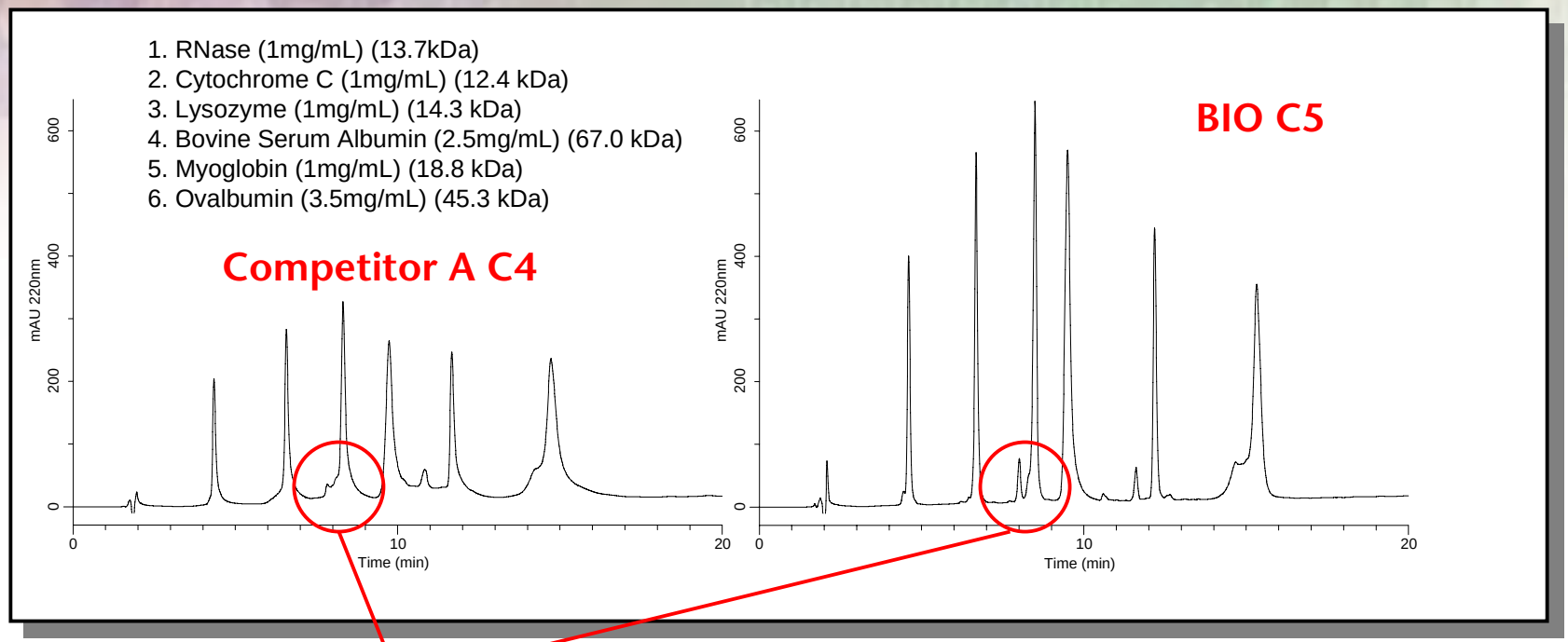
*Fig. 1 Conditions:*

**Conditions**: C4 or C5 columns, 15cm x 4.6mm, 5µm,
**Mobile Phase:** (A) 75:25, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 66:34, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 1mL/min,
**Temp**: ambient,
**Detection**: 220nm,
**Sample**: Protein mix
**Gradient**: 0-100%B in 25 mins

1. RNase (1mg/mL) (13.7kDa)
2. Cytochrome C (1mg/mL) (12.4 kDa)
3. Lysozyme (1mg/mL) (14.3 kDa)
4. Bovine Serum Albumin (2.5mg/mL) (67.0 kDa)
5. Myoglobin (1mg/mL) (18.8 kDa)
6. Ovalbumin (3.5mg/mL) (45.3 kDa)

# Demonstrating Efficiency: Proteins on C5

*BIO Wide Pore C5 has higher efficiency than popular competitive C4 phases*




Efficiency affects ability to see peaks

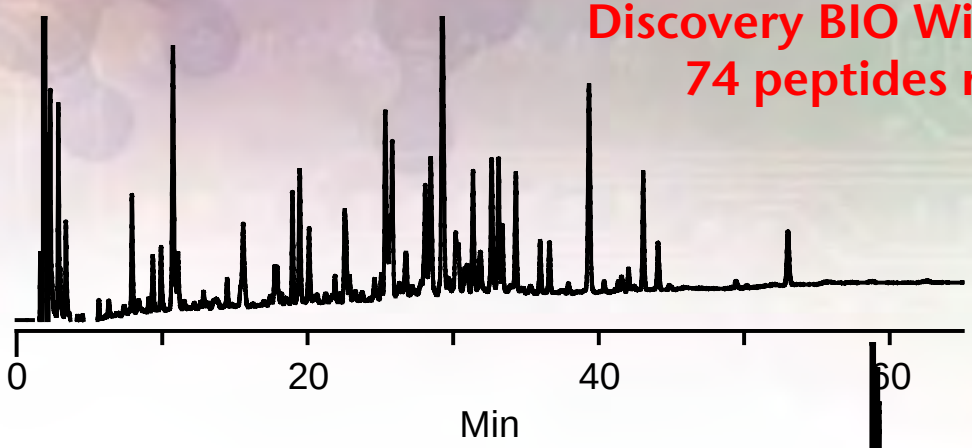
# Demonstrating Efficiency: Peptide Maps

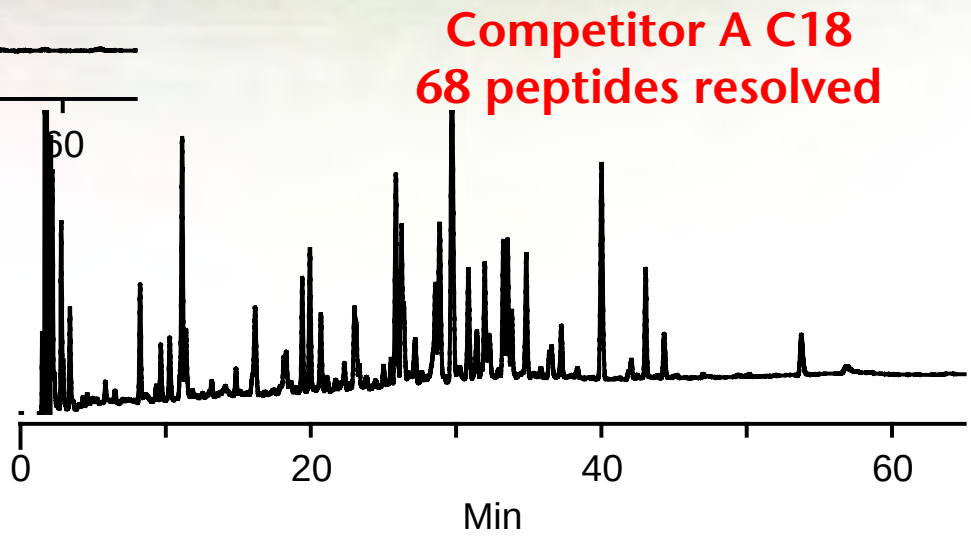
*Fig 2 Conditions BIO Wide Pore C18, peptide resolution*

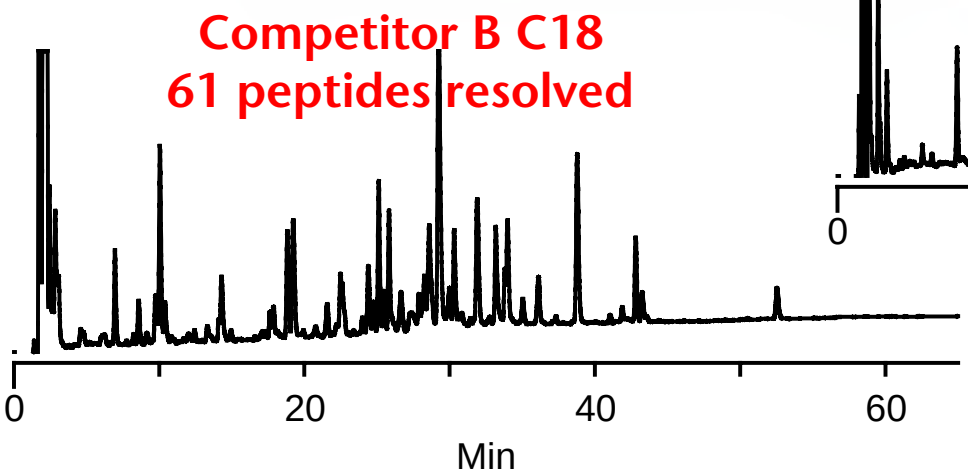
**Conditions**: C18 columns, 15cm x 4.6mm, 5µm, 300Å,
**Mobile Phase:** (A) 95:5, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 50:50, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Detection**: 215nm,
**Sample**: 50µL carboxymethylated apohemoglobin tryptic digest,
**Gradient**: 0-100%B in 65 mins

