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

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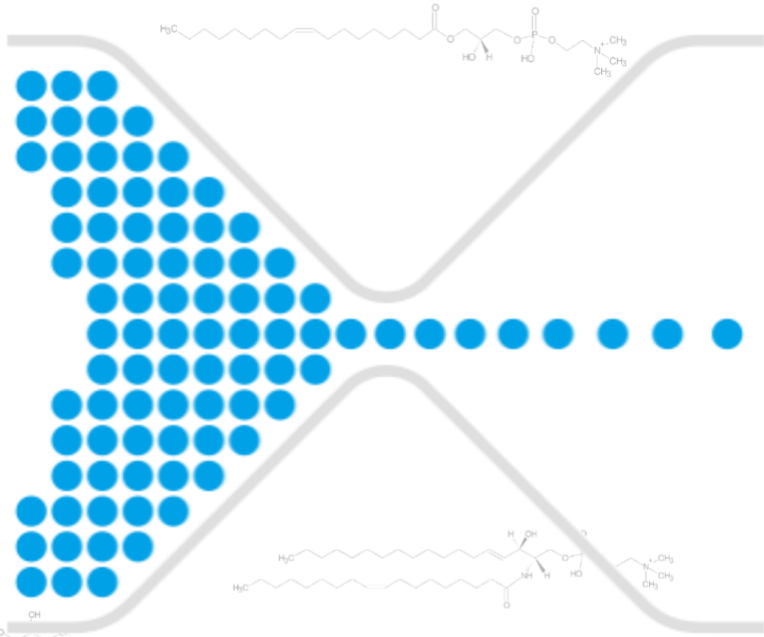
Emerging Trends in Plasma Lipidomics

- 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

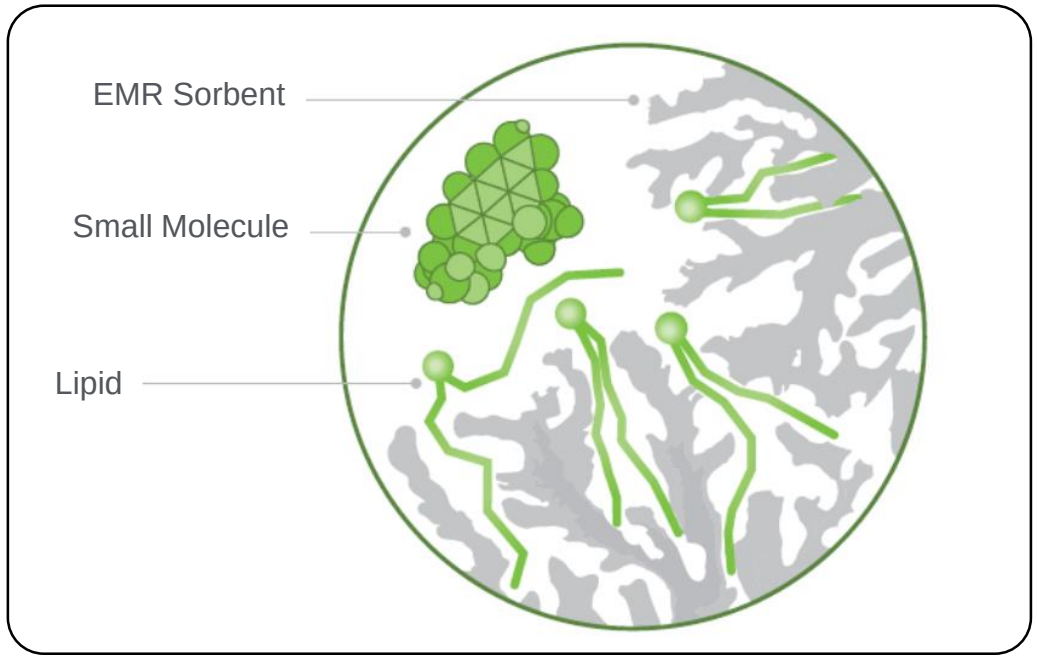
- Conventional lipid extraction protocols are based on liquid extraction methods with non-polar solvents like chloroform, MTBE or butanol.
- LLE methods require phase separation and are difficult to automate.
- More recently, a single phase butanol/methanol (BUME) method has been implemented. This method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

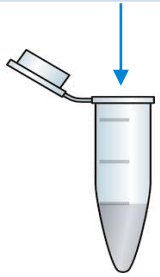
EMR-Lipidomics Sorbent

- 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



Vortex, Ultrasonicate

100µl
SRM-1950 Plasma

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

Positive Pressure Manifold 48 Processor (PPM-48)

