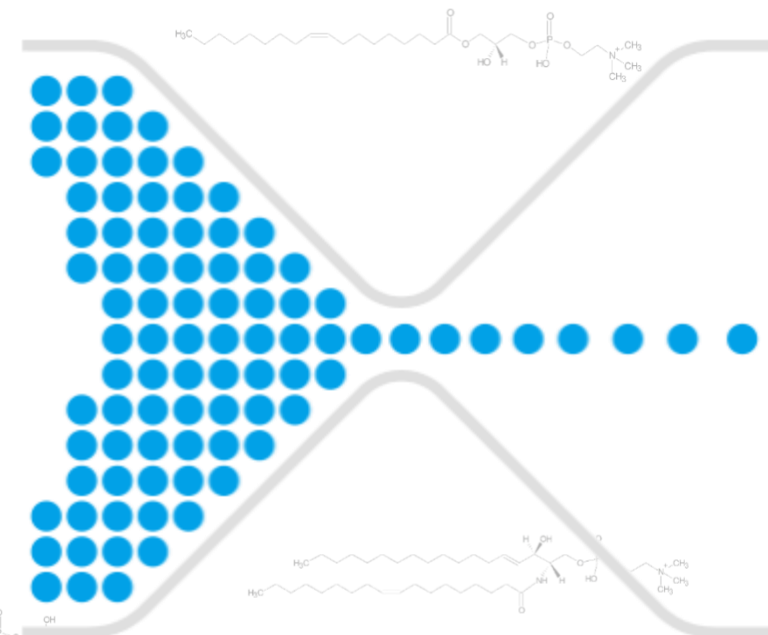


A Novel Solid Phase Sample Preparation Method for Lipidomic Analysis of Plasma Samples

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

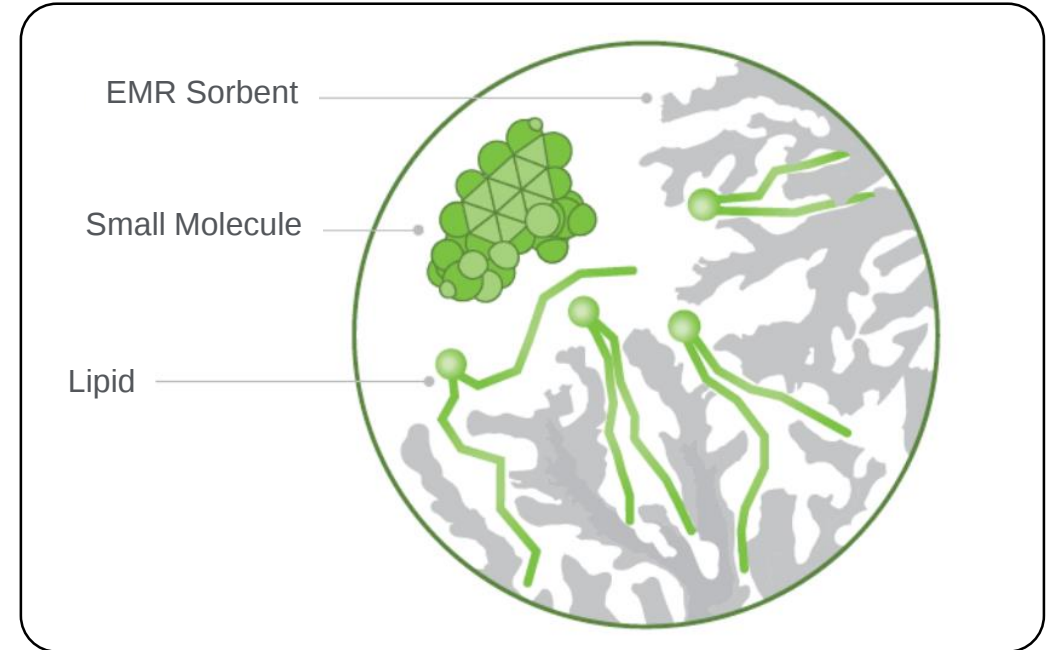
- Conventional lipid extraction protocols are based on liquid extraction methods with non-polar solvents like chloroform, MTBE or butanol.
- LLE methods require phase separation and are difficult to automate.
- More recently, a single phase butanol/methanol (BUME) method has been implemented. This method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

	Folch ¹	Bligh-Dyer ²	Maytash ³	BUME ³
Organic:MeOH:Water	8:4:3	2:2:1.8	10:3:2.5	1:1 (single phase)
Sample (mL)	0.1	0.1	0.1	0.1
Methanol (mL)	0.53	.68	0.38	0.45
Organic Solvent (mL)	1.06	.68	1.29	0.45
Water (mL)	0.4	0.62	0.32	NA
Organic Solvent:	Chloroform	Chloroform	MTBE	Butanol/MeOH 5mM Am.Acetate

1. Folch, J., et al., J Biol Chem 226, 497-509 (1957).
2. Bligh, E. G. & Dyer, W. J.. Can J Biochem Physiol 37, 911-917, doi:10.1139/o59-099 (1959).
3. Maytash, V., et al., J Lipid Res 49, 1137-1146, doi:10.1194/jlr.D700041-JLR200 (2008).
4. Alshehry, Z. H. et al., Metabolites 5, 389-403, doi:10.3390/metabo5020389 (2015).

