High Throughput Workflow for Global Metabolomic Profiling of Biological Samples with RapidFire online SPE/MS system

Michelle Romm, Ph.D.
Applications Scientist, Agilent Technologies, Inc.
What is High Throughput?

# of Samples  
N=1  
N=100  
N=1,000  
N=10,000  

RapidFire/MS  
14 secs  
23 mins  
3.8 hrs  
38.8 hrs (1.6 days)  

120X Faster  

LC/MS  
30 mins  
50 hrs  
20.8 days  
208.3 days  

12/9/2014
Why High Throughput Profiling?

Is Faster better?

When

Method Development
- pH
- Solvents/Additives
- Chemistry
- Timings/Flows

Large Population Studies
- N = 5
  - 50
  - 500
  - 5000

Better Experiments!
RapidFire/MS System
RapidFire 365 Online SPE/MS System

- **BenchBot plate handling robot**
  - 63 plate capacity (384 well plates = 24,192 samples)
  - Off-deck reach capability

- **Quaternary pumps**
  - Solvent switching capability
  - Capable of running multiple disparate methods in one batch

- **12 position automated cartridge changer**

- **Automated method development**
  - Perform multiple injections of a single sample with different SPE cartridges and mobile phases
  - C18, C8, C4, CN, Phenyl, HILIC, Graphitic Carbon
  - Simply run multiple samples overnight and select your ideal conditions the next morning

<table>
<thead>
<tr>
<th>Aspirate</th>
<th>Load/Wash</th>
<th>Elute</th>
<th>Re-equilibrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>600ms</td>
<td>3000ms</td>
<td>8000ms</td>
<td>1000ms</td>
</tr>
</tbody>
</table>

For Research Use Only. Not for Use in Diagnostic Procedures.
Method Development: Refining the Experiments

- Cartridge chemistries
- Ionization mode
- Loading solvents/times
- Elution times/solvents

One solution doesn’t fit everyone, this system allows FLEXIBILITY!
Comparison of SPE Chemistries: HILIC vs. C18 SPE/MS for a Single Feature in Urine Sample

Female Urine

HILIC

C18

For Research Use only. Not for use in diagnostic procedures.
Comparison of Chemistries: C18 vs. HILIC SPE/MS (+ESI) in Urine

- 526 metabolites annotated by C18 SPE/MS
- 270 metabolites annotated by HILIC SPE/MS
- 49 annotated metabolites common between HILIC and C18 SPE/MS

747 unique metabolites (100% reproducibility; CV<15%)

Utilize multiple unique chemistries to increase the metabolite coverage
Comparison of ESI Mode: a Small Polar Library

- ~100 pure endogeneous human metabolite standards
  - small organic acids, biogenic amines, nucleotides, amino acids
- Custom packed HILIC material

Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego
Comparison of Loading Solvents: HILIC Normal Phase

Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego
Comparison of Loading Times: Polar Analytes

Lactic Acid, -ESI

HILIC

1750ms

2250ms

3000ms

Retention of Polar Metabolites

Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego
Comparison of Elution pH

Lactic Acid, -ESI

MeOH:IPA (50:50) + 0.1% Formic Acid

MeOH:IPA (50:50) + 10mM Ammonium Acetate pH 9.0

Data obtained by Jeramie Watrous and Mohit Jain, University of California, San Diego
High-throughput Profiling: What is the Workflow?

- Human urine
- Human serum lipidome
- Milk lipidome
Human urine Metabolomics Profiling: Sample Prep

- Human urine, collected and pooled from healthy subjects (males and females), was purchased from Innovative Research Inc. (Novi, MI)
- Filter 400µL urine through a 0.22µm Ultrafree-MC® centrifugal filter units (Millipore) by centrifugation @ 12,000xg for 20min @ 4°C
- Pipette 50µL of filtered urine (10 replicates) into the Amicon Ultra-0.5mL Centrifugal Filter (3kDa Cut-off UltraceL-3 membrane) from Millipore
- Dilute with 350µL of Milli-Q quality water containing 0.2% Acetic acid, 1mM Ammonium acetate, and centrifuge @ 12000xg, 4°C for 30min to concentrate
- The flow-through represents the metabolomics sample
- Inject 10µL of flow-through for SPE/MS as well as LC/MS/MS analysis

Human urine equivalent to 1.25µL was used for the metabolomics profiling studies
TIC for 10 Human Urine Samples (C18 SPE/MS)
EIC of a Single Injection of Urine Sample on C18 RapidFire SPE/MS

Male Urine +ESI

Counts vs. Acquisition Time (min)

x10^6
EIC Overlay of a Single Feature in Urine on C18 RapidFire SPE/MS

Male Urine

For Research Use only. Not for use in diagnostic procedures.
ProFinder for High Throughput Batch Analysis of Samples