1ml cartridges

96 well plate

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

Analytical Workflow

1. Build a Database

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

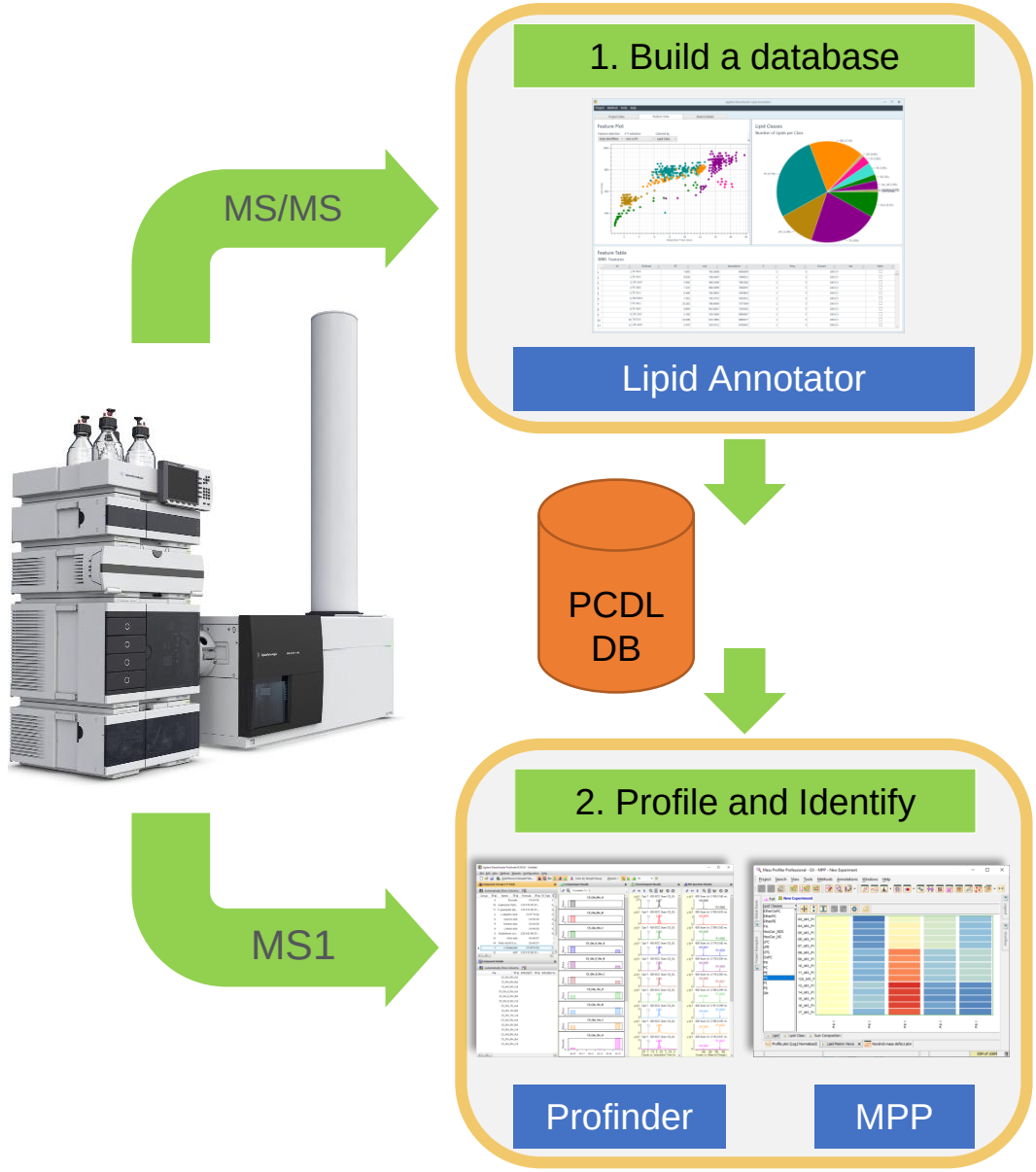
b) Data processed with Agilent Lipid Annotator. Results used to generate PCDL database with reference values of accurate mass and retention times values for each identified lipid.

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

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	60ºC
Injection volume	1µl
Autosampler temperature	5ºC
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	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

