Characterization of Antibodies using HPLC Chip and Ultra High Performance Liquid Chromatography

Jim Lau and Keith Waddell

Review of Peptide Mapping
Intact Protein Analysis
HPLC Chip Analysis of Protein Modifications
Traditional HSA protein digest: 60 minute elution of digested peptides Results found by Molecular Feature Extractor
What is Ultra High Definition?

Ultra High Definition is the combination of uncompromising performance in **All Dimensions** of the analytical measurement.

Ultra High Definition LC/MS achieves the **Maximum Qualitative** and **Quantitative Information possible**.
Agilent 1290 + 6530 Accurate Mass Q-TOF: Rapid Peptide Mapping of Proprietary IgG

100% sequence coverage for both light AND heavy chains!

TIC

ECC for Matched Peptides
Ultra High Definition
Optimizing all Analytical Dimensions

- Sensitivity
- Dynamic Range
- Linearity

- Mass Accuracy
- Resolving Power
- Acquisition Rate

- Separation Speed
- Peak Capacity

Signal Response

Chromatogram

Mass Spectrum
Molecular Feature Extractor (MFE)

• Looks at Mass spectral data first → Groups co-eluting isotopes & converts to neutral mass
• Checks that there is a chromatographic response
• Groups all charge states, adducts etc associated with a given feature into a “peak volume”
• Feature consists of an accurate neutral mass, a retention time, and a peak volume
Molecular Feature Extractor (MFE)

Transforming Data to Chemical Information

Identification, Quantification, Differential Analysis are performed on chemically qualified compound data
Agilent 1290 + 6530 or 6540 UHD Q-TOF for Rapid Peptide Mapping

- Chromatographic peak width (half-height) 0.3-0.8 secs
- MS acquisition (300-3000) at 10 spectra/sec in high resolution mode
- 98.8% sequence coverage in 1.5 min!

+5 charge state
CCTESLVNRRPCFSALTPDETYVPKAFDEK

35,000 resolution
1.5 Minute LC/MS/MS Analysis of Transferrin Digest

81% sequence coverage and 93 unique peptides in 1.5 min!!

- Red indicates matched peptides
- For transferrin, the first 19 amino acids are the signal peptide
# Ultra High Definition – What Sample Rate is Needed?

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<th>Medium 5 pts &gt; 10% Spectra/s</th>
<th>Medium 10 pts &gt; 10% Spectra/s</th>
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**Notes:**
- UHPLC: Ultra High Performance Liquid Chromatography
- FHWM: Full Width at Half Maximum
- Spectra/s: Spectra per second

**Graphs:**
- High ~ 20 spectra
- Medium ~ 5 spectra
- Medium ~ 10 spectra
- Low ~ 3 spectra
HPLC-Chip Platform Support
Supporting Workflows from Discovery to Verification

- Introduced with Integrated nano-ESI on Ion Trap for Protein ID
- Reference Mass Introduction for Ultimate Mass Accuracy on TOF & QTOF Biomarker Discovery
- Integrated with Agilent’s Triple Quad for Ultimate Sensitivity and Quantification
Integrate functional components onto a reusable, biocompatible chip

- enrichment and analytical nanocolumns,
- nanospray emitter
- fittings and connection capillaries
- directly on a reusable biocompatible polymer chip.
HPLC-Chip/MS Benefits

- Max Efficiency & Sensitivity
  - Zero dead volume for better chromatographic performance
  - LC/MS sensitivity

- Hassle-free – All-in-One
  - Laser-ablated channels
  - Analytical column
  - Enrichment column
  - Microvalve connection
  - Nano-electrospray tip
  - Micro-filters

- Maximum Uptime
  - No clogging of spray needle
  - Plug-&-Play replacement
HPLC-Chip/MS Interface: Fluid Connections to the HPLC-Chip

Side View

Rotor

Stator

Autosampler

Waste

Nanopump

(a)

Sample

from LC pump

Waste

from LC pump

(b)

LC column

inner rotor

outer rotor

Microvalve

HPLC-Chip

Agilent Technologies
HSA protein digest 7 minute elution of peptides found by Molecular Feature Extractor HPLC Chip
HSA protein digest 7 minute elution of peptides found by Molecular Feature Extractor HPLC Chip
HSA protein digest 7 minute elution of peptides found by Molecular Feature Extractor HPLC Chip
HSA protein digest 7 minute elution of peptides found by Molecular Feature Extractor HPLC Chip
Isotope fidelity for MFE extracted components
HSA protein digest 7 minute elution of peptides found by Molecular Feature Extractor
## BioConfirm Protein Analysis Peptide Matching Results

### Compound List

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<th>Mw</th>
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### Sequence Editor: Define and Match Sequences