# Demonstrating Efficiency: Peptide Maps

*BIO Wide Pore C18 resolves more peptides than competitive C18 phases*



# Demonstrating Efficiency: Synthetic Peptides

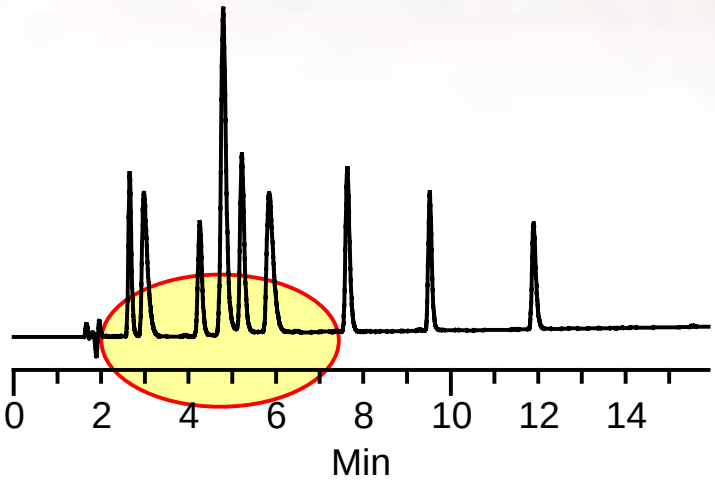
*Fig. 3 Conditions BIO Wide Pore C18 synthetic peptides resolution*

**Conditions**: C18 columns, 15cm x 4.6mm, 5µm, 300Å,
**Mobile Phase:** (A) 80:20, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 66:34, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Detection**: 220nm,
**Sample**: 10µL Sigma peptide mix (P 2693),
**Gradient**: 0-100%B in 14 mins. after 1 min. delay

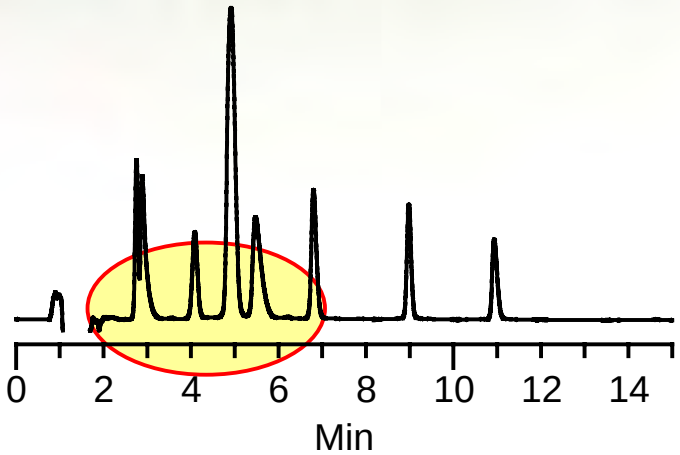
# Demonstrating Efficiency: Synthetic Peptides

*BIO Wide Pore C18 resolves these synthetic peptides better than competitive C18 phases*

**Discovery BIO Wide Pore C18**




**Competitor A C18**



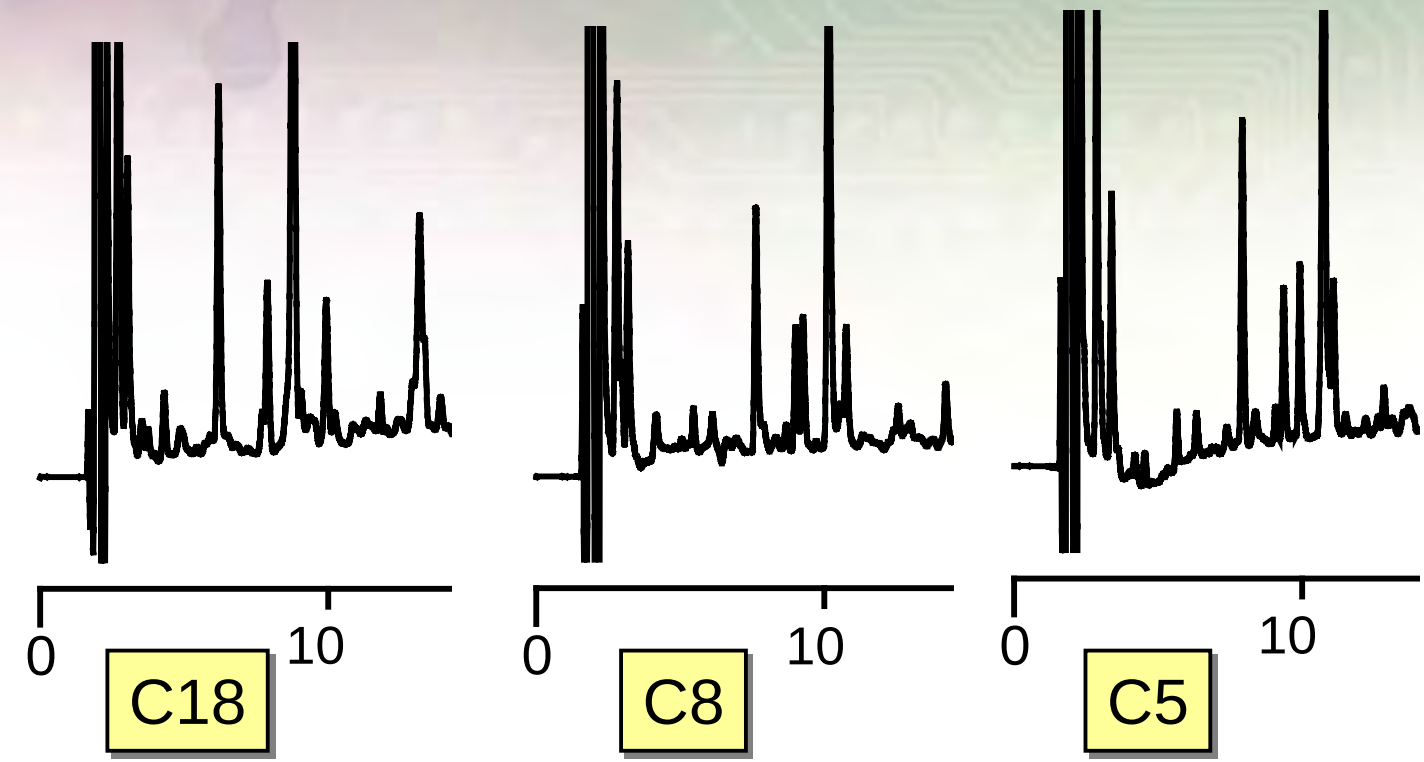
# Demonstrating Selectivity

*Fig. 4 Conditions BIO Wide Pore C5, C8, C18 selectivity*

**Conditions**: Discovery BIO Wide Pore columns, 15cm x 4.6mm, 5µm, 300Å,
**Mobile Phase:** (A) 95:5, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 50:50, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Detection**: 215nm,
**Sample**: 50µL carboxylated apohemoglobin tryptic digest,
**Gradient**: 0-100%B in 65 mins

# Demonstrating Selectivity

*BIO Wide Pore C5, C8, C18 phases offer different selectivity*



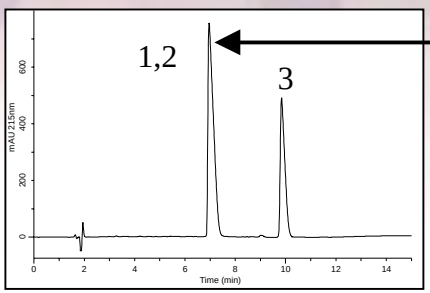
# Demonstrating Power of pH on Selectivity