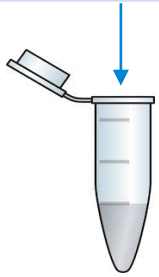
EMR-Lipidomics Sorbent

- 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

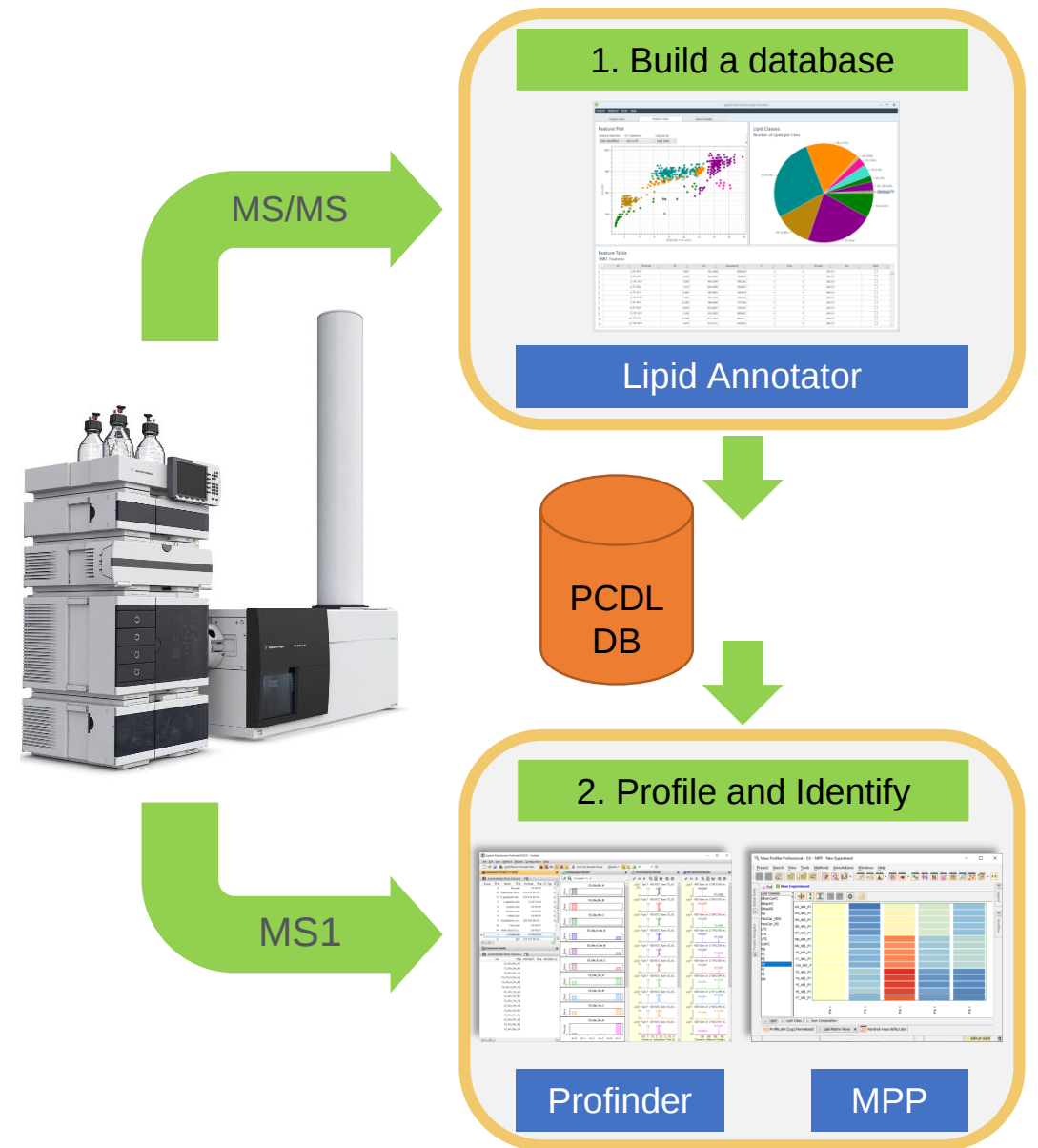
Analytical Workflow

1. Build a Database

- A pool of all samples was analyzed using iterative LC/MS/MS in positive and negative mode.
- 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

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



For more information, see Mark Sartain (MP 505)