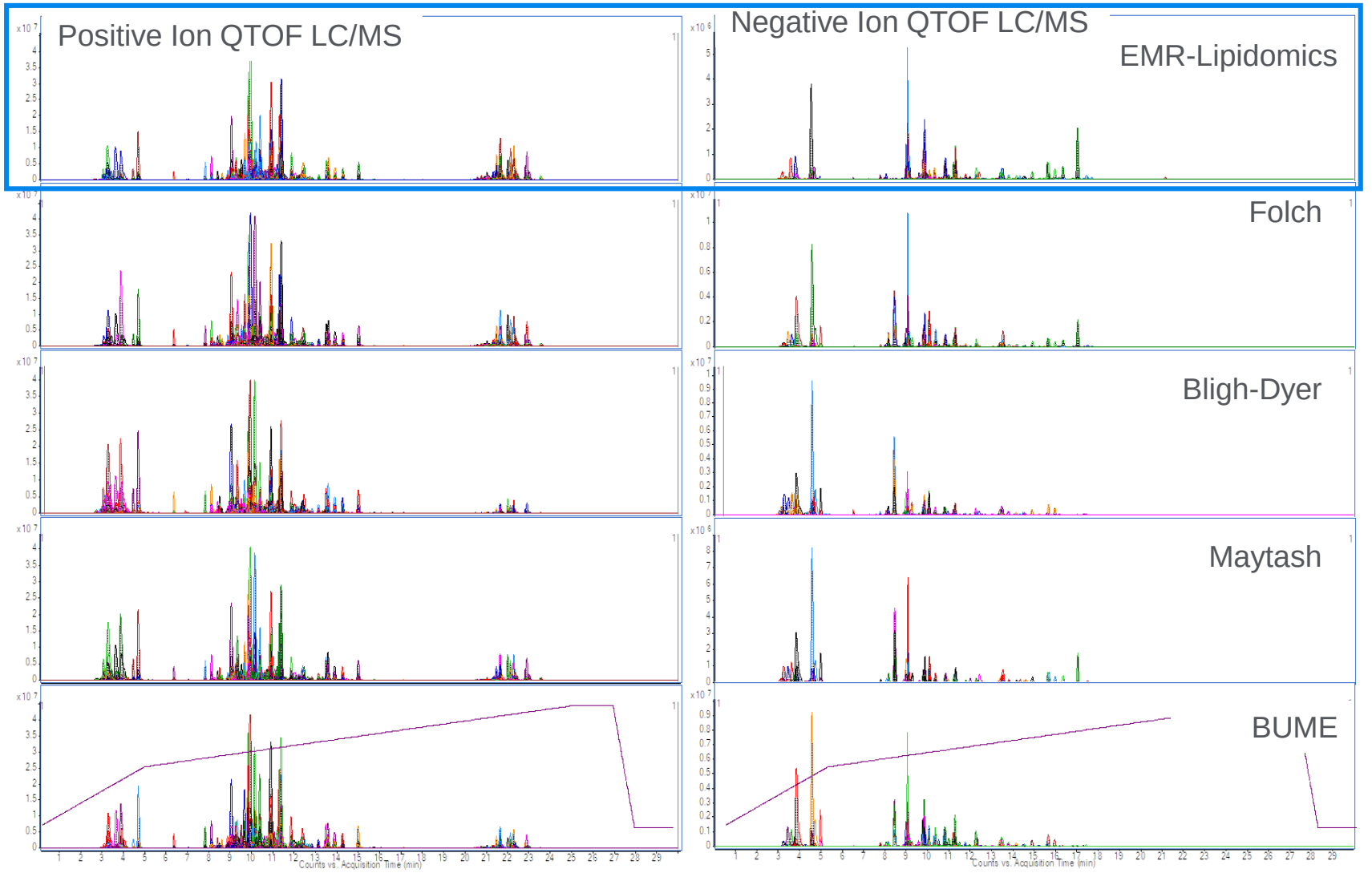
LC/MS Method

Mass Spectrometry Conditions

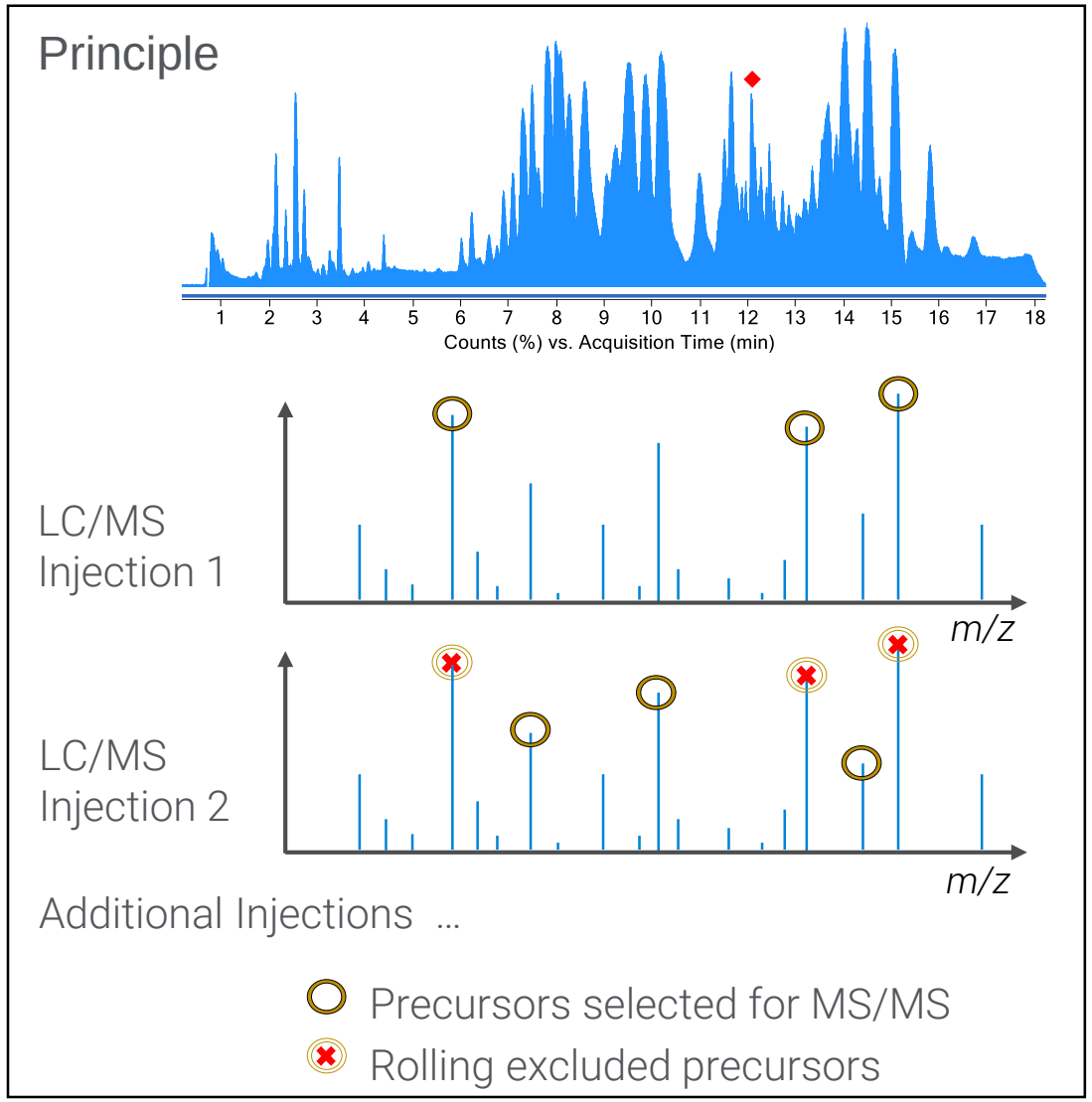
Parameter	Agilent 6545 Q-TOF with Dual Agilent Jet Stream Source
Instrument mode	2 GHz, extended dynamic range, m/z 1,700
Polarity	Positive and Negative
Gas temperature	250ºC
Drying gas (nitrogen)	11 L/min
Nebulizer gas	35 psi
Sheath gas	300°C @ 12 L/min
Capillary voltage	3,500 V (+), 3,000 V (-)
Nozzle 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(-)