<table>
<thead>
<tr>
<th>Group</th>
<th>RT (Tgt)</th>
<th>RT (med)</th>
<th>Found</th>
<th>Missed</th>
<th>%RSD (Tgt)</th>
<th>Height (med)</th>
<th>Mass (Tgt)</th>
<th>Mass (ppm)</th>
<th>RSD (Mass, ppm)</th>
<th>RT (span)</th>
<th>RT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.115</td>
<td>0.119</td>
<td>10</td>
<td>0</td>
<td>0.3</td>
<td>1817538</td>
<td>282.1632</td>
<td></td>
<td>0.4</td>
<td>0.004</td>
<td>0.151</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>File</th>
<th>Score (Tgt)</th>
<th>Score (MFE)</th>
<th>Area</th>
<th>Height</th>
<th>Mass</th>
<th>Diff (Tgt, ppm)</th>
<th>RT</th>
<th>Ions</th>
<th>Z Count</th>
<th>Z Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inj00034-MetStab C18-B1.d</td>
<td>99.52</td>
<td>100</td>
<td>4744036</td>
<td>1821442</td>
<td>282.1631</td>
<td>-0.71</td>
<td>0.12</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00035-MetStab C18-B1.d</td>
<td>99.01</td>
<td>100</td>
<td>4769787</td>
<td>1824081</td>
<td>282.1679</td>
<td>-1.46</td>
<td>0.119</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00036-MetStab C18-B1.d</td>
<td>99.38</td>
<td>100</td>
<td>4788381</td>
<td>1812891</td>
<td>282.1633</td>
<td>-0.06</td>
<td>0.118</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00037-MetStab C18-B1.d</td>
<td>99.91</td>
<td>100</td>
<td>4748982</td>
<td>1814025</td>
<td>282.1632</td>
<td>-0.3</td>
<td>0.119</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00038-MetStab C18-B1.d</td>
<td>99.43</td>
<td>80</td>
<td>4768716</td>
<td>1812210</td>
<td>282.1682</td>
<td>-0.96</td>
<td>0.118</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00039-MetStab C18-B1.d</td>
<td>99.76</td>
<td>100</td>
<td>4724047</td>
<td>1935703</td>
<td>282.1631</td>
<td>-0.45</td>
<td>0.121</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00040-MetStab C18-B1.d</td>
<td>99.59</td>
<td>100</td>
<td>4763668</td>
<td>1798236</td>
<td>282.1683</td>
<td>-0.65</td>
<td>0.119</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00041-MetStab C18-B1.d</td>
<td>99.74</td>
<td>100</td>
<td>4744520</td>
<td>1825080</td>
<td>282.1631</td>
<td>-0.62</td>
<td>0.12</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00042-MetStab C18-B1.d</td>
<td>98.8</td>
<td>100</td>
<td>4749936</td>
<td>1798375</td>
<td>282.1632</td>
<td>-0.39</td>
<td>0.119</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inj00043-MetStab C18-B1.d</td>
<td>98.87</td>
<td>100</td>
<td>4649658</td>
<td>1756119</td>
<td>282.1682</td>
<td>-0.2</td>
<td>0.121</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Mass Profiler Professional (MPP)

Filter by all 10 reps, abundance $\geq 2$, CV $\leq 15\%$

Cluster Analysis
Annotation of Creatinine in Urine Samples by C18 RapidFire SPE/MS
Select High Abundant Metabolites in Human Urine
Annotated by SPE/MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>RF-C18 Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippuric acid</td>
<td>5158031</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>3255466</td>
</tr>
<tr>
<td>Enprofylline</td>
<td>496924</td>
</tr>
<tr>
<td>Lys Asn Asp</td>
<td>420101</td>
</tr>
<tr>
<td>3-Hydroxyhippuric acid</td>
<td>410564</td>
</tr>
<tr>
<td>Pinacidil</td>
<td>359491</td>
</tr>
<tr>
<td>Nadolol</td>
<td>357228</td>
</tr>
<tr>
<td>4-(1-Hydroxy-2-(methylamino)ethyl)phenol</td>
<td>299538</td>
</tr>
<tr>
<td>Leu Gln Lys</td>
<td>287646</td>
</tr>
<tr>
<td>Triciribine</td>
<td>285932</td>
</tr>
<tr>
<td>3-Amino-2-naphthoic acid</td>
<td>277964</td>
</tr>
<tr>
<td>L-4-Hydroxy-3-methoxy-a-methylphenylanine</td>
<td>266033</td>
</tr>
<tr>
<td>1-Methylxanthine</td>
<td>262927</td>
</tr>
<tr>
<td>AFMK</td>
<td>229545</td>
</tr>
<tr>
<td>Creatinine</td>
<td>225274</td>
</tr>
<tr>
<td>4-Chloroacetophenone</td>
<td>217597</td>
</tr>
<tr>
<td>Jasmolone glucoside</td>
<td>209979</td>
</tr>
<tr>
<td>Istamycin C1</td>
<td>192524</td>
</tr>
<tr>
<td>N-Acryloylglycine</td>
<td>183487</td>
</tr>
<tr>
<td>N2,N2-Dimethylguanosine</td>
<td>175089</td>
</tr>
<tr>
<td>N(alpha)-t-Butoxycarbonyl-L-leucine</td>
<td>171812</td>
</tr>
<tr>
<td>beta-Snyderol</td>
<td>135177</td>
</tr>
<tr>
<td>2-Methylguanosine</td>
<td>126988</td>
</tr>
</tbody>
</table>
# Metlin Library Check for Isomers/Isobars