LC/MS Method

Chromatographic Conditions



Agilent 1290 Infinity II LC

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

LC/MS Method

Mass Spectrometry Conditions



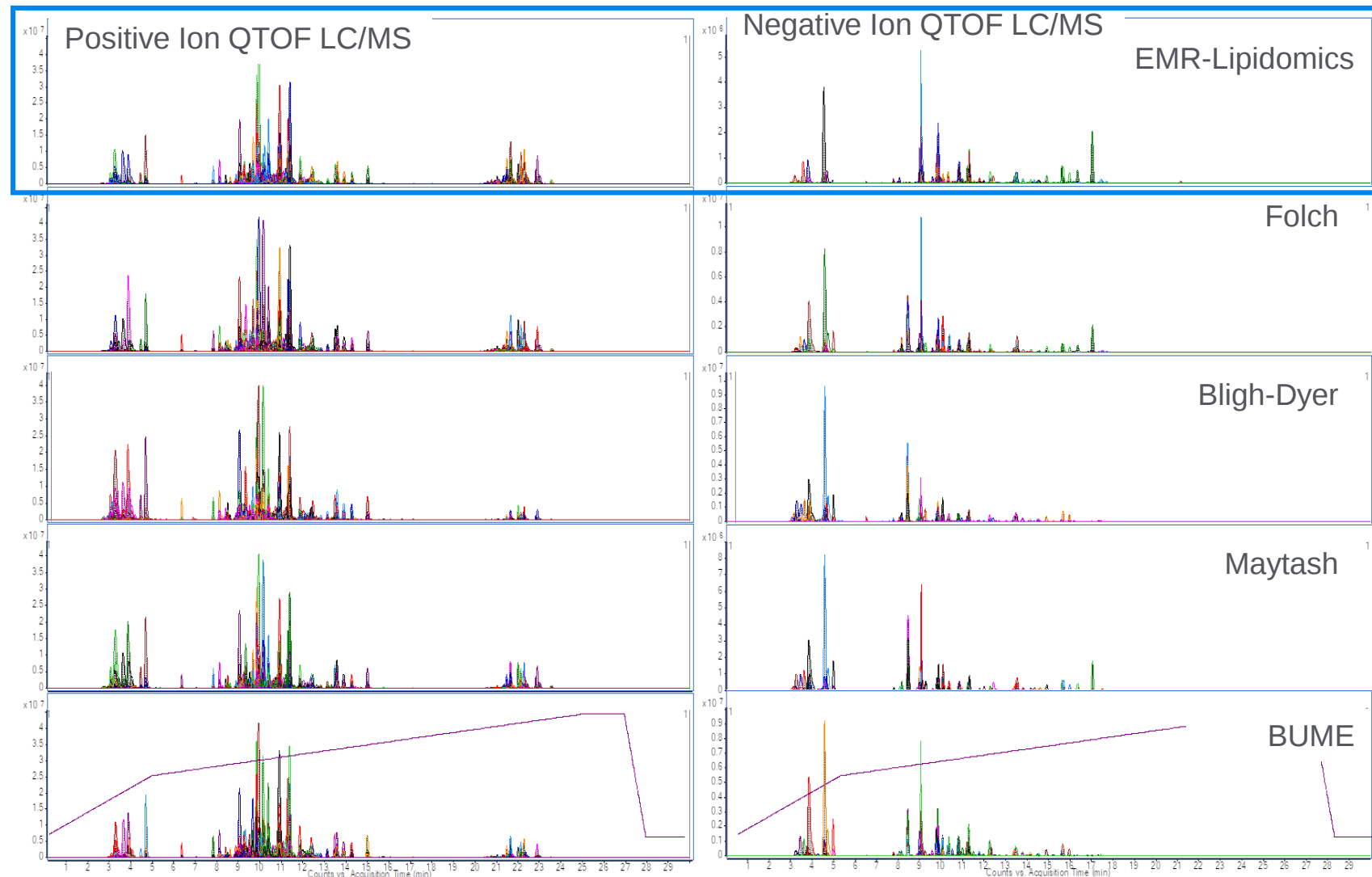
6545 LC/Q-ToF

Parameter	Agilent 6545 Q-TOF with Dual Agilent Jet Stream Source
Instrument mode	2 GHz, extended dynamic range, m/z 1,700
Polarity	Positive and Negative
Gas temperature	250°C
Drying gas (nitrogen)	11 L/min
Nebulizer gas	35 psi
Sheath gas	300°C @ 12 L/min