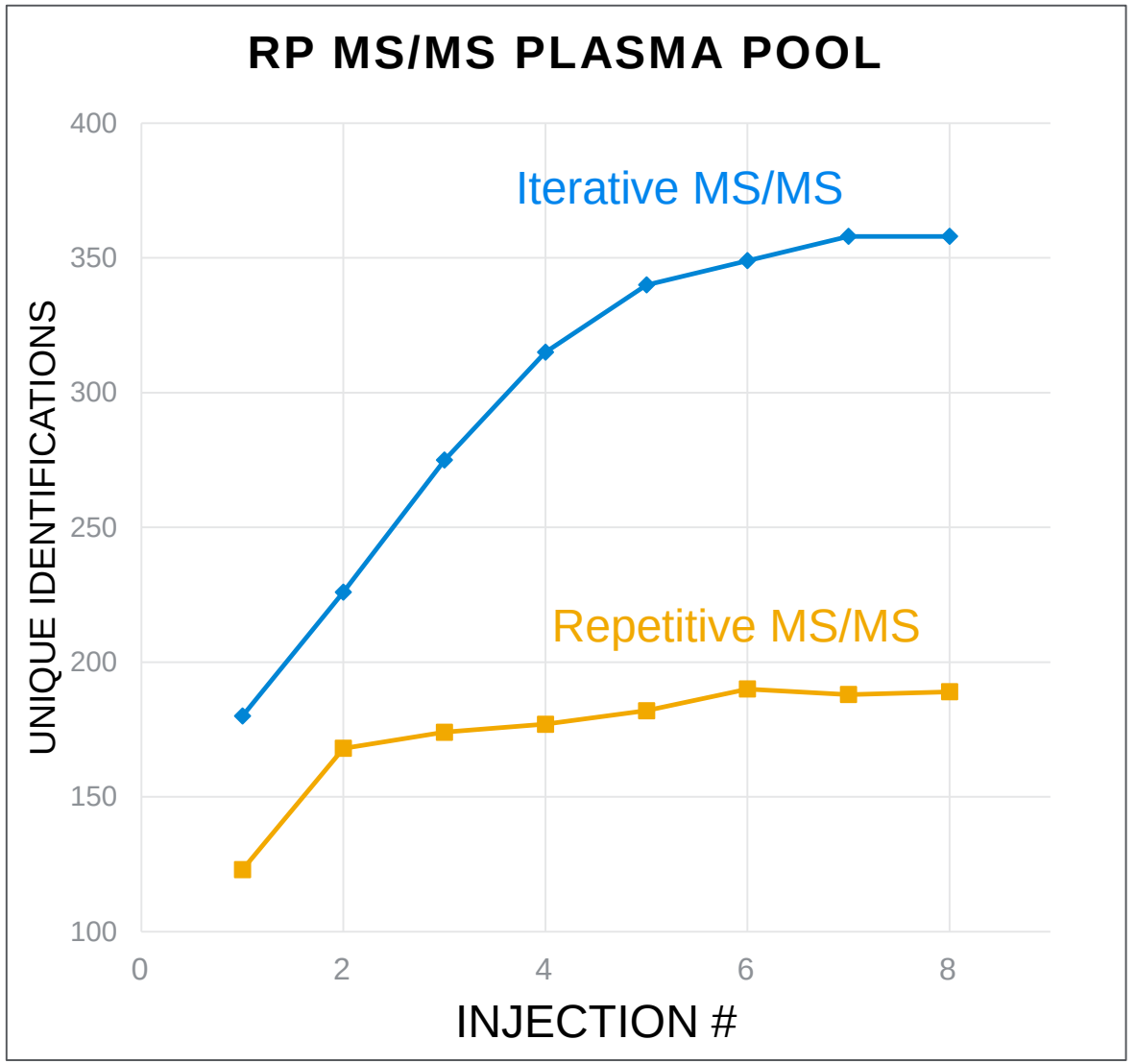
Reversed Phase UHPLC/MS

- 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)