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- **Sequence Type:** Protein Digest
- **Amino Acid List:**
  - **A:** Ala, 47
  - **R:** Arg, 26
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  - **C:** Cys, 35
  - **Q:** Gln, 20
  - **G:** Gln, 58
  - **E:** Glu, 65
  - **H:** His, 35
  - **I:** Ile, 15
  - **L:** Leu, 35
  - **K:** Lys, 60
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#### Sequence Editor

- **Sequence:**
  - **N-term:** MLVLAPEQGVPIEVLKFLVNLKPDFAKTVQSGAKKMRGK
  - **C-term:**

---

**Page 22**
BioConfirm Protein Analysis Peptide Matching Results (Sequence Coverage)
QTOF with HPLC-Chip Achieves 100 Attomole Sensitivity for Protein Digest Analysis

+ EIC(582.31, 653.36, 722.33) Scan 100attmole_BSA600nL_base_short00014.d

Abundance vs. Acquisition Time (min)
MassHunter BioConfirm
Analysis of Intact Proteins

Maximum Entropy Deconvolution

- BioConfirm Workflow
  - Integrate (MS)
  - Integrate (UV)
  - Extract (MS)
  - Deconvolute (MS): Maximum Entropy
  - Find by Molecular Feature
  - Define and Match Sequences
  - Search Database
  - Compound Report
  - Common Reporting Options

+ Chromatogram
+ Spectrum
+ General
+ Find Compounds
  - Find Compounds by Formula
  - Identify Compounds
+ Compound Automation Steps
+ Worklist Automation
+ Export
1. Intact mAb analyses - Chromatography

TIC’s of mAb 5 replicate injections of 500ng on column
Poroshell 2.1x75mm C8
80°C, 400µL/min
1. Mass Spectrum of a mAb
Maximum Entropy Deconvolution

- **Mass range:** 10000.00-17000.00 Daltons
- **Mass step:** 1.000 Daltons
- **S/N threshold:** 30.0
- **Adduct:** Proton
- **Average mass:** 90 % peak height
- **Isotope width:** Automatic 20.000 Daltons

**Compound filters**
- Minimum consecutive charge states: 5
- Minimum protein fit score: 8

- **Previous results**
  - Delete previous compounds (during Integrate and Deconvolute)

- **New results**
  - Highlight first compound
  - Highlight all compounds

- **Chromatograms**
  - Extract EIC

- **Data model**
  - Singlet width: Default
  - Peak width: 0.5 m/z
  - Resolution: 12000

- **Limits**
  - Number of iterations: 20
Deconvoluted Mass Spectrum of Intact mAb

Hexose unit Δ 162.2

Δ 1484.87

Δ 2890.81

G0

G1F
Bioconfirm: Protein Modification Matching Rules

Target protein

Protein modifications such as oxidation, sodium, potassium, immunoglobulin glycoforms (G0, G0F, G1, G1F, G2, G2F, and combinations thereof)
### mAb IgG2 Intact matching Results

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Large Molecule Feature Extractor (LMFE) - A new large molecule data mining tool
How Does LMFE Differ From Maximum Entropy Deconvolution?

Maximum Entropy Deconvolution:
- Produces deconvoluted spectra
- Works great for simple samples with known mass

LMFE:
- No knowledge of target protein mass(es) required
- Faster and produces more information for complex samples
- Drawback: No deconvoluted spectra (but can use info for MaxEnt Deconvolution)
Molecular Feature Extractor (MFE)

Transforming Data to Chemical Information

Raw data → Background noise removed → Individual m/z peaks grouped into isotope clusters → Isotope clusters grouped into molecular features

Identification, Quantification, Differential Analysis are performed on chemically qualified compound data
Overview of LMFE Algorithm

- LMFE uses a similar methodology to the previously-developed Molecular Feature Extractor (MFE) algorithm.

- This approach first finds all peaks in an LC/MS run and creates three dimensional peaks for each species.

- Subsequently, LMFE groups the peaks with the same retention time and elution profile into “coelution groups”. In the process, background compounds that do not show a true LC elution profile are removed from consideration.

- The peaks within a given coelution group will contain the different charge states of the same protein, which are subsequently grouped together by algebraic charge state deconvolution.

- While algebraic deconvolution can be challenging for very complex spectra, the charge states for a given protein will generally elute at a slightly different time than other eluting proteins even in highly complex mixtures.