## Single Search Results: 9 hits for Mass: 179.0582

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Formula</th>
<th>Mass</th>
<th>Delta Mass (ppm)</th>
<th>Action</th>
<th>Cation</th>
<th>RT (min)</th>
<th>CAS</th>
<th>ChemSpider</th>
<th>METLIN</th>
<th>KEGG</th>
<th>LMP</th>
<th>IUPAC Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippuric acid</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>495-59-2</td>
<td>14501</td>
<td>309167</td>
<td>C01556</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,8-Dihydroxy-3,4-dihydrocarboxylic acid</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>555-85-7</td>
<td>1552</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Acetaminobenzoic acid</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>555-88-1</td>
<td>2530</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Oxo-4-G-pyridylbutanoic acid</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>4182-31-8</td>
<td>5924</td>
<td>309167</td>
<td>C19569</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenochrome</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>54-06-8</td>
<td>64954</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetylserotonin</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>89-52-1</td>
<td>56327</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,6,6-Trithroxy-5,6-dihydroquinoline</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>89-52-1</td>
<td>56327</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-C-formylalaninate</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>41270-80-9</td>
<td>88422</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-[(4-Methoxyphenyl)-2-iminothiophene]</td>
<td>C8H9NO3</td>
<td>179.0582</td>
<td>0.24</td>
<td></td>
<td></td>
<td>3179-10-0</td>
<td>86550</td>
<td>309167</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
LC/MS/MS Confirmation of Hippuric acid

CID@10
CID@20
CID@40

RapidFire C18 SPE/MS +ESI

Male Urine

Hippuric acid
MS/MS @ 40
Metlin Spectral Library

MFG for MS/MS Spectral Peaks
Hippuric acid
Human Serum Lipidomics Profiling: Sample Prep

- Human serum, collected and pooled from healthy subjects (males and females), was purchased from Innovative Research Inc. (Novi, MI)
- Filter 400µL serum through a 0.22µm Ultrafree-MC® centrifugal filter units (Millipore) by centrifugation @ 12,000xg for 20min @ 4°C
- Pipette 100µL filtered serum into a Protein LoBind® Eppendorf tube
- Add 700µL ice-cold Methanol:Isopropanol, 1%Acetic Acid, vortex 30sec
- Equilibrate for 10min at room temp and Centrifuge at ~12000xg, 20min, 4°C
- The organic supernatant represents the lipidomics sample
- Inject 10µL for SPE/MS as well as LC/MS/MS analysis

Human serum equivalent to 1.25µL was used for the lipidomics studies
EIC of a Single Injection of Serum Sample on C18 RapidFire SPE/MS
Lipidomics Profiling of Human Serum by C18 SPE/MS

- 169 lipids annotated in +ESI mode
- 275 lipids annotated in –ESI mode
- 29 annotated lipids common between +ESI and –ESI modes

415 unique lipids
Milk equivalent to 1 µL was used for the lipidomics studies
Whole Milk C4 SPE/MS (+ESI) – Time Course
Whole Milk C4 SPE/MS (+ESI) – Days 1 to 7
Mass Profiler Professional (MPP)

1056 features (all 9 reps, CV ≤ 15%)

894 features (un-paired t-test, p ≤ 0.05)

534 features (Fold-change > 2)

Cluster Analysis (534 features)
Summary

- Performed “high throughput” profiling of biological samples with RapidFire SPE/MS
- RapidFire data were fully integrated and compatible with Agilent software solutions (MassHunter, ProFinder, MPP, Metlin)
- Demonstrated reproducibility and fidelity of the current high throughput workflow for data processing and analysis
  - Confirmed select metabolites/lipids by MS/MS analysis
Take Home Message…

- Fast
  - Less than 15 secs per sample
  - Facilitates quick hypothesis verification
- Flexible
  - Ability to change method parameters quickly
  - Multiple binding chemistries available
- Reproducible
  - Replicates had less than a CV <15%
  - Extracted peaks were superimposable
- High fidelity of data
  - Annotations were confirmed by LC/MS/MS
Acknowledgements

I would like to thank Jeramie Watrous and Mohit Jain from University of California, San Diego.