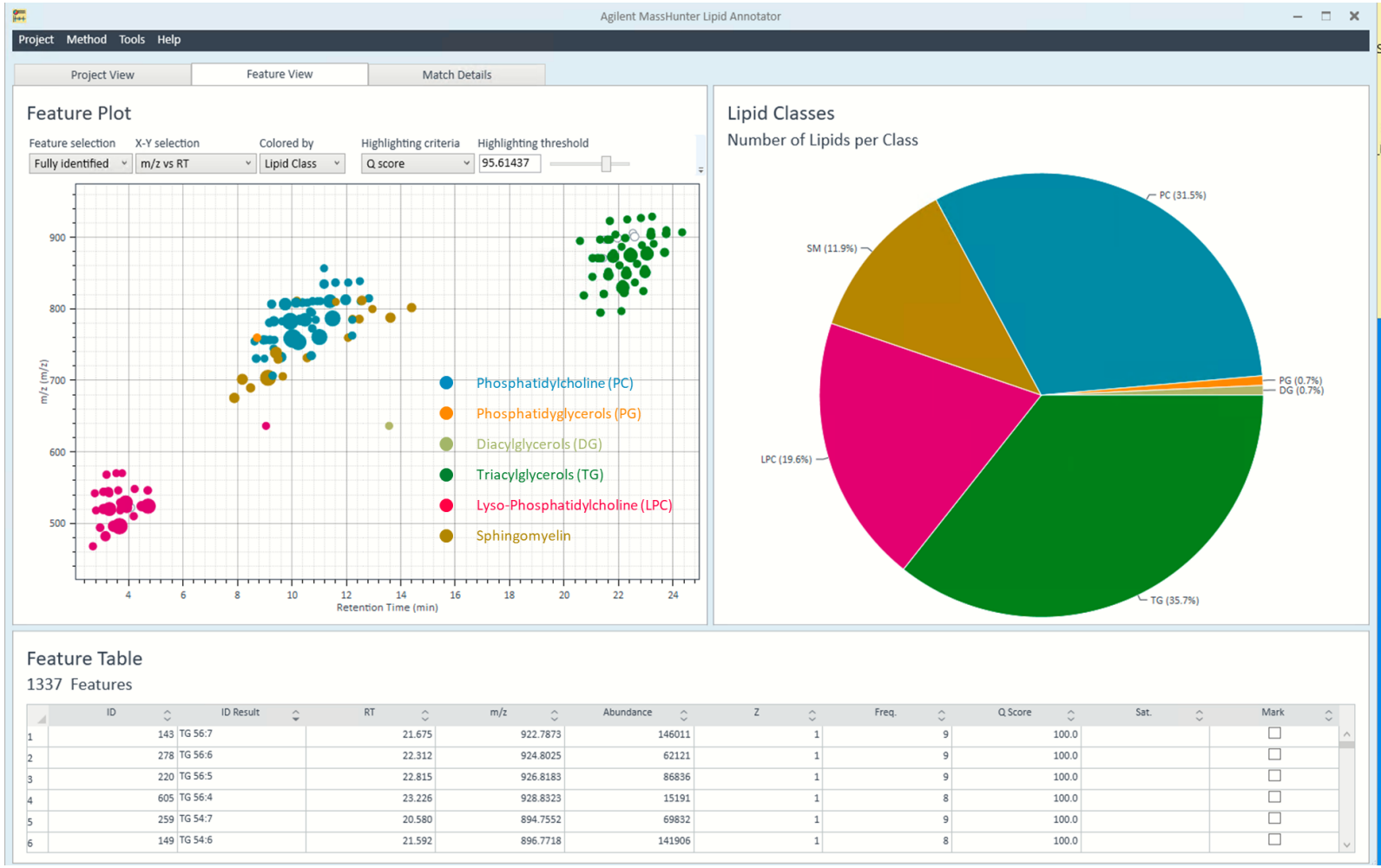
Automated Iterative MS/MS





Agilent Lipid Annotator Software

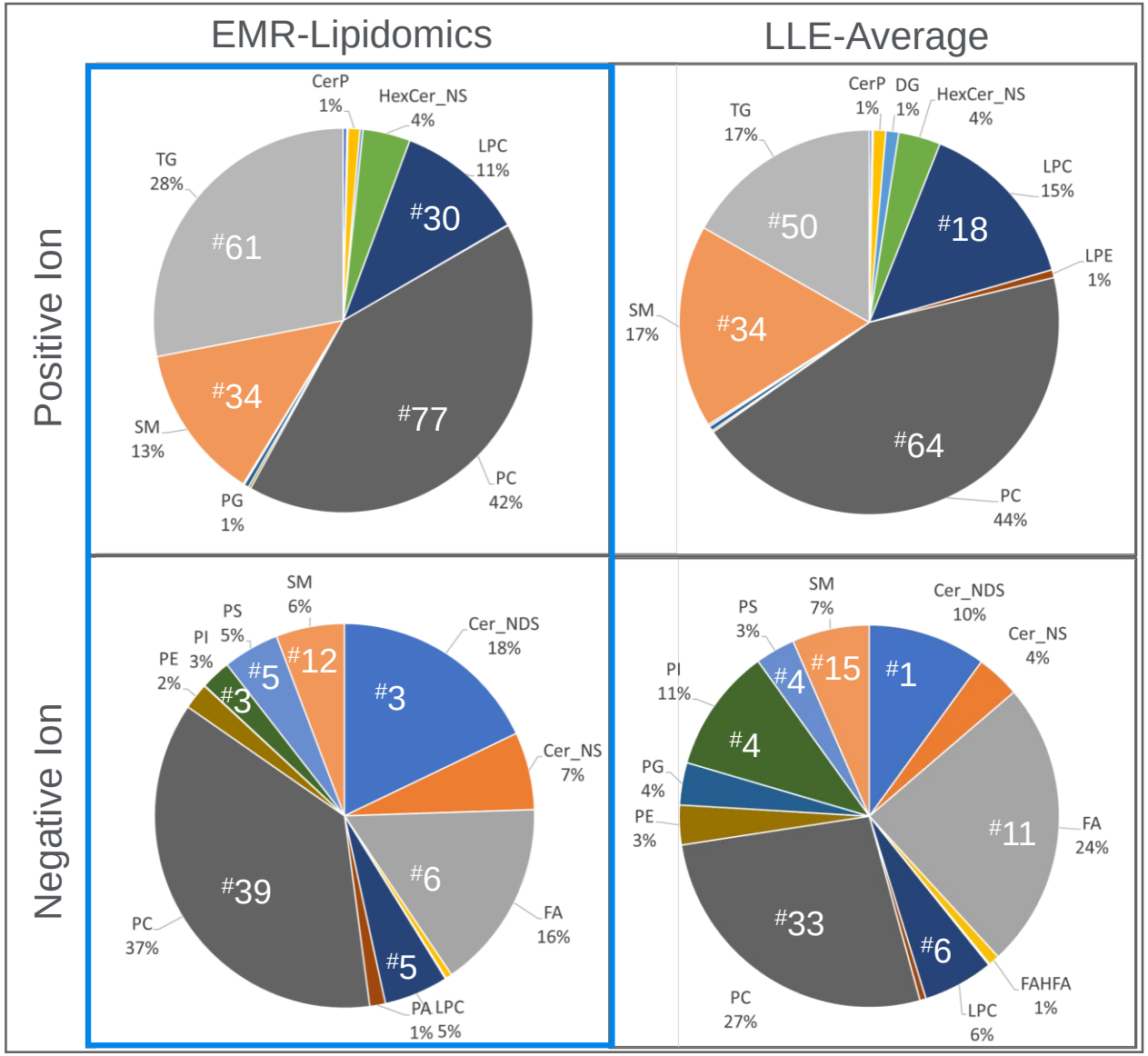
- Product ion spectral matching against *in silico*-generated databases.
- Utilizes theoretical lipid library (modified LipidBlast) developed by Kind et al.[5]
- Based on combination of Bayesian scoring, probability density and non-negative least squares fit.
- Special care to not <u>over-annotate.</u>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

