Development of an Integrated Workflow for Profiling and Semi-Quantitative Analysis of Lipids

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Introduction

The lipidome covers a range of lipids from non-polar to polar. Profiling experiments often need two or more chromatography separation schemes to cover the range of polarities: a Normal phase method and a reverse phase method. Quantitation of the annotated lipids requires additional investigation using a more targeted approach.

We describe a comprehensive analytical method using supercritical fluid chromatography (SFC) coupled to high resolution mass spectrometry for the analysis of lipids covering the whole spectrum of lipid classes in a single run.

The method is semi-quantitative - multiple internal standards covering the common lipid classes are spiked into the sample. We have developed advanced normalization tools for the reduction of data variability across samples and batches to improve the performance of multivariate statistical analysis.

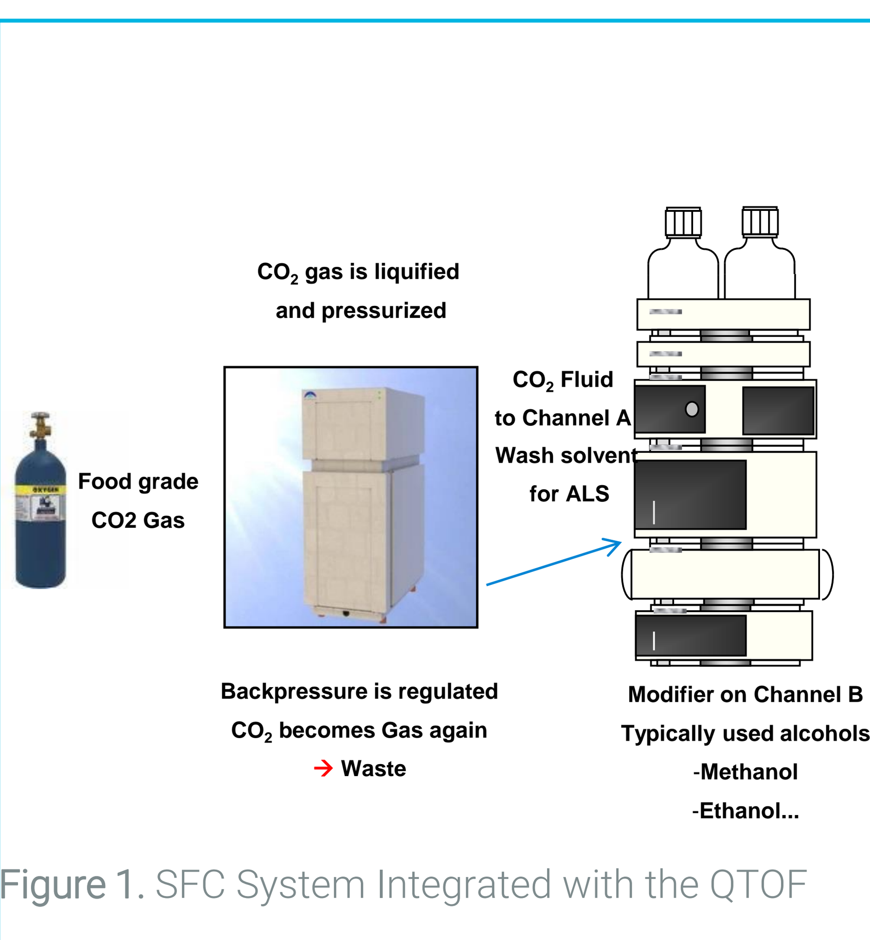


Figure 1. SFC System Integrated with the QTOF

Experimental

Mouse bone marrow derived macrophages (BMDMs) were differentiated and collected after 48 hours of stimulation with ligands to TLRs 2, 3, and 4. The cells were counted by imaging and collected by scraping. Lipids were extracted by Bligh and Dyer extraction and dried over argon until analysis.

Comprehensive lipidomic analysis was done using an Agilent 1260 Infinity SFC coupled with a 6545 LC/Q-TOF high resolution mass spectrometer (Figure 1). Nonpolar and polar lipid classes were separated within a 15-minute run time by employing two columns with different chemistries in series: EC-C18 and hybrid UPC[2] BEH. A gradient of CO_2 and methanol with ammonium acetate was used as a modifier. The separation of lipid classes using two columns in series worked well giving great chromatographic peak shapes and resolution across the diverse polarity of lipids. Figure 2 shows a clear improvement in peak shape from one column to a two column approach.

SimLipid software (PREMIER Biosoft, CA, USA) was used for identification, followed by Mass Profiler Professional for differential analysis.

