Improving HPLC Selectivity and Resolution for Protein and Peptide Separations

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September 30, 2008
Column Chromatography Methods for Peptide/Protein Separations

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

**Elution with salt or pH change**
Positively charged salt ions replace bound peptide molecules

**Fraction collection**
Positively charged sample is loaded and bound to column

**Peptides are separated according to difference in their net charge**
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
Bio-Monolith: Rapid and High Resolution Separation of Macro Bio-Molecules

- Large Proteins
- IgG Antibodies
- IgM Antibodies
- pDNA
- Adenovirus
- phage
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).
Macro Bio-Molecule Analytics

Antibody Separations


pDNA Analytics

Quantitation of Virus
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](http://www.validated.com)
Method duration: from 14.8 min to 7.4 min.

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), ST1 (4) (BioRad). Method: gradient from 0-50% buffer B in 125 cV.
Consecutive injections of a mixture of 4 test proteins (BioRad) on a CIM QA Analytical Column
Gel Filtration (Size Exclusion) Chromatography

1. big molecules cannot enter pores → fast elution
2. Small molecules enter pores → later elution

Protein/peptide sample contains molecules of different size
Column contains porous particles
Particles act as molecular sieve

Separation according to size
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

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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.
SEC Separation Mechanism

Inject

Elution volume

Mobile phase

Column packing

Pores

Macromolecule Analytes

A B C

A B C

A B C
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.
Mechanism of SEC Separation

Small Change in elution volume ---> big change in molecular weight ---> excellent HPLC equipment required!
Characteristic Elution Profiles of a Basic Protein

**CYTOCHROME C**

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
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
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
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
Effect of Column-Length on Efficiency and Resolution

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.
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.
  - Rs = 2.3
  - N = 15,680

- **TIME= 30 min.**
  - 0.5 ml / min.
  - Rs = 2.1
  - N = 11,939

- **TIME= 15 min.**
  - 1 ml / min.
  - Rs = 1.9
  - N = 8,913

- **TIME= 7.5 min.**
  - 2 ml / min.
  - Rs = 1.7
  - N = 6,623

- **TIME= 3.5 min.**
  - 5 ml / min.
  - Rs = 1.4
  - N = 3,588
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
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.
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

Elution by increasing hydrophobicity: concentration of organic solvent

Organic solvent elutes peptide at hydrophobic interaction site

Elutropic force

Water>Methanol>Acetonitrile>n-Propanol>THF

Polarity

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
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
Improved Peak Shape for Large Molecules in Solution

**Columns:** 4.6 x 150 mm, 5 μm  
**Mobile Phase:** 60% MeOH: 40% 0.1% TFA  
**Flow Rate:** 0.75 mL/min  
**Temperature:** RT  
**Detection:** UV 282 nm  
**Sample:** Tylosin (MW 916)

- The size of a molecule in solution determines which pore size column is best.
- The narrower peak width indicates unrestricted access to the pores.
Recovery of Polypeptides from ZORBAX 300SB Columns

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

Columns: 4.6 x 150 mm
Mobile Phase: 5 - 40% B in 20 min.
A: 0.1% TFA / Water
B: 0.1% TFA / ACN
Flow Rate: 1 mL / min.
Temperature: 60°C
Sample: 4 µg each protein
25 µL injection
Effect of Bonded-Phase Ligand on Recovery of a Synthetic Lipopeptide

- **300SB-CN** Recovery 59%
- **300SB-C8** Recovery 75%
- **300SB-C18** Recovery 89%

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.
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

Selectivity Impacts Resolution Most
- Change bonded phase
- Change mobile phase

\[
R_s = N^{1/4} \cdot (\alpha - 1) / \alpha \cdot k' / (k'+1)
\]

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<thead>
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<th>Plates:</th>
<th>5000</th>
<th>10000</th>
<th>15000</th>
<th>20000</th>
<th>25000</th>
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<tr>
<td>Alpha:</td>
<td>1.10</td>
<td>1.35</td>
<td>1.60</td>
<td>1.85</td>
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<td>k':</td>
<td>2.0</td>
<td>4.5</td>
<td>7.0</td>
<td>9.5</td>
<td>12.0</td>
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</table>
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

Column used
SB-C18, 2.1x150mm, 1.8µ
35% More peak capacity, more resolution

Peak Capacity 673
Starting Pressure 380 bar

SB-C18, 2.1x150mm, 3.5µ
Less peak capacity, less resolution

Peak Capacity 502
Starting Pressure 105 bar

★ Smaller particle size = sharper peaks = greater peak capacity
Typical Conditions for Separations of Peptides and Proteins on 300SB Columns

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<table>
<thead>
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</thead>
<tbody>
<tr>
<td><strong>Column:</strong></td>
<td>4.6 x 150 mm, 5 or 3.5 μm 300SB</td>
</tr>
<tr>
<td><strong>Mobile Phase:</strong></td>
<td>A: 95:5, H₂O : ACN with 0.1% TFA</td>
</tr>
<tr>
<td></td>
<td>B: 5:95, H₂O : ACN with 0.085% TFA</td>
</tr>
<tr>
<td><strong>Flow Rate:</strong></td>
<td>1 mL / min.</td>
</tr>
<tr>
<td><strong>Temp:</strong></td>
<td>35 - 40°C</td>
</tr>
<tr>
<td><strong>Initial Gradient:</strong></td>
<td>0 - 60% B in 60 min.</td>
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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
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
2. Insulin
3. Cytochrome C
4. Lysozyme
5. Parvalbumin
6. CDR
7. Myoglobin
8. Carbonic Anhydrase
9. S-100β
10. S-100α