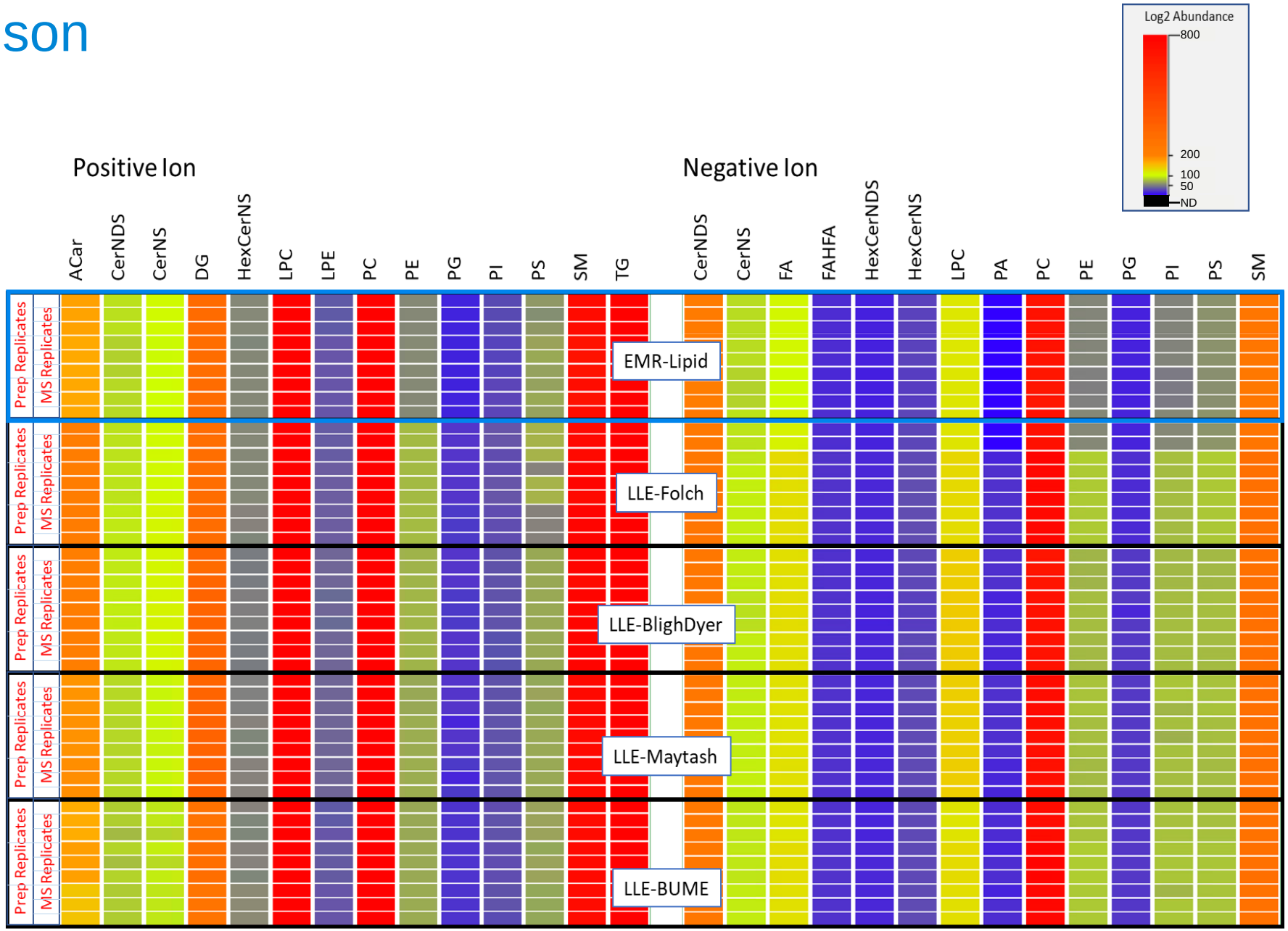
EMR-Lipidomics extraction yields lipid coverage qualitatively and quantitatively comparable to conventional LLE extraction approaches



% 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.



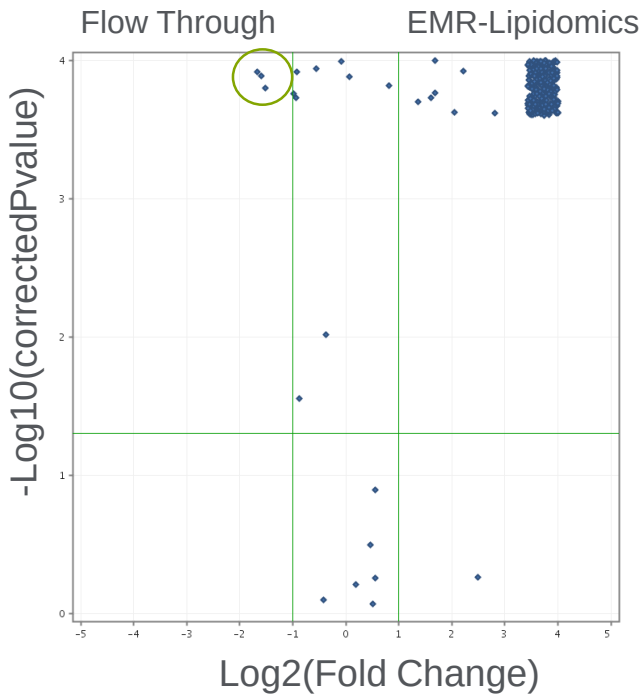
Sterols and short acyl chains are not well retained by EMR-Lipidomics Cartridge