Capillary voltage	3,500 V (+), 3,000 V (-)
Nozzle voltage	500 V
Fragmentor	160 V
Oct 1 Rf Vpp	750 V
Acquisition speed	MS-Only: 3 spectra/second (MS) Auto MS/MS: 3 spectra/second (MS), 4 spectra/second (MS/MS)
Auto MS/MS parameters	Isolation width: Narrow (~1.3 amu) Collision energy: 20, 35 eV
Reference correction	2 points at m/z 121.050873(+), 922.009798(+) 2 points at m/z 119.036320(-), 980.016375(-)

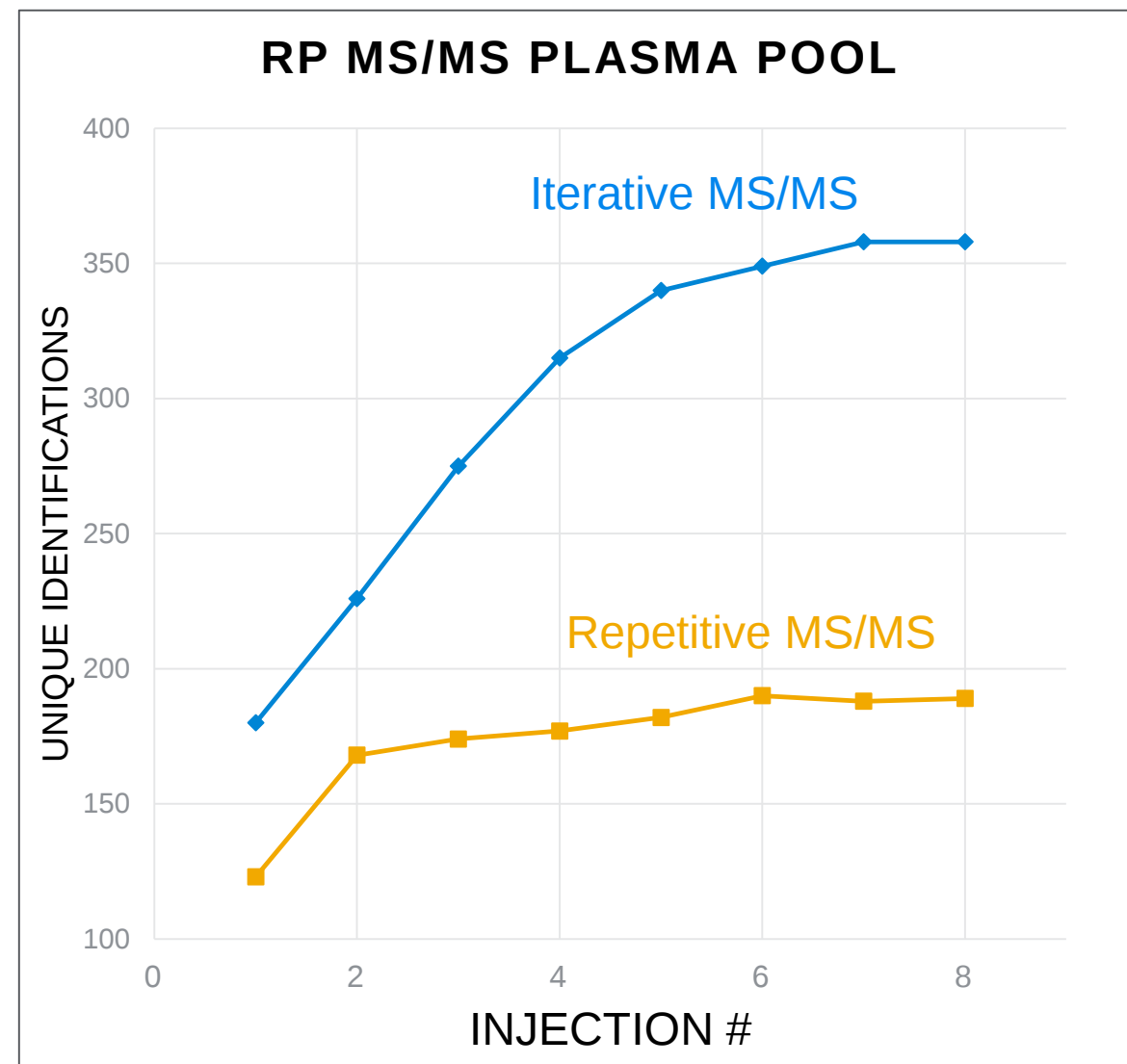
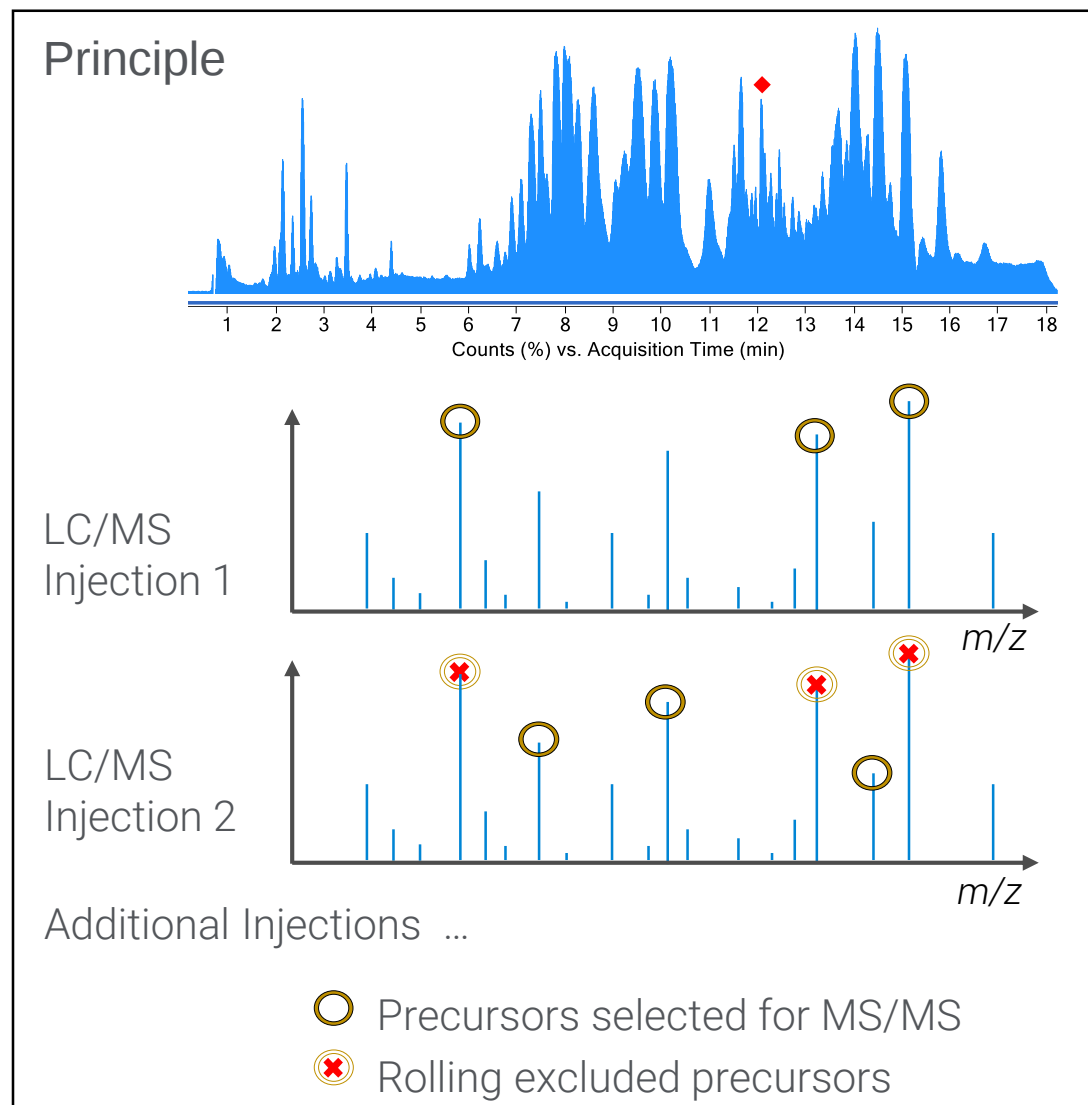
Reversed Phase UHPLC/MS

