High Performance Mass Spectrometry Begins with High Performance Separations

Our Measure is Your Success

Doug McIntyre
An HPLC is merely an inlet system for a mass spectrometer

I don’t need baseline resolution because I have the selectivity of a mass spectrometer
Is Good Chromatographic Separation Needed?

As the sample matrix becomes more complex, complete separation requires more time.

Since we can also distinguish based on $m/z$ ratio, time can be saved by sacrificing chromatographic resolution.

Use MS/MS instead of time based separation.
Incomplete Separation of Sulfa Drugs

Extract individual $m/z$ values, do SIM or choose as precursor ions for MS/MS. Assumes compound response independent of other compounds.
Ion Suppression Example – good separation

Diphenhydramine
*m/z* 256.17

Ketoprophen
*m/z* 255.10

30:70 Water/Methanol w 0.2% acetic acid
Ion Suppression Example – poor separation

Diphenhydramine
m/z 256.17

Ketoprophen
m/z 255.10

5:95 Water/Methanol w 0.2% acetic acid
Poor Separation - Qualitative Issues

Deconvolution algorithms may be unable to group ions correctly to identify components in sample

Data dependant MS/MS may not provide information on low level components

• Ion suppression may lower intensity below trigger level
• More components present than number of precursor ions selected
Pesticides in Plant Extract
Targeted Analysis - Diuron

Base Peak Chrom. of +TOF MS: from WorklistData26.wiff

Max. 4.7e5 cps.

Not apparent, even in BPC

m/z 233
Untargeted – What is present?

+TOF MS: 6.752 min from WorklistData26.wiff Agilent, subtracted (6.068 to 6.176 min)

Max. 1.0e4 counts.

m/z, amu

Rel. Int. (%)
Results from Molecular Feature Extraction

Desmidopham $\text{C}_{16}\text{H}_{16}\text{N}_{2}\text{O}_{4}$, MW 300.111

Spectrum at 6.76 min
High Performance Separations – Why Not?

The increased time to separate impacts throughput

The complexity and added hardware costs (e.g. nanoflow) are not suitable in my laboratory environment

Solution:

• Small particle size columns with hardware optimized for rapid resolution
• HPLC-chip technology for easier to use improved nanoflow separations
Fundamentals of RRLC – Zorbax 1.8μm RRHT
2003: Characteristics of sub-two Micron (STM) Columns

Increased Speed
- Run short columns with small particle size at high linear velocities
- Speed gains: 5-10x
RRLC Speed
Up to 20x faster than HPLC

4.6 x 150mm, 5µm
1.20ml/min, 40°C
Analysis Time = 11min

2.1mm x 50mm 1.8µm
1.00ml/min, 40°C
Analysis Time= 1.1min

2.1mm x 50mm 1.8µm
2.40ml/min, 95°C
Analysis Time: 0.4min

HPLC, 40°C
PW = 3.4sec

RRLC, 40°C
10x faster
PW = 0.5 sec

RRLC, 95°C
27x faster
PW = 197msec

150mm > 50mm: 3x
1.2ml/min on 4.6 > 2.4ml/min on 2.1: 10x
3 x 10 = 30x
Zorbax RRHT Columns

6 Stationary phases
1, 2.1, 3, and 4.6 mm ID
Lengths from 20 mm to 150 mm
Comparison of Typical Particle Size Distribution and Engineered PSD of RRHT Columns

Goal: Achieve lower backpressure vs. a typical PSD

- Small amount of slightly larger particles
- Lower level of fines

Engineered Bimodal Distribution:
- Only 3% compromise in resolution
- 25% gain in back pressure reduction
Does speed impact precision?
Maintaining Area Precision at High Analysis Speed

**Area Precision (n=10)**

- **Inj. Vol.: 3.0µl**
  - 0.307% RSD

- **Inj. Vol.: 1.0µl**
  - 0.569% RSD

- **Inj. Vol.: 0.5µl**
  - 0.672% RSD

**Retention Times (min):**
- Butyrophenone: 1.27
- Benzophenone: 1.28
- Valerophenone: 1.29
- Heptanophenone: 1.30
- Octanophenone: 1.31

**Peaks:**
- Acetanilide
- Acetophenone
- Propiophenone
- Benzophenone
- Valerophenone
- Hexanophenone
- Heptanophenone
- Octanophenone

The chart illustrates the area precision for different injection volumes, showing the impact of speed on precision.
RRLC Improves Resolution
Up to 60% higher Resolution