- LMFE produces a list of compounds within the Mass Hunter Qualitative Analysis software, with links to a compound spectrum containing the different charge states found for a given protein and the extracted compound chromatograms for each compound.
Instead of grouping coeluting isotopes, group coeluting charge states from an intact protein:
Large Molecule Feature Extractor (LMFE)

New algorithm invoked by calling “Find By Molecular Feature” using the target data type “Proteins (chromatographic)"
Large Molecular Feature Extractor Results

Protein compound EIC’s and TCC

Charge states for protein compound

Protein masses in compound list
TIC Overlay: Run #3 for Samples A2, A29 and A39

A2 = red
A29 = blue
A39 = black
140 minute LC/MS run of *E. coli* intact proteins:

- **Maximum entropy**: Integrate and extract 60 peak spectra, deconvolute each spectrum (90 minutes), 140 compounds
- **LMFE**: 15 minutes, 682 protein compounds
LMFE Results: Average number of features (3 runs averaged) per bacterial strain – threshold of 500 counts

• A2 - $\frac{674 + 652 + 659}{3} = 661$ features
• A29 - $\frac{691 + 700 + 658}{3} = 683$ features
• A39 – $\frac{611 + 596 + 588}{3} = 598$ features
PCA after filtering
Hierarchical Clustering
PCA Plot – From Mass Profiler professional

![PCA Plot](image-url)
Panel of Proteins (features/compounds) that differentiate the 3 samples
Example 3: Raw Data (RD) and LMFE extracted Data, cont.

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<td>Cpd 315: 47.066</td>
<td>Large Molecular Feature Extractor</td>
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</table>
Example 2 ECCs:
Example 4 ECCs:
Introduction
Agilent Chip Technology

The HPLC-Chip Cube Interface
The HPLC-Chip Cube MS interface contains the loading mechanism for chip positioning, the microvalve for nano-LC hydraulic connections and flow switching, and the nanospray ion source with camera for spray visualization. The HPLC-Chip Cube automatically and precisely positions the chip spray tip orthogonal to the MS inlet to ensure maximum sensitivity and robustness, and makes the necessary electrical connections and all hydraulic connections to the chip. The entire process is fully automated and requires no tools. Since it’s so easy to do, each individual researcher can have his or her own dedicated chip, reducing the risk of cross contamination.

HPLC-Chip Cube interface.
Sample Preparation Steps for N-Linked Glycan Characterization

**Glycoprotein**

- PNGase F
  - Glycosylamine
  - N-Glycan

**Workflow time: ~ 1 day**

- MALDI-ToF
- HPLC with MS detection
- HPLC with pulsed amperiometric detection

**Reductant**

- 2-AB

**Workflow time: 2-3 days**

- HPLC with fluorescence detection
- CE with fluorescence detection

**Labelled N-Glycan**
On-Chip Glycan Characterization in 10 Minutes

Experimental Time

\[\text{Time: 10 minutes}\]
3-layer chip for Glycan Sample Preparation & Analysis

Deglycosylation: Enzyme Reactor
Protein retention:
Glycan analysis:

Enzyme reactor 180 nL
6 second residence time for deglycosylation
Fluidic Path Configuration

A. Sample Preparation Configuration

1. Antibody sample loaded (in aqueous volatile buffer)
2. Glycans cleaved with PNGaseF
3. Glycans purified via protein retention column
4. Glycans concentrated

B. Sample Analysis Configuration

5. Nanopump gradient generated (aqueous to organic)
6. Glycans eluted
7. Glycans separated on PGC column and detected via MS
Deglycosylation Mechanism by PNGase F

1. C-N bond of glycosylated asparagine side chain is cleaved
2. Asparagine residue is converted to aspartic acid
3. Glycan is an amino glycan initially
4. The amino glycan is slowly hydrolyzed to a hydroxyl and ammonia is liberated.

Glycan Profiles from Pharmaceutical Antibody 1

0.984 amu smaller

Glycan Profiles from Pharmaceutical Antibody 2: Separation of afucosylated and fucosylated glycans

Non-fucosylated glycans

Fucosylated glycans

Counts vs. Acquisition Time (min)
Chip Enables Fast Separation of Isomers:
Chip Enables Fast Separation of Isomers:

Detection of Immunogenic glycan isomers from mAb made in Mouse NSO Cell

G2 structure
Chip Enables Fast Separation of Isomers:

Detection of Immunogenic glycan isomers from mAb made in Mouse NSO Cell

G2 structure

1786.650 amu
Comparison of Two Antibody Batches

Results:
- Profiles for batch 1 and 2 look similar.
- Batch 2 has a higher percentage and greater variety of sialylated glycan forms.
On-chip Deglycosylation and analysis of intact antibodies

- Intact mass of heavily modified proteins is difficult to measure.
- By deglycosylating the protein first, the accurate protein mass is measureable.
HPLC Chip MS for mAB analysis

• The monoclonal ANTI-FLAG M2 antibody (Sigma) was provided in two forms:
  • As purchased from Sigma (glycosylated form) – at a concentration of 1mg/ml
  • Deglycosylated form – at a concentration of 2mg/ml

