A Novel Solid Phase Sample Preparation Method for Lipidomic Analysis of Plasma Samples

Alex Apffel, Limian Zhao, Mark Sartain
Agilent Technologies, Inc.
Emerging Trends in Plasma Lipidomics

- Lipids are implicated in a wide range of key biological processes
- Mass Spectrometric based studies play a key role for:
  - Basic biological research
  - Translational studies
- Translational Research will require high throughput. Sample Preparation is a bottle-neck
- Automated sample preparation methods are an enabling technology both in terms of throughput and reproducibility
Overview of Conventional Liquid-Liquid Extraction (LLE) Methods

- Conventional lipid extraction protocols are based on liquid extraction methods with non-polar solvents like chloroform, MTBE or butanol.
- LLE methods require phase separation and are difficult to automate.
- More recently, a single phase butanol/methanol (BUME) method has been implemented. This method requires centrifugation for pelleting of precipitate.

<table>
<thead>
<tr>
<th></th>
<th>Folch(^1)</th>
<th>Bligh-Dyer(^2)</th>
<th>Maytash(^3)</th>
<th>BUME(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic:MeOH:Water</td>
<td>8:4:3</td>
<td>2:2:1.8</td>
<td>10:3:2.5</td>
<td>1:1 (single phase)</td>
</tr>
<tr>
<td>Sample (mL)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Methanol (mL)</td>
<td>0.53</td>
<td>0.68</td>
<td>0.38</td>
<td>0.45</td>
</tr>
<tr>
<td>Organic Solvent (mL)</td>
<td>1.06</td>
<td>0.68</td>
<td>1.29</td>
<td>0.45</td>
</tr>
<tr>
<td>Water (mL)</td>
<td>0.4</td>
<td>0.62</td>
<td>0.32</td>
<td>NA</td>
</tr>
<tr>
<td>Organic Solvent:</td>
<td>Chloroform</td>
<td>Chloroform</td>
<td>MTBE</td>
<td>Butanol/MeOH 5mM Am.Acetate</td>
</tr>
</tbody>
</table>

EMR-Lipidomics Sorbent

• Based on Captiva EMR-Lipid (Enhanced Matrix Removal) Material
• The EMR-Lipid sorbent is used to remove lipids from a range of sample matrices for analysis of small molecules.
• Retention of lipids is based on a combination of size exclusion and hydrophobic interaction mechanisms.
• Proteins precipitated from sample are retained by a filtration process.
Captiva EMR-Lipidomics Protocol

**Crash:** + 900μl ACN 1% MeOH

100μl SRM-1950 Plasma

Vortex, Ultrasonicate

**Rinse:** EMR-Lipidomics Cartridge

**Load:** Transfer to 1ml EMR-Lipidomics Cartridge (including precipitate)

**Wash:** 2 x 1mL with water/acetonitrile (v/v, 1:9)

**Elute:** 2 x 1mL with chloroform/methanol (v/v, 1:1)

**Dry:** N2 at 30°C

**Reconstitute:** 100μL butanol/methanol (v/v, 1:1)

**Analyze:** by MS or Store @ -20°C

3 x Preparation Replicates
- EMR-Lipidomics Cartridges
- Conventional Methods
  - Folch
  - Bligh-Dyer
  - Maytash
  - BUME

**LC/MS Samples**
- RP LC/MSMS (6545 QTOF)
- Pooled Samples
- 3 x RP LC/MS (6545 QTOF)
- Targeted Acquisition

Positive Pressure Manifold 48 Processor (PPM-48)

1ml cartridges

96 well plate

For Research Use Only. Not for use in diagnostic procedures.
Analytical Workflow

1. Build a Database
   a) A pool of all samples was analyzed using iterative LC/MS/MS in positive and negative mode.
   b) Data processed with Agilent Lipid Annotator. Results used to generate PCDL database with reference values of accurate mass and retention times values for each identified lipid.

