Two Dimensional HPLC: Theory and Applications
The mass of Earth is $6 \times 10^{24}$ kg. If Archimedes can lift 60 kg, he would need a lever with an arm ratio of $10^{23}:1$. So if the short arm is one meter long, the lever length will be $10^{23}$ meters plus one. Also, note that he would have to push the lever for $10^{20}$ meters to shift the Earth just by one millimeter.

Give me a column long enough (with enough time and enough pressure) and I can separate anything.

-Bob Giuffre
What is Two Dimensional LC

The selective transfer of a fraction (or fractions) from one chromatographic column to a secondary chromatographic column for further separation.
Why Two Dimensional LC

- Further resolution of a complex mixture that cannot be separated on a single column
- Sample cleanup by removing matrix or interfering compounds
- Increase sample throughput (two separations going on at once)
- Trace enrichment of major compounds of interest (column focusing)
- **Increased peak capacity**
- **Second dimension mobile phase for amenable to mass spectrometry**
Resolution

a Function of Selectivity, Column Efficiency, or Retention

Resolution

Selectivity Affects Resolution Most
• Change bonded phase
• Change mobile phase

\[ R_s = N^{\frac{1}{2}} / 4 \cdot (\alpha - 1) / \alpha \cdot k / (k+1) \]

Plates:
- 5000
- 10000
- 15000
- 20000
- 25000

Alpha:
- 1.10
- 1.35
- 1.60
- 1.85
- 2.1

\[ k = 2.0 \]

\[ \alpha = \begin{cases} 
1.10 & \text{for } 5000 \\
1.35 & \text{for } 10000 \\
1.60 & \text{for } 15000 \\
1.85 & \text{for } 20000 \\
2.1 & \text{for } 25000 
\end{cases} \]
Principles of Two Dimensional HPLC

- Long efficient first column retains sample components in one mode
- The eluent flows through an injection loop (this first step may include peak detection for heart cutting the sample)
- The loop content is automatically injected into a 2\textsuperscript{nd}, fast column giving rise to an orthogonal separation
<table>
<thead>
<tr>
<th>Mode Combination*</th>
<th>Application</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Column Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEC &amp; RP</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT BIO-IEX; ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>SEC &amp; RP</td>
<td>POLYMERS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT PL-GEL (VARIABLE BEDS); ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>NP &amp; RP</td>
<td>PHARMACEUTICALS METABOLICS</td>
<td>Orthogonality</td>
<td>Solvent compatibility, limited application</td>
<td>ZORBAX CYANO, AMINO, SIL; ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>RP &amp; RP</td>
<td>PHARMACEUTICALS METABOLICS</td>
<td>Miscible Solvents, broadest applications, fast speed, gradient on both dimensions, highest peak capacity</td>
<td>Strongly depends on column choice of mobile phase choice</td>
<td>ZORBAX STABLEBOND, ECLIPSE, EXTEND, BONUS RP, POROSHELL</td>
</tr>
<tr>
<td>AFFINITY &amp; RP</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity, Off-line</td>
<td>AGILENT MULTIPLE AFFINITY REMOVAL COLUMNS</td>
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<tr>
<td>SEC &amp; NP</td>
<td>POLYMERS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT PL-GEL (VARIABLE BEDS); ZORBAX CYANO, AMINO, SIL</td>
</tr>
<tr>
<td>SEC &amp; IEC</td>
<td>PROTEOMICS</td>
<td>Orthogonality</td>
<td>Low Peak Capacity</td>
<td>AGILENT BIO-SEC; AGILENT BIO-IEX</td>
</tr>
</tbody>
</table>

* Stoll et al, Jchrom A, 1168 (2007) 3-43
Road Blocks to Two Dimensional Chromatography

- Typically first dimension gradient is a long, slow gradient followed by rapid, repeated gradients on the second dimension so a very low delay volume pump is needed capable of ballistic gradients.
- Difficult to coordinate timing between first and second dimension gradient.
- Difficult to coordinate valve timing between first and second dimension.
- Difficult to coordinate heart-cutting first dimension detector with trapping valve.
- Any changes to one time table necessitates changes to all the other tables.
Without Two Dimensional Software: Complex Gradient and Valve Switch Tables

