Improving HPLC Selectivity and Resolution for Protein and Peptide Separations

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Column Chromatography Methods for Peptide/Protein Separations

Ion Exchange Chromatography	Charge
Reversed-Phase Chromatography	Hydrophobic Interaction
Gel Filtration	Molecular Size
IMAC (immobilized metal affinity chrom.)	Non-specific affinity
Affinity Chromatography	(Bio)specificity for a defined ligand
Hydrophobic Interaction Chromatography (HIC)	Hydrophobic Interaction under high salt

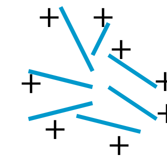
Cation Exchange Chromatography

Principle:
competitive interaction of ions: charged sample molecule competes with salt ion about fixed charges of stationary phase

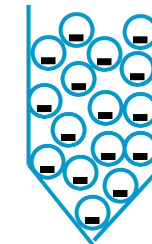
Cation exchange:
stationary phase carries negative charge, analyzed peptide molecules are positively charged (at acidic pH)

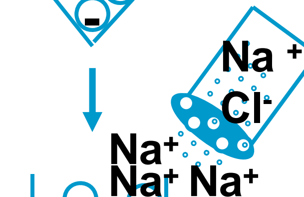
Functional groups of column are:
Sulfonic acid, sulfomethyl, sulfoethyl, sulfopropyl

Elution:
by increasing salt concentration or pH change



Positively charged sample is loaded and bound to column

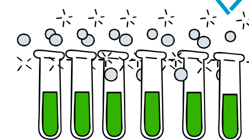




Na+
Na+ Na+

Elution with salt or pH

Positively charged salt ions replace bound peptide molecules



Fraction collection

Peptides are separated according to difference in their net charge

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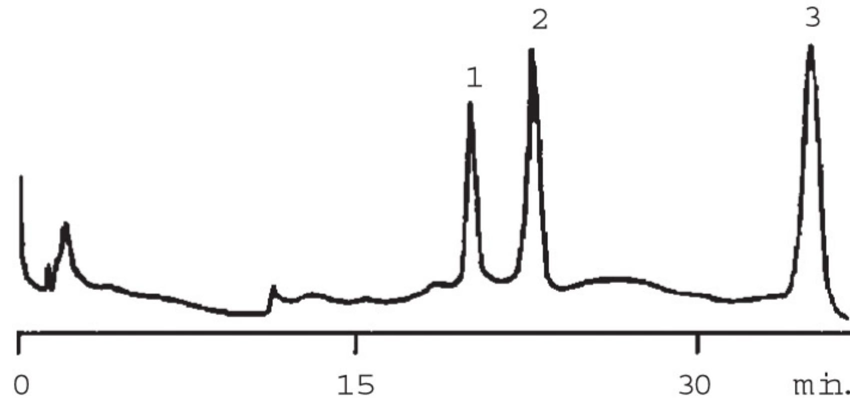
Use of Cation Exchange to Separate Basic Proteins



Column: SCX, 4.6 x 100 mm, 6.5 µm
Mobile Phase: A: 0.02 M tris, pH 7
B: 0.02 M tris in 0.5M sodium acetate, pH 7
Gradient: 0 – 100% B in 30 min.
Flow Rate: 1.0 mL/min
Detection: UV 254 nm
Sample: Basic proteins
1. RNA polymerase
2. Chymotrypsinogen
3. Lysozyme

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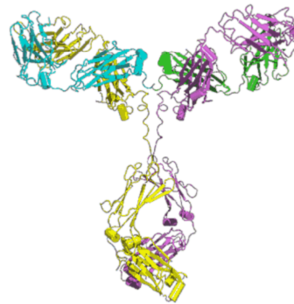
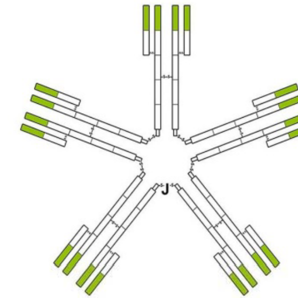
Bio-Monolith: Rapid and High Resolution Separation of Macro Bio-Molecules

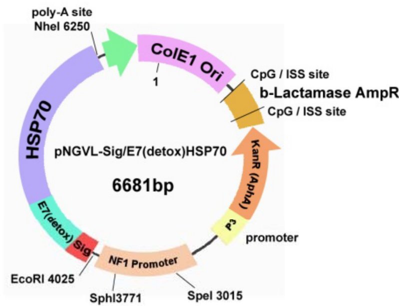
Large Proteins



IgG Antibodies

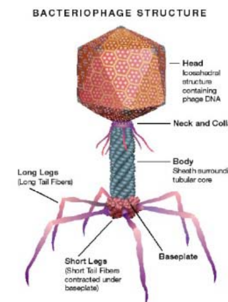


IgM Antibodies



pDNA

Adenovirus



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Another Challenge – the Size of the Molecule of Interest

Large biomolecules like large proteins, viruses and DNA

Pores too small!
- Binding mostly on outer surface
- Too small surface area

⇨ **Very low binding capacities!**

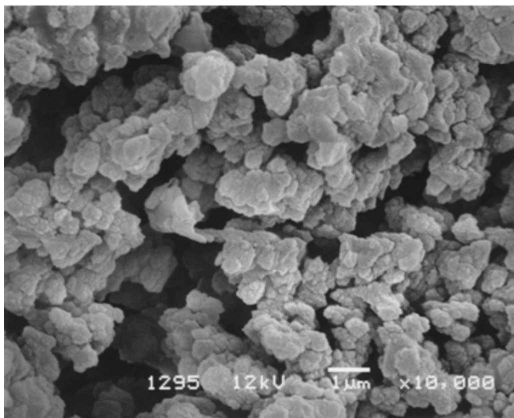
Just some pores large enough
- Some pores interconnected, still many dead end pores exist

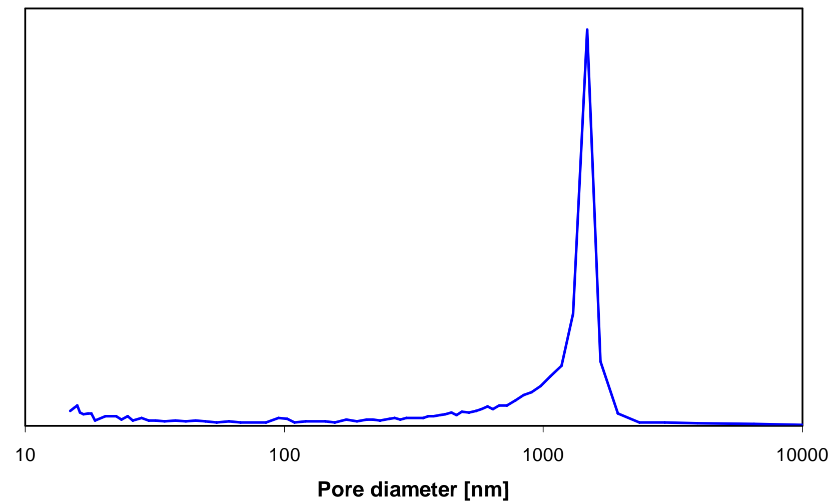
⇨ **Low binding capacities!**

CIM® Monoliths

CIM® monolithic supports are highly porous rigid polymers with:

- High porosity (over 60 %)
- Flow-through channels ("pores") having large diameter (1.5 µm)
- Uniform channel connectivity in 3D (homogeneous structure).





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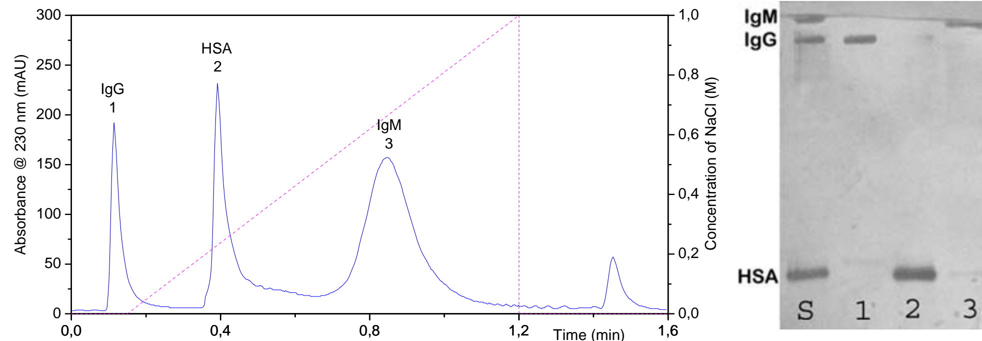
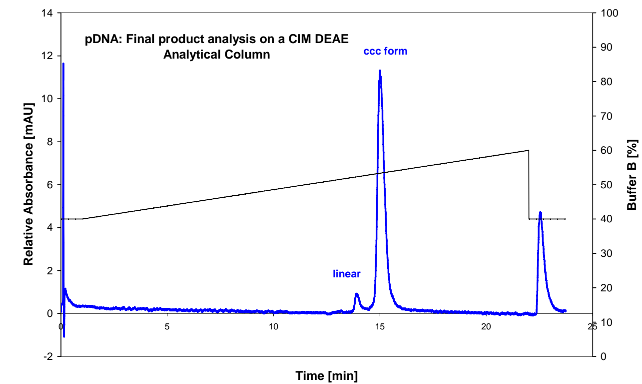
Macro Bio-Molecule Analytics

Antibody Separations

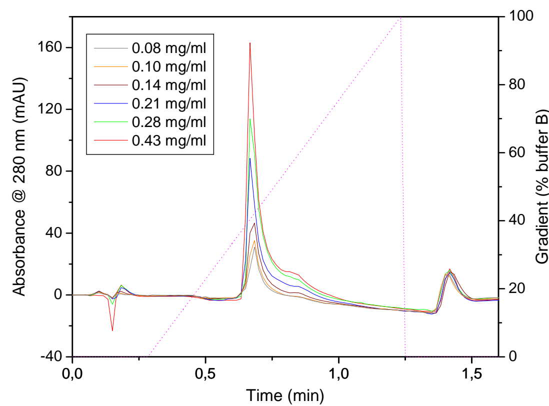


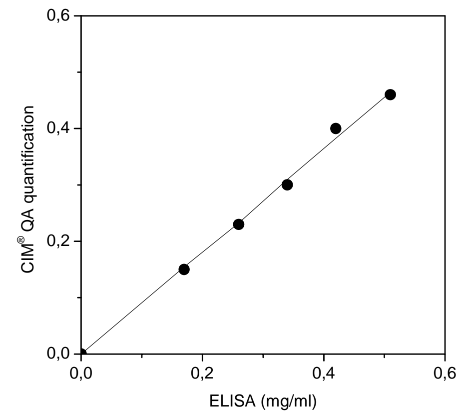
Brne et al., J. Chromatogr. A, (2007).

pDNA Analytics



Quantitation of Virus





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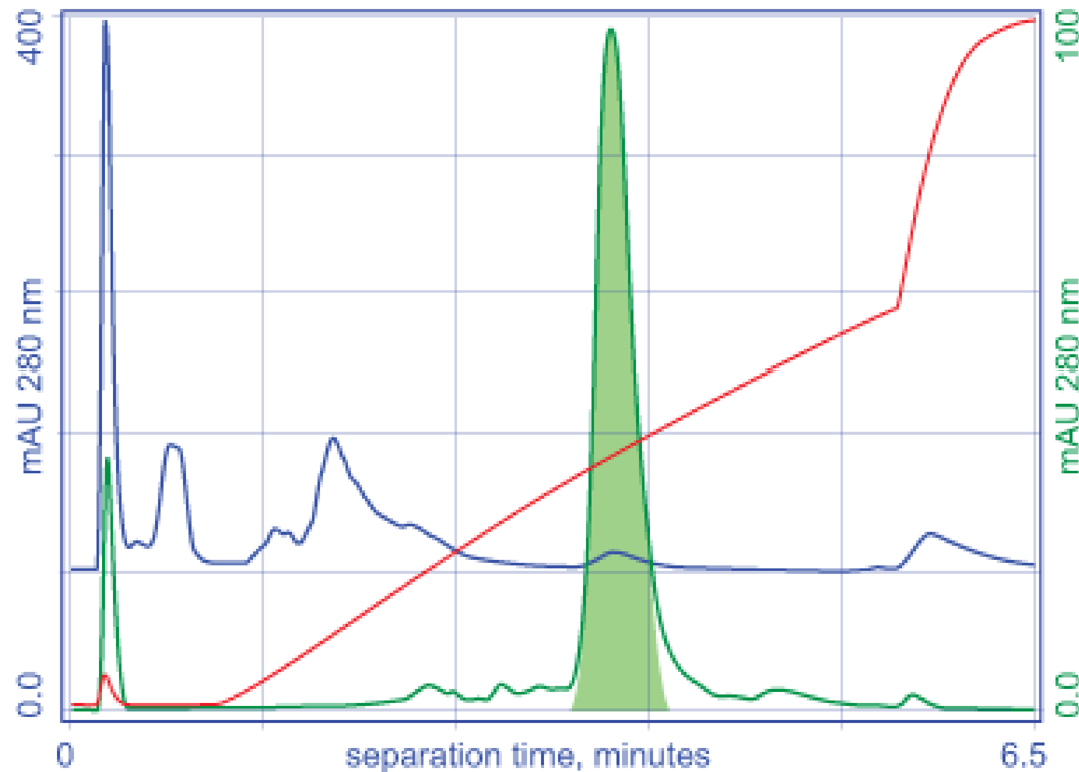
Enabling IgM Purification



Media: CIM® QA, 0.34 mL

Flow rate: 4 mL/min

Buffer A: 10 mM NaPO4, pH 7.0

Buffer B: 500 mM NaPO4, pH 7.0

Equilibrate: buffer A

Load: 100 µL

Wash: buffer A

Elute: 34 CV LG to 50% buffer B

Clean with 100% buffer B

Highlighted peak is IgM. Blue trace, IgM CCS. Green trace, CHT-purified IgM. Strong anion exchange retention is typical of IgMs.

Courtesy of Pete Gagnon, Validated Biosystems, San Clemente, USA; www.validated.com

Method duration: from 14,8 min to 7,4 min.

QA

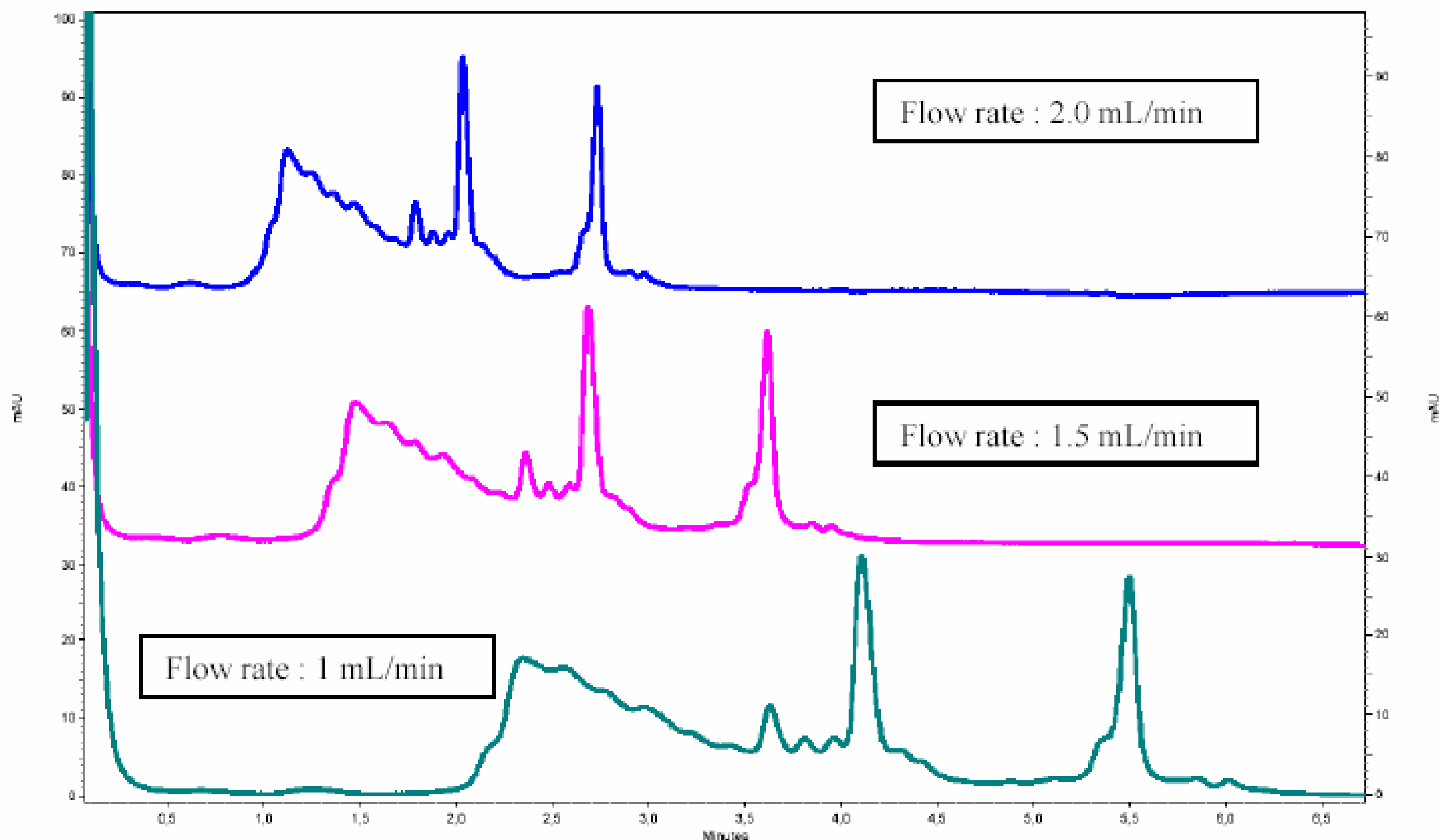


Figure 8: : Buffer A: 20 mM Tris-HCl, pH 7.4, buffer B: 20 mM Tris-HCl + 1 M NaCl, pH 7.4 Injection volume: 10 µL. Myoglobin (1), conalbumin (2), ovalbumin (3), STI (4) (BioRad). Method: gradient from 0-50% buffer B in 125 cV.