*Fig. 5 Conditions Angiotensins at neutral pH*

**Conditions**: Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm, 300Å,
**Mobile Phase:** (A) 65:35, (10mM $NH_4OAc$, pH 7):(50% $CH_3CN$ in 20mM $NH_4OAc$, pH 7), (B) 25:75, (10mM $NH_4OAc$, pH 7):(50% $CH_3CN$ in 20mM $NH_4OAc$, pH 7),
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Detection**: 215nm,
**Sample**: 6µL (10 µ g) each peptide in $H_20$,
**Gradient**: 0-100%B in 12.5 mins

# Demonstrating Power of pH on Selectivity

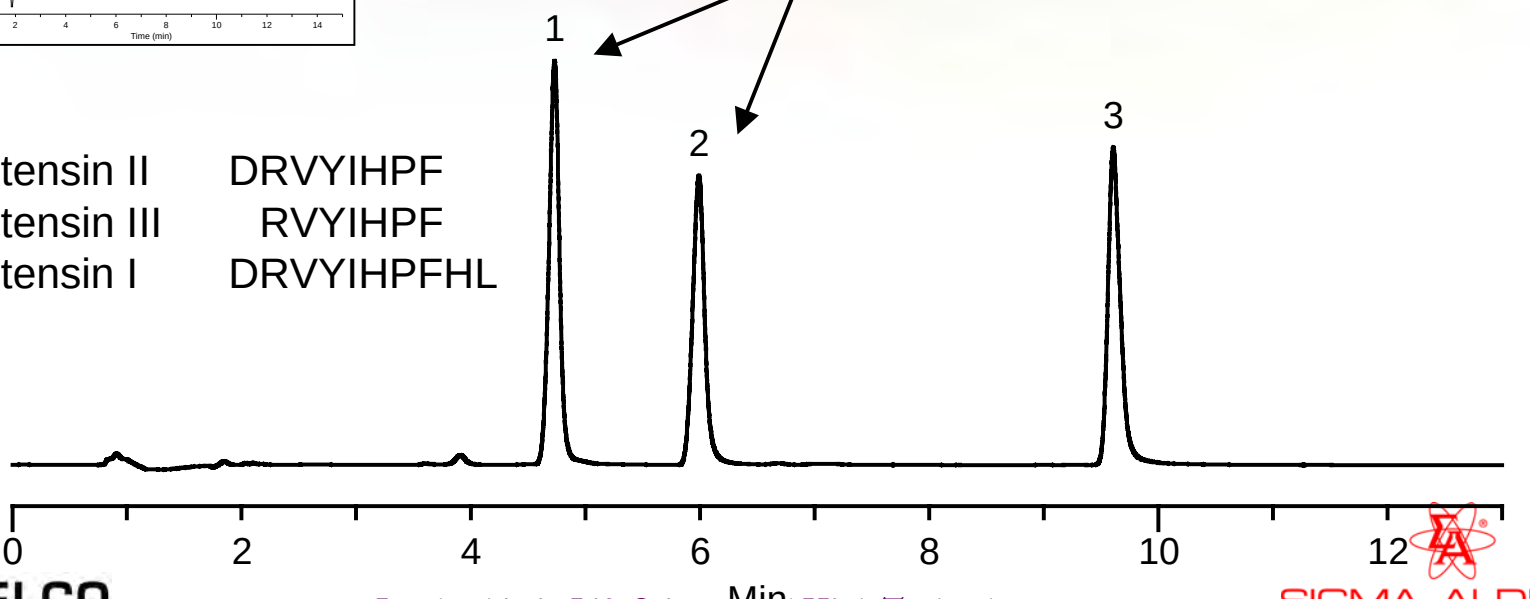
*Angiotensins resolved at neutral pH on Discovery BIO Wide Pore C18*




at pH 2, II and III are not resolved

at pH 7 they are resolved

1. Angiotensin II DRVYIHPF
2. Angiotensin III RVYIHPF
3. Angiotensin I DRVYIHPFHL



# #2 Small Sample Volumes and Proteins at Low Concentrations or Low Copy Numbers

"***The efficiency of Discovery BIO Wide Pore provides Sensitive analyses, especially when combined with capillary and microbore dimensions.***"

*Demonstrations:*

- *Higher efficiency than competitive phases.*
- *The microbore and capillary dimensions greatly enhance sensitivity, and conserve samples.*

# Demonstrating Efficiency: Proteins on C5

*Fig. 6 Conditions*

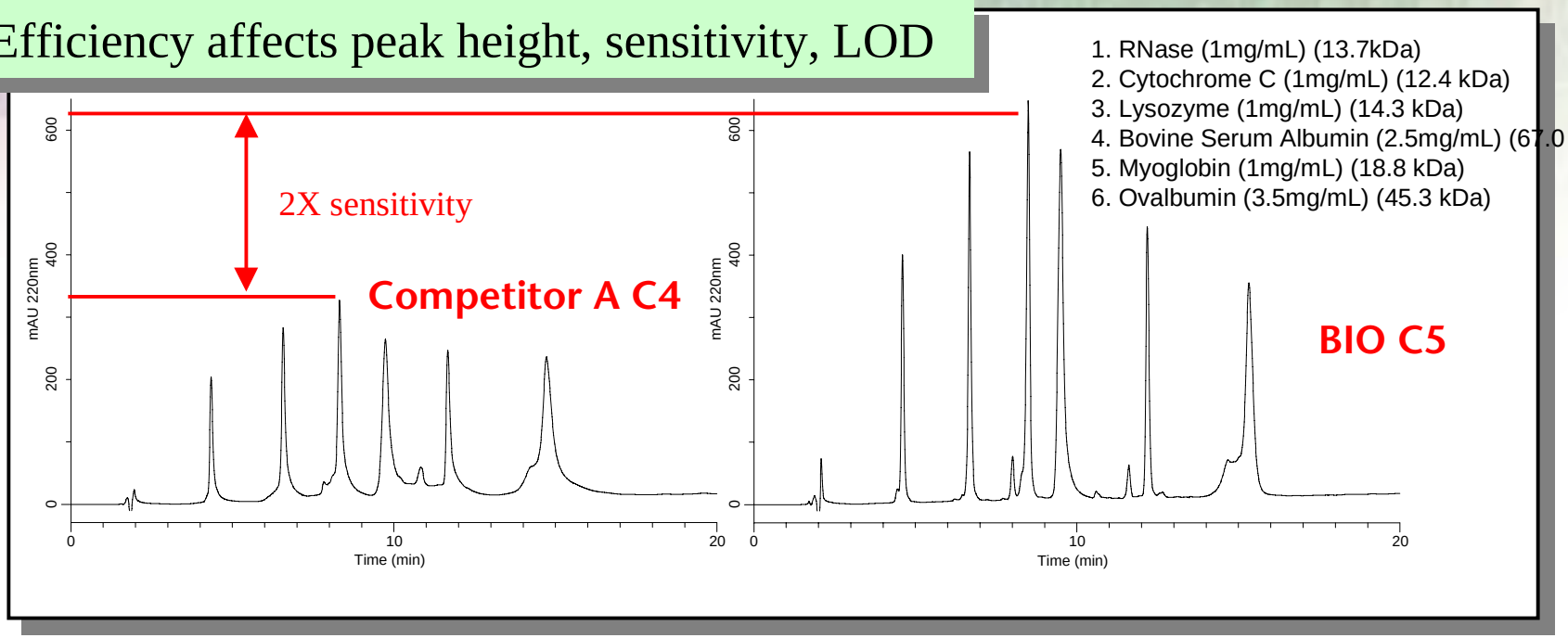
**Conditions**: C4 or C5 columns, 15cm x 4.6mm, 5µm,
**Mobile Phase:** (A) 75:25, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 66:34, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 1mL/min,
**Temp**: ambient,
**Detection**: 220nm,
**Sample**: Peptide Mix (Sigma Cat. No. P2693),
**Gradient**: 0-100%B in 25 mins

1. RNase (1mg/mL) (13.7kDa)
2. Cytochrome C (1mg/mL) (12.4 kDa)
3. Lysozyme (1mg/mL) (14.3 kDa)
4. Bovine Serum Albumin (2.5mg/mL) (67.0 kDa)
5. Myoglobin (1mg/mL) (18.8 kDa)
6. Ovalbumin (3.5mg/mL) (45.3 kDa)