**Capillary pump 1:**
gradient across analytical column

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>175</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
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<td>65</td>
</tr>
<tr>
<td>26.1</td>
<td>65</td>
<td>201.2</td>
<td>3</td>
</tr>
<tr>
<td>26.2</td>
<td>3</td>
<td>210</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>236.1</td>
<td>65</td>
</tr>
<tr>
<td>61.1</td>
<td>65</td>
<td>236.2</td>
<td>3</td>
</tr>
<tr>
<td>61.2</td>
<td>3</td>
<td>245</td>
<td>3</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>271.1</td>
<td>65</td>
</tr>
<tr>
<td>96.1</td>
<td>65</td>
<td>271.2</td>
<td>3</td>
</tr>
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<td>96.2</td>
<td>3</td>
<td>280</td>
<td>3</td>
</tr>
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<td>105</td>
<td>3</td>
<td>306.1</td>
<td>65</td>
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<td>131.1</td>
<td>65</td>
<td>306.2</td>
<td>3</td>
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<tr>
<td>166.2</td>
<td>3</td>
<td>345</td>
<td>90</td>
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</table>

**Capillary pump 2:**
gradient across SCX column

<table>
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<tr>
<th>Time</th>
<th>% B</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>135</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
</tr>
<tr>
<td>230</td>
<td>30</td>
</tr>
<tr>
<td>280</td>
<td>50</td>
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<td>295</td>
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</tr>
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<td>320</td>
<td>100</td>
</tr>
<tr>
<td>320.1</td>
<td>0</td>
</tr>
<tr>
<td>350</td>
<td>0</td>
</tr>
</tbody>
</table>

**6-port valve:**
timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>Position</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>column 2</td>
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<tr>
<td>6</td>
<td>column 1</td>
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<tr>
<td>13</td>
<td>column 2</td>
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<tr>
<td>20</td>
<td>column 1</td>
</tr>
<tr>
<td>27</td>
<td>column 2</td>
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<td>34</td>
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<td>41</td>
<td>column 2</td>
</tr>
<tr>
<td>48</td>
<td>column 1</td>
</tr>
<tr>
<td>55</td>
<td>column 2</td>
</tr>
</tbody>
</table>

**10-port valve:**
timetable

<table>
<thead>
<tr>
<th>Time</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Pos 1</td>
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<tr>
<td>5</td>
<td>Pos 2</td>
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<tr>
<td>10</td>
<td>Pos 1</td>
</tr>
<tr>
<td>15</td>
<td>Pos 2</td>
</tr>
<tr>
<td>20</td>
<td>Pos 1</td>
</tr>
<tr>
<td>25</td>
<td>Pos 2</td>
</tr>
<tr>
<td>30</td>
<td>Pos 1</td>
</tr>
<tr>
<td>35</td>
<td>Pos 2</td>
</tr>
</tbody>
</table>

- 500 mL/min
- Run time: 345 minutes, 8.9 minutes
- Injection volume: 60 mL

- 500 mL/min
- Run time: 350 min.
Two Approaches to Two Dimensional HPLC

- **Comprehensive**: All of the sample from the first column is ‘trapped & released’ on to the second column

- **Heart-cutting**: Detector in the first dimension allows components only to be trapped and released on to the second column
2D-LC

Adding 2\textsuperscript{nd} dimension

- Purpose of 2D-LC: To increase the total separation power.
- Aliquots of effluent of the 1D-column are handed to the 2\textsuperscript{nd} dimension for 2D analysis.
- Ideal case: Two columns with orthogonal separation mechanism, where separation power of 1D and 2D multiplies.
- Operation modes: Comprehensive 2D-LC and Heart-Cutting 2D-LC
Heart-Cutting 2D-LC

Principle of operation

- Fractions of specific 1D-peaks are sampled and subjected to 2D.
- Decouples 1D / 2D time scale.
- Allows for longer 2D-gradients and longer 2D-columns with higher separation efficiency.
- Better data quality for those peaks of interest.
- Use: Targeted analysis of known samples / increase confidence.
Comprehensive 2D-LC

Principle of operation

- Entire effluent of 1D-separation and thus every peak gets fully sampled and passed to 2D.
- Frequency: $\geq 2$ snippets per peak to avoid de-separation.
- Requires fast 2D-gradients (sec), short columns ($\leq 5$ cm) and high flow rates ($\geq 2 \text{ mL/min}$).
- Use: Complex / unknown samples.
Overview of Agilent Approach

- Offers both comprehensive 2DLC and heart-cutting 2DLC
- Unique features like automatically shifted gradients or peak-triggered operation
- Highest focus on simplest but still highly flexible 2DLC method set-up
- Highest flexibility on hardware set-up
  - Different pumps, autosamplers and detectors supported
  - Detectors at different positions (after 1st dimension column, after 2nd dim. column, at waste-line)
  - Different valve set-up possibilities supported
- High performance data analysis
Flow Diagram of the new 2pos/4port-duo valve

The Sampling Device / Interface

Dual loop workflow with Agilent 2 pos / 4 port Duo Valve

1. Peak sampling from 1D.

* Duo Valve specifically designed for 2D-LC Application. Two identical flow paths.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.