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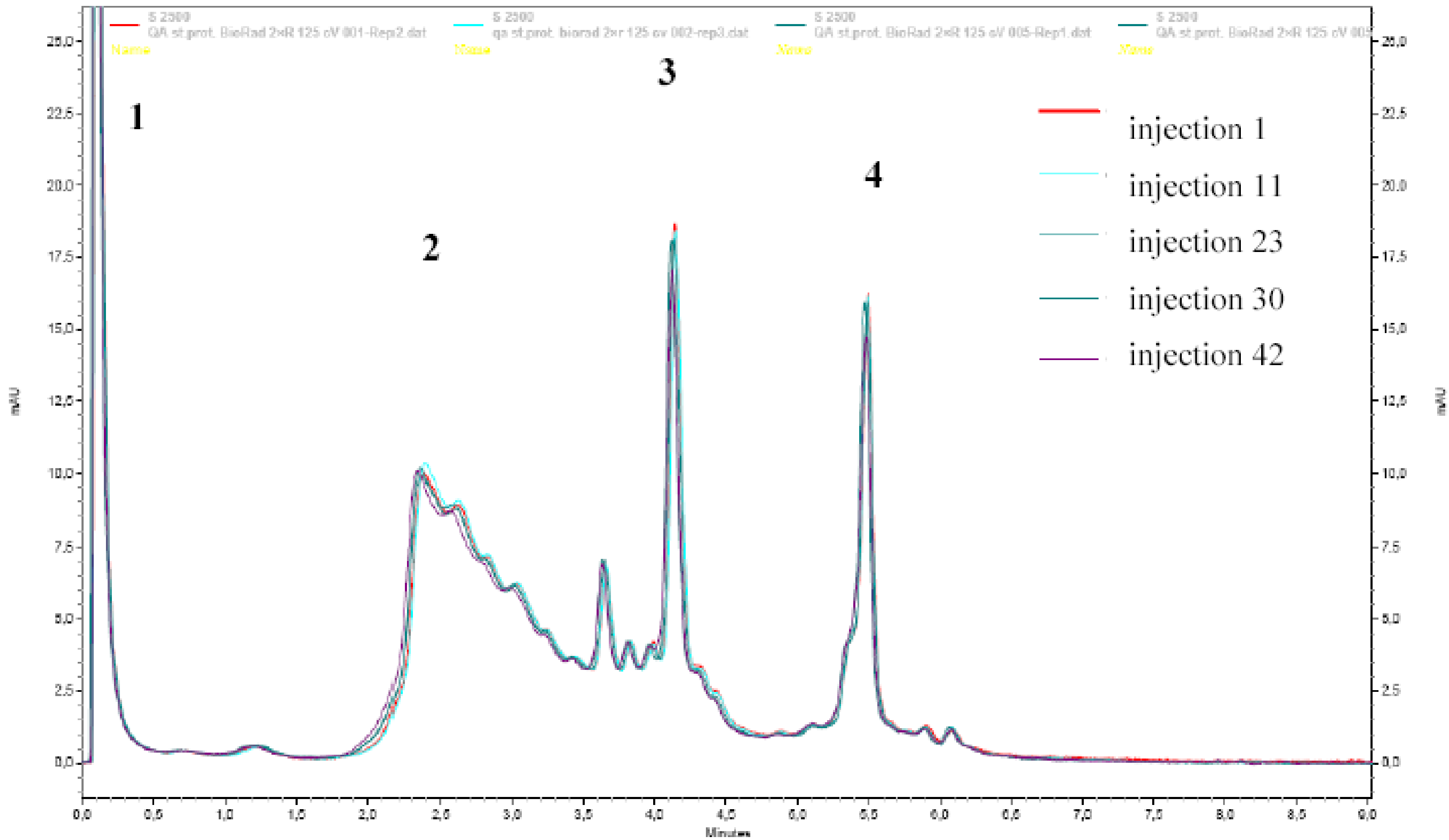
Consecutive injections of a mixture of 4 test proteins (BioRad) on a CIM QA Analytical Column



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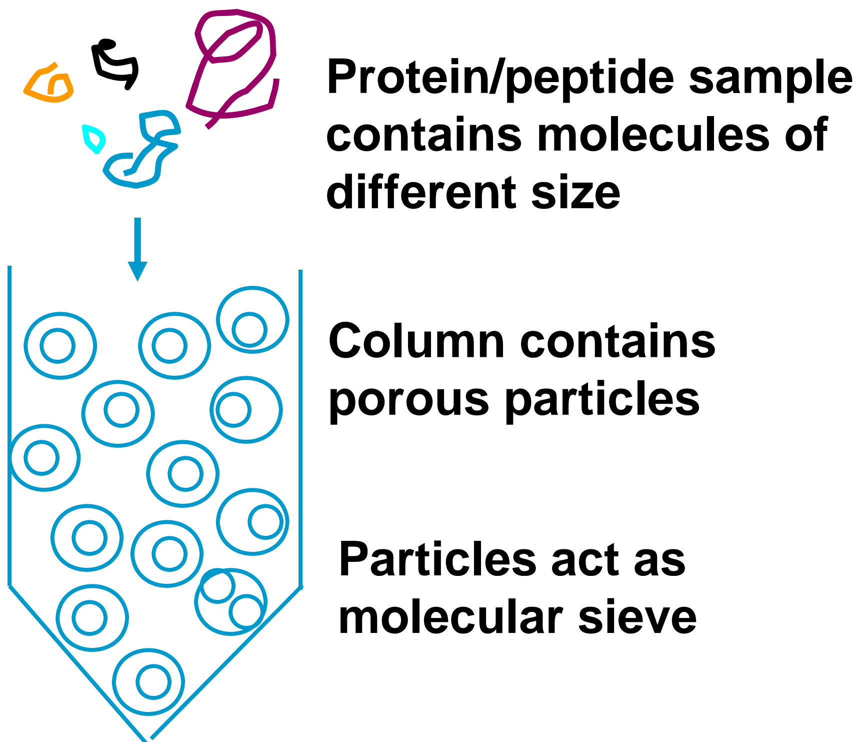
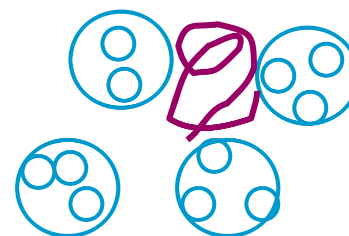
Gel Filtration (Size Exclusion) Chromatography



Separation according to size

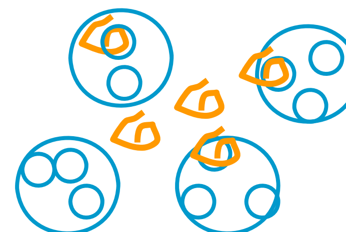
1. big molecules cannot enter pores

→ fast elution



2. Small molecules enter pores

→ later elution



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SEC Applications with Proteins

- Impurity testing (separation of monomer/dimer/aggregates)
- Molecular weight characterization – good MW accuracy (<2%) and good precision (<2% RSD) over wide MW range (1000 – 10M) possible
- Expression and folding studies
- Separation of Reaction Components and Products, (esp., antibodies, fragments, and conjugates)
- Purification
- Desalting and Exchange of Sample Buffer
- Collection of fractions under non-denaturing conditions

SEC of Proteins on ZORBAX GF-250

Separation of Albumin Monomer, Dimer and Aggregate

Column: **ZORBAX GF-250, 9.4 x 250 mm**
Mobile Phase: 0.2M Sodium Phosphate, pH 7.0,
0.1% Sodium Azide,
Detection: UV 280 nm
Sample:
1. Aggregate
2. Albumin dimer
3. Albumin

DeLeenheer, A.P. et al. *J. Pharm Sci.*, (1991) 80, 11.
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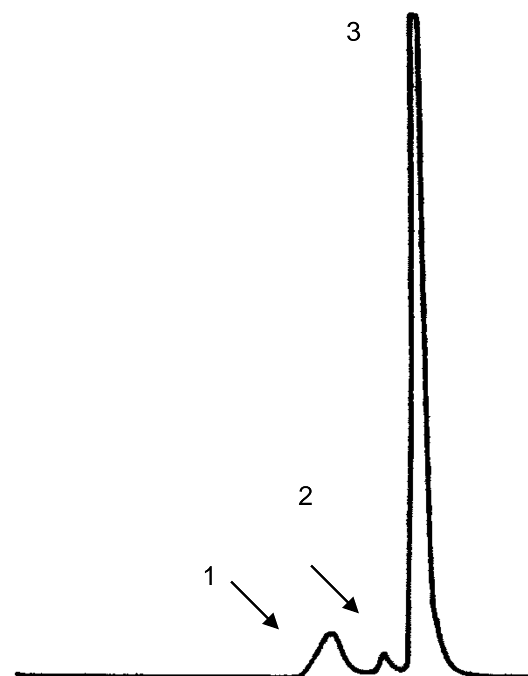

The ZORBAX GF-250/450 columns are specially treated to reduce protein sticking and for long column lifetimes
Analyze proteins with molecular weights from 4,000 – 900,000.

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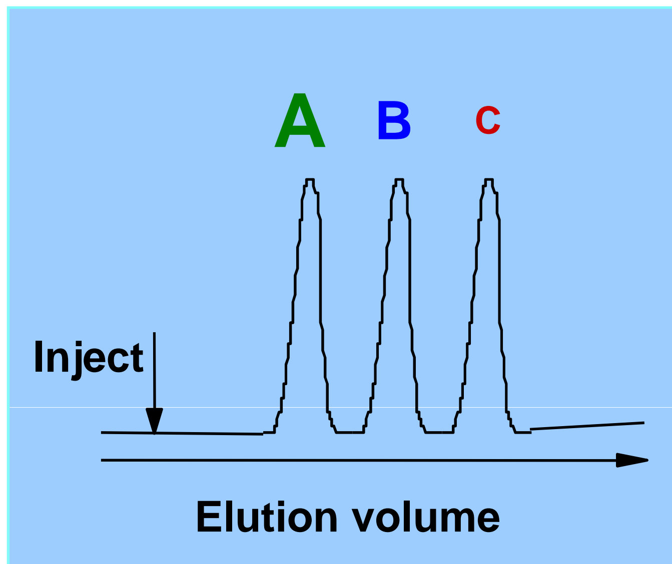
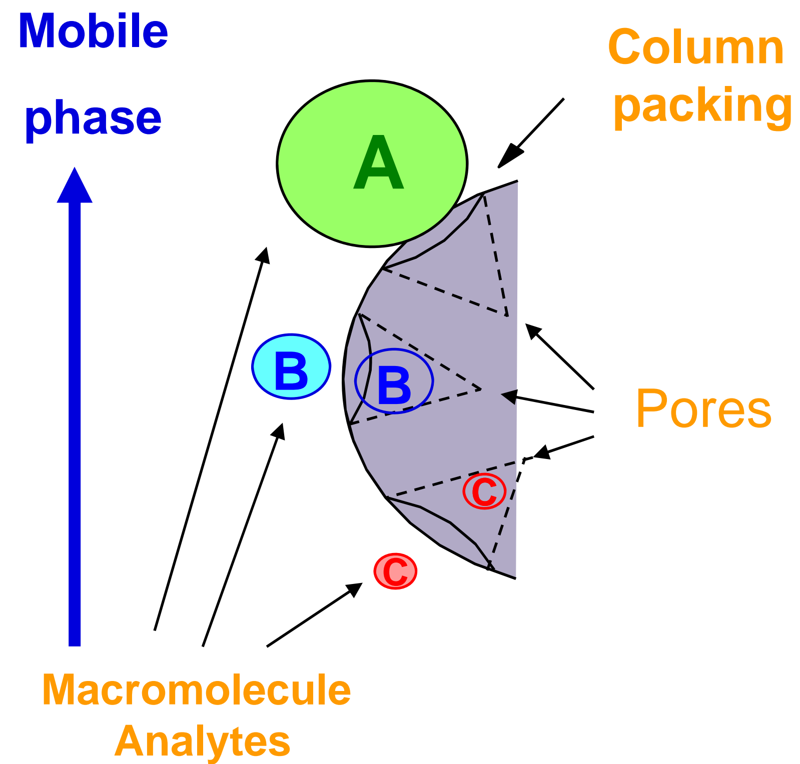
SEC Separation Mechanism





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Mechanism of SEC

Molecules are separated from largest to smallest in proportion to their molecular weight.

Very large molecules are excluded from the packed bed and elute first, in the exclusion volume.

Smaller molecules explore some of the pores of the packing and elute later.

The smallest molecules explore all of the pores and elute last, in the void volume.

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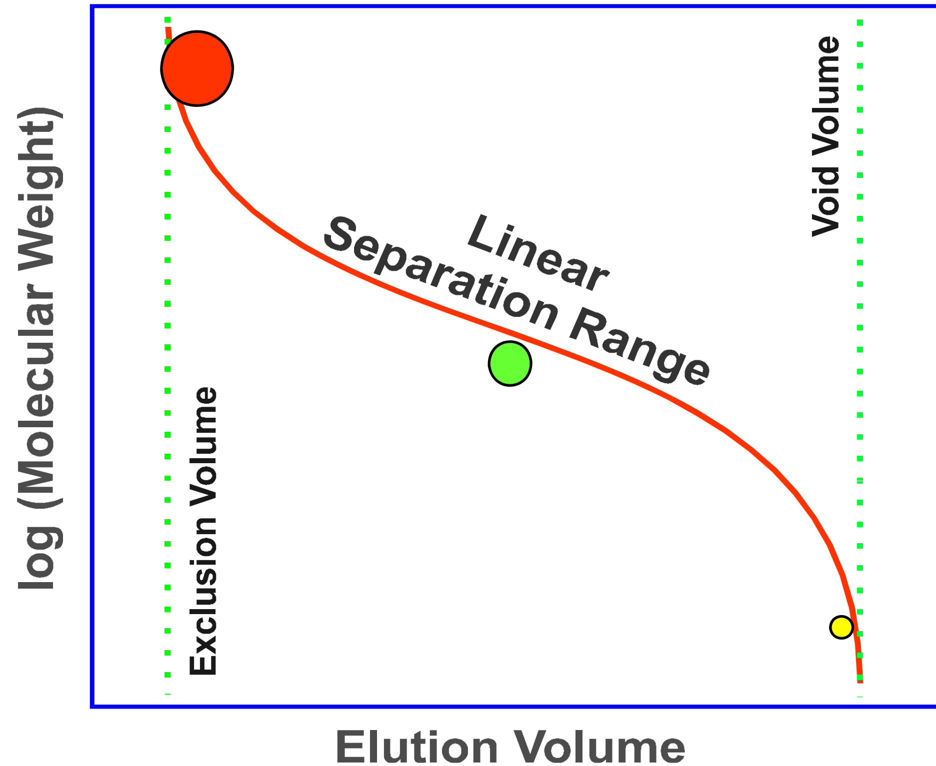
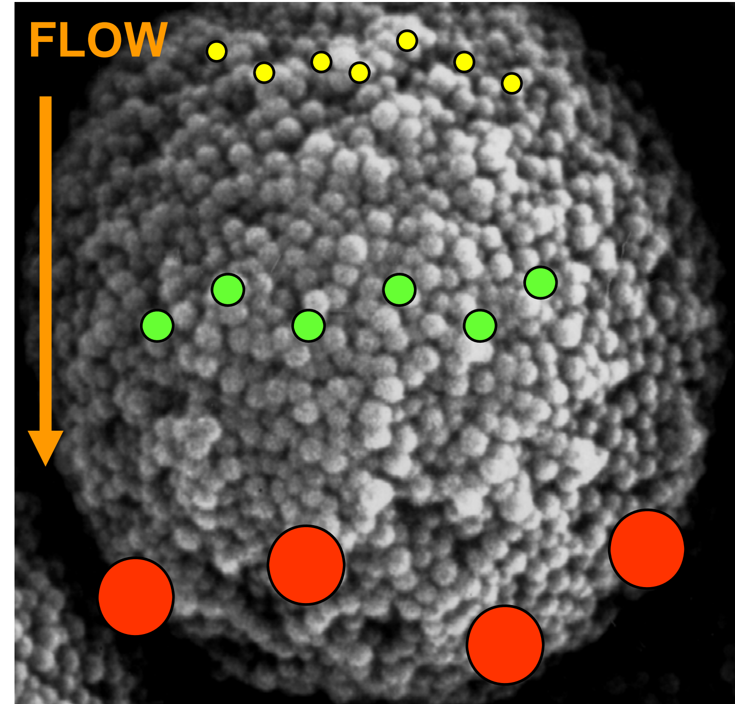
Mechanism of SEC Separation





Small Change in elution volume ---->big change in molecular weight ---> excellent HPLC equipment required!

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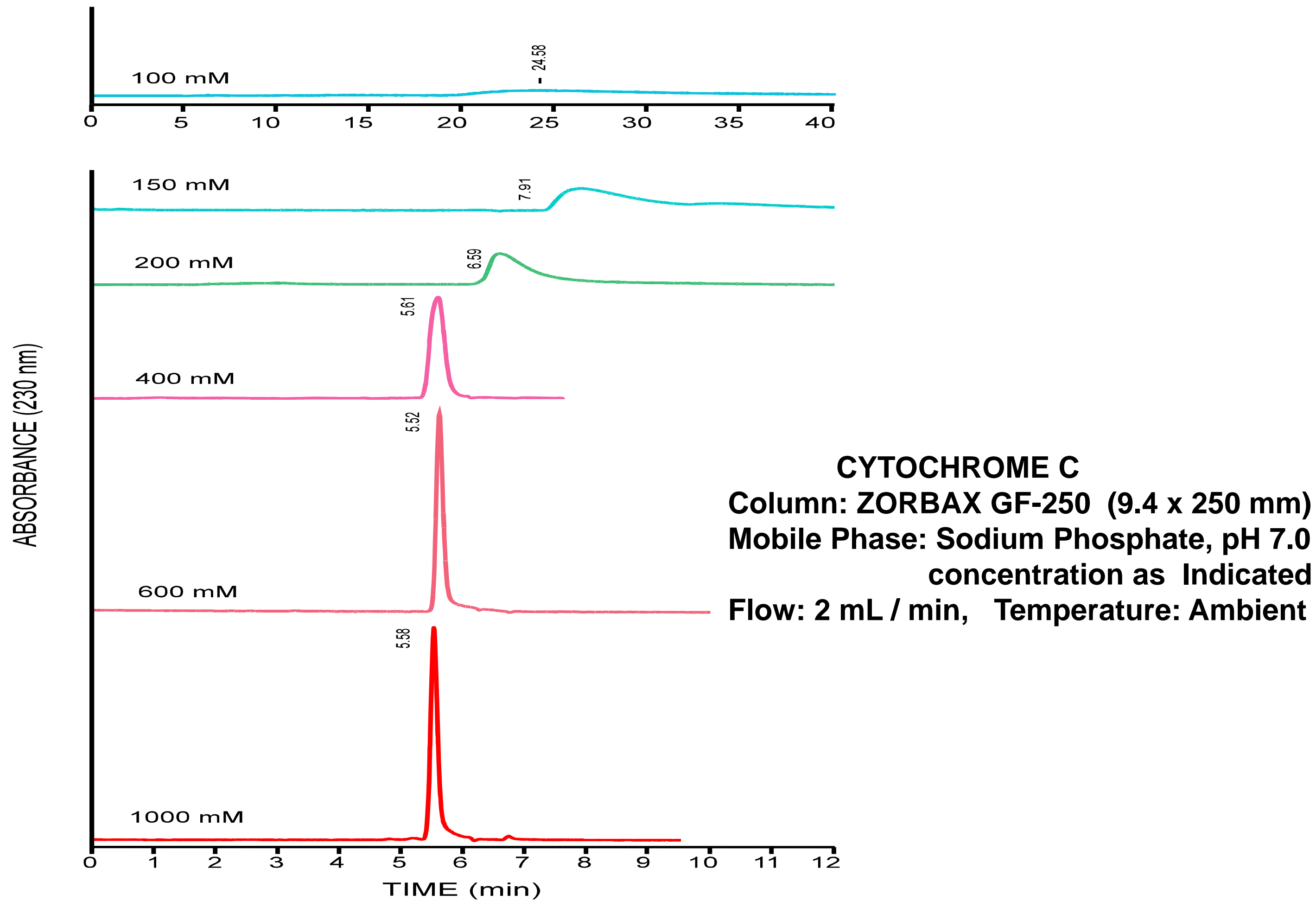
Characteristic Elution Profiles of a Basic Protein