2. Profile and Identify
   a) All individual samples analyzed by LC/MS in triplicate in positive and negative mode.
   b) Data processed in MassHunter Profinder using a Batch Targeted Feature Extraction with the PCDL database generated by Lipid Annotator.
   c) Results transferred to Mass Profiler Professional for statistical analysis

For more information, see Mark Sartain (MP 505)
LC/MS Method  
Chromatographic Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agilent 1290 Infinity II LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical column</td>
<td>Agilent InfinityLab Poroshell 120 HPH-C18 2.0x150mm, 2.7um (693775-702)</td>
</tr>
<tr>
<td>Guard column</td>
<td>Agilent Poroshell HPH-C18, 3.0 mm, UHPLC Guard (823750-928)</td>
</tr>
<tr>
<td>Column temperature</td>
<td>60°C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>1μl</td>
</tr>
<tr>
<td>Autosampler temperature</td>
<td>5°C</td>
</tr>
<tr>
<td>Needle wash</td>
<td>15 seconds in wash port (50:50 methanol:isopropanol)</td>
</tr>
</tbody>
</table>
| Mobile phase                     | A) 10mM Ammonium Acetate, 10μM Medronic Acid 9:1 water:methanol  
B) 10mM Ammonium Acetate, 2:2:6 acetonitrile:methanol:isopropanol |
| Flow rate                        | 0.6 ml/min                  |
| Gradient program                 | Time(min) %B                |
|                                  | 0.0  55                     |
|                                  | 5.0  57                     |
|                                  | 25.0 100                    |
|                                  | 27.0 100                    |
|                                  | 28.0 55                     |
| Stop time                        | 30 minutes                  |
| Post time                        | 5 minutes                   |
| Observed column pressure         | 300-600bar                  |

For Research Use Only. Not for use in diagnostic procedures.
## LC/MS Method

### Mass Spectrometry Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Agilent 6545 Q-TOF with Dual Agilent Jet Stream Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument mode</td>
<td>2 GHz, extended dynamic range, m/z 1,700</td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive and Negative</td>
</tr>
<tr>
<td>Gas temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Drying gas (nitrogen)</td>
<td>11 L/min</td>
</tr>
<tr>
<td>Nebulizer gas</td>
<td>35 psi</td>
</tr>
<tr>
<td>Sheath gas</td>
<td>300°C @ 12 L/min</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>3.500 V (+), 3.000 V (-)</td>
</tr>
<tr>
<td>Nozzle voltage</td>
<td>500 V</td>
</tr>
<tr>
<td>Fragmentor</td>
<td>160 V</td>
</tr>
<tr>
<td>Oct 1 Rf Vpp</td>
<td>750 V</td>
</tr>
<tr>
<td>Acquisition speed</td>
<td>MS-Only: 3 spectra/second (MS)</td>
</tr>
<tr>
<td></td>
<td>Auto MS/MS: 3 spectra/second (MS), 4 spectra/second (MS/MS)</td>
</tr>
<tr>
<td>Auto MS/MS parameters</td>
<td>Isolation width: Narrow (~1.3 amu)</td>
</tr>
<tr>
<td></td>
<td>Collision energy: 20, 35 eV</td>
</tr>
<tr>
<td>Reference correction</td>
<td>2 points at m/z 121.050873(+), 922.009798(+)</td>
</tr>
<tr>
<td></td>
<td>2 points at m/z 119.036320(-), 980.016375(-)</td>
</tr>
</tbody>
</table>

For Research Use Only. Not for use in diagnostic procedures.
Reversed Phase UHPLC/MS

- RP-UHPLC/MS provides fast, high-resolution acyl-chain length based separation of a wide range of lipids over many lipid classes.
- Positive and negative Ionization modes provide complementary information.
- Column stability and retention-time reproducibility enables resolution of isobaric lipid isomers.
- Within-class resolution reduces co-elution based ion suppression.
- Accurate relative quantitation will require use of isotopically labeled internal standards (e.g. Splash Lipidomix).
Automated Iterative MS/MS

Principle

LC/MS Injection 1

Precursors selected for MS/MS

Rolling excluded precursors

LC/MS Injection 2

Additional Injections ...

RP MS/MS PLASMA POOL

Iterative MS/MS

Repetitive MS/MS
Agilent Lipid Annotator Software

- Product ion spectral matching against *in silico*-generated databases.
- Utilizes theoretical lipid library (modified LipidBlast) developed by Kind et al.\(^5\)
- Based on combination of Bayesian scoring, probability density and non-negative least squares fit.
- Special care to not over-annotate.
  - Lipid sum composition is identified if specific acyl chain compositions are not confirmed by MS/MS Data.
  - Only report results supported by data!
- Produces accurate mass and retention time database (PCDL) for subsequent LC/MS searching.

EMR-Lipidomics extraction yields lipid coverage qualitatively and quantitatively comparable to conventional LLE extraction approaches.