- Pump
- Auto sampler
- 1D column
- 2D pump
- 2D detector
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
The Sampling Device / Interface

Dual loop workflow

1. Peak sampling from 1D.
2. Valve switch.
3. 2D-LC.
4. Repeat 1.- 3.
Heart-Cutting Workflow

Limit of the dual loop approach
Heart-Cutting Workflow

Limit of the dual loop approach
Heart-Cutting Workflow

Limit of the dual loop approach
Heart-Cutting Workflow

Limit of the dual loop approach
Limit of (Single) Heart-Cutting

- Skipped interesting peaks during 2D cycle.
Limit of (Single) Heart-Cutting

- Skipped interesting peaks during 2D cycle.
- There may be an additional co-eluting peak located at a different position than that where the heart cut was made.
New Multiple Heart-Cutting (MHC) Solution

From dual loop to MHC

(A) One of the loops replaced by 6-column selector valve bearing 6 loops: Parking deck cluster offering 7 sampling positions.
Solving the Real Estate Problem: Unmatched multiple heart-cutting 2D-LC Usability

Smart Valve-Loop Setup with 6 or 12 loops
→ 2D-LC valve + one or two 6/14 valves

Pre-aligned loop-valve kits, just add to the existing 2D-LC system

Online status monitoring
New Multiple Heart-Cutting (MHC) Solution

From dual loop to MHC

(A) One of the loops replaced by 6-column selector valve bearing 6 loops: Parking deck cluster offering 7 sampling positions.

(B) Parking deck cluster with 12 sampling positions.

- Parking deck clusters allow for multiple peak parking in parallel to 2D analyses.
New Multiple Heart-Cutting (MHC) Solution

Different view for better illustration
2D-LC Configuration
ChemStation Dashboard:

All modules in one dashboard can be relabeled individually, e.g. „BinPump-1st-Dim“
2D-LC System Configuration
“One screen for the entire system”

- Define 1D / 2D pump
- Define detector in the second dimension
- Define peak detector (optional)
- Select the valve(s) to be used for 2D-LC injection
- Select a possible valve / loop configuration
- Graphical representation of the selected valve / loop configuration:
  - Flow path 1D & 2D
  - Animated valve switching
Method UI
2D-LC specific parameters of the 2D-pump

Select the 2D-LC mode: comprehensive / heart-cutting

Define repetition of 2nd dimension gradient (Modulation time)

Define the gradient of the 2nd dimension

Show rollout of gradient in the 2nd dim over the runtime of the 1st dimension

Graphical editing of gradient shift

Solvent & Flow Settings
Define time window(s) where the selected 2DLC mode is active
Operation values, warnings
Close-up of 2D-gradient
Access to standard method UI of the pump
Defining shifted 2D gradients

Suitable for adjusting the 2D gradient based on the 1D gradient

=> Improve resolution in second dimension
Shifted 2D gradients

Original gradient

- **2D Gradient stop time**: 1.00 min
- **Modulation time**: 2.00 min

### Original gradient

- **Time [min]**
  - %B
  - 0.00: 20.00%
  - 1.00: 60.00%

### Gradient preview

- %B 2D: Pump
- %B 2D: 60% at 1 min
- %B 2D: 20% at 0 min
Shifted gradients

- Gradient end shifts between 60 and 80% from 5 to 20 min
- Linear interpolation
- Gradient start shifts to 40% at 10 min

Original gradient
Programming shifted gradients per time table is tedious. You can do this easily by graphical editing.
Peak triggering

time based and/or peak based

time based peaks in yellow
peak based peaks in green
Threshold or applicable peak detection settings (slope) define peak start and possible sampling start.
Valve switches if peak falls below threshold etc. or if the sampling time is exceeded (whichever comes first). The Sampling Time has been named „Loop fill time“ in version A.01.01., which is misleading.
Loop volume $V$ and 1D flow rate $F$ define loop content/sampling time $t$ in dark green area ($t = V/F$), which shows the loop content.
A reference signal can be used for predicting the sampling (estimate!) or for creating a time-based setup.
1290 Infinity 2D-LC Solution – Scalability