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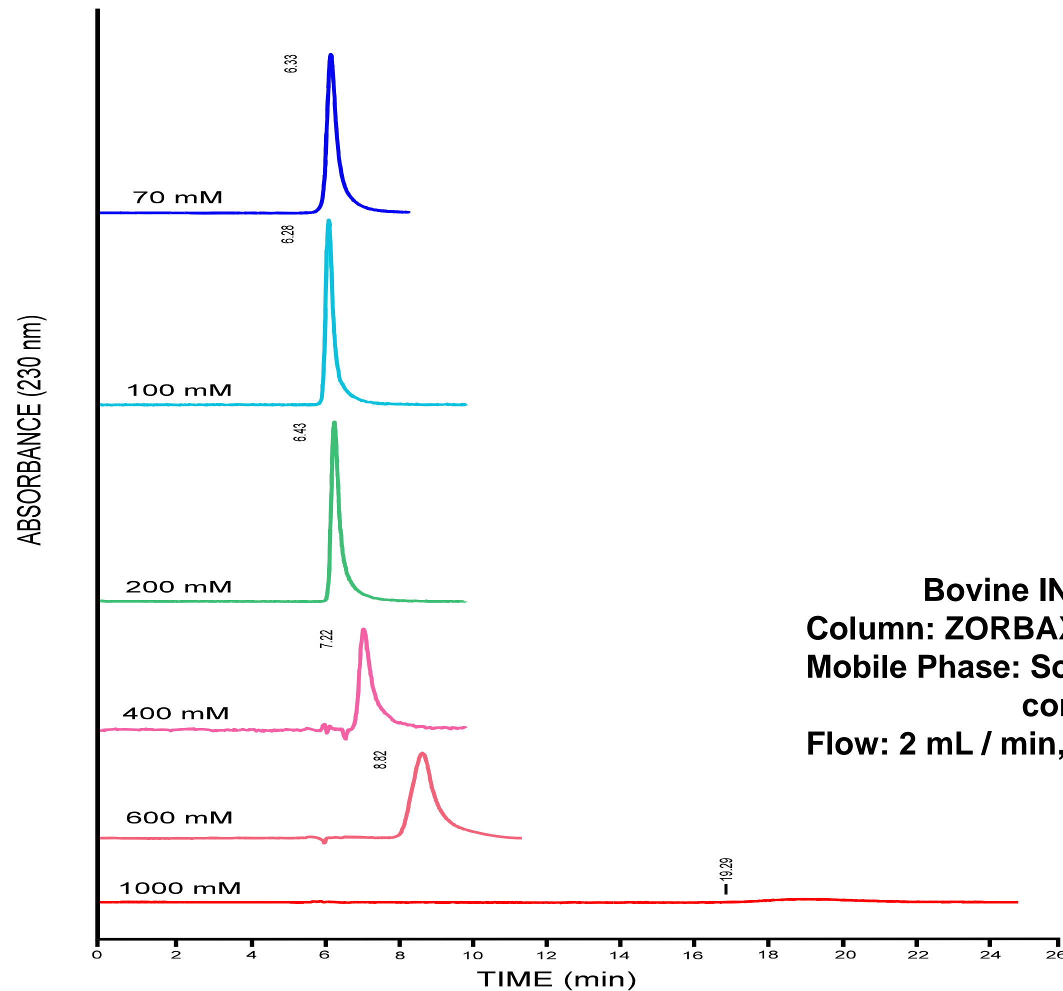
Characteristic Elution Profiles of a Hydrophobic Protein



Bovine INSULIN
Column: ZORBAX GF-250 (9.4 x 250 mm)
Mobile Phase: Sodium Phosphate, pH 7.0 concentration as Indicated
Flow: 2 mL / min, Temperature: Ambient

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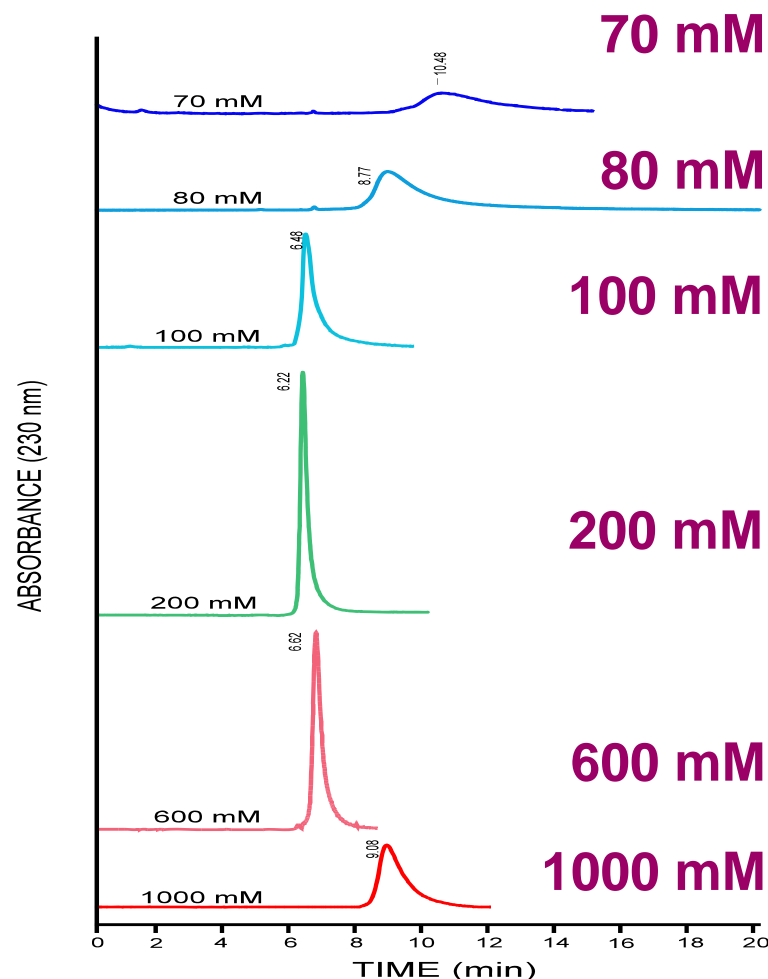
Effect of Mobile-Phase Ionic Strength on Elution of a Basic & Hydrophobic Protein

Chromatograms were collected at each of the sodium phosphate concentrations indicated.

Lysozyme (pI=10) increases in retention volume when the sodium phosphate concentration is very high or very low.

Protein exhibits both basic and hydrophobic properties.

Column: ZORBAX GF-250, 9.4 x 250 mm
Mobile Phase: Sodium Phosphate, pH 7.0
concentration as indicated
Sample: Lysozyme
Flow: 1 mL/min
Temperature: Ambient



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RESOLUTION, SPEED, LOAD,

Table 2. ZORBAX GF-250 and GF-450 Column Capacity at Different Practical Sample Concentrations.*

Column Size	Recommended Sample Injection Volume*	Injected Mass		
		0.1 mg/mL Sample	2 mg/mL Sample	20 mg/mL Sample
4.6 x 250 mm	12.5 µL	1.25 µg	25 µg	0.25 mg
9.4 x 250 mm	50 µL	5 µg	100 µg	1 mg
21.2 x 250 mm	250 µL	25 µg	500 µg	5 mg

*Assumes a sample of approximately eight peaks. Capacity may be larger with fewer peaks and/or larger Rs values.

Use injection volumes <5-10% of peak volume.

GFC is a technique where slow flow rate Is desirable

start at 0.2 ml/min for a 4.6 mm column
1.0 ml/min for a 9.4 mm column
5.0 ml/min for a 21.2 mm column

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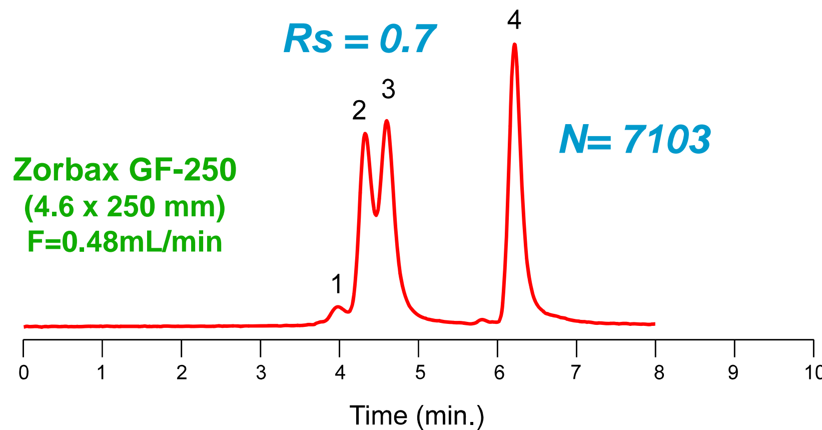
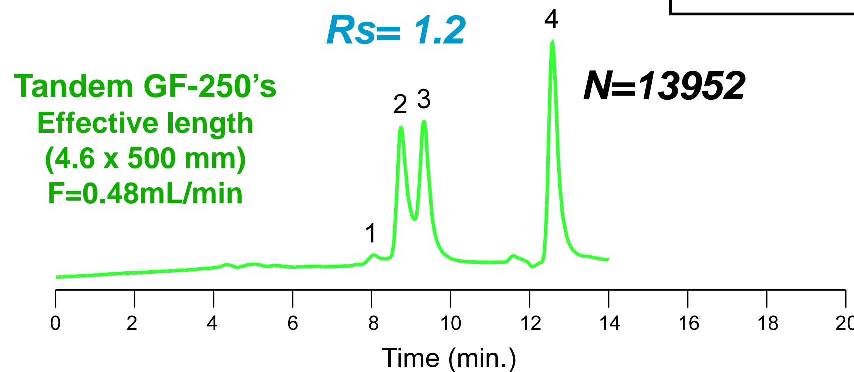
Effect of Column-Length on Efficiency and Resolution



1. BSA-dimer
2. BSA
3. Ovalbumin
4. Sodium Azide



Increasing column-length increases theoretical plate values (N), resulting in improved resolution (Rs). This is seen in the increased N for sodium azide, and in the increased Rs for BSA and Ovalbumin. The tradeoff is increased run time due to column-length and increased back-pressure, limiting an increase in flow rate.

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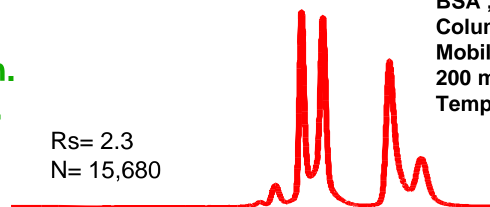
Effect of Flow Rate on Resolution and Plates in SEC

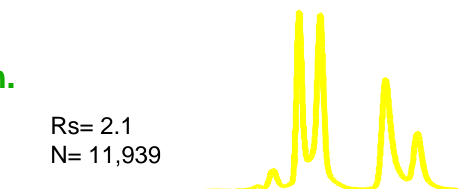
An increase in flow rate results in faster separation. Resolution (Rs) and theoretical plates (N) can be affected by decreasing flow rate. Values of Rs and N for BSA and Ovalbumin are shown. Both Rs and N decrease as flow rate is increased.

Protein Mix:
BSA , Ovalbumin, Lysozyme, Azide
Column: Zorbax GF-250
Mobile Phase:
200 mM Sodium Phosphate, pH 7.0
Temperature: Ambient

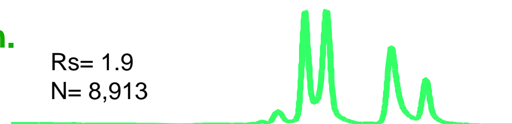
TIME= 60 min.
0.25 ml / min.



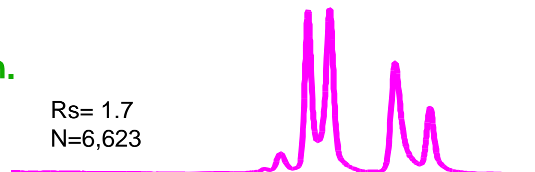
TIME= 30 min.
0.5 ml / min.



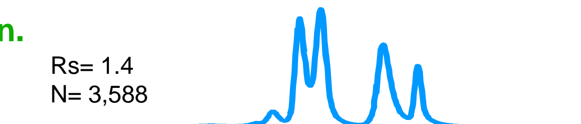
TIME= 15 min.
1 ml / min.



TIME=7.5 min.
2 ml / min.



TIME= 3.5 min.
5 ml / min.



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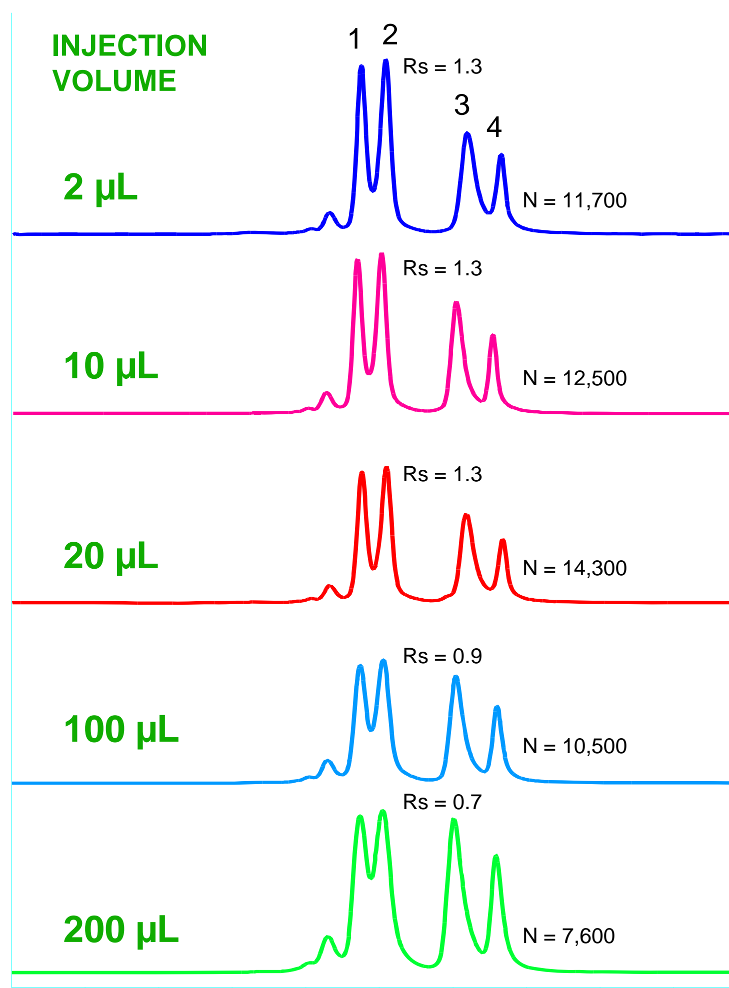
Effect of Injection Volume on Separation Efficiency in SEC

Increasing volumes of a protein mixture were injected onto a Zorbax GF-250 size-exclusion column. Values of Rs for peaks 1 and 2, and Plates (N) for peak 4 are shown. Rs and N are decreased dramatically for the 100 and 200 µl injections.

Protein Mix:
BSA, Ovalbumin, Lysozyme, Azide
Column: ZORBAX GF-250
Mobile Phase:
200 mM Sodium Phosphate, pH 7.0
Flow: 1 mL / min, Temp.: Ambient



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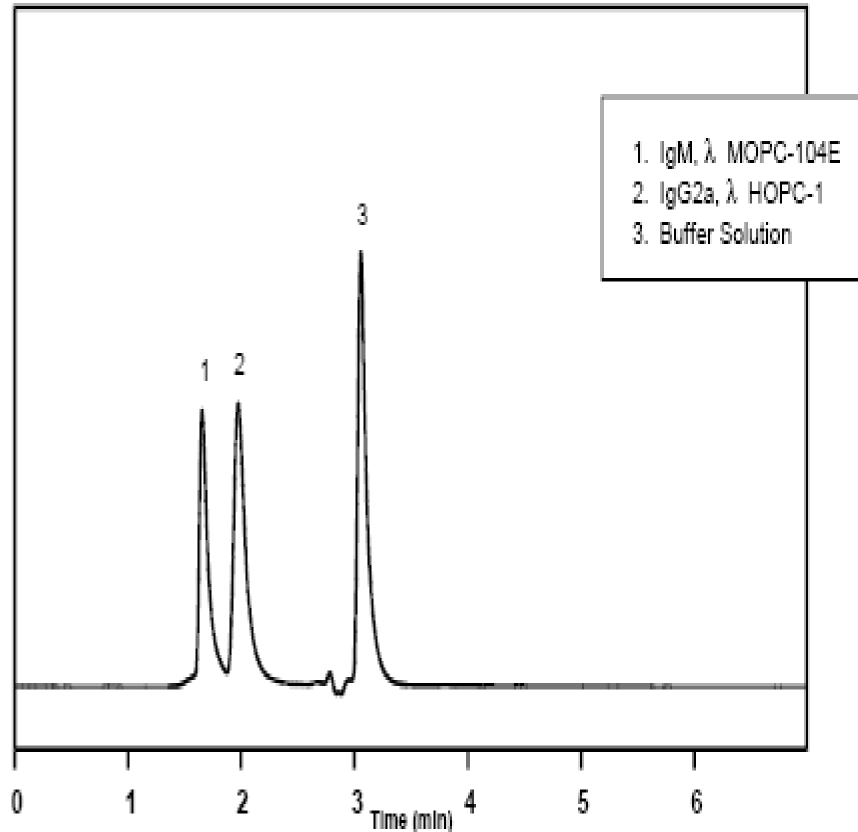
Fast Antibody Separations



FAST SEPARATION OF IgM AND IgG ANTIBODIES BY SIZE-EXCLUSION CHROMATOGRAPHY

Antibodies play a critical role in modern biotechnical research. The high specificity and affinity of antibodies for the analyte make them useful for molecular targeting, detection, and immunoassays. Use of antibodies often includes separation of small amounts of conjugated and non-conjugated forms, purification from reactants, and exchange of buffer components. As a result of the differences in molecular size of the components, size-exclusion chromatography is a powerful tool, well-adapted to these separations.

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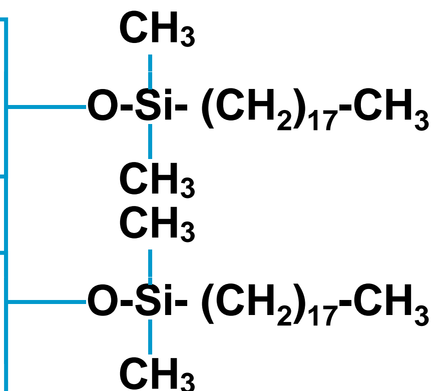
Reversed Phase Chromatography

Reversed phase: historical term :“nonpolar hydrocarbon chains are attached to polar groups”

Stationary phase: silica gels with hydrocarbon chains between 1 and 18 carbon atoms (C1 to C18)

For peptides: C18 phases are most popular

Mobile phase: organic solvent; ion pair reagent

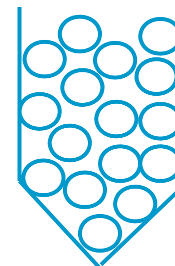


Elutropic force

Water>Methanol>Acetonitrile>n-Propanol> THF

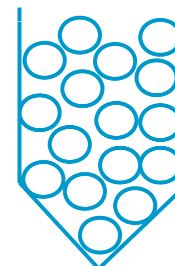
Polarity

Peptide sample retains on nonpolar hydrocarbon chains of column stationary phase

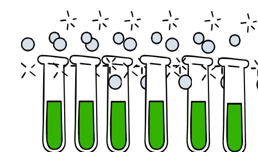


Elution by increasing hydrophobicity:

concentration of organic solvent



Organic solvent elutes peptide at hydrophobic interaction site



Fraction collection

Reversed Phase Columns for Separations of Proteins and Peptides

Requirements

- Wide pore - 300Å for unrestricted access to bonded phase
- LC/MS compatible bonded phases at low and high pH – low bleed, high performance
- Multiple bonded phases for method optimization
- Many configurations for LC/MS compatibility, small sample sizes and 2-D HPLC for proteomics

Columns available

- 300StableBond
- 300Extend
- Poroshell 300SB
- Configurations from nano to prep

Separations of Proteins and Peptides
300SB and 300Extend Columns

Reversed-phase separations of high molecular weight molecules require wide-pore (300Å) columns

StableBond 300SB columns

- Ideal for analysis of proteins and peptides at low pH
- Typically used with TFA Containing Mobile Phases
- Formic or acetic acid can also be used for MS analysis

300Extend-C18 columns

- Ideal for analysis of proteins and peptides at mid and high pH
- Ammonium hydroxide is ideal mobile phase for LC and LC/MS
- Can be used from pH 2 – 11.5 to improve resolution

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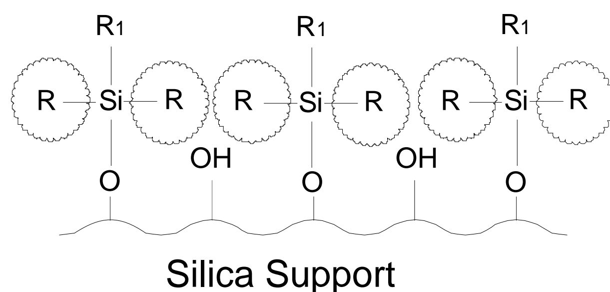
ZORBAX 300SB and 300Extend-C18 Columns for the Analysis of Proteins and Peptides