# Demonstrating Efficiency: Proteins on C5

*BIO Wide Pore C5 has higher efficiency than popular competitive C4 phases*

Efficiency affects peak height, sensitivity, LOD



# Demonstrating Sensitivity: Cap/MB Dimensions

*These parameters vary with the square (or inverse square) of column radius.*

Chromatographic parameters relative to 4.6mm ID columns

| | Flow rates (volumetric) | Injection volume | Sensitivity | Mobile phase used |
|---|---|---|---|---|
| 4.6 mm ID | 1 | 1 | 1 | 1 |
| 3 mm ID | 0.42 | 0.42 | 2.4 | 0.42 |
| 2.1 mm ID | 0.21 | 0.21 | 4.8 | 0.21 |
| 1 mm ID | 0.047 | 0.047 | 21.2 | 0.047 |
| 0.5 mm ID | 0.012 | 0.012 | 84.6 | 0.012 |
| 0.32 mm ID | 0.0048 | 0.0048 | 206.6 | 0.0048 |

uses less sample

more sensitive

# #3 Need for Detailed Characterization

***"Discovery BIO Wide Pore phases are bleed-free and designed for LC-MS. Often purified sample is needed for further characterization. Discovery BIO Wide Pore phases are completely scalable from analytical to preparative for easy, reliable scale-up."***

*Demonstrations:*

- *If you use LC-MS, there is no bleed, and you can use very low levels of TFA and have good peak shape.*
- *If you need to isolate and purify proteins or peptides, analytical separations are completely scalable on Discovery BIO Wide Pore preparative columns.*

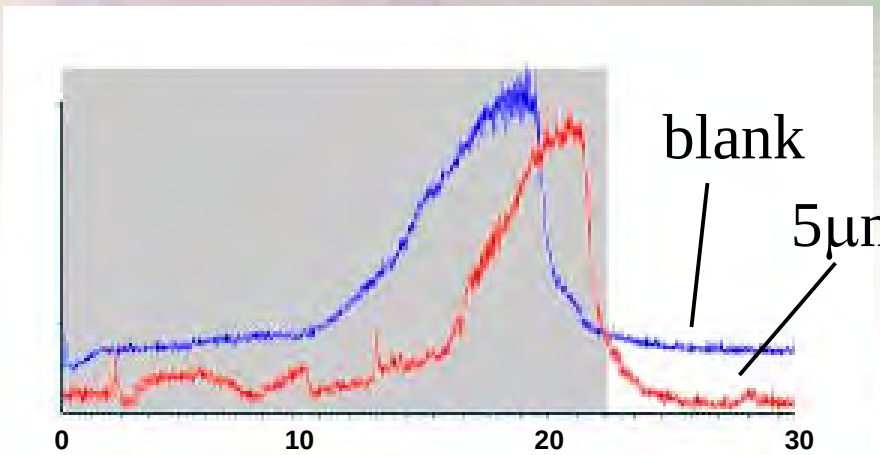
# Demonstrating Sensitivity: LC-MS Compatible

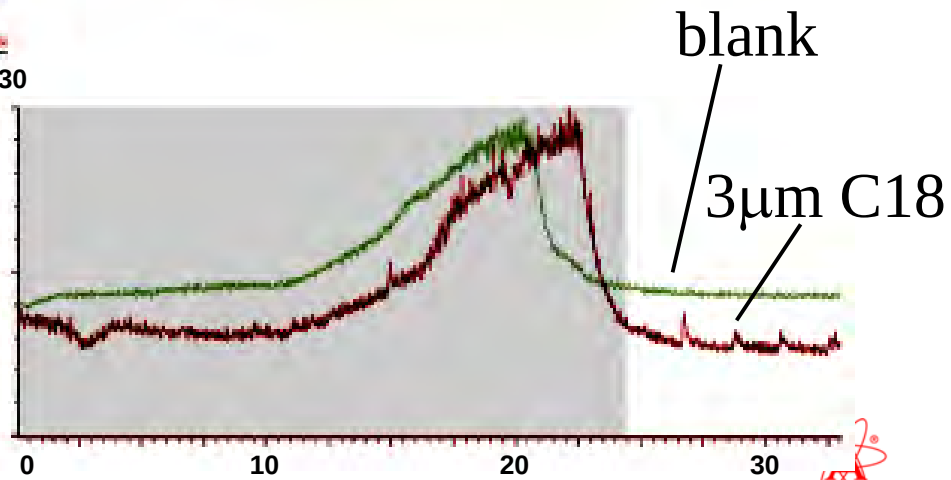
*Fig. 7 Conditions Column bleed*

**Conditions**: Columns, 15cm x 4.6mm,
**Mobile Phase:** (A) 0.1%TFA in $H_2O$, (B) 0.1% TFA in $CH_3OH$,
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Gradient**: 0-100% B in 15 mins, 100% B 5 mins, 0% B 10 mins

# Demonstrating Sensitivity: LC-MS Compatible

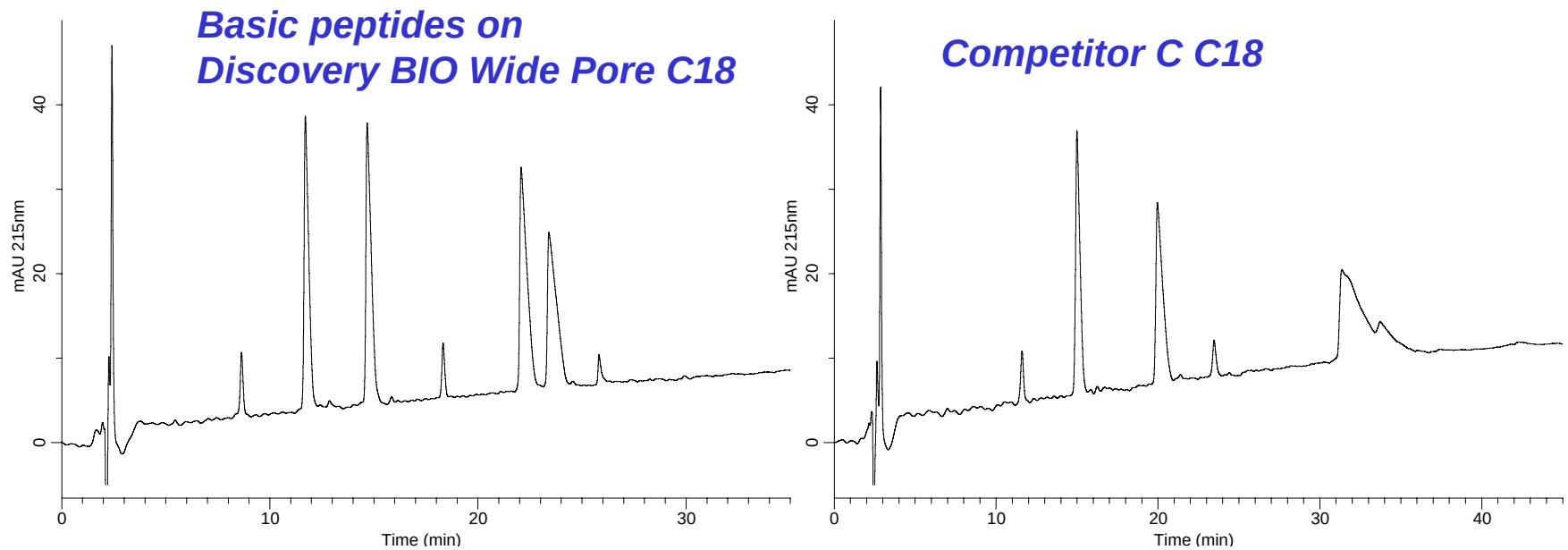
*Discovery BIO Wide Pore phases are bleed-free*