**Multiple heart-cutting**

Multiple parts/peaks of the 1D effluent are injected online onto 2D system

Smart valve setup needed to „park“ the peaks before analyzing in 2nd dimension
Comprehensive 2D-LC
Phenolic compounds in Virgin Olive Oil

• Antioxidants in Virgin olive oil associated with health and nutritional benefits

• Antioxidants represented by tocopherols, carotenoids, chlorophylls and hydrophilic phenols

• Phenolic compounds in olive oil dependant on olive cultivar, fruit ripening, geographic and technological conditions

• Hydrophilic phenols include phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans
Multiple Heart-cutting 2D-LC
Lipid Composition of Blood Plasma and Induced Sputum Samples

Analysis of intact lipids in biological matrixes (plasma, sputum). Group type separation in the first dimension using HILIC, reversed phase in the second. Analysis by QQQ MS.

• Besides their well-known role in cell membranes and energy household, lipids are also involved in many biological processes and are associated with various diseases such as diabetes and obesity.

• Lipids exist in different classes with different physicochemical and biological properties. Each class contains many members that differ in the length of the fatty acid side-chain and the number of double bonds, among others.

Data from Agilent Application Note 5991-5532EN
Lipid Composition
Onedimensional Separations

<table>
<thead>
<tr>
<th>Lipid class (abbreviation)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids (FFA)</td>
<td>A</td>
</tr>
<tr>
<td>Monoacylglycerols (MG)</td>
<td>B</td>
</tr>
<tr>
<td>Diacylglycerols (DG)</td>
<td>C</td>
</tr>
<tr>
<td>Triacylglycerols (TG)</td>
<td>D</td>
</tr>
<tr>
<td>Cholesterol (Chol)</td>
<td>E</td>
</tr>
<tr>
<td>Cholesterol esters (CE)</td>
<td>F</td>
</tr>
<tr>
<td>Ceramides (CER)</td>
<td>G</td>
</tr>
<tr>
<td>Glycoceramides (Hex-CER)</td>
<td>H</td>
</tr>
<tr>
<td>Glyconceramides (Hex$_n$-CER)</td>
<td>I</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>J</td>
</tr>
<tr>
<td>Phosphatidylethanolamines (PE)</td>
<td>K</td>
</tr>
<tr>
<td>Glycosphingolipids (GSL)</td>
<td>L</td>
</tr>
<tr>
<td>Phosphatidylserines (PS)</td>
<td>M</td>
</tr>
<tr>
<td>Phosphatidylcholines (PC)</td>
<td>N</td>
</tr>
<tr>
<td>Sphingomyelins (SM)</td>
<td>O</td>
</tr>
<tr>
<td>Lysophospholipids (LysoPL)</td>
<td>P</td>
</tr>
</tbody>
</table>

HILIC

Reversed Phase
Lipid Composition
1st Dimension HILIC Separation

Column: Agilent RX-SIL, 1.0 x 150 mm, 3.5 µm (custom made)
Mobile phase A: 0.1 % formic acid in acetonitrile
Mobile phase B: 20 mM ammonium formate in acetonitrile/methanol/water, 50/20/30, v/v
Gradient: at 0 min 30%B, at 2 min 60%B, at 4 min 70%B, at 5 min 100%B, at 12 min 100%B
Flow rate: 40 µL/min
Col.temp.: 40 °C
Inj. volume: 1 µL
Lipid Composition
2nd Dimension Reversed Phase Separation

Column: Agilent ZORBAX Eclipse Plus C18 RRHD, 2.1 x 100 mm, 1.8 µm
Mobile phase A: 20 mM ammonium formate/0.1% formic acid in methanol/water, 10/90, v/v
Mobile phase B: 20 mM ammonium formate/0.1% formic acid in acetonitrile/methanol/isopropanol, 20/30/50, v/v
Gradient: at 0 min 40%B, at 6.5 min 100%B, at 8.5 min 100%B, at 10 min 40%B
Flow rate: 0.6 mL/min
Col.temp.: 60 °C
MS detection: QTOF Mass range m/z 200–1,700
Lipid Composition
Power of 2D-LC