Customer Example
Isocr. Impurity Method
Zoom of critical time range @ 7min

4 Impurities
2 Not Baseline Separated!

4.6 x 150, 5um
93 bar
N = 7259
R_S = 1.15
S/N = 42

7 Impurities
6 Not Baseline Separated!

4.6 x 150, 3.5um
165 bar
N = 14862
R_S = 1.37
S/N = 50

7 Impurities
All 7 Baseline Separated!

4.6 x 150, 1.8um
490 bar
N = 28669
R_S = 1.80 (+ 57%)
S/N = 44
Gradient Analysis: Simpler/Faster/No Resolution Loss
Process Monitoring of Bis-Phenol A in Industrial Feed Stocks

Current Analysis: Acquired from process site

- Column: Supelco LC-18 4.6 x 250mm, 5um
- Mobile Phase: A:0.025% H3PO4 B:MeCN C:MeOH
- Flow: 2 mL/min.
- Detector: VWD
- Wavelength: 240 nm initial, 280 nm @ 4 min
- Temperature: 35°C
- Sample size: 20 µL

50 min. @ 2 mL/min

High Throughput Analysis – 7X Faster

- Column: ZORBAX Eclipse XDB-C18 4.6x50mm, 1.8µm
- Mobile Phase: A:0.1% formic B:MeCN:MeOH (200:800)
- Flow: 1 mL/min.
- Detector: (DAD) 280 nm
- Temperature: 25°C
- Sample size: 2 µL

7.5 min. @ 1 mL/min
Rapid Resolution System – Peptides
Increased Peak Capacity for Complex Samples on Shorter Columns

2.1x150mm, 5μm
P/N 883700-922
70 min gradient
0.2 mL/min
120 peaks
60 mins

2.1x50mm, 1.8μm
P/N 822700-902
10 min gradient
0.5mL/min
125 peaks
10 mins!

2.1x50mm, 1.8μm
P/N 822700-902
30 min gradient
0.5mL/min
156 peaks!
25 mins

Conditions: Mobile Phase A: Water w/ 0.1% TFA, B: ACN w/0.1% TFA, Gradient 2%B to 50%B, Temperature: 50°C
Detection: UV 214 nm  Sample: HSA Tryptic Digest
Elevated Temperature: Reduces Pressure and Expands Column Choices

Column: SB-C18, As described below  
Mobile Phase: A: 0.1% TFA, 5% MeCN, (v/v) B: 0.08% TFA, 95% MeCN (v/v)  
Sample: 0.1 mg/ml of cardiac drugs  
Temperature: 70°C  
Flow: 2 mL/min. gradient  
Detection: 230, 16 nm

- **R_s = 4.3**  
  4.6 x 250 mm, 3.5 µm  
  P=221 bar  
  Rs = 5.6

- **R_s = 3.6**  
  4.6 x 150 mm, 1.8 µm  
  P=418 bar

- **R_s = 2.4**  
  4.6 x 50 mm, 1.8 µm  
  P=164 bar

<table>
<thead>
<tr>
<th>% B</th>
<th>50 mm</th>
<th>150 mm</th>
<th>250 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>3.5</td>
<td>10.5</td>
<td>17.5</td>
</tr>
<tr>
<td>60</td>
<td>4</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>12.5</td>
<td>4.01</td>
<td>12.01</td>
<td>20.01</td>
</tr>
</tbody>
</table>
The 1200 RRLC System

600 Bar capability at up to 5 mL/min for pump, sampler, and valves in column compartment

Column compartment limit increased to 100 degrees

Significant improvements in delay volume and dispersion for reduced run time and improved peak shape
Fast Chromatography and MS Detection

Acquisition rate of MS analyzer
Flow path from column to MS
Duty cycle issues
• Switching sources (APCI/ESI)
• Switching Polarities
• Alternating collision energies
• Number of MRMs
Analyzer Acquisition Rates

Quadrupole 10,000 u/sec
Ion Trap 25,000 u/sec
TOF 20 – 40 spectra/sec
MS/MS (scan) 2 – 8 scans/sec
MRM 50 – 100/sec

**Issue:** How to get good data at fast acquisition rates?
Effect of Acquisition Rate on Resolution

UV Signal, 40 Hz

1 spectrum/sec

2 spectra/sec

4 spectra/sec

6 spectra/sec

20 spectra/sec

0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45 min
Quadrupole MS Detection and Fast Acquisition

At typical energy values (5eV) it takes an ion 60 – 200 µsec for an ion to pass through a quadrupole mass filter.

For unit mass resolution, quadrupole is stepped in ~0.1 u increments.

As quadrupole is stepped faster, ions become unstable, less transmission.

If ion energy is increased, less mass filtering occurs and resolution suffers.
Quadrupole Lag Factors

Ion exits quadrupole at different mass setting than when it entered. Depends on \( m/z \) value and scan speed.

