Ultra-High Sensitivity in Triple Quadrupole LC/MS/MS Performance

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with contributions from
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Frank Kuhlman, Ning Tang
The Agilent 6460: Cutting Edge QQQ Performance

With…

Agilent Jet Stream Technology
Unmatched sensitivity
Workflow improvements
Faster method development

- 100fg reserpine sensitivity with less than 10% RSD !!!
- Fast Pos/Neg switching
- Faster MRMs and more MRMs per time segment
- New Optimizer software enables faster MS/MS method development
Breaking the “fg barrier” with the new 6460A Triple Quad LC/MS/MS

Performance of Agilent QQQ models

Femtogram barrier

6460
6410-High Sensitivity
6410

Femtograms

10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
Agilent 6460 QQQ Performance

Shattering the Femtogram Barrier – 500 Attograms

Verapamil, 500 attograms

Breakthrough Sensitivity
6460 triple quadrupole with Agilent Jet Stream technology breaks the femtogram barrier, shown here with 500 attograms of verapamil injected on-column, using unit resolution for both Q1 and Q3.
Agilent 6460 QQQ Performance –
500 attograms verapamil and extended dynamic linear range

Five Decades of Linearity
6460 triple quad with Agilent Jet Stream technology exhibits outstanding performance with 5 decades of linearity from sub-femtogram to 100 picograms of verapamil injected on-column.
Why focus on improving the efficiency of ESI?

Ionization and Transmission Efficiency in an Electrospray Ionization–Mass Spectrometry Interface
Jason S. Pagea, Ryan T. Kellya, Keqi Tanga and Richard D. Smith,
Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington, USA

Journal of the American Society for Mass Spectrometry
Volume 18, Issue 9, September 2007, pages 1582-1590

“Ion transmission efficiency, also defined as the fraction of ES current that enters the mass analyzer, has traditionally been limited by losses at the mass spectrometer inlet and at the skimmer [[7] and [21]]. It has been estimated that only about one out of every $10^3–10^5$ analyte ions generated by ESI at atmospheric pressure is actually detected using present instrument designs [[7], [10] and [22]].”
Agilent Jet Stream* Ion Generation

Gas Dynamics View

The super-heated sheath gas collimates the nebulizer spray and creates a dramatically “brighter” source.

*N Patent Pending
Agilent Jet Stream Ion Generation

*Thermal Dynamics View*

This plot is a simulation showing the **thermal profile** of the Agilent Jet Stream technology.

Thermal energy is focused to the nebulizer spray.

- **Super-heated N₂ sheath gas**
- **Nebulizer N₂ gas** (near sonic velocity)

Thermal focusing produces the most efficient desolvation and ion generation possible!

*Patent Pending*
Agilent Jet Stream In Action
Observing Thermal Gradient Focusing

Temperature 25°C at t = 0 min to 400°C at t = 8 min
Narrower spray beam, higher concentrations

Less light scattering shows increased droplet desolvation and high ion production.

Stop Temperature $400^0\text{C}$ at $t = 8\text{ min}$
Agilent Jet Stream animation
Agilent Jet Stream available on 6460 QQQ and 6530 QTOF

What else is new for these systems?

**Agilent Jet Stream***

**Resistive Capillary***

Increased turbo stage 1 pumping conductance

*Patent Pending*
Reserpine sensitivity comparisons

6460 QQQ: > > Signal/Noise vs. 6410 QQQ for 500 femtogram injections on column
Noise = 3x RMS noise

6410 QQQ
SNR = 36:1

New 6460 QQQ
SNR = 354:1
10x improvement
Limits of Detection (Estrone 3- sulfate) in Negative Ion Mode

100 fg on-column
LOD 25 femtogram

- MRM (349.1 -> 269.2) 02_E3S_100fg-r001.d
Noise (PeakToPeak) = 8.00; SNR (0.420min) = 12.1

100 fg on-column
LOD 0.9 femtogram!

6410

6460 Sub-Femtogram LOD!

Noise (RMS) = 3.15; SNR (0.330min) = 898.3

6460  Sub-Femtogram LOD!
6460 Triple Quad compared to 6410 Triple Quad

6410 Triple Quad:

Tramadol (LOD 23 fg)

Propoxyphene (LOD 33 fg)

Phencyclidine (LOD 54 fg)

6460 Triple Quad:

Tramadol (LOD 3 fg)

Propoxyphene (LOD 2 fg)

Phencyclidine (LOD 3.5 fg)

6460 8 x more S/N

6460 16 x more S/N

6460 15 x more S/N
Five Order of Linearity with new 6460 Triple Quad
Alprazolam 22 femtograms to 22 nanograms!