Merged EICs

- 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)

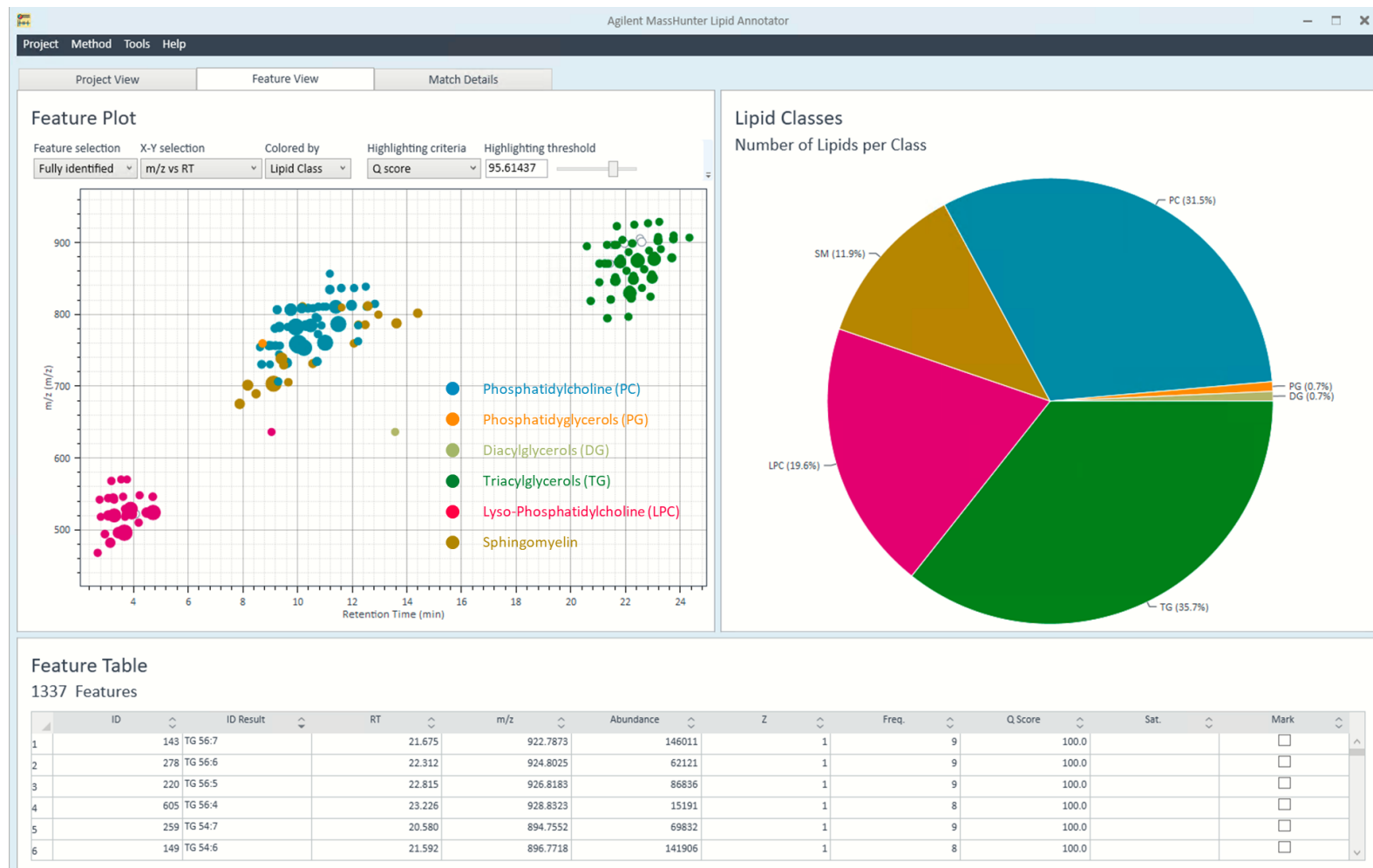


Automated Iterative MS/MS



Agilent Lipid Annotator Software

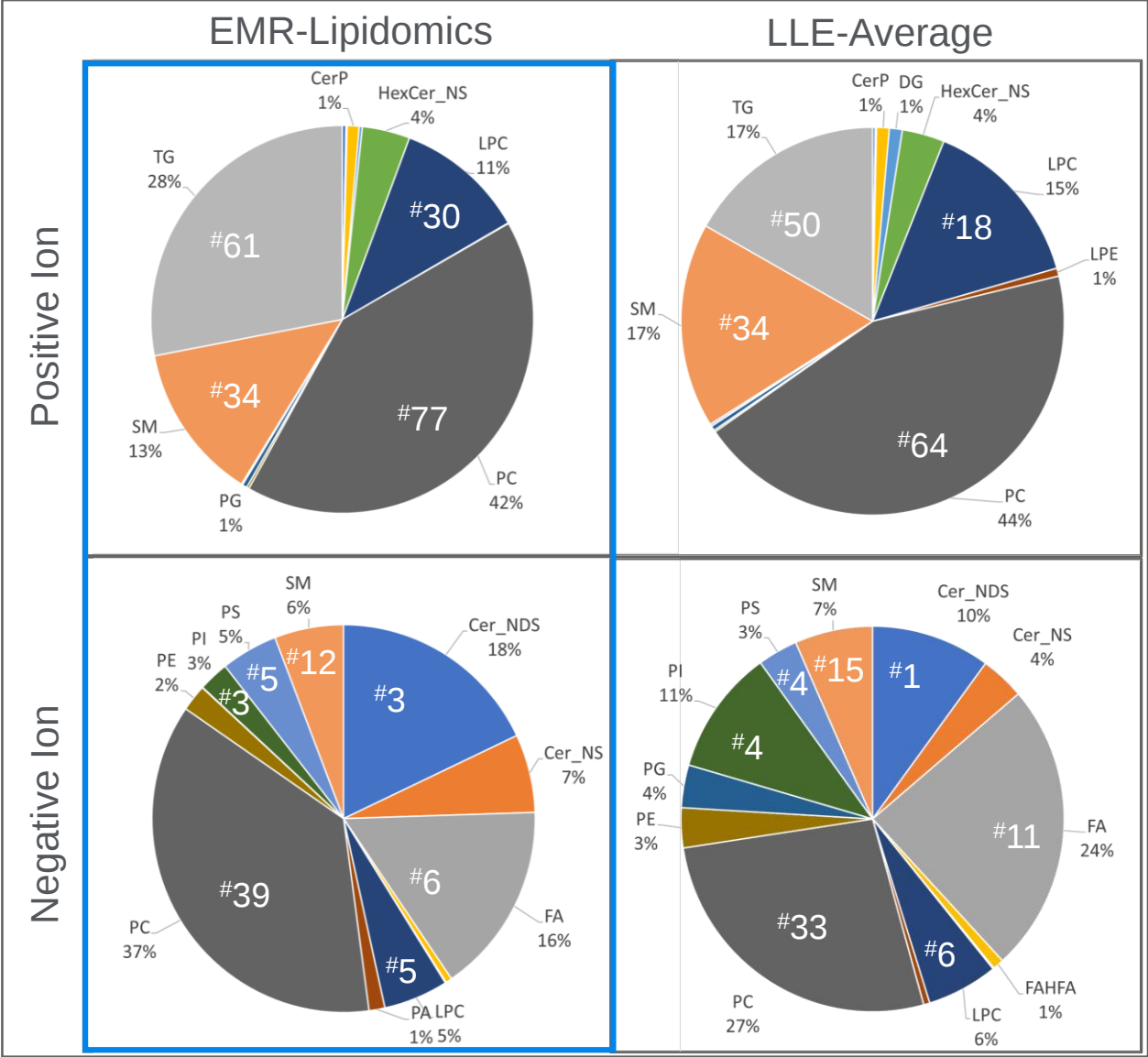
- Product ion spectral matching against *in silico*-generated databases.
- Utilizes theoretical lipid library (modified LipidBlast) developed by Kind et al.⁵
- Based on combination of Bayesian scoring, probability density and non-negative least squares fit.
- Special care to not over-annotate.
 - Lipid 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.