<table>
<thead>
<tr>
<th>Scans/sec</th>
<th>Samples</th>
<th>Ion Energy (eV)</th>
<th>Step Size</th>
<th>Quad setting when ion exits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>8</td>
<td>5</td>
<td>0.1</td>
<td>499.9</td>
</tr>
<tr>
<td>4.5</td>
<td>2</td>
<td>5</td>
<td>0.1</td>
<td>499.6</td>
</tr>
<tr>
<td>8.3</td>
<td>1</td>
<td>10</td>
<td>0.1</td>
<td>499.5</td>
</tr>
<tr>
<td>16.5</td>
<td>1</td>
<td>15</td>
<td>0.2</td>
<td>499.2</td>
</tr>
</tbody>
</table>

Agilent automatically calculates lag factors to avoid need for recalibration when scan speed changes.
Innovations in Front-End Ion Optics Deliver Better Sensitivity Across a Broad Mass Range

10X sensitivity advantage

Key components contributing to sensitivity

- Dielectric capillary
- Small diameter octopole ion guide
- High frequency RF octopole
- Lens 2 RF (transmission of higher masses)
- Hyperbolic post-filter and quadrupole
Achievable Scan Speed

Depends on interscan delay time

- 23 msec for pos/neg switching
- 50 msec to switch between ESI and APCI
- 10 msec for all other modes

Example:
- Scanning 100 – 600 requires 50 + 10 = 60 msec so 16.6 spectra/sec possible
Optimizing Instrument Set-up

Ultra high throughput and shortest cycle-times by using high temperature (80°C) and high flow rate (1.8mL/min) and Alternating Column Regeneration (ACR)

Best MS peak shape with direct (one piece) connection between flow cell and nebulizer
Agilent 6140 Offers High Speed and High Quality Performance

Chromatographic parameters:
- Column: Zorbax SB C-18 RR 2.1x50mm
- Solvent Composition: 5% ACN to 95% over 3 min at 1.0 ml/min.

MS parameters:
- Scan 100 – 900 m/z
- Speed 10,000 m/z per sec.

1.4 sec FWHM
11 scans/sec

Proper Chlorine Isotope Pattern
Fast Polarity Switching and Fast Scanning

• New capillary that is metallized and more conductive
• Pos/neg switching speed now 20 msec
• Firmware (in combination with software) includes improved filtering calculations
Ultra Fast Scan, m/z 100 – 400, 9.2 cycles/sec, 2.1 x 50 x 1.8 micron SB-C8 column
Water/Methanol w 0.02% formic acid, 1 mM ammonium acetate, 15%B – 605B at 1 ml/min

Pos/Neg Switching with Sulfa Mix

PWHM = 1 sec
Spectra for Sulfachloropyridazinone (C_{10}H_{9}ClN_{4}O_{2}S)

*MSD1 SPC, time=0.630 of C:\CHEM32\1\DATA\POSNEG\SULFAS100008.D  MM-ES+APCI, Fast Scan, Frag: 140
Max: 110608

*MSD2 SPC, time=0.631 of C:\CHEM32\1\DATA\POSNEG\SULFAS100008.D  MM-ES+APCI, Fast Scan, Frag: 140
Max: 68375

All Isotopes Present
MS Peak Broadening

Direct connection between UV and nebulizer, new capillary

< 15% peak broadening

*DAD1 A, Sig=272,16 Ref=360,100 (TEST\SULFASIM000004.D)
MSD1 TIC, MS File (D:\CHEM32\1\DATA\TEST\SULFASIM000004.D)
Peak width comparison UV/MS

Realistic scenario: Flow from column through UV cell to MS sprayer. UV cell and capillary to sprayer add peak broadening to MS signal.

Contribution UV cell: ~ 20%
Contribution capillary: ~ 45%
The Agilent Multi-Mode Source

Flow rates up to 2 mL/min
- ideal for 2.1 and 3 mm ID columns
Can do ESI and APCI simultaneously
- No switchover time
Use methanol instead of ACN for close to universal ionization
- Must deal with higher viscosity
Fast LC/MS/MS Analysis of Drugs in Oral Fluids

Cocaine

MDMA (“Ecstasy”)

d5- MDMA

Methamphetamine
d5- Methamphetamine

Amphetamine
d5- Amphetamine

Agilent Technologies
High Performance MS, High Performance Separations
Extracted Ion chromatograms - 100 Compounds by QqQ

100 pg on column or 0.01 ppm (Baby food levels)
TOF and Fast Chromatography: 20 Hz (50 msec)