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

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

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

46 to 48 peaks, 1/20 analysis time

Data courtesy of:
Novartis Pharma, Biotechnology, Basel
Dr. Kurt Forrer
Patrik Roethlisberger
More Poroshell Bonded Phases Provide Selectivity Options to Enhance Resolution:

Poroshell 300SB-C18
2.1 x 75 mm

Poroshell 300SB-C3
2.1 x 75 mm

Samples:
1. Angiotensin II
2. Neurotensin
3. RNase A
4. Insulin B Chain
5. Insulin
6. Cytochrome C
7. Lysozyme
8. Myoglobin
9. Carbonic Anhydrase

Column: Agilent Poroshell (2.1 x 75 mm); Temp.: 70 °C; Flow: 0.5 mL/min; Det: UV 215 nm
Mobile Phase: A = 0.1% TFA/H₂O, 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!
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

After 495 mL

After 13680 mL
LC/MS Sensitivity vs. Mobile Phase Modifier
TFA vs. Acetic Acid

A significant increase in sensitivity was observed using 5% acetic acid instead of TFA.
Reducing TFA concentration to 0.001% improved sensitivity very little.
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 NH₄OH in water
B: 20 mM NH₄OH 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 Aβ 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.

NH₄OH Conditions, 25°C
A- 20 mM NH₄OH in water
B- 20 mM NH₄OH 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 β Sequences:
Asp Ala Glu Phe Arg His Asp Ser Gly Tyr
Glu Val His His Gln
Lys Leu¹⁷ Val Phe Phe Ala Glu Asp Val
Gly Ser Asn Lys Gly Ala
Ile Ile Gly Leu Met Val Gly Gly³⁸ Val Val⁴₀
Ile Ala⁴² Thr⁴³-COOH

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

Reference 1

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

Comparison of TFA and NH₄OH 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

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

NH₄OH Conditions:
A- 20 mM NH₄OH in water
B- 20 mM NH₄OH in 80% AcN

Reference 5
Peptide RP-HPLC/ESI-MS Using NH₄OH 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)

- 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 NH₄OH mobile phase.

Reference 2
Angiotensins Separation at High and Low pH on Extend-C18

**Acidic Conditions**
A- 0.1% TFA in water
B- 0.085% TFA in 80%ACN

**Basic Conditions**
A- 10 mM NH₄OH in water
B- 10 mM NH₄OH in 80%ACN

Column: ZORBAX Extend-C18, 2.1 x 150 mm, 5 µm
Agilent 1100
MSD: Pos. Ion ESI
Vf 70V, Vcap 4.5 K
N₂=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)
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, N₂-35 psi, 12L/min., 325°C     Sample: Angiotensin I, II, III 2.5 μL (50 pmol each)

• Acidic conditions can not resolve all three Angiotensins.
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 NH₄OH in water  B: 10 mM NH₄OH in 80% ACN  
LC/MS: Pos. Ion ESI – Vf 70V, Vcap 4.5 kV, N₂-35 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.
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.
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 \( \sim 10^4 \) 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
Two or more modes of HPLC with independent physical characteristics

- Ion Exchange/Affinity/SEC
- Reverse Phase

**OFF-Line or ON-Line**

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

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

Applications
- Superior for global proteomics
- Small, basic and hydrophobic proteins
Proteomics With 2D-LC/MS - Workflow

1. cell disruption
2. prefractionation
3. solubilization
4. sample clean up

Cell disruption leads to free proteins (10^4 to 10^5). Tryptic digest converts these proteins into peptides (10^5 to 10^6). These peptides are then separated by HPLC: 1st dimension - ion-exchange, 2nd dimension - reversed phase. The separation and isolation process involves mass spectrometry (Mass Spec) for identification. The data from Mass Spec is analyzed (Data Analysis).
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 \( L = 90 \text{ min} \), peak width \( 4 \sigma \sim 25s \)
- \( n_c = 216 \)

- Total Peak capacity : 1. Dim \(*\) 2. Dim

\[ 15 \times 216 = 3240 \]

3. Dimension Mass Spec

- 7 \(*\) 3240 = 23,000 peptides
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

Data Analysis

Eluted and separated peptides are directly analyzed by MS/MS

MS/MS Data

Mass Spec

Waste

SCX (SEC)

RP

Protein mixture

Peptides

Digest

pH < 3

ICAT
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 program (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:
2D-LC Method Diagram – RPLC/MS

Wash C8 TRAPPING COLUMN:

Analyse Sample
2-D LC/MS Analysis of Peptide Mix

Met-Enkephalin

Angiotensin II

alpha MSH (Melanocyte stimulating hormone)
2D-HPLC: Cation Exchange and Reversed Phase Overlay of Direct-injected Standard vs. KCl Fractions

- 0.4m KCl
- 0.1m KCl
- RP-only

Peak markers:
- Neurotensin 8-13
- Met-Enkephalin

mAU vs. time in minutes.

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

1ul inj. and preconcentrated

0.025M KCl
0.1M KCl
0.3M KCl
1M KCl
2-D Analysis of Myoglobin Tryptic Digest
- 100 and 1000 nL injection volumes

Pre-column: ZORBAX 300SB 0.3 x 5 mm, 3.5 μm
+ Zorbax SCX 0.3 x 35 mm
Column: Zorbax 300SB 0.3 x 35 mm, 3.5 μm
Mob.phase: H₂O .025%FA/CH₃CN .0225% FA grad.
Flow: 5 μL,
Main flow: 200 μL

DAD1, A, Sig=206,10 Ref=450,80 (PEP-PROT/MYO1U-2.D)
*DAD1 A, Sig=206,10 Ref=450,80 (PEP-PROT/MYO-3.D)

Trapping Volume
0.025M KCl

1000nl inj.
100nl inj.
Agilent Technical Support

LC Column Support

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

Select option 4, then option 2 for LC.

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