Improvements in Mass Spectrometry for Life Science Research – Does Agilent Have the Answer?

Ashley Sage PhD
What is Mass Spectrometry?

A powerful analytical chemistry technique that is used to:

– elucidate the structure and chemical properties of molecules
– quantify trace levels of compounds in complex matrices

Detection of compounds can be accomplished with very minute quantities

A mass spectrometer does not actually measure the molecular mass directly, but rather the mass-to-charge ratio (m/z) of the ions formed from the molecules
Mass Spectrometry – could it ever be routine?

Aston’s Spectrograph
Circa 1937

Mid 70s onwards

Circa 1954
Optimizing all Analytical Dimensions
- consider the variables

- Signal Response
  - Sensitivity
  - Dynamic Range
  - Linearity

- Chromatogram
  - Separation Speed
  - Peak Capacity

- Mass Spectrum
  - Mass Accuracy
  - Resolving Power
  - Acquisition Rate

- Software
  - Data Mining
  - Analysis
  - Differential Profiling
MS today – it’s come a long way, but what to use?

Single Quad

Triple Quad

TOF/Q-TOF
Data Output – *its only software!*

1980’s data output
Obtaining Meaningful Data.....*but what do you want to find?*

_Hunts Needle in a Haystack_

How long does it take to find a needle in a haystack? Jim Moran, Washington, D.C., publicity man, recently dropped a needle into a convenient pile of hay, hopped in after it, and began an intensive search for (a) some publicity and (b) the needle. Having found the former, Moran abandoned the needle hunt.
MassHunter Compound-Centric Data Processing

Acquire Data
LC/MS+GC/MS

Find Compounds
- Find Compounds MFE
- Find Compounds LMFE (Proteins)
- Find Compounds Auto MS/MS
- Find Compounds Targeted MS/MS
- Find Compounds By Formula
- Find Compounds (GC/MS)

Compare Compounds
- Compare compounds between samples
  - Metabolite ID
  - Mass Profiler
- Compare compounds between 2 sample sets
  - Mass Profiler
  - Mass Profiler Pro
- Compare compounds between >2 sample sets
  - Mass Profiler Pro

Identify Compounds
- Protein DB Search
  - Spectrum Mill
  - Mascot
  - Others (via mzXML, mzData)
- Accurate Mass and RT (AMRT) Database Search
  - Endogenous Metabolites (METLIN)
  - Food, Forensics, Environmental
- Spectral Library Search
  - LC/MS/MS Library Search
  - NIST GC/EI-MS Library
  - Fiehn GC/EI-MS Library
- Molecular Formula Generation (MFG)
  - Via accurate mass MS and MS/MS

Fully automated

Agilent Technologies
## Typical Applications for Mass Spectrometry

<table>
<thead>
<tr>
<th>Small Molecule Analysis</th>
<th>Clinical Research &amp; Diagnostics</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Mass confirmation</td>
<td>- Inborn errors</td>
</tr>
<tr>
<td>- Structural elucidation by MSMS</td>
<td>- Therapeutic drugs etc</td>
</tr>
<tr>
<td>- Impurity Profiling</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolism Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Metabolite ID</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolomics</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Sample comparison</td>
</tr>
<tr>
<td>- Metabolism pathways</td>
</tr>
<tr>
<td>- Biomarker discovery</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteomics</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Intact protein analysis</td>
</tr>
<tr>
<td>- Peptide analysis and quant</td>
</tr>
<tr>
<td>- Biomarker discovery</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food Safety &amp; Environmental</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Water quality testing</td>
</tr>
<tr>
<td>- Residue analysis</td>
</tr>
<tr>
<td>- Soil testing</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Forensics</th>
</tr>
</thead>
<tbody>
<tr>
<td>- drugs of abuse</td>
</tr>
<tr>
<td>- Sports testing</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pharmacokinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Drug discovery &amp; delivery</td>
</tr>
</tbody>
</table>

And the list goes on.........
Small Molecule Accurate Mass – using TOF MS with Database Searching
The Advantage of Accurate Mass Measurement
- increased specificity

$C_{33}H_{40}N_{2}O_{9}$ has a protonated ion at 609.28066

Quadrupole MS reports mass to +/- 0.1Da = 165 ppm

High Resolution MS reports to <2ppm

Possible Formulas (C,H,N,O)

- 165 ppm 209
- 10 ppm 13
- 5 ppm 7
- 3 ppm 4
- 2 ppm 2

0.7Da FWHM
40,000 FWHM
Isotopic Interpretation – aid deconvolution

Correct Identity
\( C_{19}H_{17}N_5S \)

Elements Used to Calculate Formulas

<table>
<thead>
<tr>
<th>Element</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>N</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>
Metabolite Identification

Precursor Isotope Pattern and Fragment Mass Agreement

Buspirone monohydroxy metabolite

MS spectrum

MS/MS spectrum

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

Counts (%) vs. Mass-to-Charge (m/z)
Metabolism – important part of Pharmaceutical Development