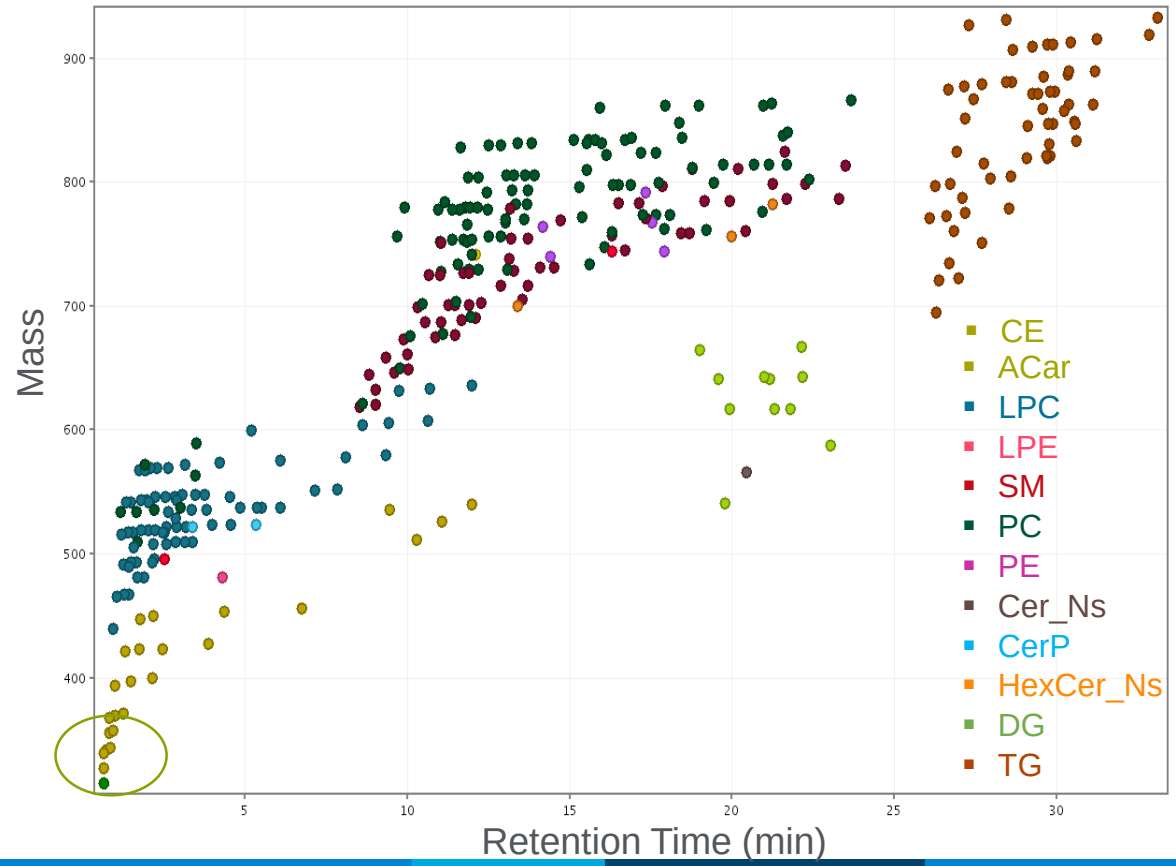
EMR-Lipidomics retention mechanism requires acyl chain stearic/hydrophobic interaction.

∴ 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*

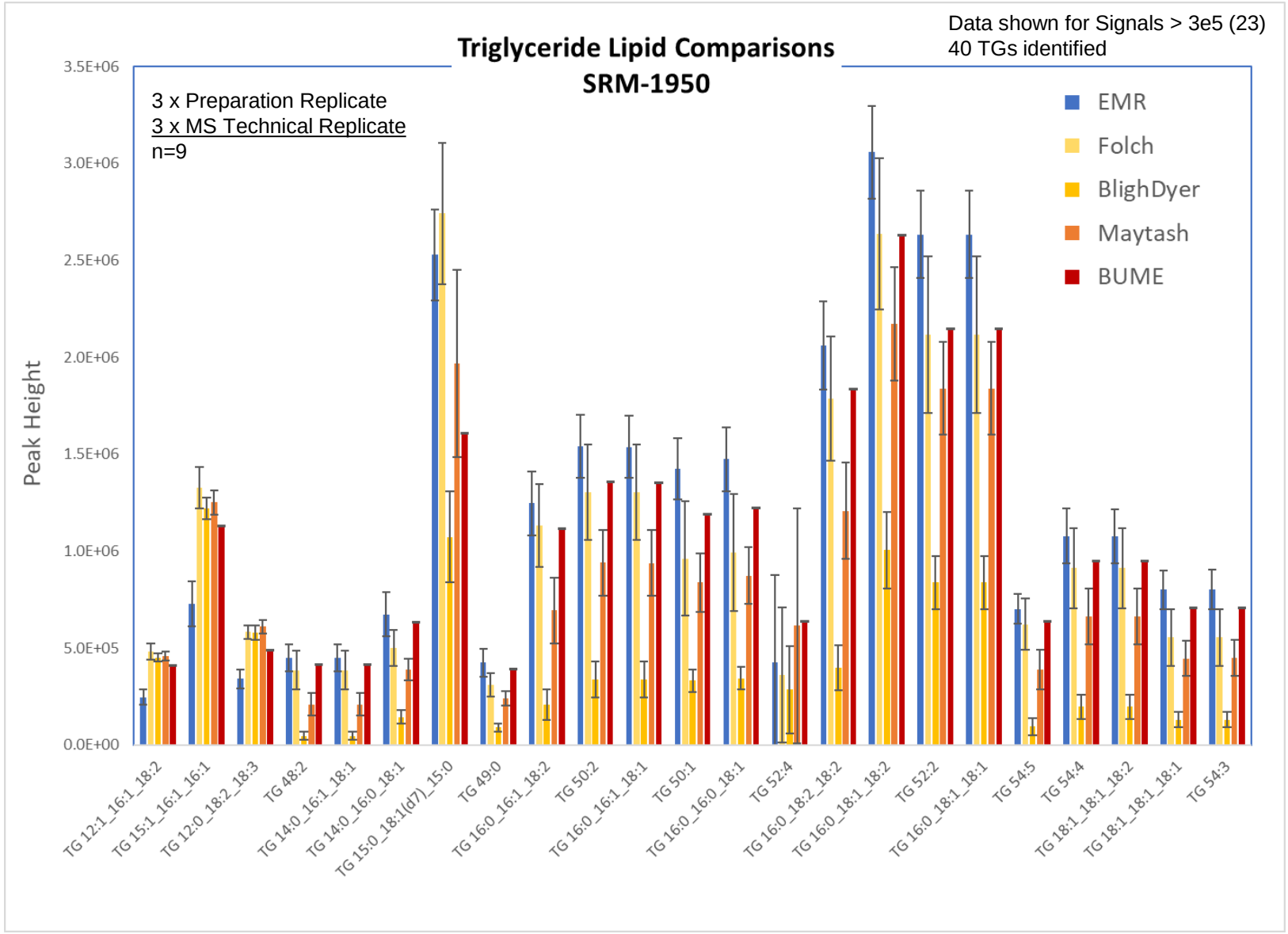
**recovered in flow through for analysis*