300SB

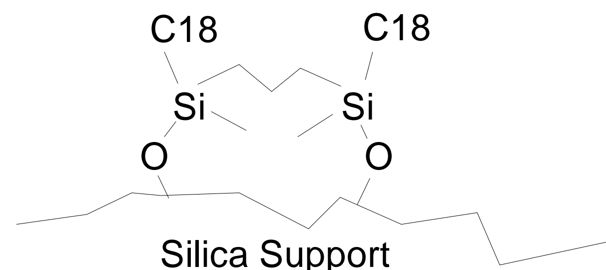
- Four different bonded-phases, 300SB-C18, C8, CN, and C3 for selectivity optimization
- Extremely stable at low pH
- Use with TFA, Formate and Acetate mobile phases
- Stable at high temperature – up to 80 - 90°C

300Extend-C18

- Uses unique bidentate-C18 bonded phase for long lifetime at high pH
- Double endcapped
- Can also be used at low pH
- Ammonium hydroxide mobile phase good for high pH LC and LC/MS



Silica Support



Silica Support

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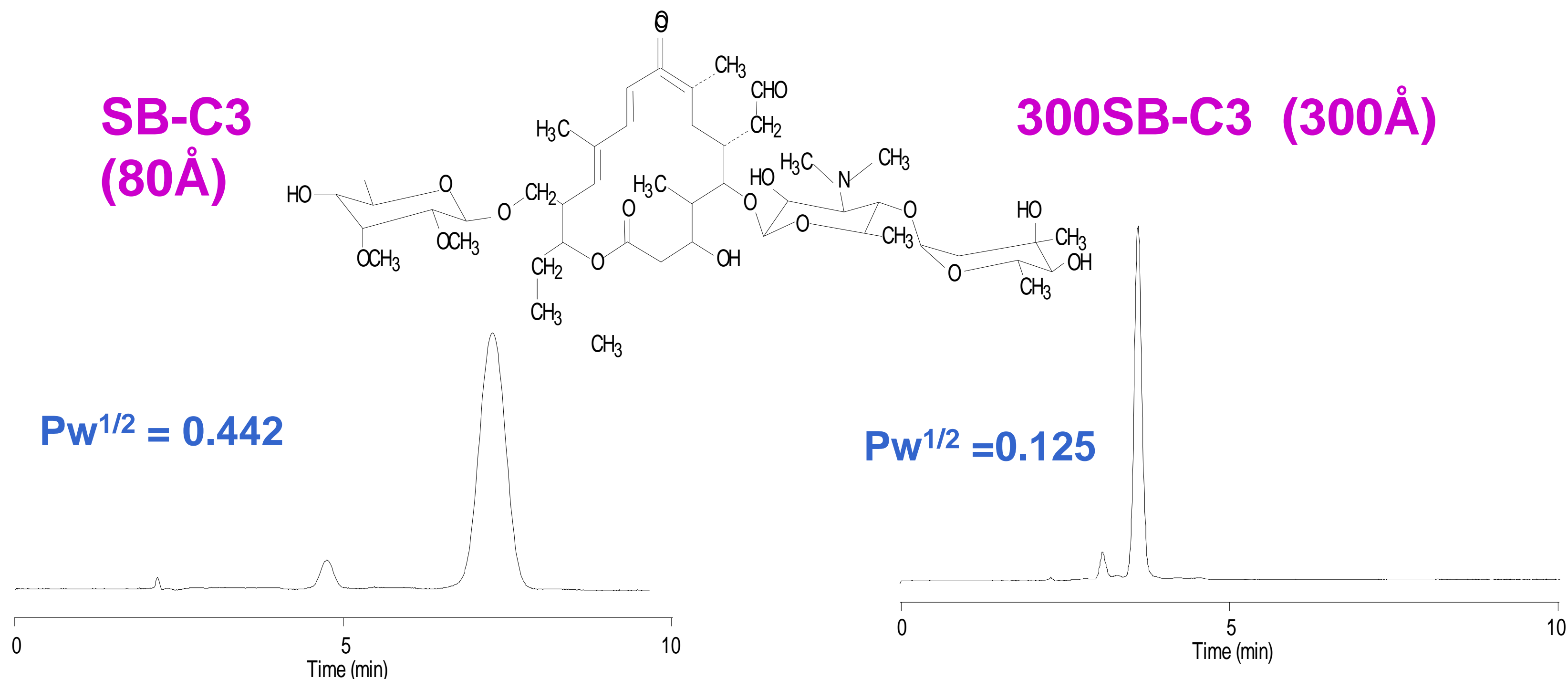
Improved Peak Shape for Large Molecules in Solution



- The size of a molecule in solution determines which pore size column is best.
- The narrower peak width indicates unrestricted access to the pores.

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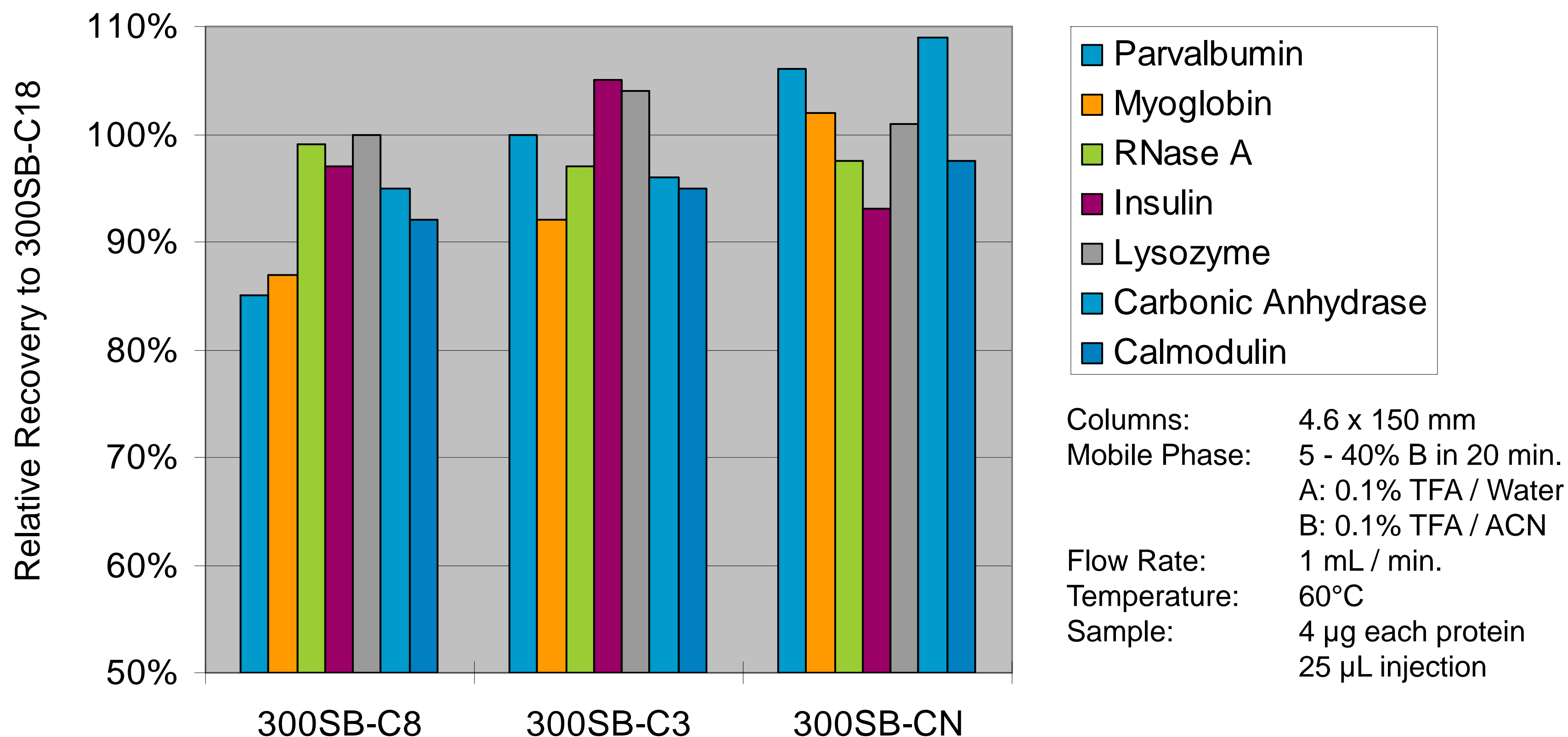
Recovery of Polypeptides from ZORBAX 300SB Columns



- **Recovery may vary depending on bonded phase.**
- **All 300SB bonded phase generally provide good recovery.**

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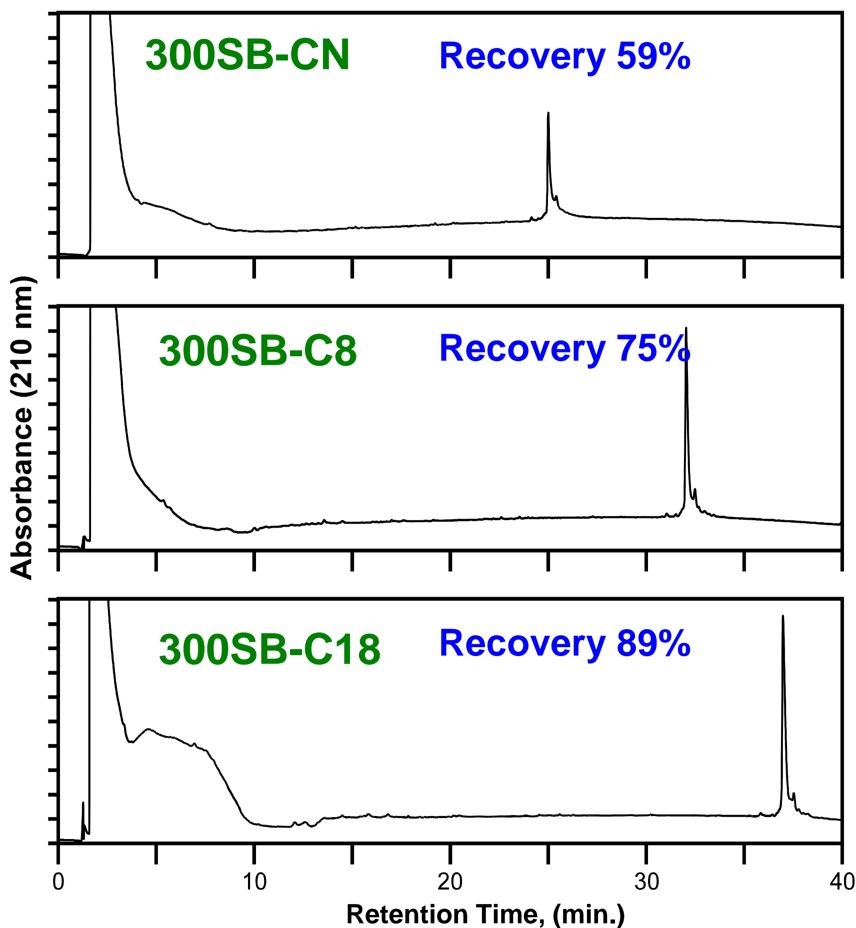
Effect of Bonded-Phase Ligand on Recovery of a Synthetic Lipopeptide



Conditions:
Column: ZORBAX 300SB, 4.6 x 150 mm, 5 µm
Mobile Phase: A – 0.1% TFA/water, B – 0.1% TFA/can
Gradient: 10 - 90 % B in 40 min
Flow Rate: 1 mL/min
Temperature: 60°C,
Sample: 15 µL (15 µg) of peptide in 0.1% TFA/DMSO

- **Recovery on each bonded phase is not always predictable so evaluate different bonded phases.**

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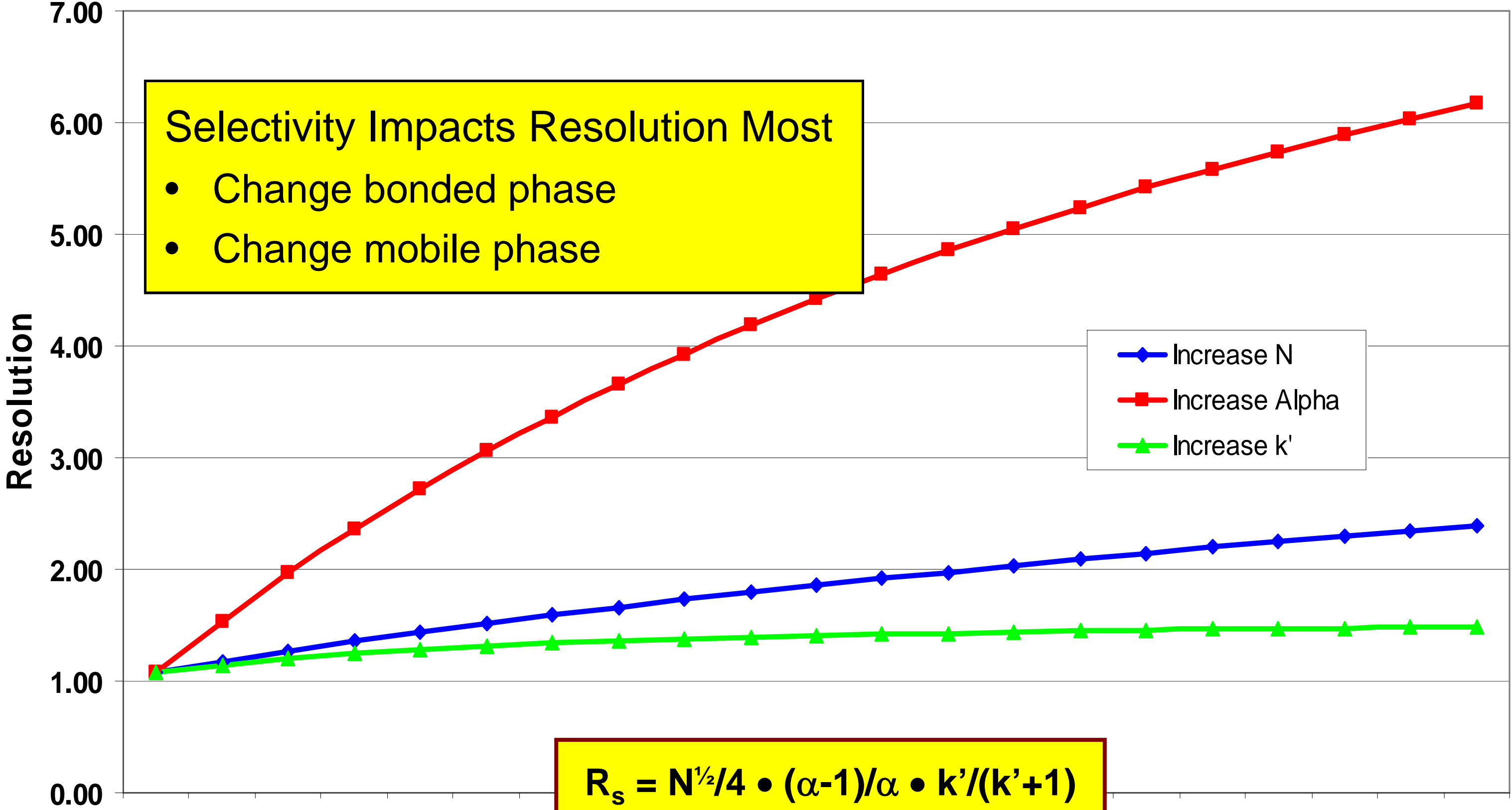
Each Controlling Factor Can Be Combined to Define and Calculate Resolution

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'}{k' + 1}$$

Theoretical Plates ***Selectivity*** ***Retention***

Resolution as a Function of Selectivity, Column Efficiency, or Retention



Plates:	**5000**	**10000**	**15000**	**20000**	**25000**
Alpha:	**1.10**	**1.35**	**1.60**	**1.85**	**2.1**
k':	**2.0**	**4.5**	**7.0**	**9.5**	**12.0**

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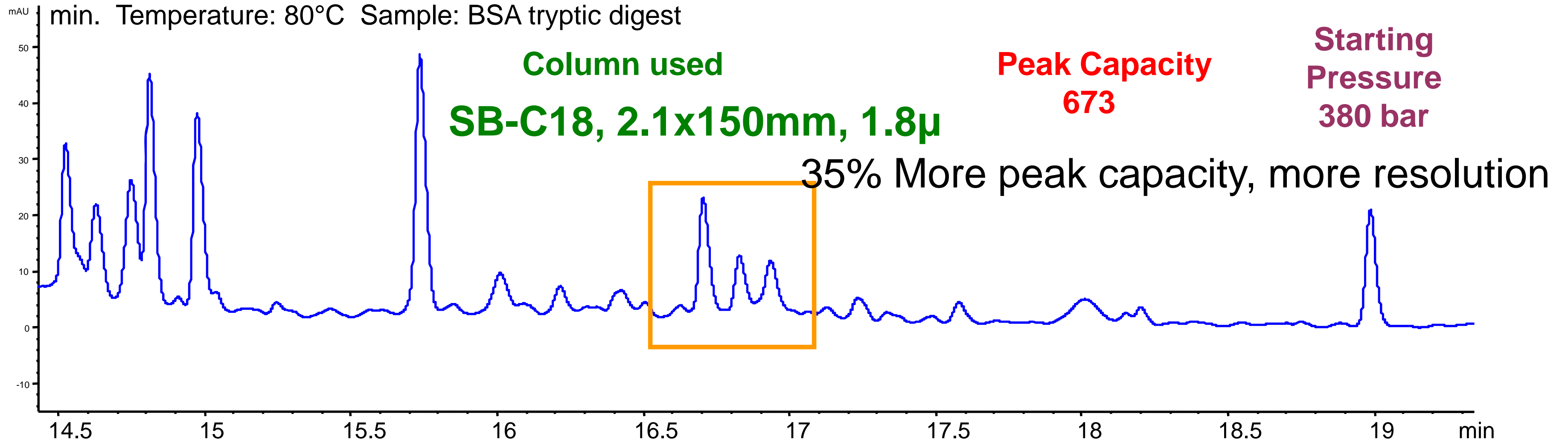
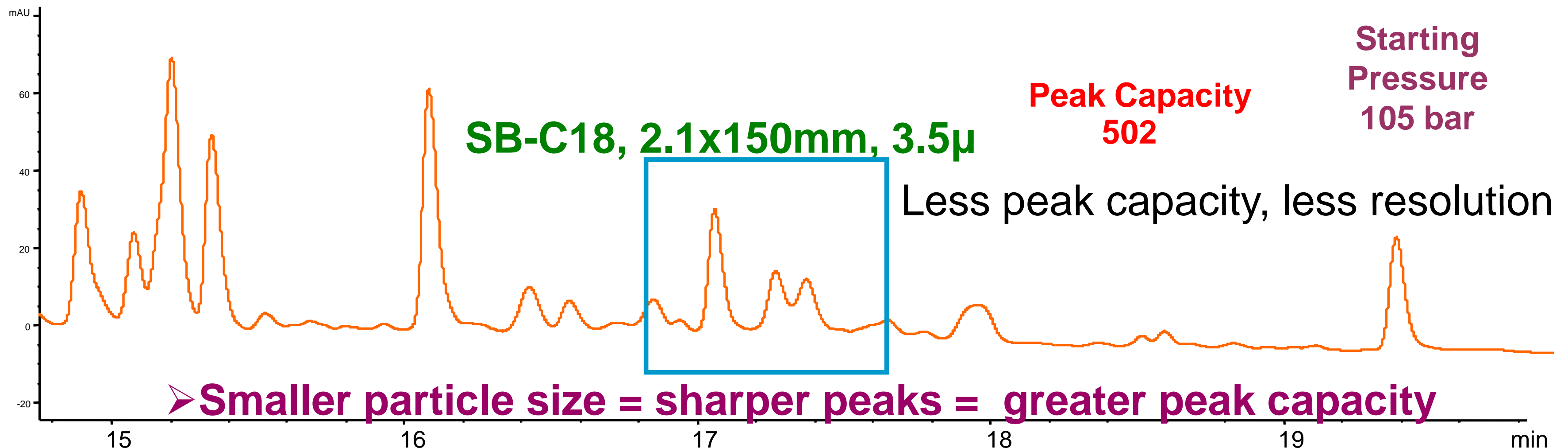
Particle Size - More Peak Capacity with 1.8 um RRHT Columns - Peptide Map of BSA

Conditions: Columns: as listed, Mobile Phase: A:0.1% TFA in Water B:0.08% TFA in ACN Gradient: 5% B to 60%B in 25 min. Temperature: 80°C Sample: BSA tryptic digest





