Multi-Dimensional Liquid Chromatography for Shotgun Proteomics

4th Multidimensional Chromatography Workshop
Toronto (January, 2013)

Herman C. Lam, Ph.D.
Calibration & Validation Group
MDLC for Shotgun Proteomics

- Introduction
  - General concepts
  - Advantages
  - Challenges in implementation

- Applications
  - Shotgun proteomics
Multidimensional chromatography

TLC

LC

Fractions

1st Dimension

2nd Dimension

Unresolved

Resolved

Peak capacity

- Peak capacity $P$ is the number of peaks that would fit into a separation space

  - Separation performance and utilization of separation space

<table>
<thead>
<tr>
<th>Isocratic</th>
<th>$P = 1 + \frac{\sqrt{N}}{4} \ln \left( \frac{T_f}{T_1} \right)$</th>
<th>Gradient</th>
<th>$P = \frac{\sqrt{N}}{4} \left( \frac{T_f}{T_1} - 1 \right)$</th>
</tr>
</thead>
</table>

- Estimation: $P = \frac{(t_f - t_1)}{W}$
  - $t_f =$ time for the final peak,
  - $t_1 =$ time for the void peak
  - $W =$ average peak width
Peak capacity

- For a multidimensional chromatographic system
  - \( P = P_1 \times P_2 \times P_3 \)
  - \( P_1 \) = peak capacity of the first dimension
  - \( P_2 \) = peak capacity of the second dimension
  - \( P_3 \) = peak capacity of the third dimension

- Assumptions: The separation dimensions are orthogonal and no peak broadening
Orthogonality

- Correlation between the mode of separation
  - High orthogonality: low correlation between the modes of separation (e.g., SCX and reversed phased)

- Geometric orthogonality concept

(A) Non orthogonal system, 10% area coverage represents 0% orthogonality.
(B) Hypothetical orthogonal system, full area coverage.
(C) Random, ideally orthogonal, system, area coverage is 63% representing the 100% orthogonality.

LC Column Orthogonality and Solvent Compatibility

**Orthogonality**

**Solvent compatibility**
Challenges for implementation of MDLC

- **Off-line**
  - Very tedious
  - Sample lose

- **On-line**
  - Different separation mechanisms at work
    - Optimization of D₁ and D₂
    - Solvent compatibility
  - Multiple pumps and complex switch valve systems
  - Long total run time: For 2D separation \((T_{d1} + nT_{d2})\)
    - \(T\) - Run time
    - \(n\) = number of fractions from D₁
  - Volume of data and data analysis
Proteomics

- Proteomics deals with the large-scale determination of gene and cellular functions directly at the protein level.

- Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and their functions.

![Diagram of proteomics approaches: Bottom up and Top down](image)
Shotgun proteomics

Trypsin: Cleaves at lysine and arginine, unless either is followed by proline in C-terminal direction.

Peptide sequences:
- SPAFDSIMAETLK
- AFDSLPPDIHE
- GGILAQPFLIIK
- IIGHFYDDWCPL

Proteins
Challenges of MS proteomics

- The complexity of biological samples (great dynamic range, huge numbers of proteins)
  - $10^{7-8}$ in human cells, and at least $10^{12}$ in human plasma
  - Dynamic range of a LC-MS is about $10^{4-6}$

- Ion suppression when peptides co-elution

- The limited scanning speed of MS

Signal is suppressed
1. Not being picked for MS/MS
2. Poor MS/MS spectrum
Advantages for MDLC

- Increase in peak capacity to reduce sample complexity
  - Enable the analysis of very complex biological samples
- Eliminate interferences
- Can be automated for on-line applications
SCX-RP 2D combination

- High orthogonality and relatively easy to implement
- MudPIT (Multi Dimensional Protein Identification Technology)

Low resolution in the SCX separation

- Peptides carryover to subsequent fractions

High pH RP - Low pH RP System

- Reversed phase separation is based on hydrophobicity
- Orthogonality is related to differences in retention of peptides in RP under different pH
  - Different charges of amino acid side chain at different pH
- RP-RP provides comparable performance to SCX-RP with a higher peak capacity in the first-dimension
  - Salt free eluents - minimized risk of ion suppression
High pH RP - Low pH RP System

Step 1: Partial filling of the loop with fraction eluted from the 1st Dimension high pH RP column
Step 2: Transfer to 2nd Dimension low pH RP column
Step 3: Elution from 2nd Dimension low pH RP column
Step 4: Refill the loop with low pH mobile phase
High pH RP - Low pH RP System

- Analysis of Mouse embryonic fibroblasts and Zebra fish embryo lysates

A. Mouse embryonic fibroblasts

B. Zebra fish embryo
HILIC-RP combination

**Challenges:**

- Solvent compatibility and peptide solubility

---

HILIC-RP combination

- Rat pheochromocytoma lysate
3DLC High pH RP-SCX- Low pH RP

- Evolved from using SCX as trapping column to separation column

![Diagram showing 3DLC process](image_url)
Analysis of STO mouse cell digest

- 3D RP-SCX-RP (8 high-pH RP fractions x 3 SCX fractions, 24 fractions in total)
- In comparison, 2D RP-SCX (as trap column)-RP (12 high-pH fractions)

No. of unique peptide 
(15523 vs. 26391) 
70.0% increase

No. of proteins 
(2985 vs. 3613) 
17.4 % increase

Protein sequence coverage 
2D 13.8% (5.2 peptides/protein) 
3D 18.9% (7.3 peptides/protein)
Peptide separation in 3D RP-SCX-RP

Correlation of hydrophobicity across fractions

Charge separation in SCX fractionation

Peptide carryover

Sufficient for tryptic peptides (+2,+3 and +4)
Proteome profiling of S. cerevisiae

Capillary-/nano-flow 3D RP-SCX-RP

1st Dimension
Capillary-flow

2nd Dimension
Capillary-flow

3rd Dimension
Nano-flow

Peptide identification

Total: 23953

Protein identification

Total: 2916

~ 50% protein of yeast proteome
Outlook and Summary

- MDLC has a key role in the analysis of highly complex samples in proteomics applications
- Suitable of Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) base quantitative proteomics applications
- Future areas of MDLC research in Dr. I.K. Chu Group
  - Other column combinations
  - Runtime reduction
  - 4DLC separation
  - Applications on system biology
Acknowledgements

Dr. Ivan Chu and members of his research group at the University of Hong Kong

- Dr. Ricky Ng
- Dr. Ricky Kong
- Dr. S. O. Siu
- Dr. Maggie Lam
- Yun Zhao
- Edward Lau
- Henry Law
- Quan Quan