# Demonstrating Sensitivity: No TFA

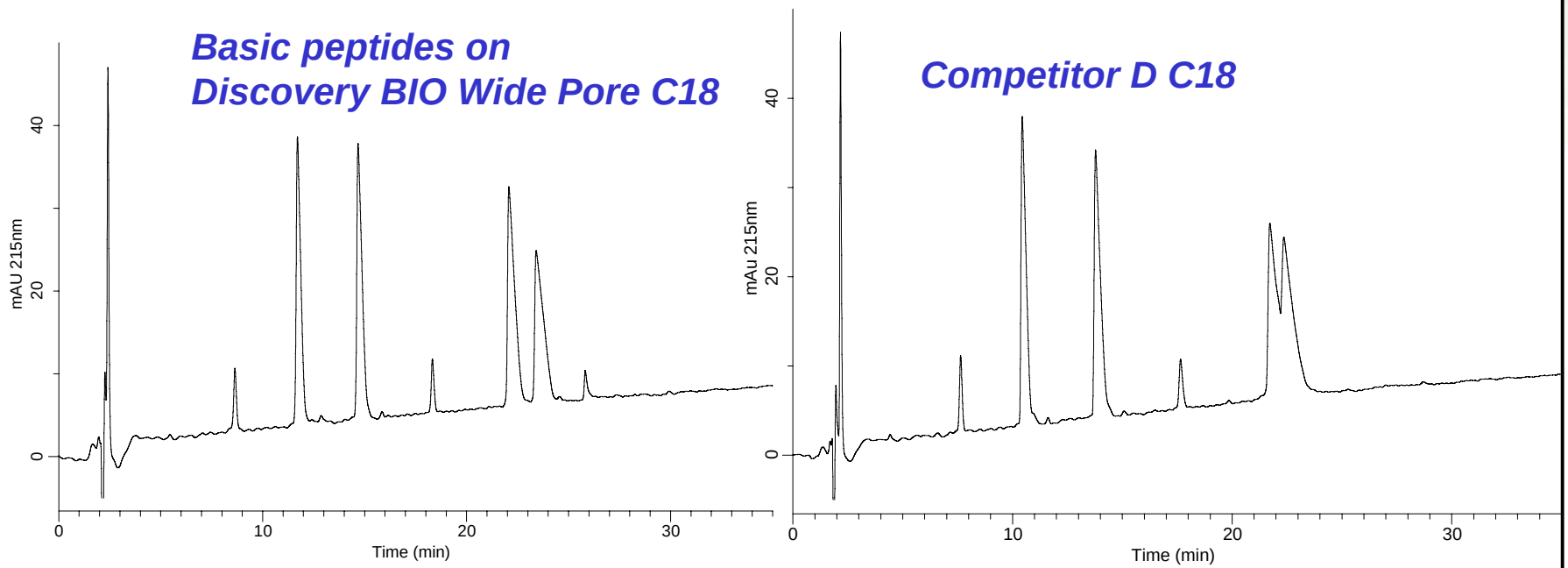
*Discovery BIO Wide Pore can be used without TFA, increasing LC-MS sensitivity.*




Columns: 15cm x 2.1 (or 2.0) mm, 5µm, Mobile Phase A: water/25mM HCO2H B: 50:50, (water/25mM HCO2H) : (MeCN/20mM HCO2H), Flow: 0.208 (or 0.189) mL/min, Gradient: 15 to 60%B in 45 min

# Demonstrating Sensitivity: No TFA

*The low surface activity of Discovery BIO Wide Pore is evident without TFA in the mobile phase, even compared to shielded phases.*




Columns: 15cm x 2.1 (or 2.0) mm, 5µm, Mobile Phase A: water/25mM $HCO_2H$ B: 50:50, (water/25mM $HCO_2H$) : (MeCN/20mM $HCO_2H$), Flow: 0.208 mL/min, Gradient: 15 to 60%B in 45 min

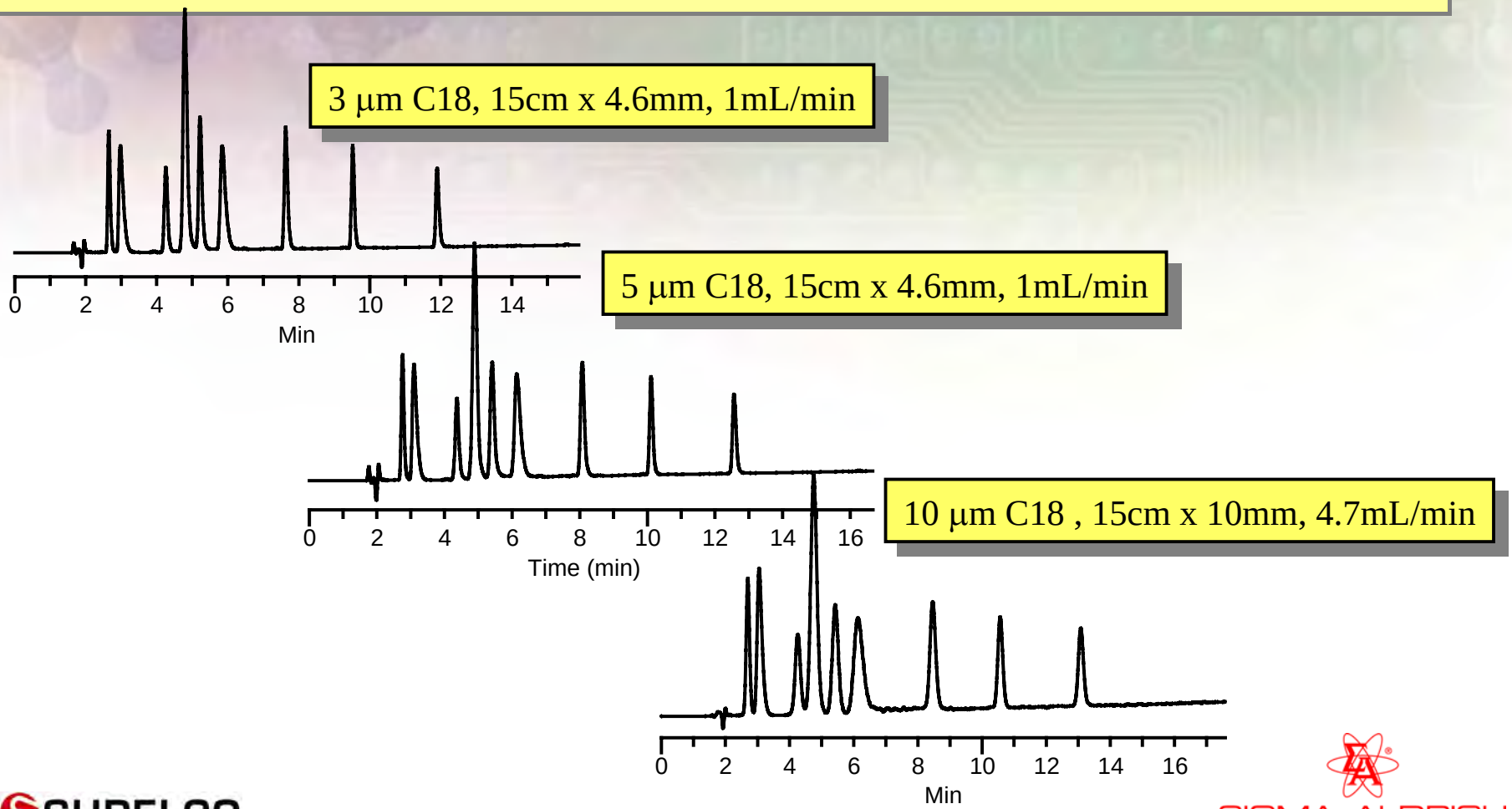
# Demonstrating Scale-Up

*Fig 10 Conditions Reproducibility of 3, 5 10um phases*

**Mobile Phase:** (A) 80:20, $H_2O$:$CH_3CN$ containing 0.1%TFA, (B) 66:34, $H_2O$:$CH_3CN$ containing 0.1%TFA,
**Flow Rate**: 6.02cm/sec,
**Temp**: 30°C,
**Detection**: 215nm,
**Sample**: Peptide Mix (Sigma Cat. No. P 2693),
**Gradient**: 0-100%B in 9 column volumes

# Demonstrating Scale-Up

*Reproducible separations on 3, 5, and 10µm Discovery BIO Wide Pore*



# #4 Maintaining the Separation (Trouble-Free Operation)

***"The stability and reproducibility of Discovery BIO Wide Pore phases permit reliable, trouble-free routine and long term operation."***