5) Kind, T. et al., Nature methods 10, 755, doi:10.1038/nmeth.255

For more information, see Jeremy Koelmel (ThOA am 9:10)

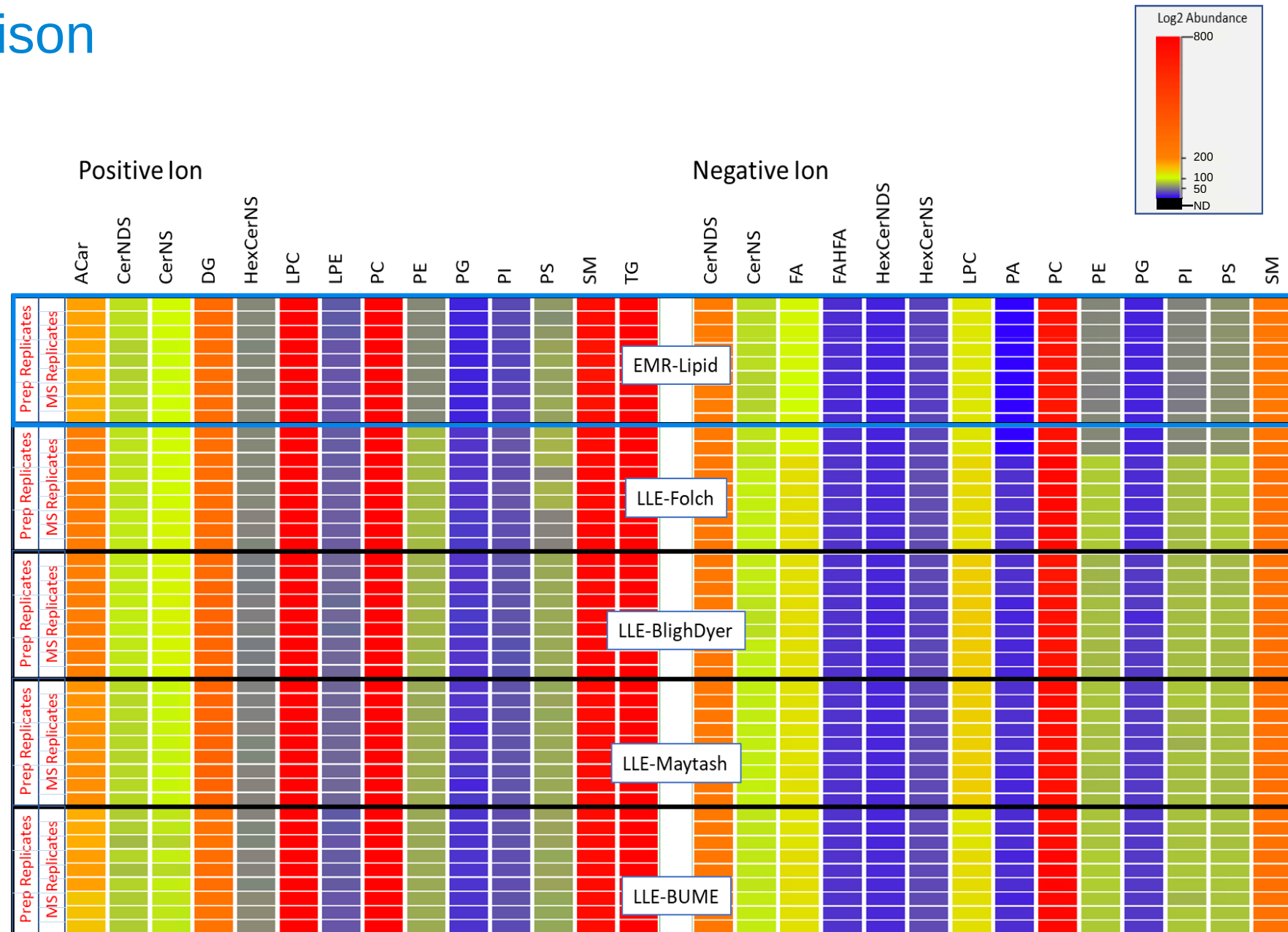
EMR-Lipidomics extraction yields lipid coverage qualitatively and quantitatively comparable to conventional LLE extraction approaches



% 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.



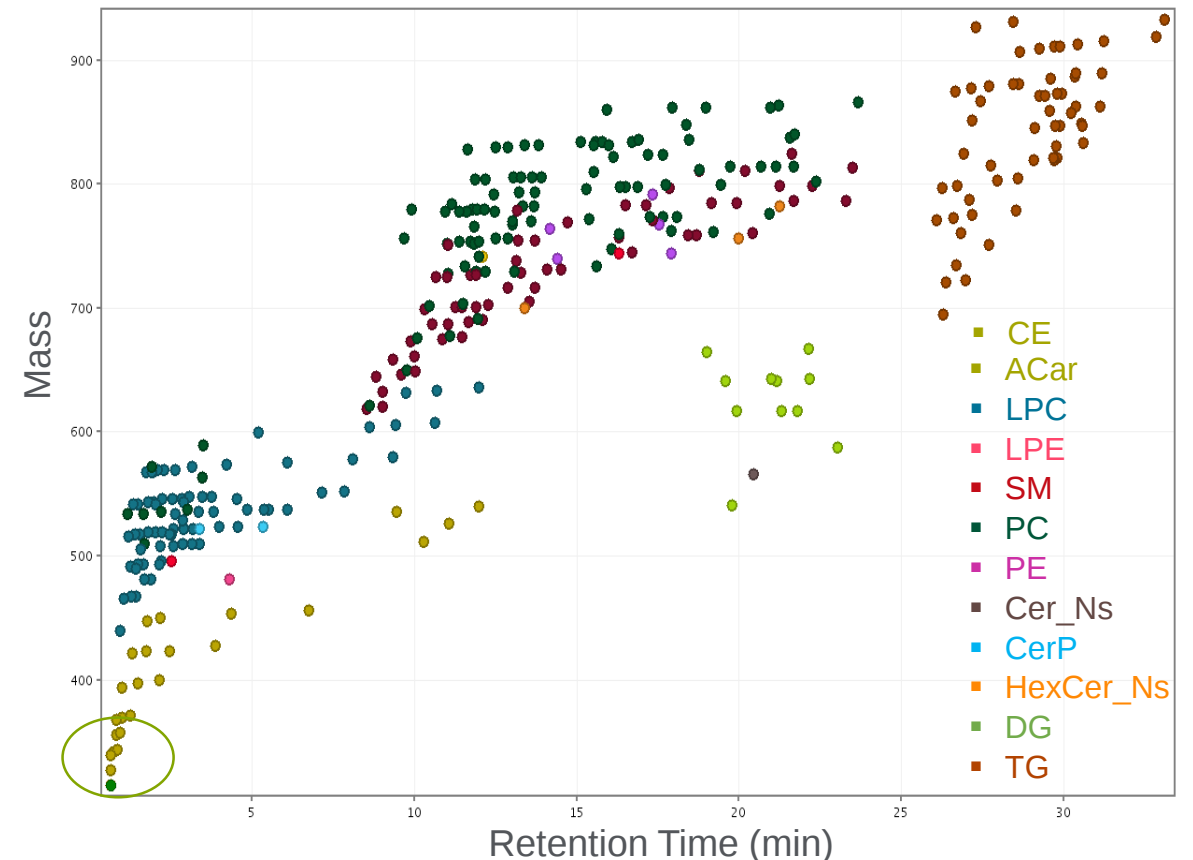
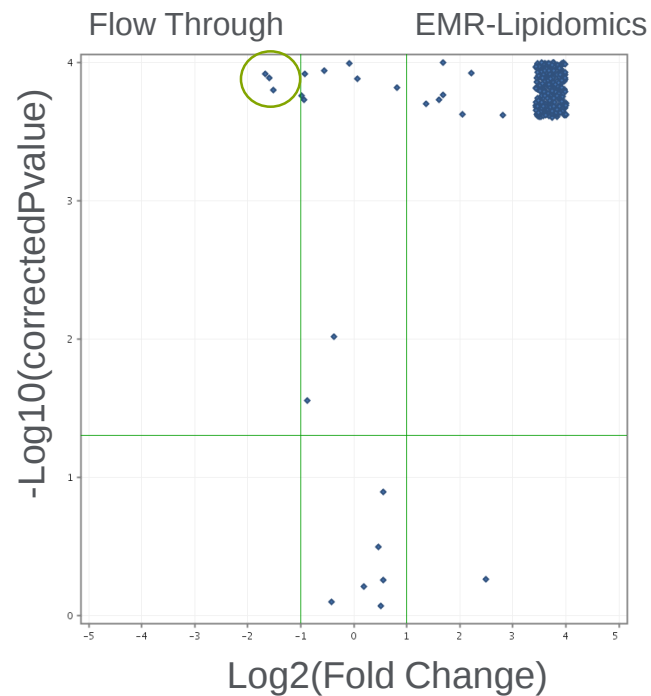
Sterols and short acyl chains are not well retained by EMR-Lipidomics Cartridge