% by summed area
# individual species
Lipid Class Comparison

- There are differences in abundance within lipid classes between LLE methods and the EMR-Lipidomics method.
- There are differences in abundances within lipid classes between individual LLE methods.
- EMR-Lipidomics shows quantitatively higher levels of TG, PC, and LPC.
- EMR-Lipidomics method shows quantitatively lower levels of FA, PE, PI, PS.
Sterols and short acyl chains are not well retained by EMR-Lipidomics Cartridge

EMR-Lipidomics retention mechanism requires acyl chain stearic/hydrophobic interaction.

*: Unretained* by EMR-Lipidomics Sorbent:
- Short chain acyl carnitines (positive ion)
- Sterols (e.g. cholesterol) *not shown*
- Short chain fatty acids (negative ion) *not shown*
  *recovered in flow through for analysis*

For Research Use Only. Not for use in diagnostic procedures.
Different sample preparation techniques show different selectivities

- Absolute abundances of extracted lipids show variation as a function of the extraction method.
- The variation between EMR-Lipidomics method and specific LLE methods is comparable to the differences between individual LLE Methods.

Data shown for Signals > 3e5 (23) 40 TGs identified

For Research Use Only. Not for use in diagnostic procedures.
EMR-Lipidomics protocol improves reproducibility for manual sample preparation.
**EMR-Lipidomics Method yields improved reproducibility**

<table>
<thead>
<tr>
<th></th>
<th>EMR-Lipidomics</th>
<th>LLE Folch</th>
<th>LLE Bligh-Dyer</th>
<th>LLE Maytash</th>
<th>LLE BUME</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LC/MS Replicates</strong></td>
<td>6.4%</td>
<td>6.3%</td>
<td>5.8%</td>
<td>7.1%</td>
<td>6.0%</td>
</tr>
<tr>
<td><strong>Extraction Replicates</strong></td>
<td>9.4%</td>
<td>12.2%</td>
<td>22.6%</td>
<td>11.2%</td>
<td>19.8%</td>
</tr>
</tbody>
</table>
EMR-Lipidomics method simplifies and accelerates sample processing

<table>
<thead>
<tr>
<th>Feature</th>
<th>EMR-Lipidomics</th>
<th>Liquid-Liquid Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coverage</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Selectivity ¹</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Time (batch of 1-48 Samples)²</td>
<td>30 minutes</td>
<td>60-90 minutes</td>
</tr>
<tr>
<td>Ease of Use</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>&lt;10% RSD</td>
<td>10-20% RSD</td>
</tr>
<tr>
<td>Ease of Automation</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Selective isolation of lipids in complex matrix
2. Not including 1-2 hours N₂ Drying time.
Conclusions

• EMR-Lipidomics solid phase extraction yielded lipid coverage qualitatively and quantitatively comparable to conventional LLE approaches.

• EMR-Lipidomics method shows improved ease-of-use and reproducibility compared to conventional LLE approaches.

• The combination of Agilent LC-QTOF MS/MS, Lipid Annotator and Mass Profiler Professional provides a complete untargeted lipidomics workflow. The addition of the EMR-Lipidomics Method for sample preparation further enhances that workflow.

• Future development will focus on automation of the EMR-Lipidomics Protocol
Acknowledgements

Thanks to the help and contributions from:

- Agilent Technologies
- Limian Zhao
- Mark Sartain
- Christine Miller
- Dan Cuthbertson
- Derick Lucas
- Genevieve Van de Bittner
- Laurakay Bruhn
- Sarah Stow
- Sheher Mohsin
- Prof. Xianlin Han and Dr. Chunyan Wang, University of Texas Health Science Center at San Antonio

For more information, see:

- **MP 505**: A New Lipidomics Software Workflow Demonstrates Disrupted Lipogenesis Induced with Drug Treatment in Leukemia Cells – Mark Sartain
- **ThOA am 9:10**: Lipid Annotator: a Rapid, Accurate, and User-Friendly Software for Comprehensive LC-HRMS/MS Lipidomics – Jeremy Koelmel
- **ThP 398**: Lipid Annotator: a Rapid, Accurate, and User-Friendly Software for Comprehensive LC-HRMS/MS Lipidomics – Sarah Stow
- **TP 039**: Proteomic and lipidomic analysis reveals altered fatty acid metabolism in the liver of the symptomatic Niemann-Pick, type C1 mouse model – Melissa Pergande
First Level Arial: 21 pt sentence case
• Second Level Arial: 19 pt sentence case
  – Third Level Arial: 17 pt sentence case
  • Fourth Level Arial: 17 pt sentence case
    – Fifth Level Arial: 15 pt sentence case