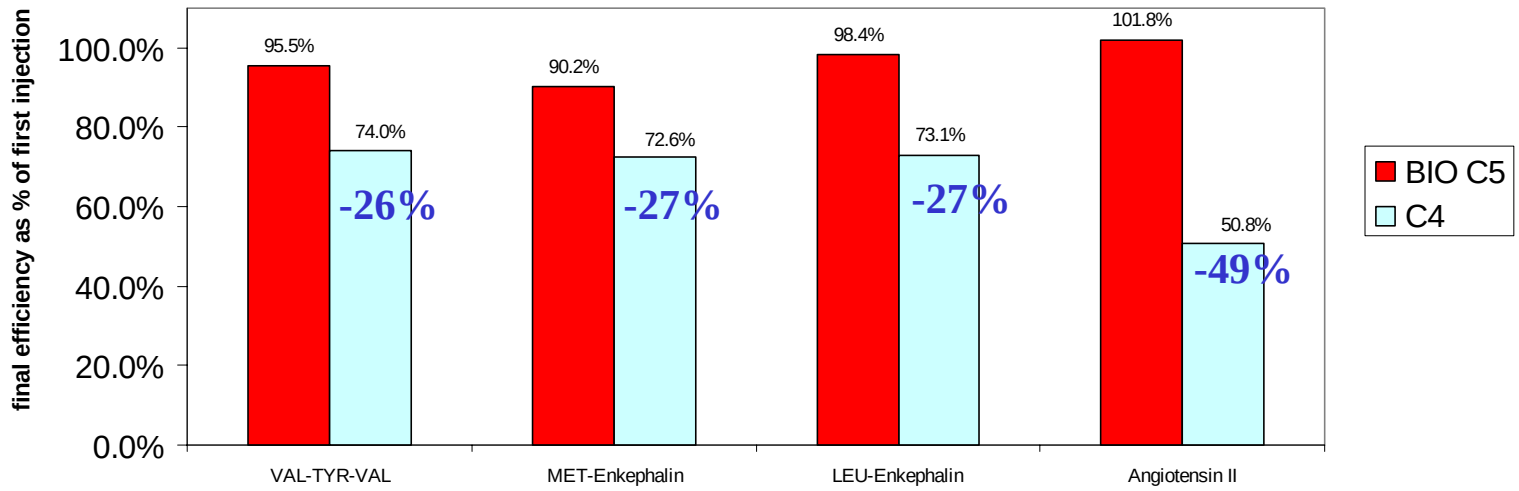
*Proof:*

- *Run-to-run reproducibility and excellent column lifetime at low and high pH are characteristics of Discovery BIO Wide Pore phases.*
- *Batch-to-batch reproducibility is a very important concern. We have designed Discovery BIO Wide Pore phases to have guaranteed reproducibility.*

# Demonstrating Stability at pH 2

*Enhanced stability at low pH of BIO C5 vs. popular C4*

*Change in efficiency after 25,000 column volumes (15L, 5 days) of 0.5% TFA in water:$CH_3CN$*



# Demonstrating Column Lifetime at pH 2

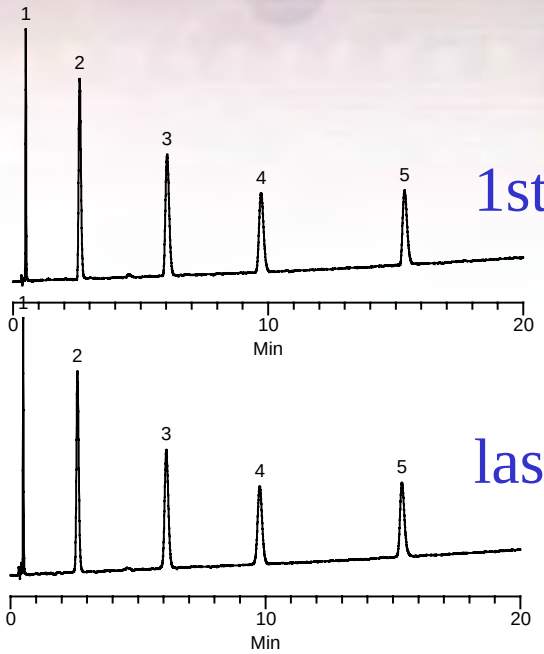
*Fig. 12 Conditions: BIO Wide Pore C5 stability at pH 2*

**Discovery BIO Wide Pore C18 Stability**
**Conditions**: Discovery BIO Wide Pore C18, 5cm x 4.6mm, 5µm
**Mobile Phase:** (A) 5:95, $H_2O$:$CH_3CN$ containing 0.5%TFA, (B) 25:75, $H_2O$:$CH_3CN$ containing 0.5%TFA
**Flow Rate**: 2mL/min
**Temp**: 70°C
**Detection**: 220nm, **Sample**: Peptide Mix (Sigma Cat. No. H 2016)
**Gradient**: 2-24%B in 22 mins, 100%A for 8 mins

**Column volume (CV) calculation:**
CV = (0.7) $\pi r^2 L$ = (0.7) $\pi(0.23)^2 5$ = 0.6mL
40,000 CV = 24,000mL
Time: (40,00mL)(1min/2mL)(1hr/60min)(1day/24hr) = 8 days

# Demonstrating Column Lifetime at pH 2

*BIO Wide Pore C18 stability at pH 2, 70°C, 40,000 column volumes (24L, 8 days)*




**No change in selectivity or peak shape on Discovery BIO Wide Pore C18 after 40,000 column volumes of 0.5% TFA at elevated temperature (70°C).**

**1. Gly-Tyr**
**2. Val-Tyr-Val**
**3. Met-Enkephalin**
**4. Lue-Enkephalin**
**5. Angiotensin II**

# Demonstrating Column Lifetime at pH 11.5

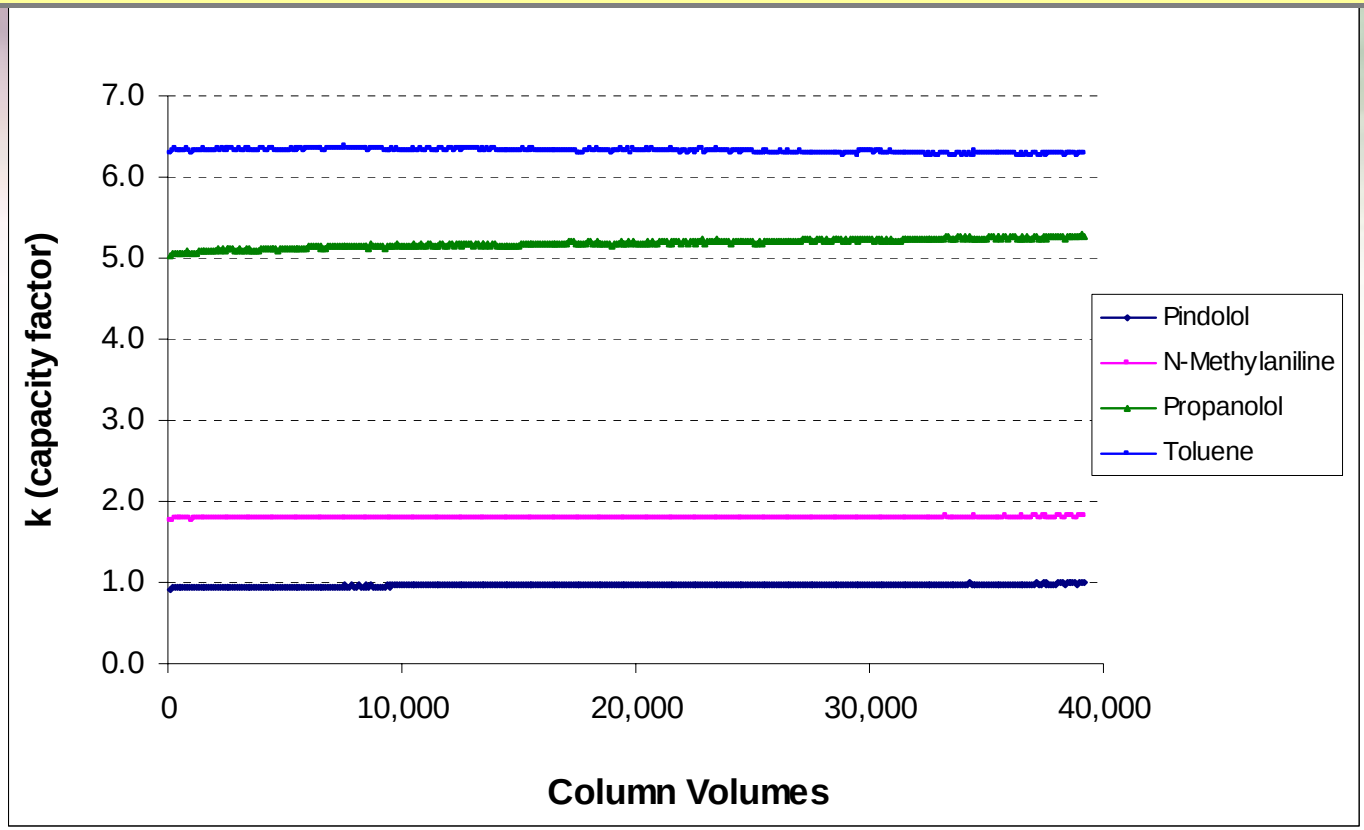
*Fig 13 Conditions: BIO Wide Pore C18 stability at pH 11.5*

**Discovery BIO Wide Pore C18 Stability**
Column #35136-03
5μm, 50x4.6mm
(65:35) 50mM **pH 11.5** Pyrrolidine-HCl : Acetonitrile
2mL/min
35°C
UV254nm
5 μL injection every 30 minutes