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

∴ Unretained* by EMR-Lipidomics Sorbent:

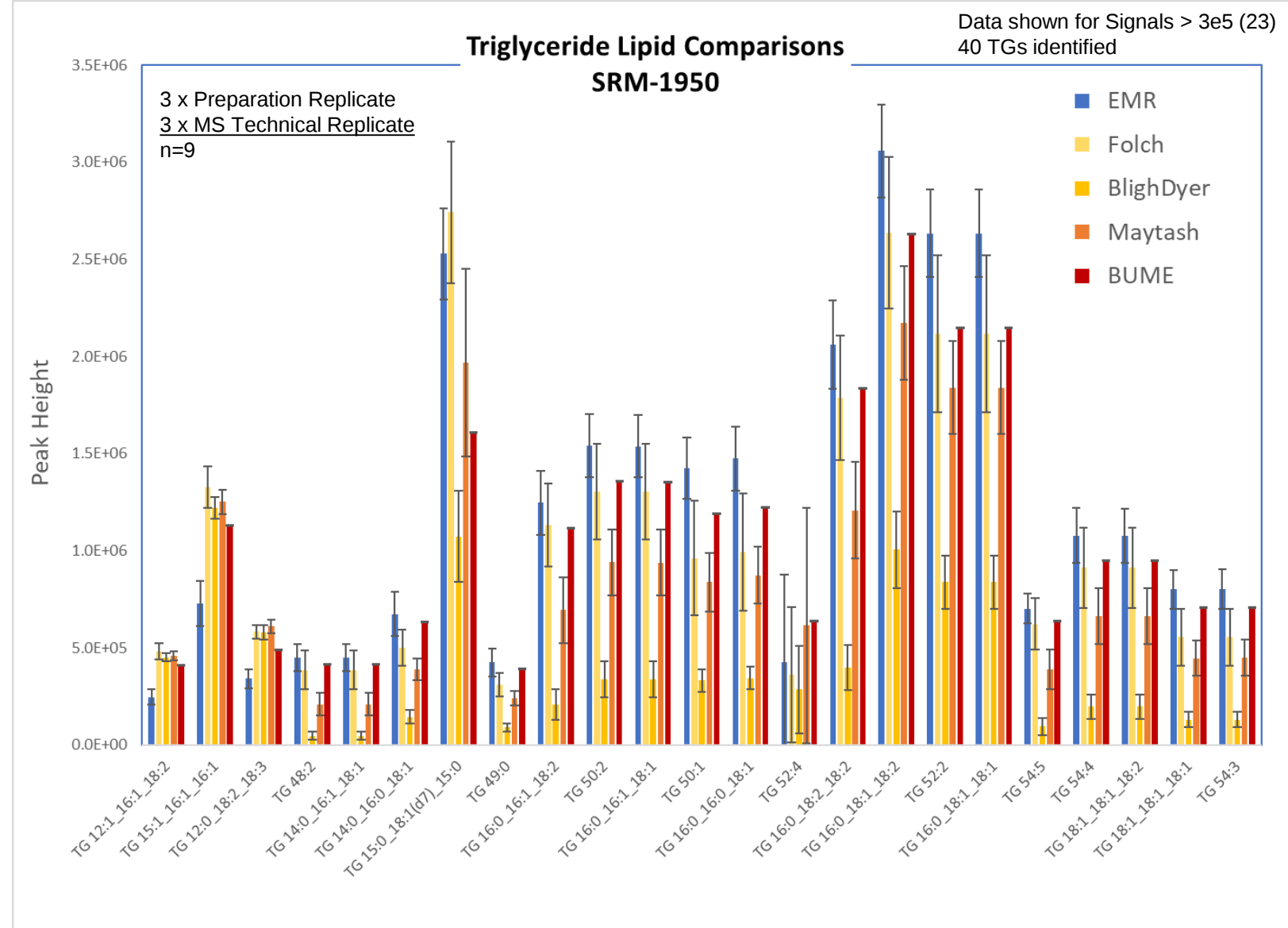
- Short chain acyl carnitines (positive ion)
- Sterols (e.g. cholesterol) *not shown*
- Short chain fatty acids (negative ion) *not shown*