12 calibration levels

\[ y = 0.13371x - 0.000076 \]
\[ R^2 = 0.99912 \]
Agilent Jet Stream Performance
Ruggedness & Reproducibility – 6460 QQQ

500 Injections of Alprazolam in Spiked Human Plasma Extract, ~ 10hrs.

External Standard = 3.76% RSD for areas

Internal Standard = 1.45 % RSD for amount
6460 QQQ performance: some additional application examples
Agilent Jet Stream Performance - Ruggedness

Minimal Effect of Matrix on Response

Results show 4 therapeutics in analyzed in solvent and plasma

Relative Response: 100.3 - 116.7%
Cyprodinil: LOD 21 femtograms with 100 msec +/- switching and 5 msec dwell times

Positive ion

100 fg, S/N=14

Blank

+ MRM (226.0 -> 118.0) 11_50MRM_Blk-r001-r002.d
Noise (PeakToPeak) = 2.92; SNR (2.35min) = 14.0

Agilent Technologies
QQQ talk LCMS US Tour Fall 2008
Chloramphenicol: LOD 50 femtograms with 100 msec +/- switching and 5 msec dwell times

250 fg, S/N=15.7

Negative ion

Counts vs. Acquisition Time (min)
Agilent 6460 QQQ

*Trace analysis of pesticides*

Solvent Std, 1uL injection, 50ppt (50 fg each on-column)
Agilent 6460 QQQ

Trace analysis of pesticides

Herbicides in Spinach Matrix - 1uL injection, 100ppt level (100 fg each on-column)
Agilent MassHunter Workstation

More Innovations in Mass Spectrometry Software
MassHunter Optimizer
Automated MRM Method Development Software

Traditional MS/MS method development:
• Manual optimization of even three parameters, for dozens of compounds
  => MANY Days to WEEKS of tedious, interactive work

WITH Optimizer:
• Optimization can be fully automated for multiple compounds
  => One or a few DAYS of unattended work!

Compound-specific optimization for MRM experiments
• Selection and optimization of precursor and product ions
• Supports optimization with multiple methods (e.g. pos then neg)
• Creation of a compound database with optimized parameters for re-use
MassHunter Optimizer
Advantages over previous solutions

Utmost flexibility via support of all common optimization modes:
- Manual infusion (syringe pump)
- Automatic infusion (via loop injection at lower flows)
- Flow injection analysis without column (FIA)
- Analysis with column (multiple compounds per run)

Optimization WITH column for highest success:
- Infusion or FIA can result in 20% of cmpds not optimizing
- Optimizer is designed for injection analysis WITH column using fast LC.

MassHunter “Scheduled MRM”
Increased Utility and Performance

New applications require quantitation of 100 – 1000 compounds in one MRM method!

• Food and environmental analysis (e.g. pesticides)
• Targeted quantitation of proteins via peptides (proteomics)

WITHOUT Scheduled MRM:
• Need to manually set up multiple time segments to maximize dwell time
• Tedious to set up; problematic if changes in retention times

WITH Scheduled MRM:
• Automatic setup of overlapping time segments without user intervention
• Fewer MRMs per unit time results in longer dwell time => incr sensitivity
• Unaffected by chromatographic time shifts
Scheduled MRM

*Increased Utility and Performance*

2x shorter cycle times better for narrow chromatographic peaks, more analytes, longer dwell time per analyte.
Application example for new software features – Optimizer and Scheduled MRM

Peptide quantitation from protein digests for protein biomarker studies

• SpectrumMill Peptide Selector to choose useful peptides
• QQQ Optimizer to create MRM methods for quantitation
• HPLC-Chip MS/MS for rapid and reliable quant with small sample quantities
• MassHunter Quantitative Analysis for efficient data processing
Agilent Proteomics Biomarker Workflow

- Extraction
- Depletion
- Fractionation
- Proteolytic Digest
- Candidate Biomarker Identification
- Validation