XIC of +TOF MS: Experiment 2, from hilowfrag01.wiff

Max. 3.8e5 cps.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low Frag error</th>
<th>High Frag error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfamethizole</td>
<td>-1.44</td>
<td>-2.73</td>
</tr>
<tr>
<td>Sulfachloropyridazine</td>
<td>-1.92</td>
<td>-2.17</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>-0.89</td>
<td>-0.44</td>
</tr>
<tr>
<td>Sulfadimethoxine</td>
<td>0.00</td>
<td>-0.55</td>
</tr>
</tbody>
</table>

Fast scanning TOF provides simultaneous:

- High resolution high speed separation – 30 secs
- High mass accuracy – 1-2 ppm
- High information content – high frag CID
- APCI and ESI for broader coverage
## Test-Mixture

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Formula</th>
<th>[M+H]^+</th>
<th>Molecular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>0.058µg/µl</td>
<td>C_{14}H_{22}N_{2}O_{3}</td>
<td>267.1709</td>
<td>![atenolol.png]</td>
</tr>
<tr>
<td>Primidone</td>
<td>0.085µg/µl</td>
<td>C_{12}H_{14}N_{2}O_{2}</td>
<td>219.1134</td>
<td>![primidone.png]</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>0.062µg/µl</td>
<td>C_{15}H_{25}N_{2}O_{3}</td>
<td>268.1913</td>
<td>![metoprolol.png]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.125µg/µl</td>
<td>C_{27}H_{38}N_{2}O_{4}</td>
<td>455.2910</td>
<td>![verapamil.png]</td>
</tr>
<tr>
<td>Beclomethasone dipropionate</td>
<td>0.075µg/µl</td>
<td>C_{28}H_{37}ClO_{7}</td>
<td>521.2306</td>
<td>![beclomethasone.png]</td>
</tr>
</tbody>
</table>

21nmol on col., 38nmol on col., 23nmol on col., 27nmol on col., 14nmol on col.
MS with 40 Cycle/s, 5-90%B Gradient in 0.65min

Peak capacity of >40 in 39 sec in the MS chromatogram!!!

H2O/ACN
Flow =1.8ml/min
5-90%B in 0.5min
Stop time =0.65min
80°C, ACR
MS 40Hz
100-1000Da

Flow chart:
- Atenolol: 0.34s
- Primidone: 0.36s
- Metoprolol: 0.36s
- Verapamil: 0.42s
- Beclomethasone-dipropionate: 0.36s

Agilent Technologies
High Performance MS, High Performance Separations
Result from Formula Confirmation Report

Merged XIC at : 267.170

<table>
<thead>
<tr>
<th>Formula</th>
<th>Compound</th>
<th>Mass</th>
<th>Peak RT (min)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14H22N2O3</td>
<td>Atenolol</td>
<td>266.16304</td>
<td>0.21</td>
<td>3.55628 E4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Abd. (cts)</th>
<th>Ion Mass</th>
<th>Meas. Mass</th>
<th>Error (mDa)</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M+H]+</td>
<td>63089.32</td>
<td>267.17032</td>
<td>267.17016</td>
<td>-0.15448</td>
<td>-0.58</td>
</tr>
</tbody>
</table>

LC: Water/ACN(0.1%TFA), 5-95%B in 0.7min, 60°C, 1.5ml/min; UV-Detection: 214nm & 254nm, 80Hz MS-Detection: 120-1200Da, 30Hz, Split 1:3
High Throughput for Chemical Library Analysis

Pharma-Company in Germany (1200 RRLC beta-test site)

**Application: Re-analysis of screening hits**

- 2µl of compound as a DMSO solution
- Dilution by an injector program **directly before analysis** to prevent decomposition of compound
- Fast LC on 2.1mm x 50mm SB C18, 1.8µm
- Detection by **DAD** and **TOF** for accurate mass
- No ACR yet, but that would be the final goal

**Throughput: several thousand compounds per week**
High Throughput for Chemical Library Analysis

Analysis of 140 screening compounds under ultra-fast LC conditions (90 s cycle time → 1000 samples/day):

Results from automated formula confirmation report – no manual interaction!

2 outliers not shown, 16 compounds could not be ionized by ESI+

LC: Water/ACN(0.1%TFA), 5-100%B in 0.7min, 60°C, 1.5ml/min
UV-Detection: 210 – 500 nm, 80Hz MS-Detection: 120-1200Da, 8Hz, Split 1:7.5
Injection: 1µl, online sample dilution by injector program (determined the cycle time!)
High Throughput for Chemical Library Analysis

Mass error histogram of the analysis of 140 real screening compounds under ultra-fast LC conditions (90 s cycle time → 1000 samples/day):

Specs: ±3ppm

>80% of the population between ±1.5ppm

2 outliers not shown, 16 compounds could not be ionized by ESI+
All the Performance
All the Time