Separation of Phosphatidylethanolamines (PE) and Phosphatidylcholines (PC)
Lipid Composition Results (Example)

**A**

BPC(+) Fraction 1

**B**

Extracted features

<table>
<thead>
<tr>
<th>Feature</th>
<th>Formula</th>
<th>$t_r$ (min)</th>
<th>m/z</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\text{C}<em>{55}\text{H}</em>{101}\text{O}_2\text{N}$</td>
<td>11.45</td>
<td>871.7629</td>
<td>TG(52:4)NH$_3$</td>
</tr>
<tr>
<td>2</td>
<td>$\text{C}<em>{55}\text{H}</em>{103}\text{O}_2\text{N}$</td>
<td>11.61</td>
<td>873.7786</td>
<td>TG(52:3)NH$_3$</td>
</tr>
<tr>
<td>3</td>
<td>$\text{C}<em>{47}\text{H}</em>{79}\text{NO}_2$</td>
<td>11.71</td>
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<td>CE(20:4)NH$_3$</td>
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<tr>
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<td>875.7942</td>
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<td>TG(52:1)NH$_3$</td>
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<tr>
<td>7</td>
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<td>12.1</td>
<td>667.6287</td>
<td>CE(18:1)NH$_3$</td>
</tr>
</tbody>
</table>
Comprehensive 2D-LC Analysis of *E.coli* Tryptic Digest and Intact Protein

Peptide analysis after tryptic digestion and intact protein analysis using comprehensive 2D-LC on the same columns in the first and second dimension.

- In the field of protein analysis, sample complexity is enormous. It is not uncommon to be confronted with samples containing hundreds of proteins, and following digestion, thousands of peptides.

- Combination of SCX with RPLC for the analysis of *E.coli* tryptic digest and intact protein.

- Both intact protein and peptide analyses were done with the same instrument and column configuration using DAD and Q-TOF LC/MS.

Data from Agilent Application Note 5991-5179EN
### Analysis of E.coli Tryptic Digest and Intact Protein Peak Capacity for Tryptic Digest

<table>
<thead>
<tr>
<th>Gradient time [min]</th>
<th>Elution window [min]</th>
<th>Peak capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>70 – 75</td>
<td>10 – 63</td>
<td>1927</td>
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<tr>
<td>140 – 150</td>
<td>10 – 112</td>
<td>3138</td>
</tr>
<tr>
<td>210 – 225</td>
<td>10 – 158</td>
<td>3750</td>
</tr>
</tbody>
</table>

Separation by IEX (Bio SCX, NP 10)

Separation by RP (300SB-C18)
Analysis of E.coli Tryptic Digest and Intact Protein Peptide Analysis

Identity of the spots was determined by searching the MS/MS spectra against the *E. coli* protein database (SWISS-PROT) using Agilent MassHunter Spectrum Mill software.
## Analysis of E.coli Tryptic Digest and Intact Protein Peptide Analysis

<table>
<thead>
<tr>
<th>Spot</th>
<th>Peptide</th>
<th>Protein accession</th>
<th>Protein description</th>
<th>m/z</th>
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<tbody>
<tr>
<td>1</td>
<td>TTDVTGTIELPEGVEMVMPGDNK</td>
<td>EFTU1_ECO24</td>
<td>Elongation factor Tu 1</td>
<td>849.4184 (3+)</td>
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<tr>
<td>2</td>
<td>DFEAQLASTETOVGNEPLKL</td>
<td>ZNUA_ECOLI</td>
<td>High-affinity zinc uptake system protein ZnuA</td>
<td>754.3768 (3+)</td>
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<tr>
<td>3</td>
<td>DTTTIDGVGEAAIQR</td>
<td>CH60_ECO24</td>
<td>60 kDa chaperonin</td>
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<td>GFSGEDATPALEGADVVLISAGVAR</td>
<td>MDH_ECOBW</td>
<td>Malate dehydrogenase</td>
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<td>5</td>
<td>FGVSAAAAVAVAPAAGPEAEEK</td>
<td>RL7_ECO24</td>
<td>50S ribosomal protein L7/L12</td>
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<td>6</td>
<td>DLVESAPAALK</td>
<td>RL7_ECO24</td>
<td>50S ribosomal protein L7/L12</td>
<td>557.3104 (2+)</td>
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<td>7</td>
<td>AVAAVNGPIAQALIGK</td>
<td>ENO_ECOBW</td>
<td>Enolase</td>
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<td>8</td>
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<td>SKP_ECOLI</td>
<td>Chaperone protein Skp</td>
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<td>9</td>
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<tr>
<td>15</td>
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<td>METE_EODH</td>
<td>5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase</td>
<td>469.9215 (3+)</td>
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</tbody>
</table>
Analysis of E.coli Tryptic Digest and Intact Protein Peptide Analysis