SAMPLE1

SAMPLE2

DATA

Candidates

Extraction

Identification

6520 QTOF

6410 QQQ

Our measure is your success.
High Throughput : 2D-Gel Spot

5 min run time

Counts vs. Acquisition Time (min)

<table>
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<tr>
<th>#</th>
<th>Filename</th>
<th>z Score</th>
<th>Forward Score</th>
<th>SPI (%)</th>
<th>Spectrum Intensity</th>
<th>Sequence Map</th>
<th>RT (min)</th>
<th>m/z Measured (Da)</th>
<th>MH* Matched (Da)</th>
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Using Spectrum Mill Peptide Selector for Optimizing MRM Transitions

Perform in silico digest

Filter digestion product peptides as desired by user (mass, composition)

Predicts best product ions from most likely MS/MS cleavage for each peptide

Can provide Uniqueness of the peptide sequence in the database: helps select peptides most likely to provide unique information
SM Peptide Selector – Results for Catalase

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Acc #</th>
<th>RP</th>
<th>RT</th>
<th>MH+</th>
<th>m/z</th>
<th># DB peps</th>
<th>Start AA</th>
<th>End AA</th>
<th>Prev. Sequence</th>
<th>Next</th>
<th>b&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Y&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Y&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Caside of Aas, Gln</th>
<th>Naside of Pro</th>
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<tr>
<td>Catalyst (EC 1.11.1.6)</td>
<td>P04762</td>
<td>13.63</td>
<td>984.5109</td>
<td>492.7591</td>
<td>1</td>
<td>243 251</td>
<td>(GkLNLPEGACGR</td>
<td>(LAQ)</td>
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<td>(LVL)</td>
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<td>(CAM)</td>
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<td>1805.93</td>
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</table>

The matched peptides cover 12% of the protein.
MassHunter Optimizer – Compound(s) setup

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Group</th>
<th>Formula</th>
<th>Nominal Mass</th>
<th>Vial Number</th>
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<td>HSA peptide KVP</td>
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<td>C72H126N20023</td>
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<td>C52H34N12C17</td>
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<td>P1-E1</td>
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</tbody>
</table>

Project Name: HSA5peptides-1pm...
Optimizer Status: Complete

Sample Introduction:
- Injection built without column
- Automatic infusion using Loop injection
- Manual infusion using syringe

Fragmentor Ramp:
- Course: From 0 to 100, By 10 (Max 8 ramp steps)

Collision Energy Ramp:
- Course: From 0 to 45, By 10 (Max 5 ramp steps)
- Fine: Step [This takes more time]
MassHunter Optimizer – optimization results for peptides

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Method</th>
<th>Precursor Ion</th>
<th>Fragmentor</th>
<th>Product Ion</th>
<th>Collision Energy</th>
<th>Abundance</th>
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Agilent HPLC-Chip/QQQ LCMS Technology
Nanospray chip configuration brings new era in high sensitivity quantitation

Sensitivity: down to low amol
Dynamic range: up to $10^5$
HPLC-Chip/MS Interface: making nanoESI almost routine

Stator

Rotor

inner rotor

outer rotor

Microvalve

Autosampler
Waste
Nanopump

Sample
from LC pump

Waste
from LC pump

Nanopump

HPLC-Chip

Side View
Chromatographic Performance
Protein Digest Mixture

Reduced MS complexity + reduced ionization competition = improved ID
Limit of Quantitation in the Low Amol Range
Peroxidase 10 amol to 10 fmol spiked into 1 µg human serum

Counts vs. Acquisition Time (min)
External Quantitation Curve of Peroxidase Peptide DTIVNELR From 10 amol to 10 fmol Spiked into Human Serum

\[ y = 5287.8283 \times x + 29.6860 \]

\[ R^2 = 0.99866123 \]
Excellent Reproducibility of MS Response
HSA Peptide LVNEVTEFAK from 10 amol to 1 pmol (n=6)

All RSDs are within 15%
New 6460 Triple Quad LC/MS

- Breaks the femtogram barrier for some compounds
- New Technology – Agilent Jet Stream
- Higher Signal Strength, great RSDs even in low fg region
- New Acquisition Software - Scheduled MRMs
- New Method Development Tool – MH Optimizer
- **Agilent is committed to continuous innovation in:**
  - Quadrupole technology
  - Brighter sources
  - Innovative software