➢ Smaller particle size = sharper peaks = greater peak capacity

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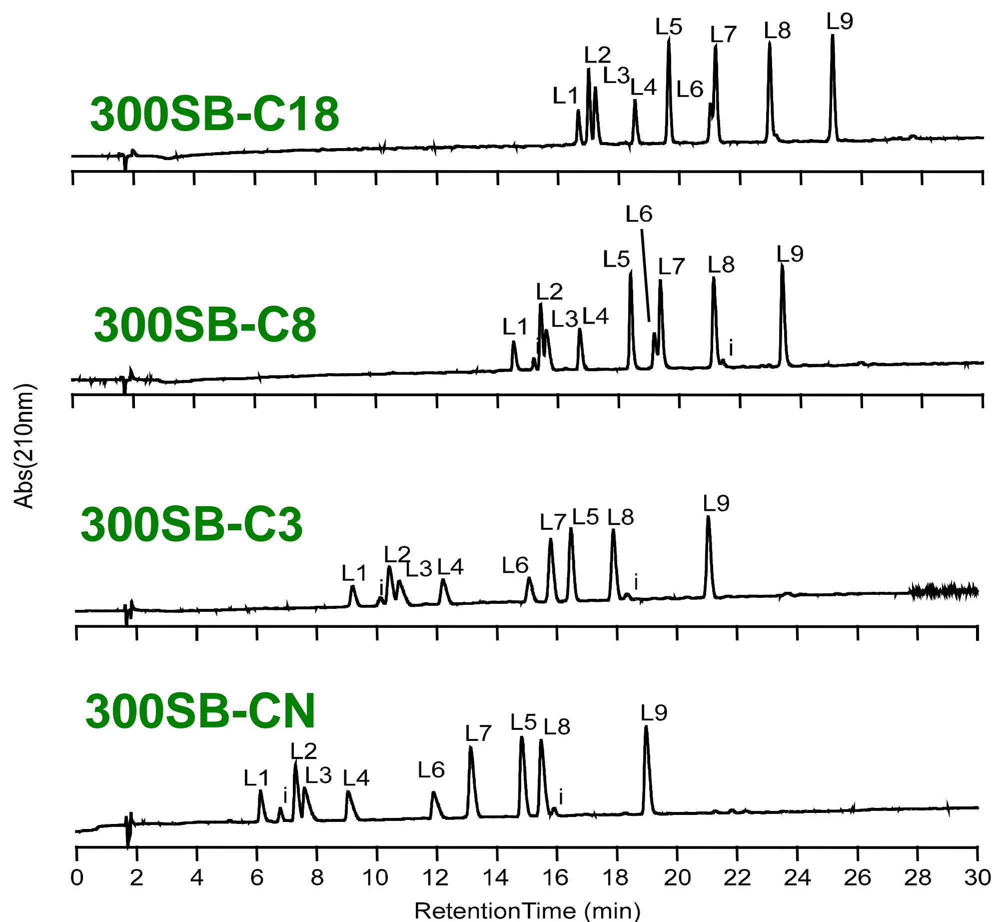
Typical Conditions for Separations of Peptides and Proteins on 300SB Columns

Column:	4.6 x 150 mm, 5 or 3.5 μm 300SB
Mobile Phase:	A: 95:5, H_2O : ACN with 0.1% TFA
	B: 5:95, H_2O : ACN with 0.085% TFA
Flow Rate:	1 mL / min.
Temp:	35 - 40°C
Initial Gradient:	0 - 60% B in 60 min.

Comparison of Small Peptide Selectivity Differences on 300SB Bonded Phases



Conditions:

Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 µm
Mobile Phase: Gradient, 0 - 26% B in 30min.
A = 0.1% TFA in Water
B = 0.1% TFA in Acetonitrile
Temperature: 40°C
Sample: 2 µg of each peptide
Flow Rate: 1.0 mL / min.
Detection: UV-210nm

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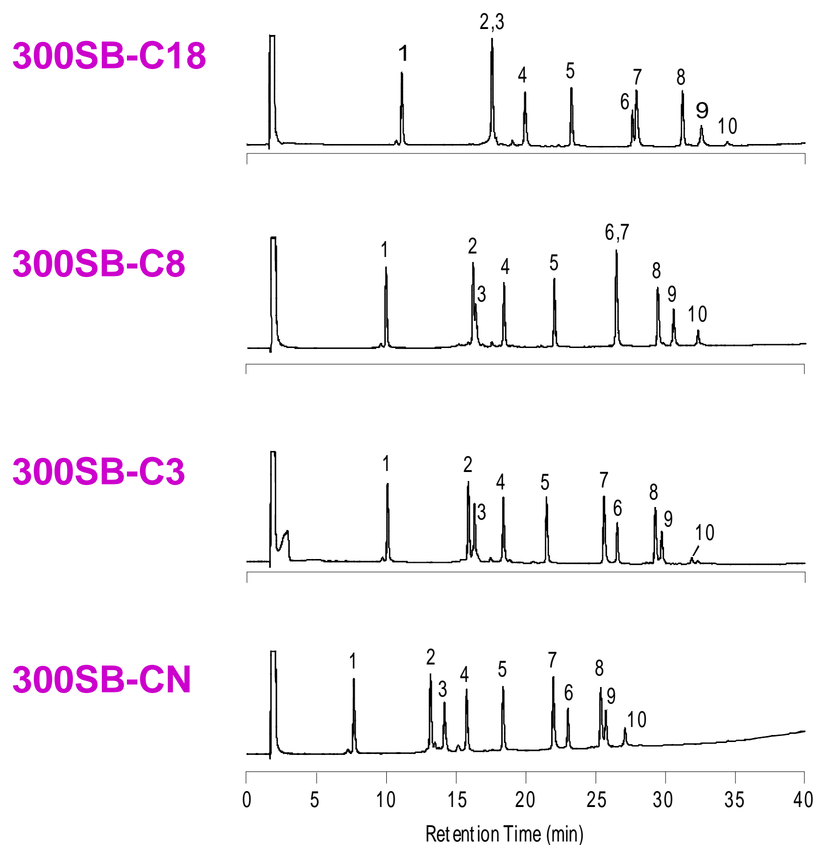
Comparison Separation of Large Polypeptides on 300SB Bonded Phases



Columns:	**ZORBAX StableBond 300SB**	
	4.6 x 150 mm, 5 µm	
Mobile Phase:	Linear Gradient, 25- 70% B in 40 min	
	A: 0.1% TFA in Water	
	B: 0.09% TFA in 80% ACN/20% water	
Flow Rate:	1.0 mL/min	
Temperature:	60°C	
Sample:	3 µg each protein	
	1. RNase	6. CDR
	2. Insulin	7. Myoglobin
	3. Cytochrome C	8. Carbonic Anhydrase
	4. Lysozyme	9. S-100β
	5. Parvalbumin	10. S-100α

- Four different 300SB bonded phases allow selectivity optimization of proteins.

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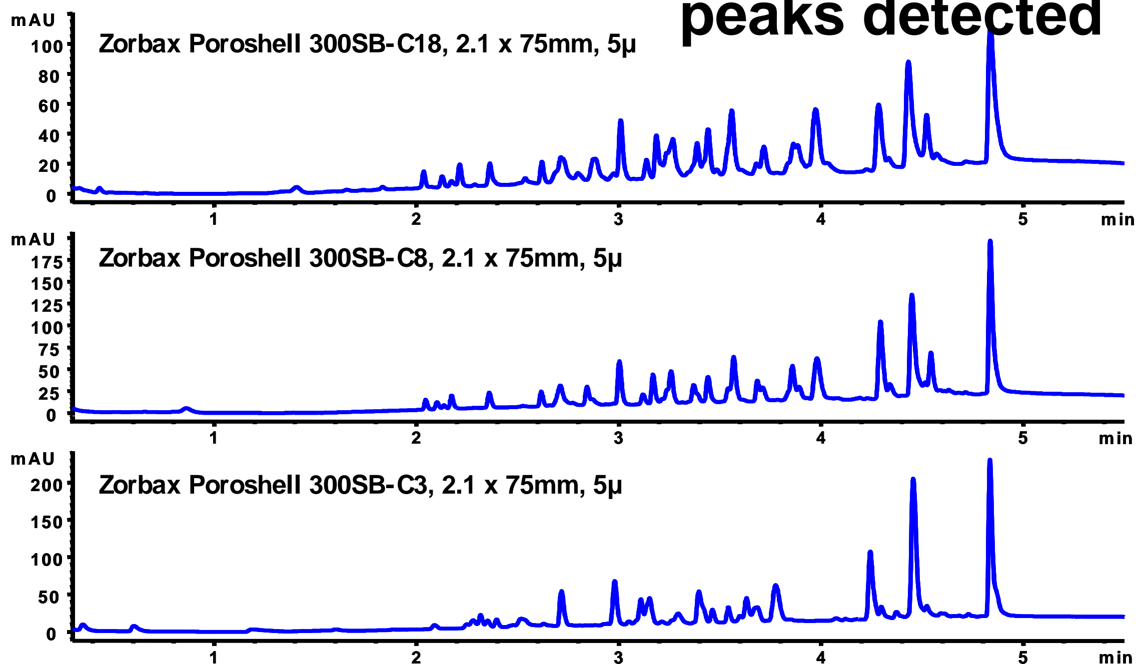
Ultra High Speed HPLC Peptide Maps of a Monoclonal Antibody on Several Zorbax Poroshell Phases

Original method – 120 min t_G using a C18 4.6 x 250 mm – 57 peaks detected



Conditions: Mobile phase A = 0.1% TFA in water Mobile phase B = 0.1% TFA in ACN; Temperature: 70°C; Detection: VWD, 210nm; Injection: 10 µl Lys-C digest of Human Monoclonal Antibody; Flow: 1.0 ml/ min; Gradient: 0 min, 0% B; 5.5 min, 55% B; 5.6 min, 55% B; 7.0 min, 0% B

Aligned

46 to 48 peaks, 1/20 analysis time

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer
Patrik Roethlisberger

- Zorbax Poroshell technology facilitates ultra-fast HPLC analysis of peptides

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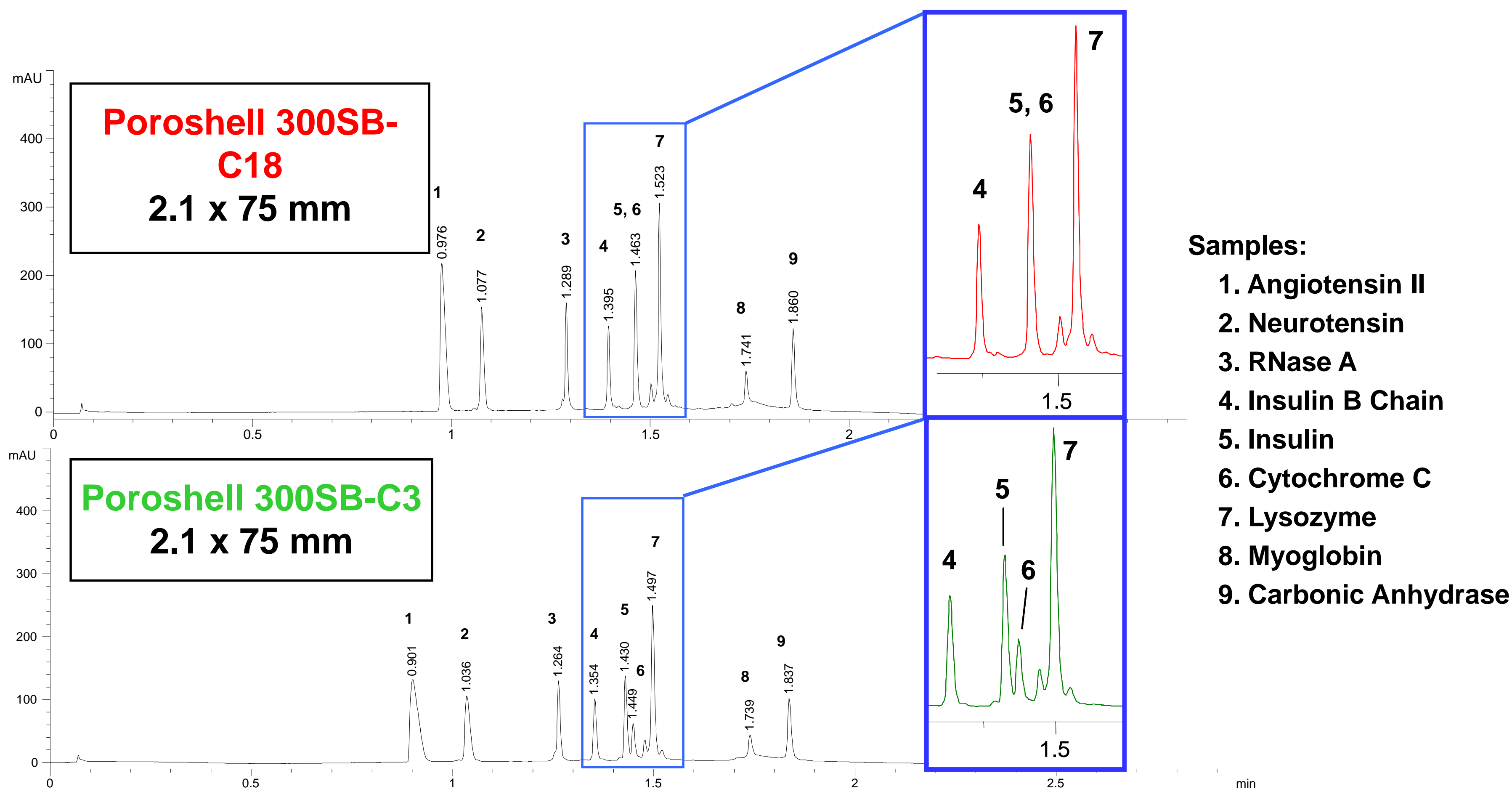
More Poroshell Bonded Phases Provide Selectivity Options to Enhance Resolution:



Column: Agilent Poroshell (2.1 x 75 mm); Temp.: 70 ^{0}C; Flow: 0.5 mL/min; Det: UV 215 nm
Mobile Phase: A= 0.1% TFA/H_2O, B= 0.07% TFA/ACN; Gradient: 5-100% B in 3.0 min

- Changing from SB-C18 to SB-C3, within the Poroshell family results in resolution of peaks 5 and 6, still in 3 min!

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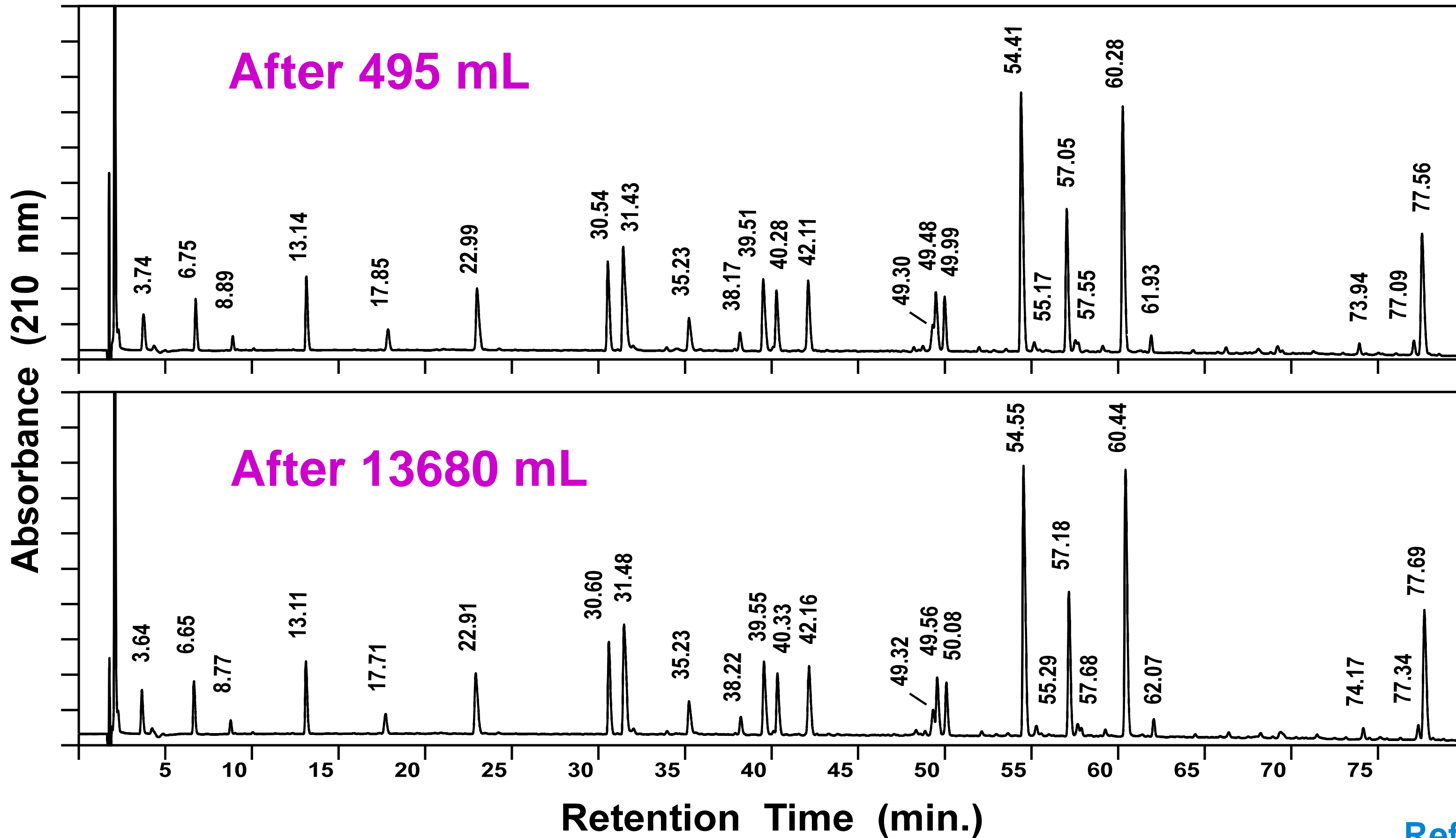
Extended Column Lifetime of ZORBAX 300SB-C8 rhGH Tryptic Digest

Column: ZORBAX 300SB-C8, 4.6 x 150mm Mobile Phase Gradient 0 - 60% in 120 min.A= 0.1% TFA in Water, B= 0.086% TFA in ACN Temp.: 40°C Flow Rate: 1mL/min. Det. UV 210nm Sample: 50 µg of rhGH Tryptic Digest



Reference 3

000336P1.PPT

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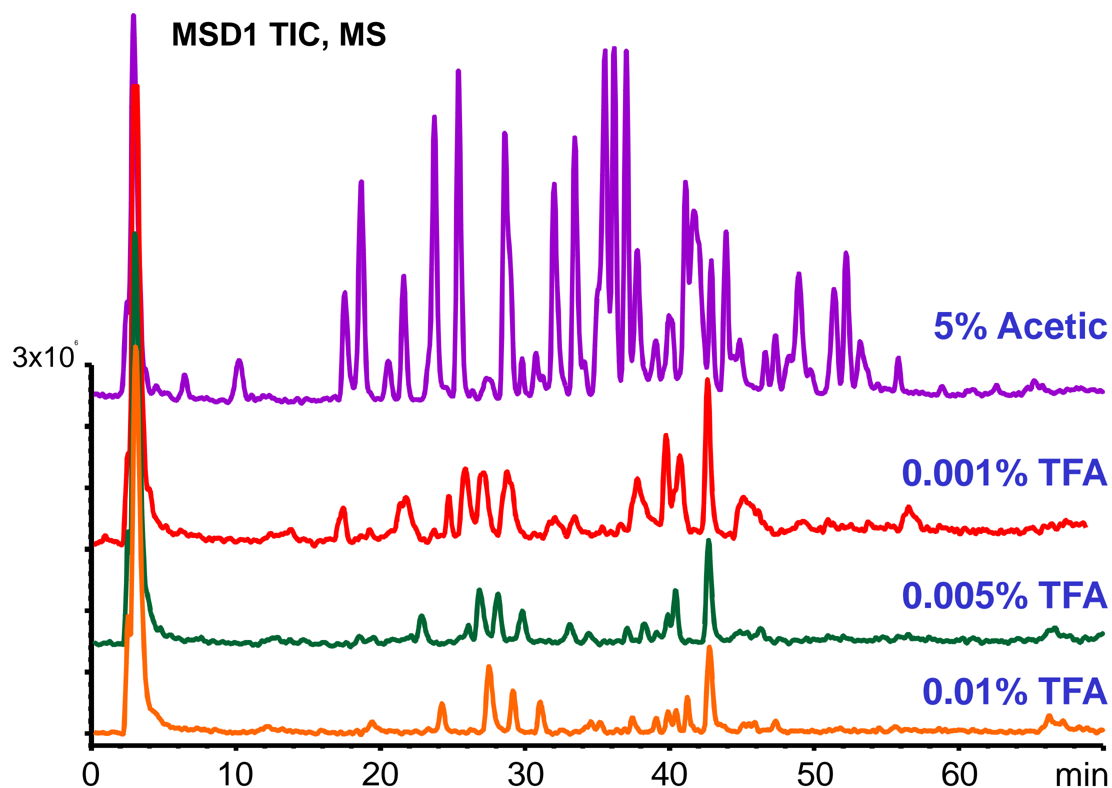
LC/MS Sensitivity vs. Mobile Phase Modifier

TFA vs. Acetic Acid



Column:	**ZORBAX 300SB-C3**
	2.1 x 150 mm, 5 µm
Mobile Phase:	Gradient: 0% B hold for 5 min.
	0 – 40% B in 55 min.
	40 – 100% B in 20 min
	A: 5% acetic acid
	B: Acetonitrile
Flow Rate:	0.2 mL/min
Instrument:	Agilent 1100 MSD
Sample:	GluC Digest of BSA

Reference 1

- A significant increase in sensitivity was observed using 5% acetic acid instead of TFA.
- Reducing TFA concentration to 0.001% improved sensitivity very little.