**Column volume (CV) calculation:**
CV = (0.7) $\pi r^2 L$ = (0.7) $\pi(0.23)^2 5$ = 0.6mL
40,000 CV = 24,000mL
Time: (24,000mL)(1min/2mL)(1hr/60min)(1day/24hr) = 8 days

# Demonstrating Column Lifetime at pH 11.5

*BIO Wide Pore C18 stability at pH 11.5, 40,000 column volumes (24L, 8 days)*



# Demonstrating Column Lifetime at pH 8

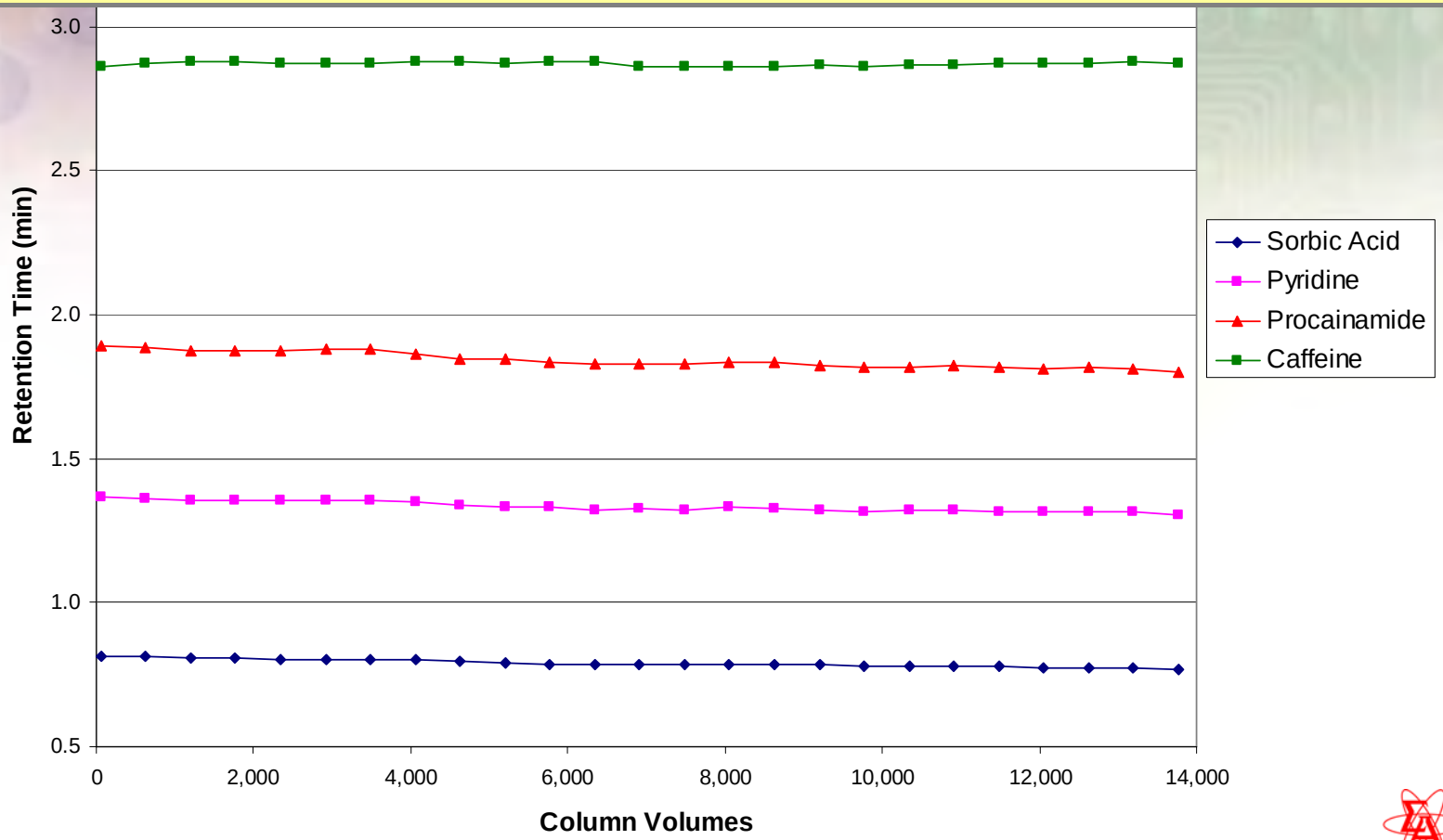
*Fig. 14 Conditions: BIO Wide Pore C5 stability at pH 8*

**Discovery BIO Wide Pore C5 Stability**
Column #11797
5μm, 50x4.6mm
(95:5) 25mM PO4 **pH 8.00**: MeOH
2mL/min
35°C
UV254nm
5 μL injection
950 psi

**Column volume (CV) calculation:**
CV = (0.7) $\pi r^2 L$ = (0.7) $\pi(0.23)^2 5$ = 0.6mL
14,000 CV = 8,400mL
Time: (8,400mL)(1min/2mL)(1hr/60min)(1day/24hr) = 3 days

# Demonstrating Column Lifetime at pH 8

*BIO Wide Pore C5 stability at pH 8, 14,000 column volumes (8L, 3 days)*



# Demonstrating Reproducibility

*Fig 15 Conditions Reproducibility of Discovery BIO Wide Pore phases*

## C18

Discovery BIO Wide Pore C18, 15cm x 4.6mm, 5µm,
**Mobile Phase:** (A) 80:20, 10mM $NH_4OAc$ (pH 6.8):$CH_3OH$,
**Flow Rate**: 1mL/min,
**Temp**: 35°C,
**Detection**: 254nm

## C5

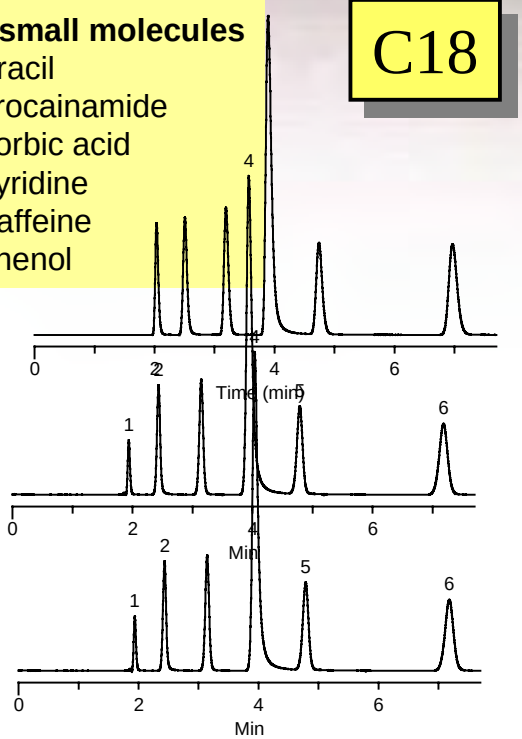
Discovery BIO Wide Pore C5, 15cm x 4.6mm, 5µm,
**Mobile Phase:** (A) 81:19, $H_2O$:$CH_3CN$ containing 0.1% PFPA, (B) 62:38, $H_2O$:$CH_3CN$ containing 0.1% PFPA,
**Flow Rate**: 1mL/min,
**Temp**: 30°C,
**Detection**: 2154nm

# Demonstrating Reproducibility

*Reproducibility of Discovery BIO Wide Pore phases for both small and large molecules.*

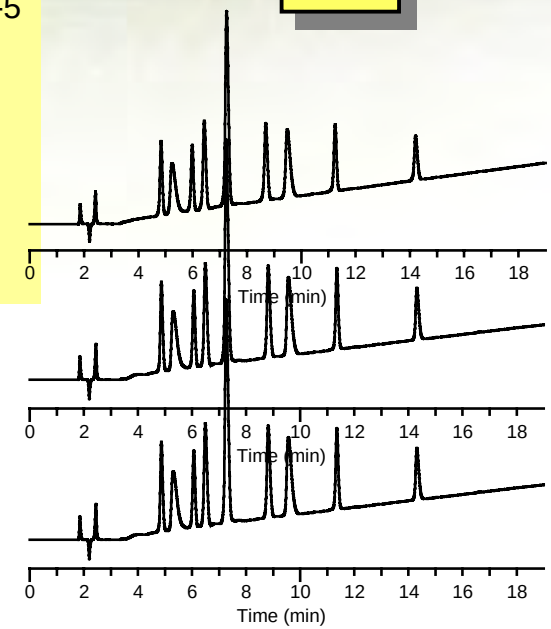
**For small molecules**
1. Uracil
2. Procainamide
3. Sorbic acid
4. Pyridine
5. Caffeine
6. Phenol