N-Oxidation

Clzapine-N-oxide

Hydroxylation

Demethylation

Thiomethylation

3-Hydroxyclozapine

Parent: Clozapine

3-Thiomethylclozapine

3-Hydroxynorclozapine

Norclozapine

3-Thiomethylnorclozapine
Accurate Mass MS/MS Database and Library

- Database of over 7500 compounds/metabolites with MSMS spectra
- MS/MS spectra are collected in positive and negative ion mode
- MS/MS spectra are produced from the isolated mono-isotopic ion
- Fragmentation data is collected at three collision energies: 10, 20 and 40eV
- Matching is done used forward and reverse database searches
- Match Quality score ranks search results
Case 353-10 – Unknowns Analysis (forensics)

- MS Database Search
  - Isomers can’t be distinguished,
  - Accurate mass and isotopic pattern allows for empirical formula confirmation

- Benzoylcegonine?
- Roletamide?
- Norcocaine?
- ???

- Dexamisole?
- Tetramisole?
- ???

- Cocaine?
- Fenoterol?
- Hydromorphinol?
- Scopolamine?
- ???
Case 353-10 – Unknowns Analysis (forensics)

- MSMS Library search
  - Isomers are identified
  - MSMS spectra containing structural information

- Benzoylecgonine?
- Roletamide?
- Norcocaine?
- Cocaine?
- Fenoterol?
- Hydromorphinol?
- Scopolamine?
- Dexamisole?
- Tetramisole!
Agilent Solutions for Metabolomics
Metabolomics is the comparative analysis of endogenous metabolites found in biological samples:

- **Compare** two or more biological groups
- Find and identify potential biomarkers
- Look for biomarkers of toxicology
- Understand biological pathways
- Discover new metabolites

Metabolites are the by-products of metabolism

- Range of physico-chemical properties
- Classes: Amino acids, Sugars, organic acids, fatty acids, lipids…
- Molecular weights upto 1000Da
Agilent Metabolomics Workflow

- **Separate & Detect**
  - GC/MSD
  - GC-QQQ

- **Feature Finding & Quantitate**
  - MassHunter Qual
    - AMDIS or Find by chromatographic deconvolution
  - MassHunter Qual
    - MFE, Find by Formula, Find by Ion

- **Alignment & Statistics**

- **Identify**
  - Mass Profiler (MP)
  - ID Browser
  - Mass Profiler Professional (MPP)

- **Pathways**
  - Pathway module Cytoscape

- **LCMS**
  - LC-TOF/QTOF
  - LC-QQQ

- **GCMS**
  - GC-MSD
  - GC-QQQ
Differential Analysis & Visualization - Software

Mass Profiler
- Performs pair wise differential analysis
- Designed for TOF data only
- Simple t-test
- METLIN Personal database is integrated

Mass Profiler Professional
- Simple or complex data sets
- Performs many types of statistical analyses
- Numerous visualizations
- Import and process data from Agilent: GC/MS, LC/TOF, LC/Q-TOF, LC/QQQ data)
- Identify metabolites using integrated ID browser

25,157 unique mass features
59 up-regulated unique mass features
PCA analysis of all Red Blood Cell samples reveals separation based on pH of extraction solvent.
Pathway analysis in MPP showing differential abundances for three compounds in the urea cycle:

- L-Arg (Arginine)
- Ornithine
- Citrulline

Infected Blood cells

Non infected
Intact Protein Analysis
Intact Protein Analysis

Check QC of manufacture

• Molecular weight confirmation
• Check impurities

Determine protein modifications

• Glycosylation etc

Measurement of therapeutic antibodies (monoclonal antibodies)
MassHunter BioConfirm – Intact Protein Analysis

Configurable Workflow for Easy Data Interpretation

Compound List based on Protein Sequence

Chromatogram

Spectrum Deconvolution Results

Agilent Technologies
Typical LCMS Analytical Conditions

LC Conditions
1200 Binary SL pump + Degasser
1200 SL Autosampler
1200 SL Diode Array
Thermostatted Column Compartment

Column: Poroshell 300SB – C8, 1.0x75mm, 5µ

Mobile Phase:
(A) Water + 0.05% TFA
(B) Acetonitrile + 0.05% TFA

Flow Rate: 0.25mL/min
Injection Volume: 10µL

LC Gradient: 95% A to 95%B over 10 mins
Myoglobin – Mass Spectrum
Horse Heart Myoglobin – MaxEnt Deconvolution

0.51ppm mass measurement

MW = 16951Da
Structure and Modifications of Antibodies

- Analyze intact antibody
- Analyze deglycosylated antibody (enzymatic)
- Analyze reduced antibody (light and heavy chains)
- Analyze Fab and Fc regions (papain cleavage)