**recovered in flow through for analysis*

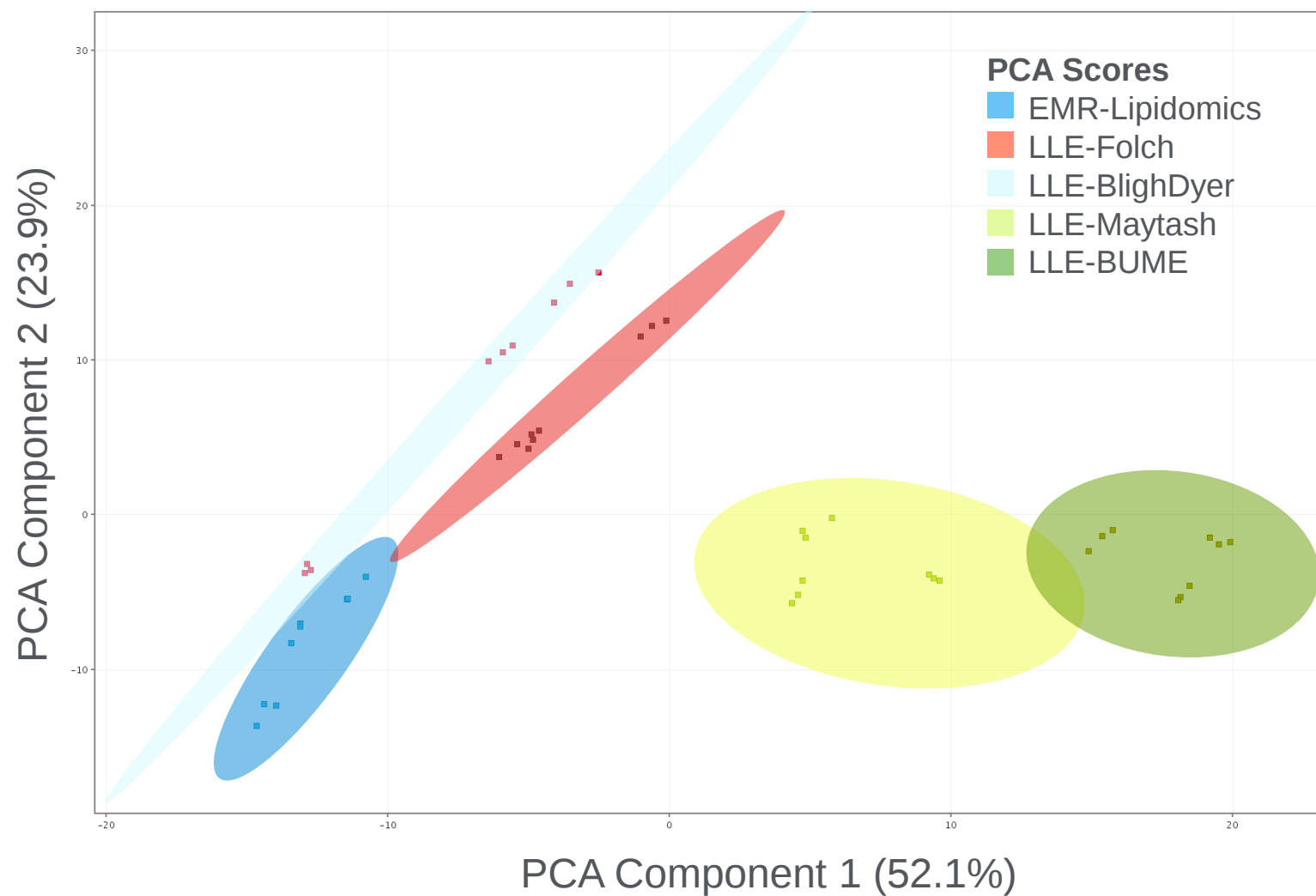


Different sample preparation techniques show different selectivities

- 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.



EMR-Lipidomics protocol improves reproducibility for manual sample preparation.



EMR-Lipidomics Method yields improved reproducibility

Average Peak Area RSD (All Identified Features)					
	EMR-Lipidomics	LLE Folch	LLE Bligh-Dyer	LLE Maytash	LLE BUME
LC/MS Replicates	6.4%	6.3%	5.8%	7.1%	6.0%
Extraction Replicates	9.4%	12.2%	22.6%	11.2%	19.8%

EMR-Lipidomics method simplifies and accelerates sample processing

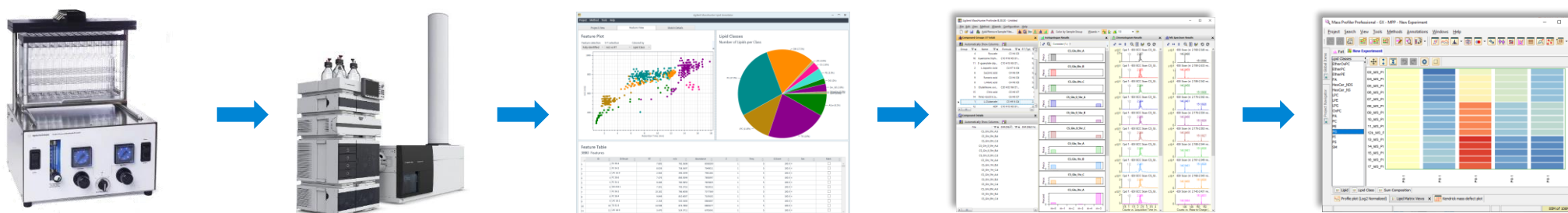
	EMR-Lipidomics	Liquid-Liquid Extraction
Coverage	+++	+++
Selectivity ¹	+++	+++
Time (batch of 1-48 Samples) ²	30 minutes	60-90 minutes
Ease of Use	++	-
Reproducibility	<10% RSD	10-20% RSD
Ease of Automation	+++	-

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

Title Slide with Image

Arial: 28 pt

Title Case

Subtitle Arial: 21 pt
sentence case

Presenter's Name Arial: 18 pt
Presenter's Title Arial: 16 pt Manually Formatted
Date Arial: 16 pt Manually Formatted

Content Slide Arial: 28 pt Title Case

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