C18




**For peptides:**
1. Arg$^{8}$-vasopressin
2. Bradykinin, frag 1-5
3. Oxytocin
4. Met-enkephalin
5. LHRH
6. Leu-Enkephalin
7. Bradykinin
8. Bombesin
9. Substance P

C5



# Choosing a Discovery® BIO Phase

## Discovery BIO Wide Pore C18

Peptides
Protein digests (mapping)
Small proteins (<5kd)

## Discovery BIO Wide Pore C8

Intermediate hydrophobicity polypeptides
Small proteins

## Discovery BIO Wide Pore C5

Large polypeptides (>20kd)
Hydrophobic peptides
Large proteins

# When to use a BIO column

| | | |
|---|---|---|
| Proteins | C5 | Phases |
| Hydrophobic peptides | C5 or C8 | |
| Peptide mapping | C18 or C8 | |
| Scouting (method development) | C8 | |
| LC-MS | 3 micron or 5 micron | Particles |
| Fast analysis, or high- throughput applications | 3 micron | |
| Peptide mapping | 3 micron or 5 micron | |
| Analytical HPLC | 3 micron or 5 micron | |
| Preparative | 10 micron | |
| LC-MS | 2.1mm or smaller | ID |
| Peptide mapping | 4.6mm, 4.0mm, 2.1mm | |
| Analytical HPLC | 4.0mm, 4.6mm | |
| Preparative | 10mm, 21.2mm | |
| Low level detection or limited sample volume | 0.32mm, 0.5mm, 1.0mm | |

# Conclusion: The BIO Story

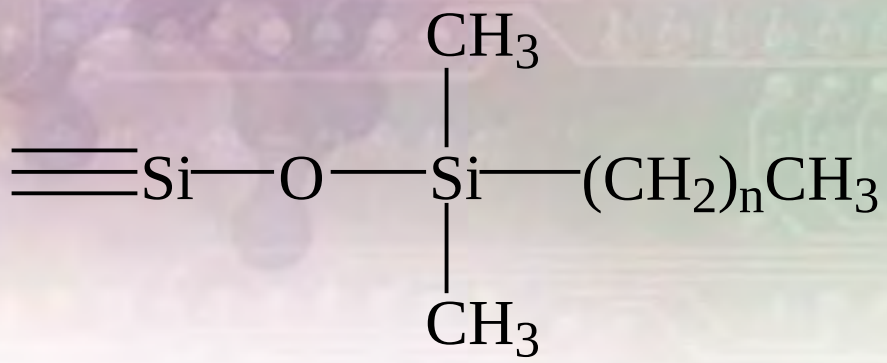
*"Discovery BIO Wide Pore HPLC columns and capillaries provide sensitive, stable, efficient, reproducible separations of proteins and peptides. The different phase chemistries provide unique selectivity increasing your resolution options. Separations are completely scalable from analytical to prep. The low-bleed feature and microbore and capillary dimensions make them ideal for LC-MS applications."*

**Discovery BIO Wide Pore meets the challenges of today's protein and peptide separations.**

# Supelco


***"We are Committed to the Success of Our Customers through Science, Technology, and Service."***

# Discovery® BIO Wide Pore Chemistry




C18: n=17
C8: n=7
C5: n=4

- What: Alkylmethylsilyl on 300Å pore silica
- How: Hydrophobic (van der Waals, dispersive) interactions
- Why:
  - Specifically designed for protein/peptide analysis
  - Matched selectivity across particle sizes for ease of scalability
  - Exceptional resolution for peptide analysis and purification
  - Highly stable to ensure excellent run to run reproducibility and long column life
  - Ideally suited for LC-MS; no detectable bleed

# Discovery BIO Wide Pore Column Testing

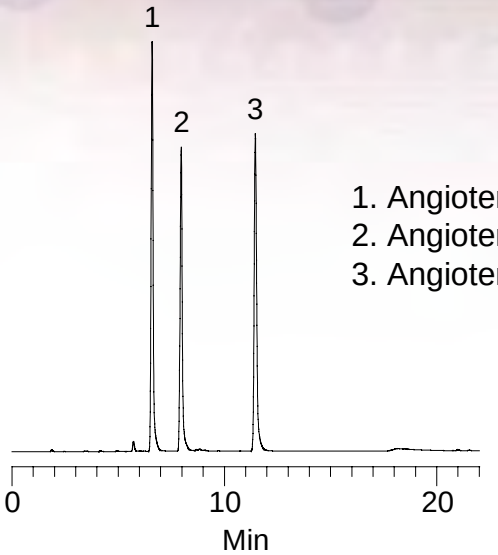
| | Efficiency (plates/m): | | |
|---|---|---|---|
| ID (mm) | 3µm | 5µm | 10µm |
| 0.32* | >95,000 | >70,000 | n/a |
| 0.50* | >95,000 | >70,000 | n/a |
| 1.0 | >88,000 | >62,000 | n/a |
| 2.1 | >84,667 | >53,333 | n/a |
| 4.0 | n/a | >66,667 | n/a |
| 4.6 | >110,000 | >80,000 | >35,200 |
| 10 | n/a | >84,000 | >35,333 |
| 21.2 | n/a | n/a | >35,000 |

* the 5cm are >90,000pl/m

# Discovery® BIO Wide Pore C8

***Intermediate hydrophobicity between a C18 and C4/C5. Ideal for the analysis and purification of peptides, polypeptides, and small proteins.***

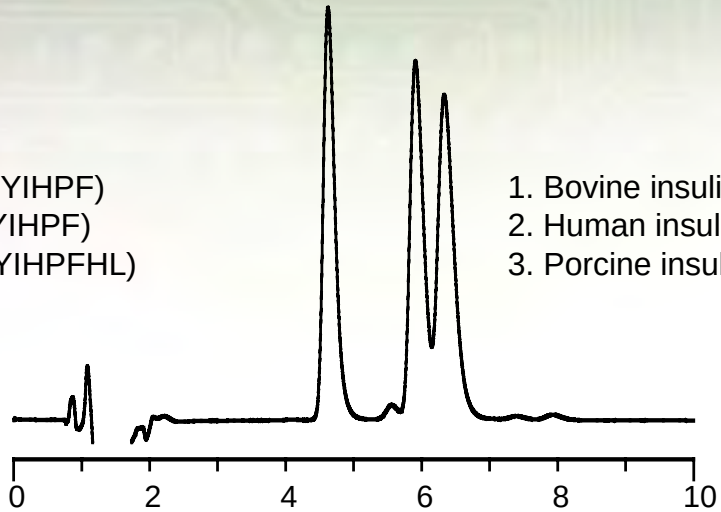
## Resolution of Angiotensins at Neutral pH




1. Angiotensin II (1.67g/L) (DRVYIHPF)
2. Angiotensin III (1.67g/L) (RVYIHPF)
3. Angiotensin I (1.67g/L) (DRVYIHPFHL)

Conditions: Discovery BIO Wide Pore C8, 15cm x 4.6mm, 5µm; Mobile Phase: (A) 10mM $NH_4H_2PO_4$/ $NH_4OH$, pH 7; (B) 50:50, (20mM $NH_4H_2PO_4$/ $NH_4OH$, pH 7):MeCN; Flow Rate: 1mL/min; Temp: 30°C; Detection: 215nm; Injection: 6µL; Gradient: 30-60% B in 15 min

## Resolution of Insulins from Various Species




1. Bovine insulin (5µg)
2. Human insulin (5µg)
3. Porcine insulin (5µg)

Conditions: Discovery BIO Wide Pore C8, 15cm x 4.6mm, 5µm; Mobile Phase: (A) 71:29, (0.1% TFA in $H_2O$):(0.1% TFA in MeCN); (B) 68:32, (0.1% TFA in $H_2O$):(0.1% TFA in MeCN); Flow Rate: 1mL/min; Temp: 30°C; Detection: 215nm; Injection: 50µL; Gradient: 0-100% B in 30min

# Resolution: The Separation Objective

$$R_S = (1/4)\{(\alpha - 1)/\alpha\}N^{1/2}\{k/(1 + k)\}$$

*To improve resolution between peaks we have three options:*

>> Increase selectivity (peak spacing) -- by changing the chemistry of the phase and the types of interactions that can occur between it and the analytes

>> Increase efficiency (narrow the peaks) -- by reducing adsorption and dispersion that lead to band broadening

>> Increase retention -- by increasing time analyte spends on the bonded phase

***Discovery BIO Wide Pore leverages all three to maximize resolution.***