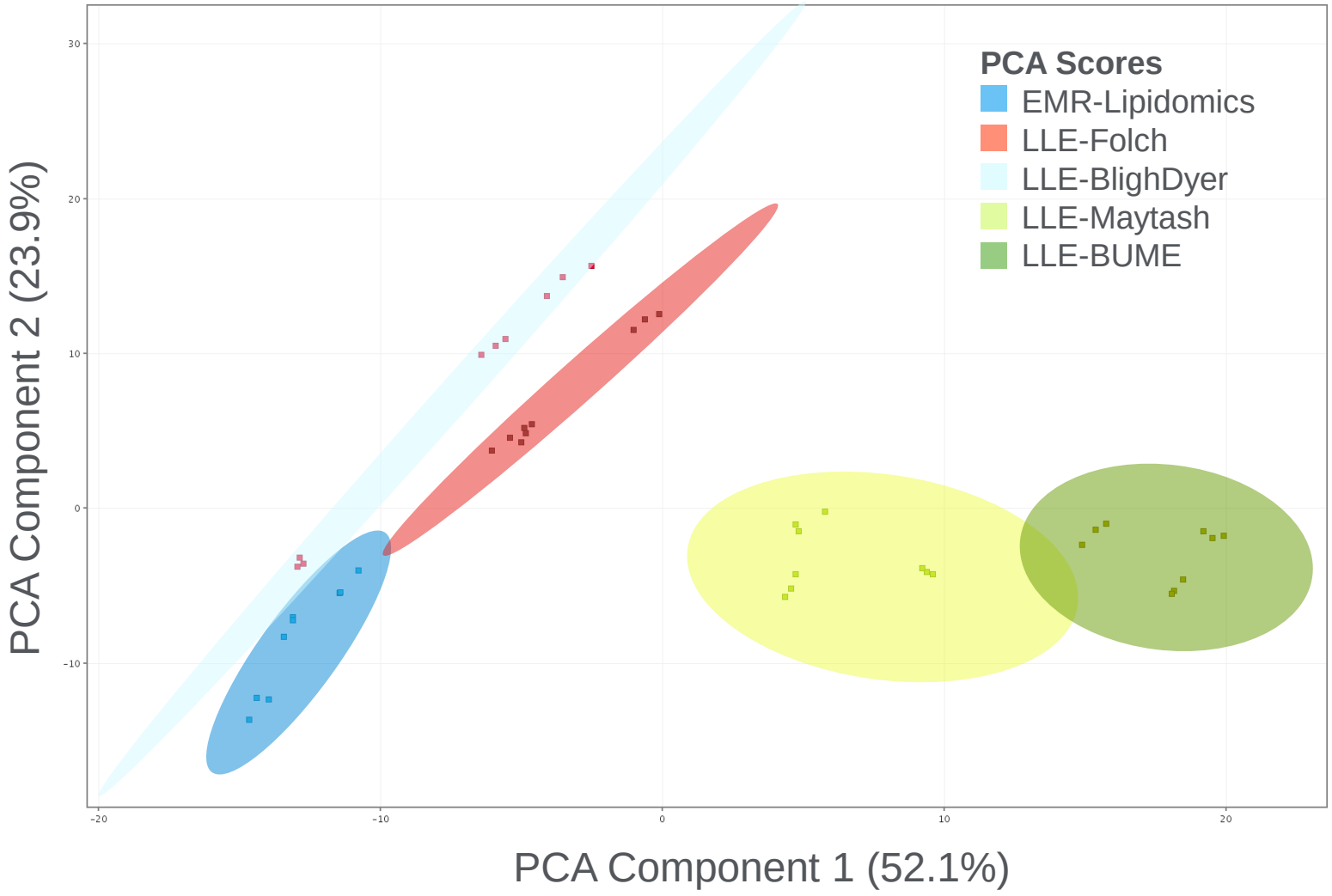


Different sample preparation techniques show different selectivities

- 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%

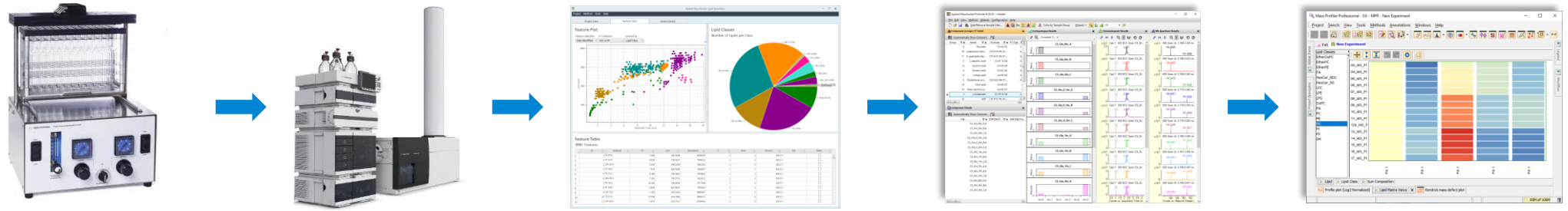
EMR-Lipidomics method simplifies and accelerates sample processing

	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 N_2 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

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