• The glycosylated mAB was diluted 100-fold with 0.1% formic acid, while the deglycosylated mAB was diluted 400-fold with 0.1% formic acid and varying amounts of each were injected on column. Triplicate runs were performed for each concentration.
TIC of Glycosylated and Deglycosylated mAB Runs (3 ng on column):
Glycosylated mAB: Charge State Dilution Series

100pg = 670 amoles

200pg = 1.34 fmoles

Counts vs. Mass-to-Charge (m/z)

Blank
Glycosylated mAB: Charge State Dilution Series (Con’t)

- 600pg = 4.02 fmoles
- 1ng = 6.71 fmoles
- 3ng = 20.14 fmoles
- 9ng = 60.42 fmoles
Glycosylated mAB: Deconvoluted Spectra Dilution Series

100 pg = 670 amoles

200 pg = 1.34 fmoles

Blank
Glycosylated mAB: Deconvoluted Spectra Dilution Series (Con’t)

600pg = 4.02 fmoles

1ng = 6.71 fmoles

3ng = 20.14 fmoles

9ng = 60.42 fmoles
Deconvoluted Glycosylated mAB: Reproducibility of Triplicate 600pg Runs
Glycosylated mAB: Reproducibility and Linearity of 148570.94 Glycoform:

<table>
<thead>
<tr>
<th>Mass</th>
<th>Peak Height</th>
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<tbody>
<tr>
<td>148571.15</td>
<td>2704</td>
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<td>148570.89</td>
<td>2852</td>
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<tr>
<td>148571.17</td>
<td>3008</td>
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Avg: 148571.07, Std.Dev: 0.16, (1.05 PPM), (5.33 %CV)

<table>
<thead>
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<th>Mass</th>
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Avg: 148571.14, Std.Dev: 0.17, (1.17 PPM), (2.73 %CV)

<table>
<thead>
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<tbody>
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<tr>
<td>148570.61</td>
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Avg: 148570.69, Std.Dev: 0.11, (0.71 PPM), (3.00 %CV)

<table>
<thead>
<tr>
<th>Mass</th>
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<tbody>
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Avg: 148571.21, Std.Dev: 0.17, (1.17 PPM), (1.02 %CV)

Avg Std. Dev. = 1.49 PPM ; Avg. %CV = 3.07
Deglycosylated mAB: Charge State Dilution Series

Blank

50pg = 345 amoles

200pg = 1.38 fmoles
Deglycosylated mAB: Charge State Dilution Series (Con’t)

- 600pg = 4.14 fmomles
- 1ng = 6.91 fmomles
- 3ng = 20.74 fmomles
- 9ng = 62.22 fmomles
Deglycosylated mAB: Deconvoluted Spectra Dilution Series:

- Blank
- 50pg = 345 amoles
- 200pg = 1.38 fmoles
Deglycosylated mAB: Deconvoluted Spectra Dilution Series (Con’t)

600pg = 4.14 fmoles

1ng = 6.91 fmoles

3ng = 20.74 fmoles

9ng = 62.22 fmoles
Deglycosylated mAB: Reproducibility and Linearity

<table>
<thead>
<tr>
<th>50 pg Runs</th>
<th>Deconvoluted</th>
<th>Mass</th>
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<tbody>
<tr>
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<td>2</td>
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<tr>
<td>Avg</td>
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<tr>
<td>Std.Dev</td>
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(6.91 PPM) (6.44 %CV)

<table>
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<td>Avg</td>
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<td>Std.Dev</td>
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(2.17 PPM) (1.60 %CV)

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<tr>
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(1.07 PPM) (1.11 %CV)

<table>
<thead>
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<td>Avg</td>
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<td>Std.Dev</td>
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(1.11 PPM) (0.81 %CV)

<table>
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<tr>
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(0.89 PPM) (0.54 %CV)

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(0.66 PPM) (1.04 %CV)

Avg Std. Dev. = 2.13 PPM ; Avg. %CV = 1.92
Glycan Analysis

- Novel Glycan chip allows retention of glycan species
- Excellent resolution
- Switch back in a minute for reverse phase separation

Base Peak Chromatograms of N-linked Glycans From Human Serum

- 10% Fraction
  - Neutral Oligosaccharides
- 20% Fraction
  - Neutral & Acidic Oligosaccharides
- 40% Fraction
  - Acidic Oligosaccharides
Ultra High Definition LCTOF and LCQTOF MS offer us the opportunity to study proteins in a manner that is faster (UHPLC MS), more sensitive (HPLC Chip MS) and more thorough (MFE and LMFE coupled with experiment specific HPLC Chip MS) than ever before.