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Typical Conditions for Separations of Peptides and Proteins on 300Extend-C18 Columns at High pH

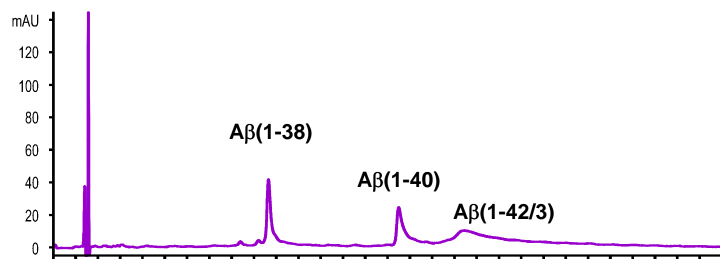
Column:	**4.6 x 150 mm, 5 or 3.5 μm 300Extend-C18**
Mobile Phase:	**A: 20 mM NH_4OH in water**
	B: 20 mM NH_4OH in 80% ACN
Flow Rate:	**1 mL / min.**
Temp:	**25 - 30°C**
Initial Gradient:	**5 - 60% B in 30 min.**

- At low pH use the same conditions as on the 300SB columns.

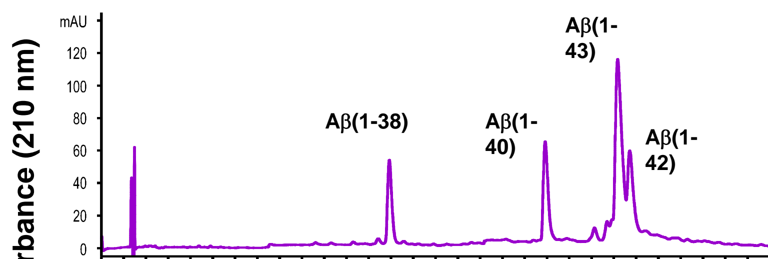
High pH Can be Used for Separating Hydrophobic or Other Low-Solubility Peptides

Comparison of Aß Peptide RP-HPLC Separations at Low and High pH

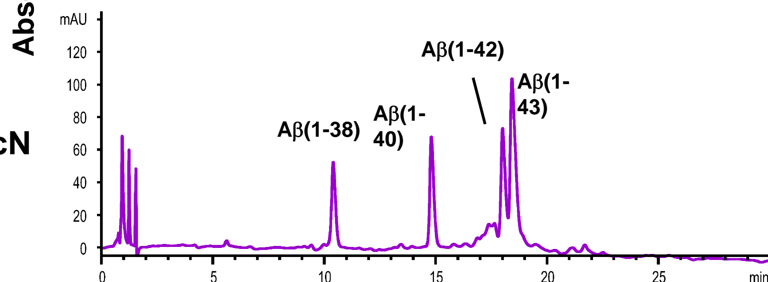
TFA Conditions, 25°C
A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
33-45%B in 30 min.



TFA Conditions, 80°C
A- 0.1% TFA in water
B- 0.085% TFA in 80%AcN
29-41%B in 30 min.



NH_4OH Conditions, 25°C
A- 20 mM NH_4OH in water
B- 20 mM NH_4OH in 80%AcN
26-38%B in 30 min.



Column:	**ZORBAX 300Extend C18**
	2.1 x 150 mm, 5 µm
Flow Rate:	0.25 mL/min
Sample:	5 µL sample (100 pmol each)

Amyloid ß Sequences:

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln

Lys Leu^{17} Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala

Ile Ile Gly Leu Met Val Gly Gly^{38} Val Val^{40} Ile Ala^{42} Thr^{43}-COOH

Reference 1

- **High pH and room temperature improve peak shape, recovery and change selectivity.**

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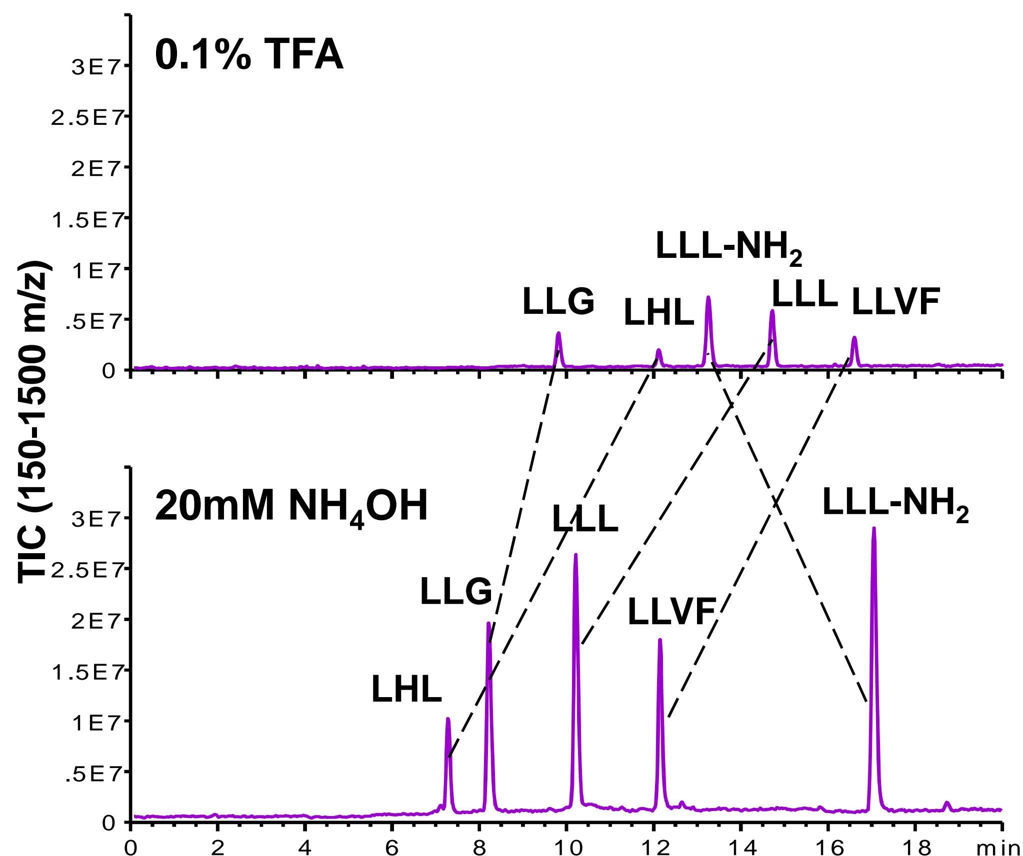
Use Extend-C18 for Different Selectivity at High and Low pH

Comparison of TFA and NH_4OH For Peptide RP-HPLC \ ESI-MS Analysis



Column: **ZORBAX Extend C18, 2.1 x 150 mm**
Flow rate: 0.25mL/min
Temp: 25°C
Gradient: 5-60% B in 20 min;
LC/MS: Pos. Ion ESI- Vf 70V, Vcap 4.5 kV, N2- 35 psi, 12L/min, 300°C
4 µL (50 ng each peptide);

TFA Conditions:
A- 0.1% TFA in water
B- 0.085% TFA in 80% AcN

NH_4OH Conditions:
A- 20 mM NH_4OH in water
B- 20 mM NH_4OH in 80% AcN

Reference 5

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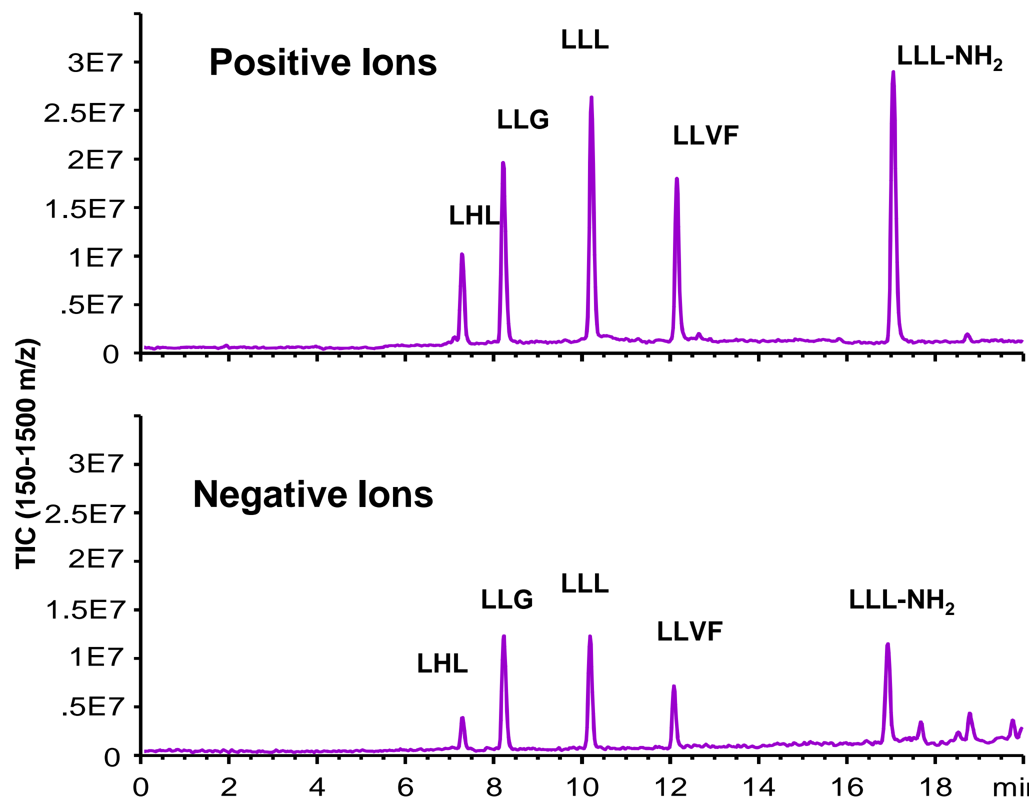
Peptide RP-HPLC/ESI-MS Using NH_4OH Mobile Phase Yields Positive and Negative Ion Spectra



Column:	**ZORBAX Extend C18 2.1 x 150 mm, 5 µm**
Flow rate:	0.25mL/min
Temp:	25°C
Gradient:	5-60% B in 20 min
LC/MS:	Pos. Ion ESI- Vf 70V, Vcap 4.5 kV, N2- 35 psi, 12L/min, 300°C
Sample:	4µL (50 ng each peptide)

Reference 2

- The Extend-C18 column is ideal for LC/MS of proteins and peptides at high pH
- Both positive and negative ion MS are possible with NH_4OH mobile phase.

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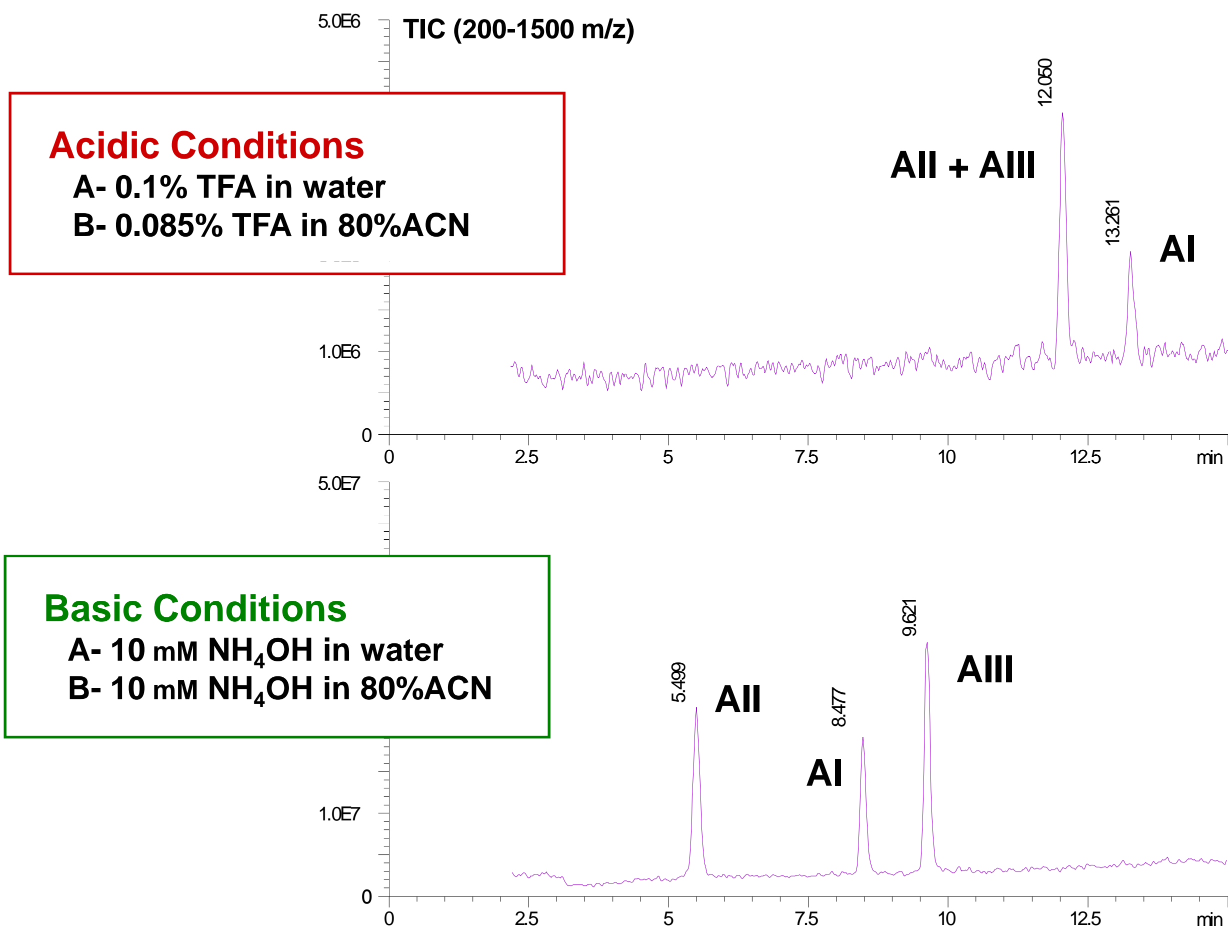
Angiotensins Separation at High and Low pH on Extend-C18



Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 µm

Agilent 1100	
MSD:	Pos. Ion ESI
	Vf 70V, Vcap 4.5 Kv
	N_2=35psi, 12L/min.
	325°C
Gradient:	15-50%B / 15 min.
	0.2 mL/min
Temp:	35°C
Sample:	2.5 µL
	(50 pmol each)

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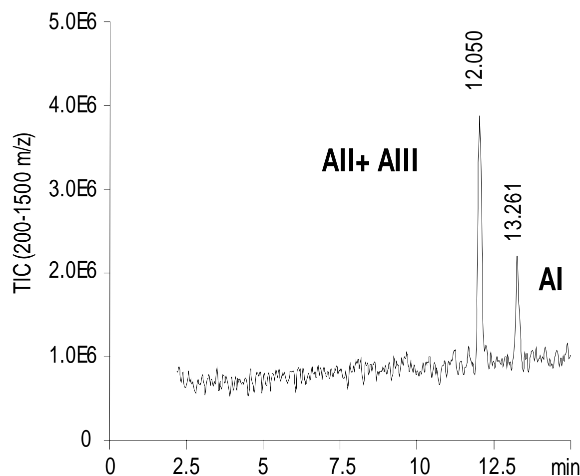
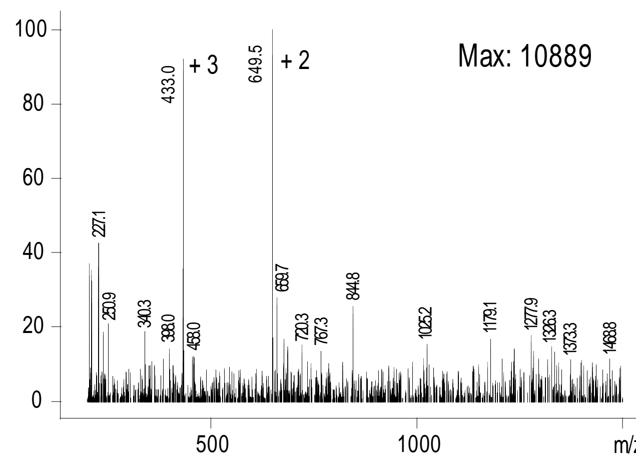
LC/MS Bonded-Phase Selection
Low vs. High pH on Extend-C18

Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 μm Flow Rate: 0.2 mL/min Temperature: 35°C
Mobile Phase Gradient: 15-50% B in 15 min. A: 0.1% TFA in water B: 0.085% TFA in 80% ACN
LC/MS: Pos. Ion ESI – Vf 70V, Vcap 4.5 kV, N_2-35 psi, 12L/min., 325°C Sample: Angiotensin I, II, III 2.5 μL (50 pmol each)





• Acidic conditions can not resolve all three Angiotensins.