<table>
<thead>
<tr>
<th>Code</th>
<th>Oligosaccharide structure</th>
<th>Average mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2F</td>
<td><img src="G2F" alt="Oligosaccharide structure" /></td>
<td>1789.64</td>
</tr>
<tr>
<td>G1F</td>
<td><img src="G1F" alt="Oligosaccharide structure" /></td>
<td>1697.49</td>
</tr>
<tr>
<td>G0F</td>
<td><img src="G0F" alt="Oligosaccharide structure" /></td>
<td>1445.35</td>
</tr>
<tr>
<td>G0</td>
<td><img src="G0" alt="Oligosaccharide structure" /></td>
<td>1239.21</td>
</tr>
</tbody>
</table>

- Fucose – 146 Da
- Mannose – 162 Da
- N-Acetylgalactosamine – 203 Da
- Galactose – 162 Da
Mass Spectrum of a mAb – HPLC-Chip/MS of 10 ng
On-column

Poroshell SB300-C18
Mwt = 149,500Da
Intact mAb Analysis - Intact and Deglycosylated

Deglycosylated (sugar groups removed enzymatically)
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.
LCMS Analysis of Therapeutic Oligonucleotides

Agilent 6520 Q-TOF (-ve ion)

**Column:** C18, at 35°C  
**Mobile Phase:** HFIP/TEA/ Methanol
High Resolution & Accuracy is Key

Me-phosphonate-DNA PS

CsAvGvTsCvAsGsTvAsCcGcT

S: phosphorothioate
V: methylphosphonate thioate
Peptide Biomarker Analysis
Biomarker ID and Quant Workflow

**Step-1 Q-TOF**
- Run samples on Q-TOF for protein ID in data-dependent MS/MS mode.

**Step-2 Spectrum Mill**
- Search QTOF data using Spectrum Mill
- Use Spectrum Mill MRM Selector to create a list of MRM transitions with RT

**Step-3 QQQ**
- Import the MRM list into QQQ Acquisition software
- Run samples on QQQ in Dynamic MRM mode

**Step-4 Mass Profiler Pro**
- Integrate the MRM chromatograms
- Import quantitation results into MPP to perform statistical analysis
Q-TOF LCMS for Peptide Mapping

- Chromatographic peak width (half-height) 0.3-0.8 seconds
- MS acquisition (300-3000) at 10 spectra/sec in high resolution mode
- 98.8% sequence coverage
LC/MS/MS Analysis of Serotransferrin Digest

81% sequence coverage and 93 unique peptides

- Red indicates matched peptides
- For transferrin, the first 19 amino acids are the signal peptide

Protein Name: Serotransferrin precursor - Bos taurus (Bovine)
Species: BOVIN
6490 vs 6460 for Peptide Quant, 2 mm id Column
Human Serum Albumin Peptide (LVNEVTEFAK, 575.5 → 937.5)

**Quantifier**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>6490-iFunnel + AJS</th>
<th>6460 + AJS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 fmol</td>
<td>25 pmol</td>
<td>25 pmol</td>
</tr>
</tbody>
</table>

**Calibration Curves**

- 1 fmol → 25 pmol

**Poroshell 120**

2.1 x 150 mm column at 0.5 mL/min
6490 – Technology Enhancements

• iFunnel Technology
  – Agilent Jet Stream Ion Generation
  – Hexabore capillary
  – Dual ion funnel (DIF) technology
    • Two stages for ion focusing and gas removal
    • Improvements for wide $m/z$ range transmission

• Collision Cell
  – Hexapole field axial focusing curved collision cell
    • Tapered cell structure for increased ion acceptance at entrance
    • Reduction of ionizer generated noise

• Improved Quad Drive Electronics
  – Improved Quad DC frequency response
  – Higher RF power capability
  – Quad drive frequency increased to 1.4 MHz
High and Low Pressure Funnels
Steroid Analysis – using ESI

HPLC Method

Column: Poroshell 120EC 2.1 x 150 mm, 2.7 um
Injection volume: 10 µl
Column Temp: 50°C
Mobile Phases: A: 0.05 mM Ammonia soln: 10% Methanol
B: Methanol
Flow rate: 0.4 ml/min
Gradient:

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>0.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>10.5</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

15 minute run
Steroids Chromatogram, Std 8

17β Estradiol

17α Estradiol
β-Estradiol lowest std: 0.02 ng/ml

T1 s/n 236.9

T2 s/n 165.3
Food Safety - 300 Pesticides in 15 min.

600 MRM Transitions acquired
Agilent and the Future.....?

Continued LC, MS hardware and software development
  – QTOF development for increased sensitivity for biomarker coverage

Increased productivity with software workflow
  – Study Manager concept for multi-user environment
  – Structure related visual aids for mass spec interpretation

Next Generation Mass spectrometer......what ever that may be?
The future of mass spectrometry........
Thank You