MS/MS spectra of some selected Peptides.
Analysis of E.coli Tryptic Digest and Intact Protein
Protein Analysis

Separation by IEX (Bio SCX, NP 10)

Separation by RP (300SB-C18)

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Molecular weight</th>
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<tr>
<td>2</td>
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<td>3</td>
<td>1,881.07</td>
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<tr>
<td>4</td>
<td>5,503.77</td>
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<tr>
<td>5</td>
<td>5,203.41</td>
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<tr>
<td>6</td>
<td>7,271.69</td>
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<tr>
<td>7</td>
<td>9,119.89</td>
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<td>8</td>
<td>3,852.13</td>
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<td>9</td>
<td>5,457.61</td>
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<td>10</td>
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<td>11</td>
<td>18,214.02; 20,840.06; 22,524.54</td>
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<tr>
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<td>13</td>
<td>15,692.94, 15,770.05</td>
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<td>14</td>
<td>9,226.56</td>
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<td>15</td>
<td>9,535.85</td>
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Analysis of E.coli Tryptic Digest and Intact Protein Protein Analysis

Raw and deconvoluted spectra of spots 2 and 6.
Comprehensive 2D-LC Analysis of Complex N-Glycans in Biopharmaceuticals

Analysis of complex glycan pattern of EPO after enzymatical release and labelling of glycans bei 2D-LC with Fluorescence Detection.

- Erythropoietin (EPO) is a 30,400 Da glycoprotein hormone that regulates the production of red blood cells (erythropoiesis).
- The molecule consists of a 165 amino acid single polypeptide chain and a complex carbohydrate addition.
- The glycosylation of EPO is highly variable because it contains multiple glycosylation sites, each of which can have a wide variety of glycan structures. This results in a huge complexity of glycan structures that is referred to as microheterogeneity.

Data from Agilent Application Note 5991-5349EN
Analysis of Complex N-Glycans in Biopharmaceuticals
EPO Structure

The glycosylation portion of EPO consists of three N-linked glycosylation sites at Asn 24, 38, and 83, and one O-linked glycosylation site at Ser 1261.

Each of the three N-linked glycans is likely to contain up to four sialic acids. The amount of NeuAcs in EPO has a huge influence on the molecule’s net charge, which is used to classify EPO isoforms.

EPO structure with four examples of N-glycans typically occurring in EPO.
Comprehensive HILIC/WAX 2D-LC separation of EPO, showing highly orthogonal separation. The IEX chromatography in the second dimension reveals the charge pattern of the glycans.
Comprehensive 2D-LC Analysis of Monoclonal Antibody Digests

Peptide mapping for identity and purity assessment of Trastuzumab by comprehensive 2D-LC using SCX/RP and HILIC/RP combinations.

- Trastuzumab, marketed as Herceptin, is a 150 kDa large molecule used in the treatment of HER2 positive metastatic breast cancer.
- Following trypsinolysis, over 100 peptides with varying physicochemical properties present in a wide dynamic concentration range are expected.
- This Application reports on the combination of strong cation exchange (SCX) and RPLC and on HILIC and RPLC, two combinations shown to provide good orthogonality towards the analysis of peptides.

Data from Agilent Application Notes 5991-2880EN and 5991-4503EN
Analysis of Monoclonal Antibody Digests
Structure and amino acid sequence

- 62 identity peptides
- Modifications
- Incomplete and aspecific cleavages
- ...

> 100 peptides
Analysis of Monoclonal Antibody Digests
SCX or HILIC in the First Dimension Provide Different Selectivity
Analysis of Monoclonal Antibody Digests
Analysis of Nonstressed and Oxidatively Stressed Trastuzumab
Analysis of Monoclonal Antibody Digests
Analysis of Nonstressed and Oxidatively Stressed Trastuzumab
Summary

- Two Dimensional HPLC can elucidate complex samples in an automated mode
- Agilent solution allows easy programming to develop and modify two dimensional setups
- Visualization tools allows for fast interrogation of data sets