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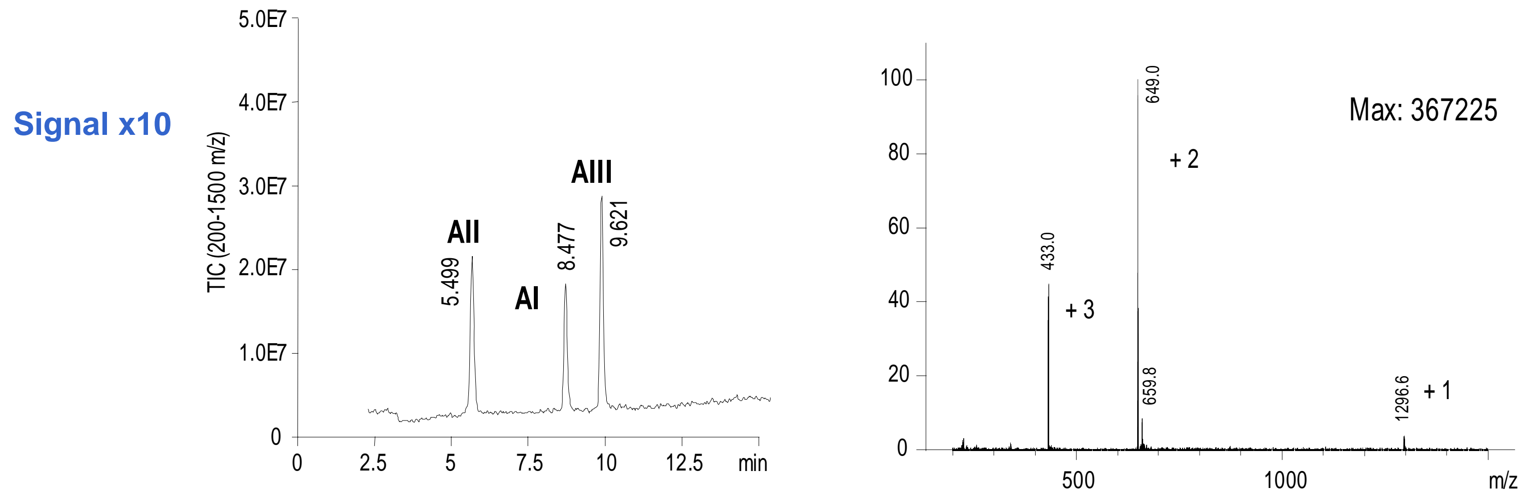
LC/MS Bonded-Phase Selection at High pH

Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 μm Flow Rate: 0.2 mL/min Temperature: 35°C
Mobile Phase Gradient: 15-50% B in 15 min. A: 10 mM NH_4OH in water B: 10 mM NH_4OH in 80% ACN
LC/MS: Pos. Ion ESI – Vf 70V, Vcap 4.5 kV, N_2-35 psi, 12L/min., 325°C Sample: Angiotensin I, II, III, 2.5 μL (50 pmol each)



At high pH all 3 Angiotensins are resolved and the mass spectrum shows improved spectral clarity.

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Conclusions

Ion exchange chromatography, size exclusion chromatography and reversed phase HPLC columns are the most popular choices for the analysis of proteins and peptides.

A variety of reversed phase columns make optimization of protein separations possible. And a variety of column configurations – length and id make it easy to find the right column for any size sample.

Movement towards faster analytical separations, smaller sample sizes, and more sensitive detection are needed for increased sample throughput and proteomics applications.

Proteomics applications use the same columns and chromatographic techniques, but with so many proteins present in each sample, 2-D techniques (2-D LC/MS) and nano scale reversed phase columns are necessary.

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BONUS DISCUSSION
2 – Dimensional HPLC

- Why You Might Want To Use It
- How It Works
- What Equipment You Will Need

HPLC of Proteins for Proteomics and Other Complex Protein Mixtures

Goal – to analyze many proteins at once in a single sample

Requires 2-D techniques to obtain information on all proteins present – use 2 different LC techniques or 2 different techniques

- HPLC – ion exchange and reversed phase
- Gel electrophoresis –1-D and 2-D techniques
- MS for identification

Proteins (mixtures of ~ 10^4 to 10^5 different proteins) are present at low –high levels – requires high sensitivity techniques

- Nano LC columns for maximum sensitivity
- High sensitivity LC/MS

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2D/Multidimensional HPLC Principle

Two or more modes of HPLC with independent physical characteristics

- Ion Exchange/Affinity/SEC
- Reverse Phase

OFF-Line or ON-Line

- ON-line more automatable
- OFF-line allows more solvent flexibility, cleaner samples and possibly more sample capacity
- OFF-line still has unattended LC/MS

OFF-Line

- Run first dimension and collect peaks
- Inject fractions on second dimension or reverse phase/MS
- Run 2D, then spot to or inject into MS

ON-Line

- Automate both separations on a single instrument with direct interface to MS

2-D-HPLC for Proteomics

Advantages

Most sensitive for low abundance proteins

Easier automation
- proteins stay in liquid
- fraction collection
- sample preparation

Flexibility
- separation technique
- chemistry

Time - faster

Application for most types of proteins

Concentrates sample

Direct coupling to MS

Limitations

Less comparative data

Digestion prior to separation

Applications

- **Targeted (functional) proteomics**
- **Small, basic and hydrophobic proteins**
- **Peptide maps**

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2-D Gel Electrophoresis for Proteomics

Advantages

High resolution of complex protein samples (> 1000 proteins)

Historical method of choice

Runs in parallel

pI and MW info from method

Samples can be stored during method

Applications

- **Superior for global proteomics**
- **Small, basic and hydrophobic proteins**

Limitations

Labor intensive and time consuming

Difficult to handle and automate

Samples are "in gel"

poor ability to handle proteins that are
- hydrophobic
- basic
- small or large

Low abundant proteins are masked by high abundant proteins

Sample loading capacity limited

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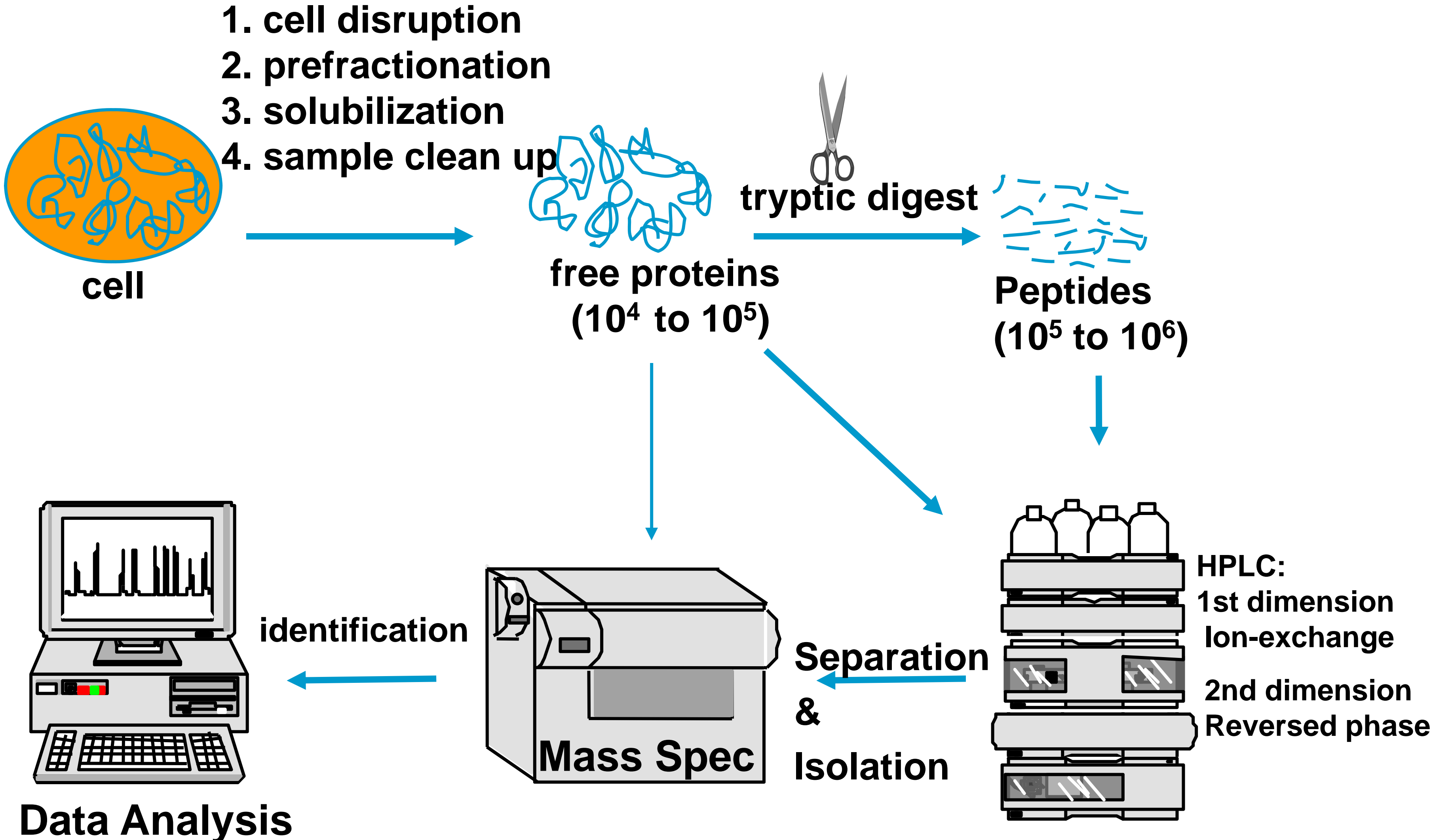
Proteomics With 2D-LC/MS - Workflow



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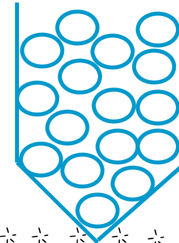
Applications of 2-D-LC/MS Proteomics

- **Mining**
  → - identify as many proteins as possible
- **Protein expression profiling**
  → - differentiation, disease status, developmental state
- **Mapping of protein modifications**
  → - phosphorylation, glycosylation etc.
- **Protein-network mapping**
  → - search for interacting proteins, complex identification

Replacement and/or supplementation of 2-D-Gel electrophoresis

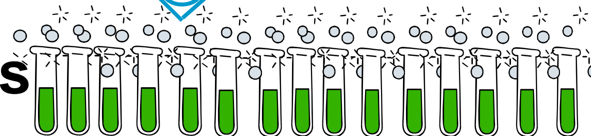
Theoretical Resolving Power of 2D-LC/MS

1. Dimension



e.g. cation exchange, gel filtration

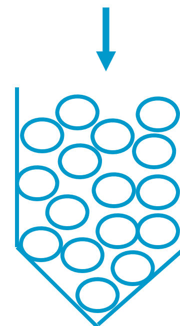
15 fractions



2. Dimension
(15 x 2. Dimension)

Gradient run time L = 90 min,
peak width 4 σ ~25s

n_c = 216



e.g. reversed phase

Peak capacity $n_c = L/(4\sigma)$

L total elution time

σ average standard deviation of peaks

Total Peak capacity : 1. Dim * 2. Dim → 15 * 216 = 3240

3. Dimension Mass Spec → 7 * 3240 = 23,000 peptides

(Wolters Et Al

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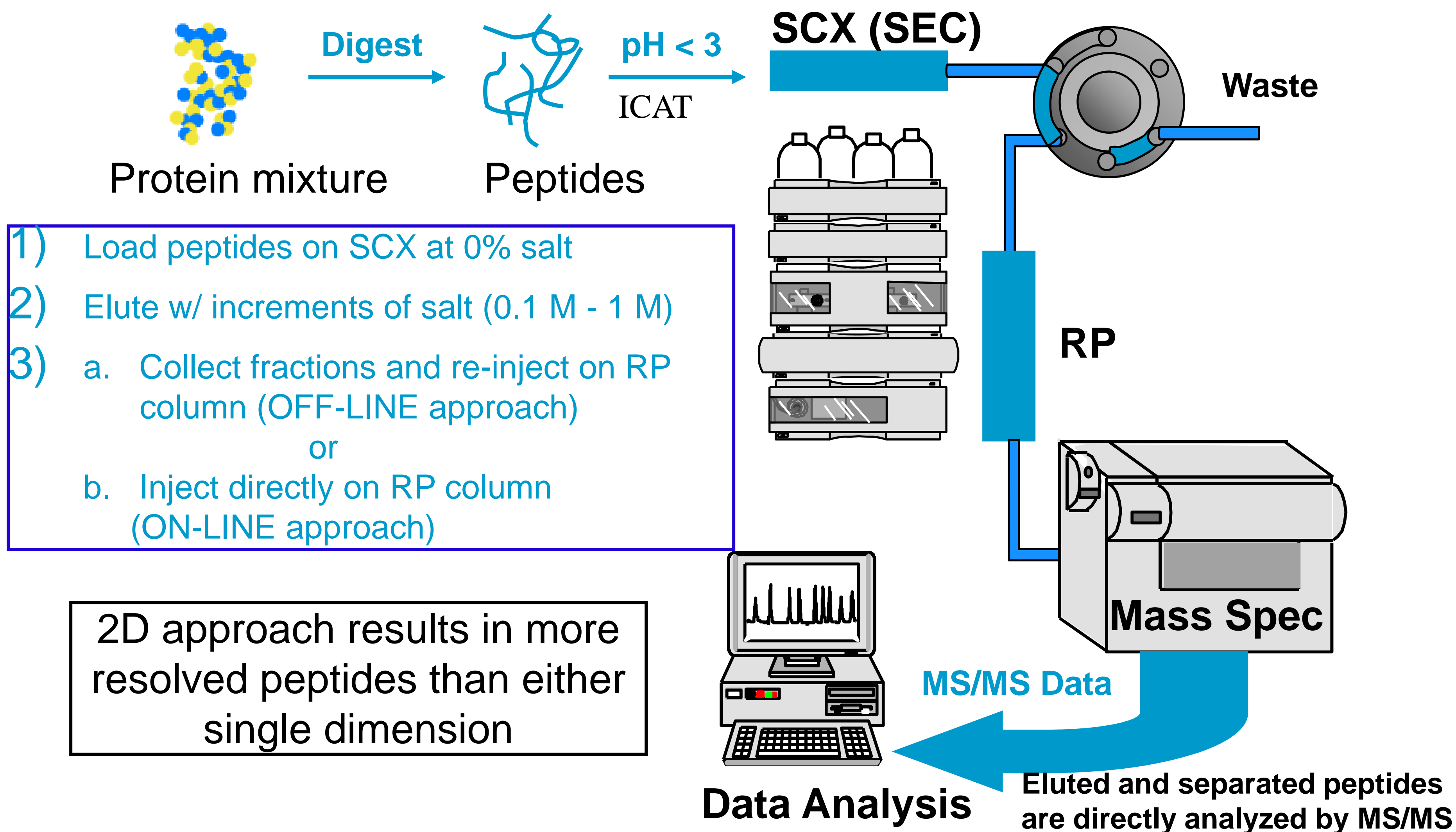
2-D HPLC: Cation Exchange and Reversed Phase Chromatography



1) Load peptides on SCX at 0% salt
2) Elute w/ increments of salt (0.1 M - 1 M)
3) a. Collect fractions and re-inject on RP column (OFF-LINE approach)
   or
   b. Inject directly on RP column (ON-LINE approach)

2D approach results in more resolved peptides than either single dimension

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2D-LC Example Method

Ion exchange trapping column

0.3 x 35 mm Poly-LC SCX, Flow rate 20 µl/min (5 mM ammonium acetate, 0.04 % formic acid in water)

2 µl injection of sample, 8 µl injection of salt gradient using injector progam (ammonium acetate)

Reversed Phase trapping/enrichment column

0.3 x 35 mm ZORBAX 300SB-C18, Flow rate 20 µl/min (5 mM ammonium acetate, 0.04 % formic acid in water)

5 minutes loading

Used when loading large volumes of dilute peptides/proteins in high salt or detergent matrix

Trapping columns used to remove salts and detergents prior to MS

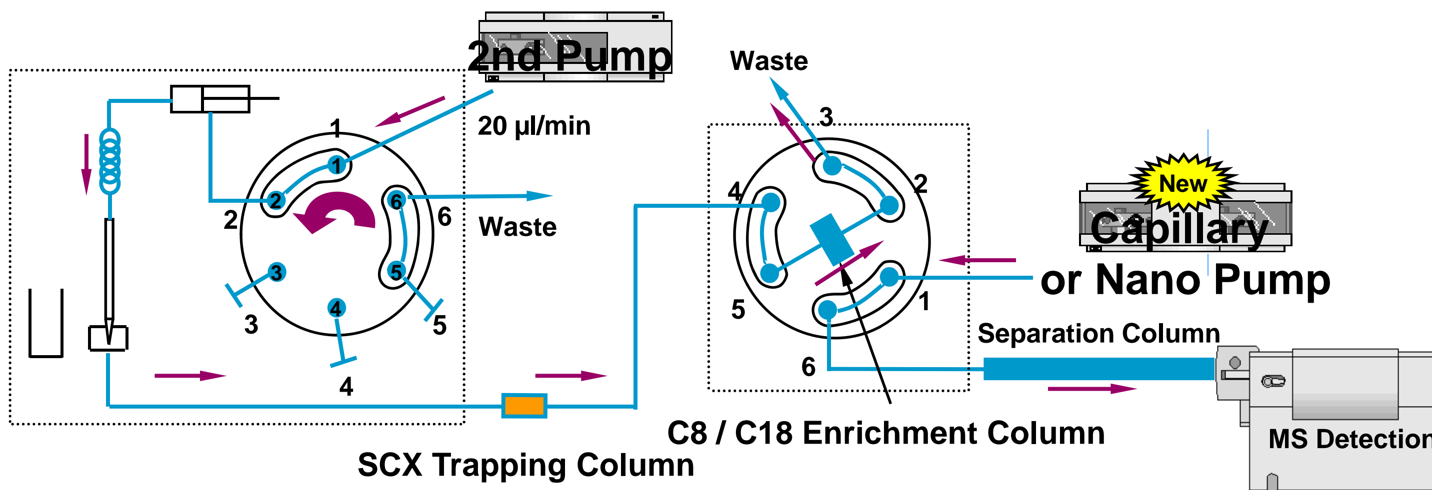
Analytical column

0.3 x 150 mm ZORBAX 300SB-C18, Flow rate: 4 µl/min

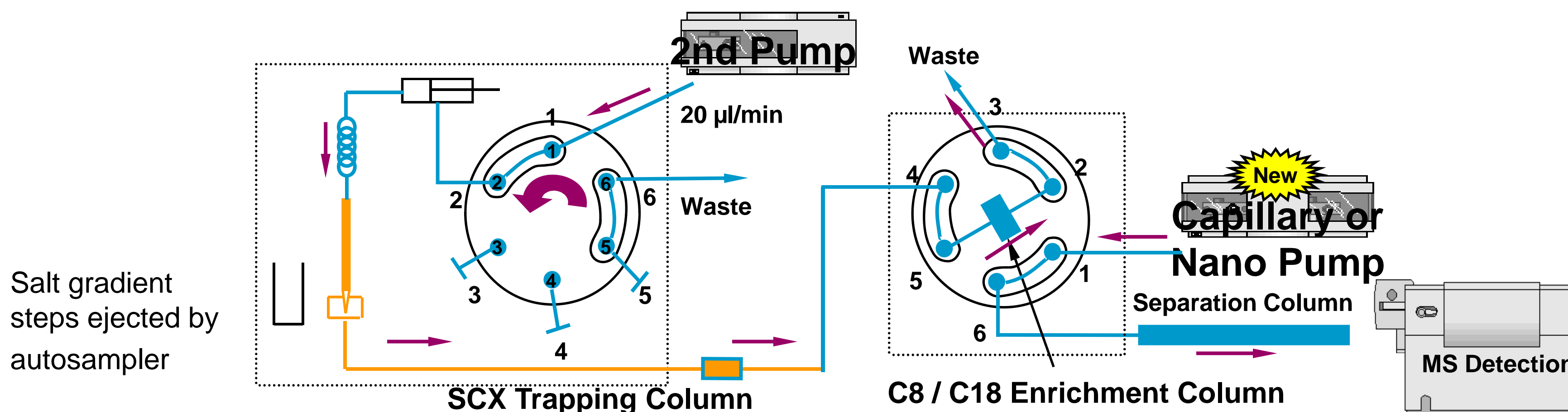
Gradient: A: 0.1 % formic acid, B: Acetonitrile + 0.1% formic acid, Start at 5% B, 3 % B per minute

2D-LC Method Diagram - SCX

LOAD SAMPLE ON SCX TRAPPING COLUMN:



STEP ELUTE SAMPLE ON from SCX to C8 TRAPPING COLUMN:



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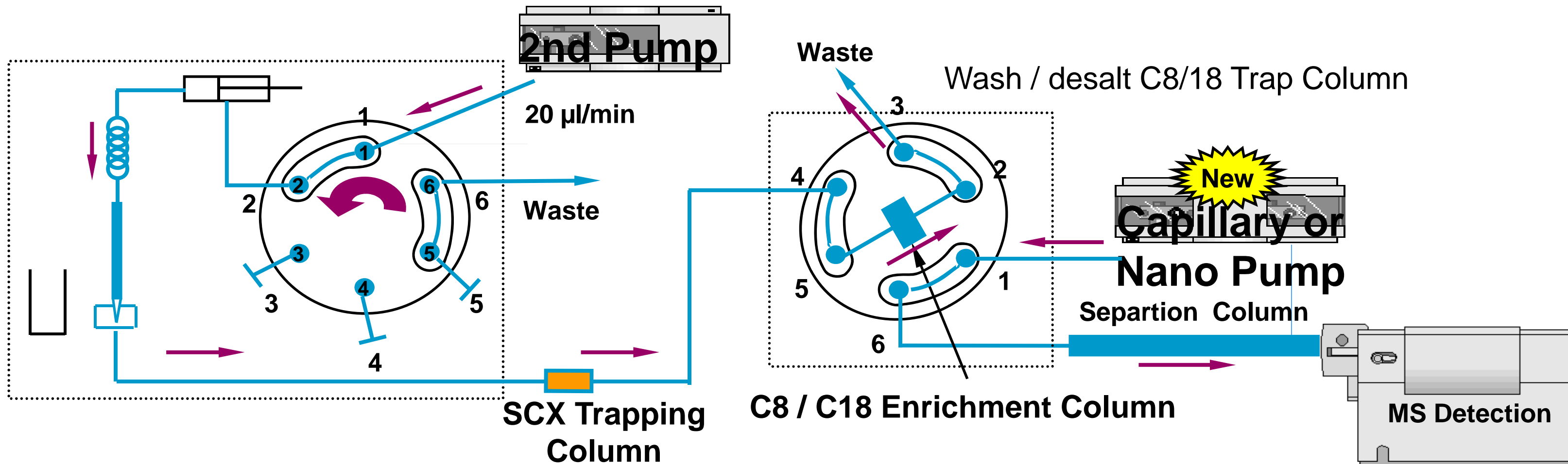
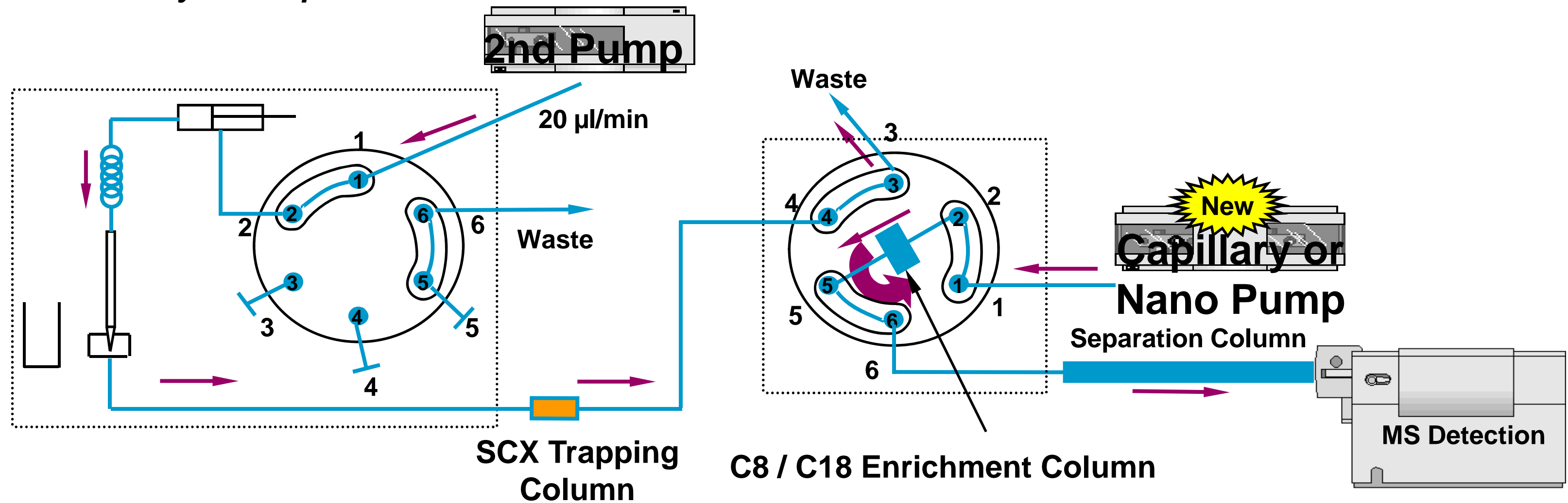
2D-LC Method Diagram – RPLC/MS

Wash C8 TRAPPING COLUMN:



Analyze Sample



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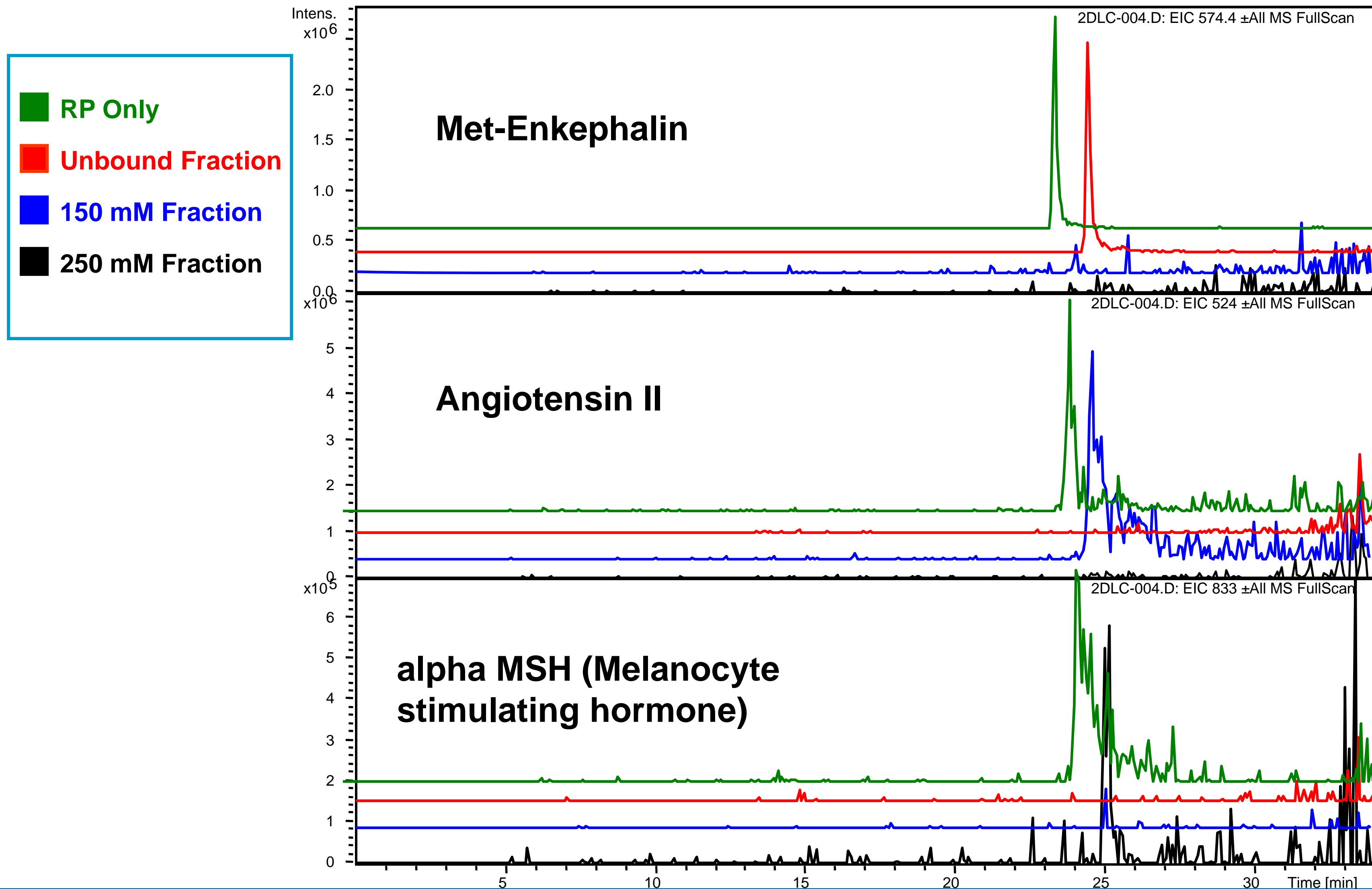
2-D LC/MS Analysis of Peptide Mix



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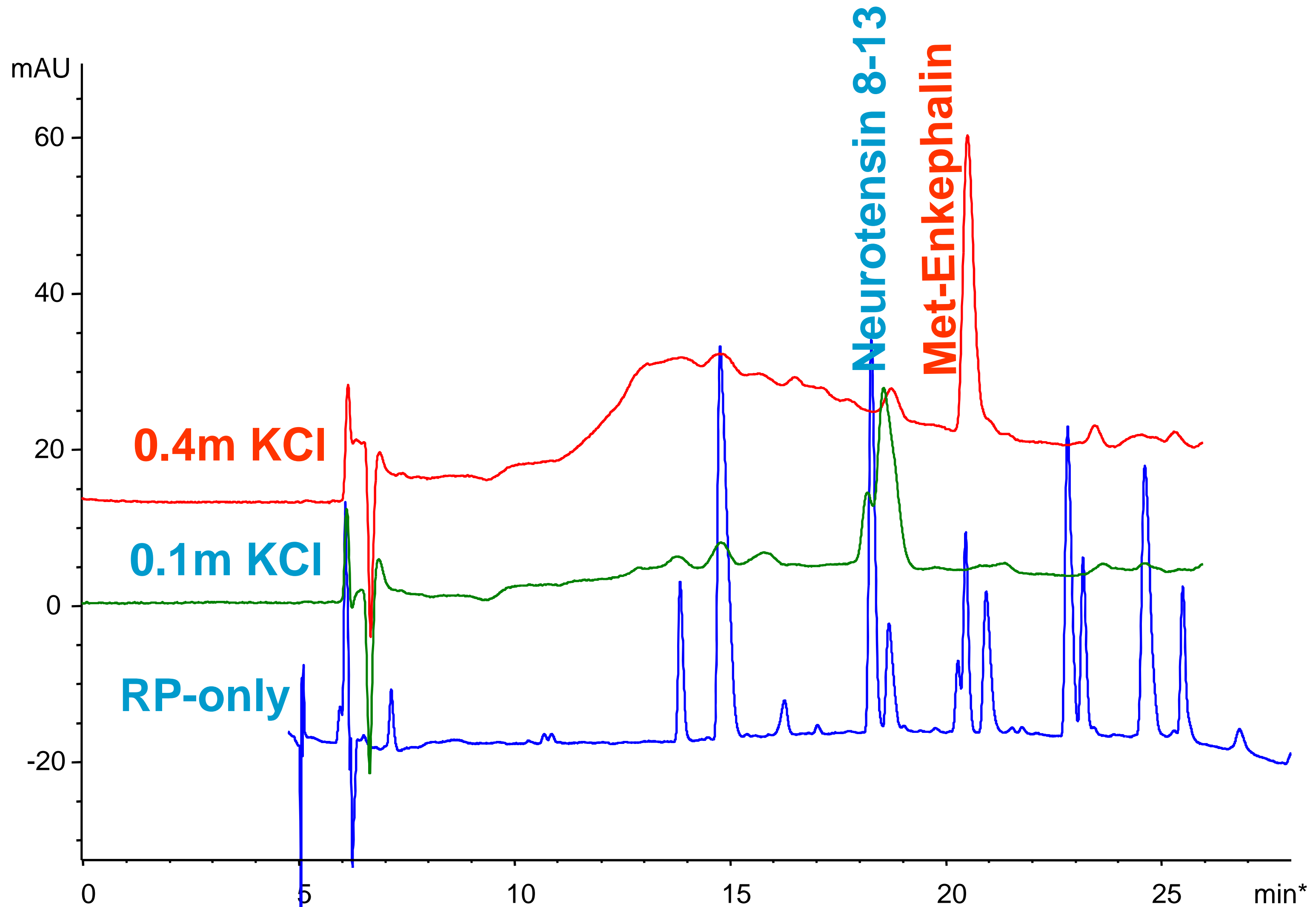
2D-HPLC: Cation Exchange and Reversed Phase
Overlay of Direct-injected Standard vs. KCl Fractions



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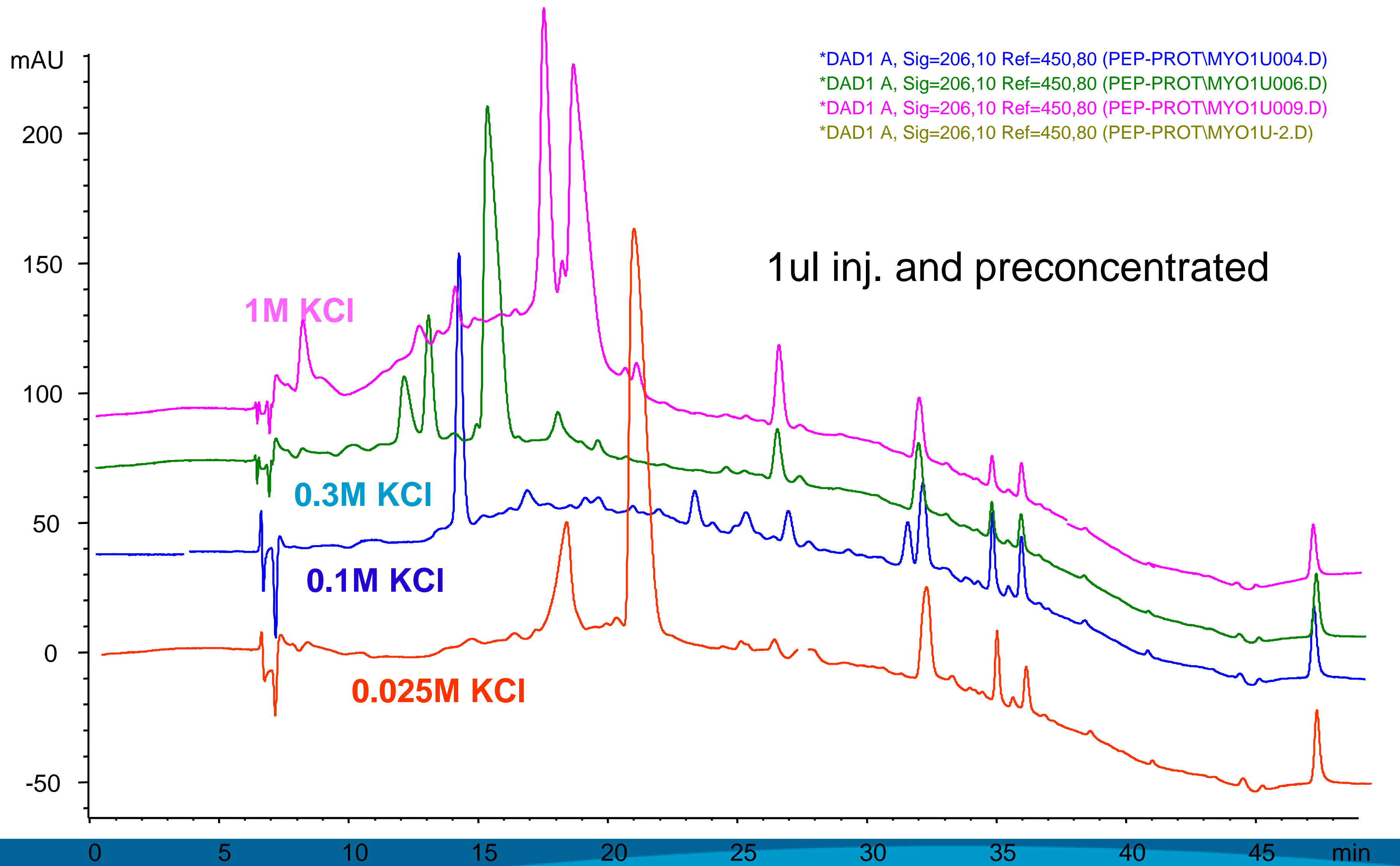
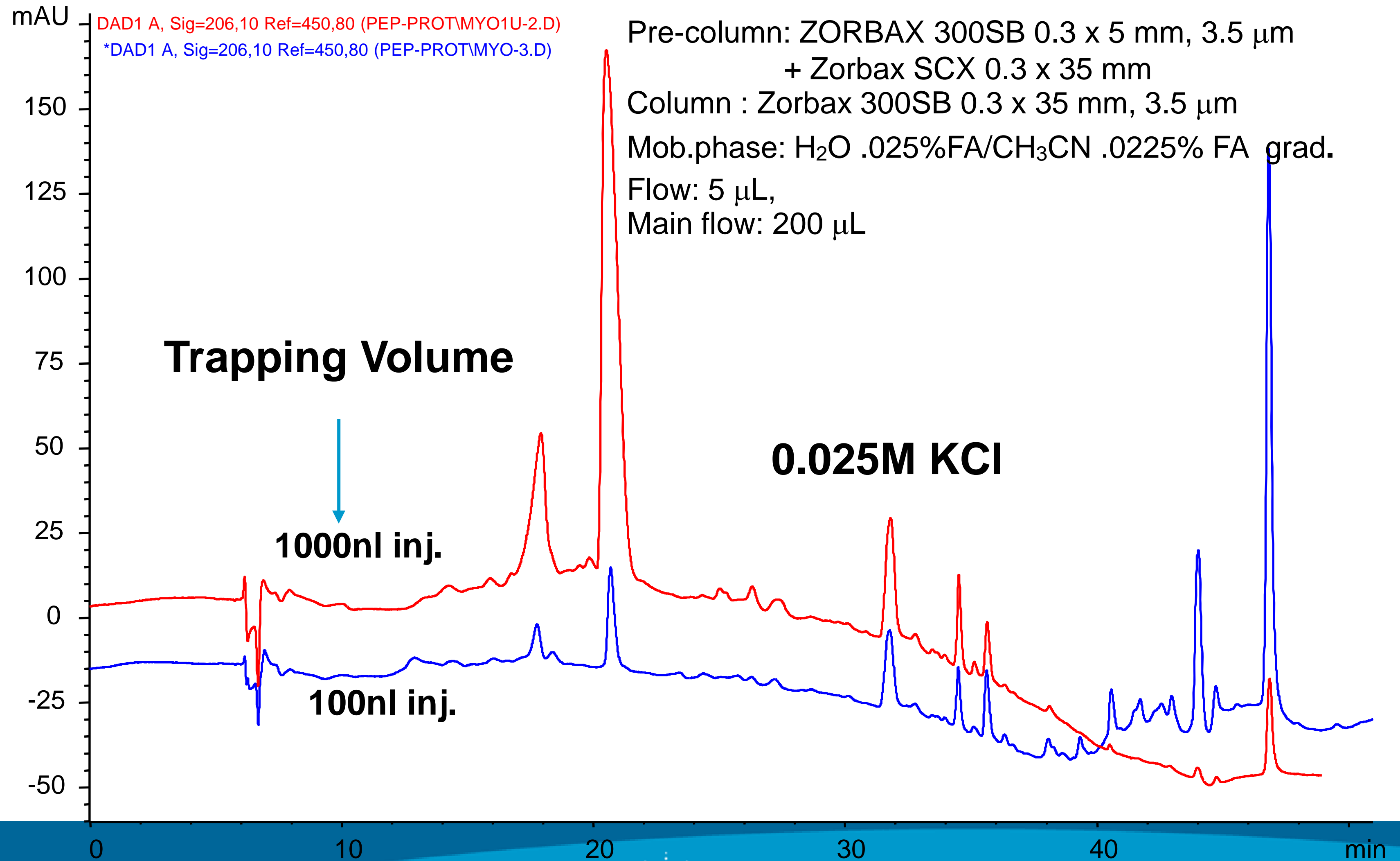
2-D Analysis of Myoglobin Tryptic Digest
Elution at Different KCl Concentrations



2-D Analysis of Myoglobin Tryptic Digest
- 100 and 1000 nL injection volumes



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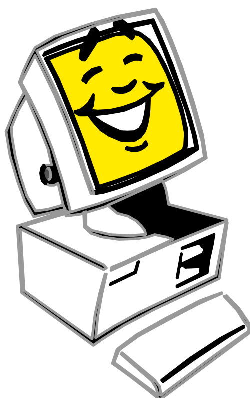
Agilent Technical Support

LC Column Support

800-227-9770 (phone: US & Canada)

Select option 4, then option 2 for LC.

www.agilent.com/chem



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