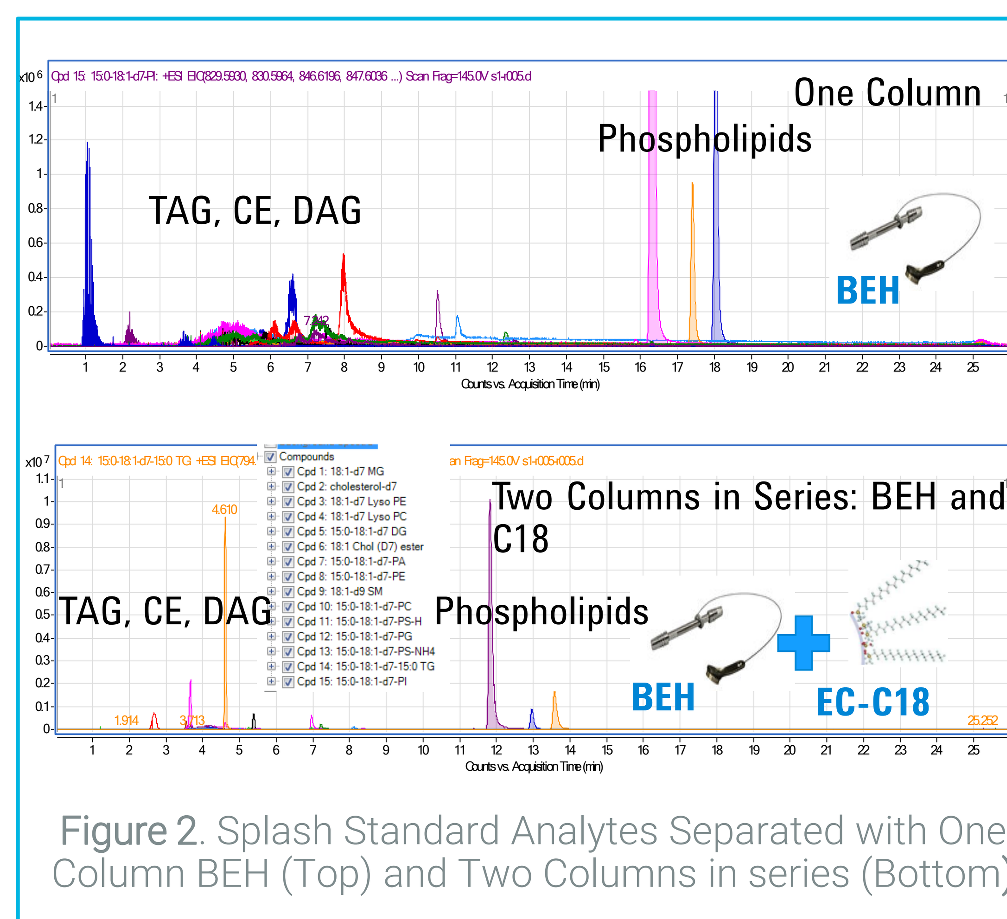


Figure 2. Splash Standard Analytes Separated with One Column BEH (Top) and Two Columns in series (Bottom)

Results and Discussion

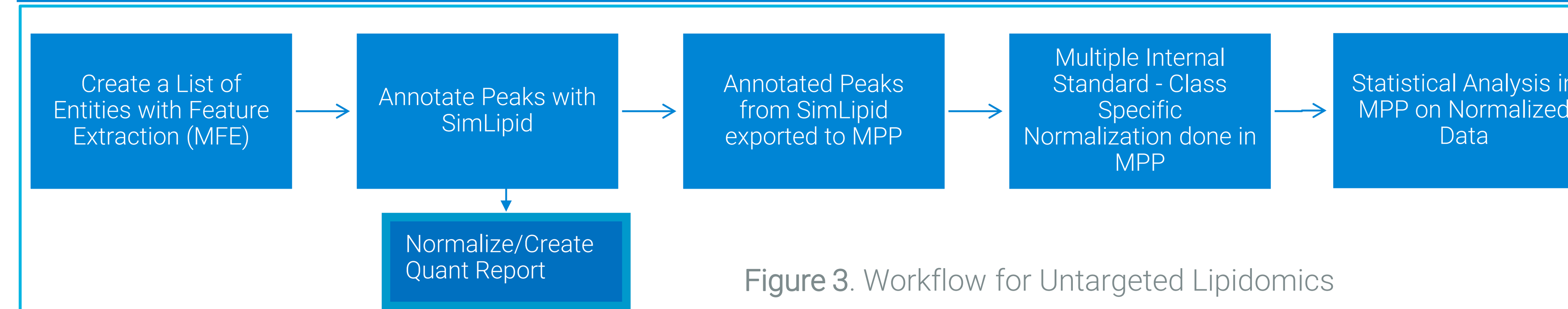


Figure 3. Workflow for Untargeted Lipidomics

The peak list of entities generated using Molecular Feature Extraction (MFE) were subjected to SimLipid MS/MS database search for identification (Figure 4). The search was performed using 5 ppm error tolerances for both the precursor and product ions. The program annotated 431 unique lipid species between the samples . Table 1 shows the identity of the lipids and their frequency.

Lipid Class	Frequency
PC	119
TAG	78
PE	72
DAG	40
PI	26
Phosphosphingolipids	24
PS	21
PG	16
Ceramides	12
Sterols	9
Neutral glycosphingolipids	8
Fatty esters	2
Other Sphingolipids	2
PA	2

Table 1. List of unique lipids found between the sample groups and their frequency.

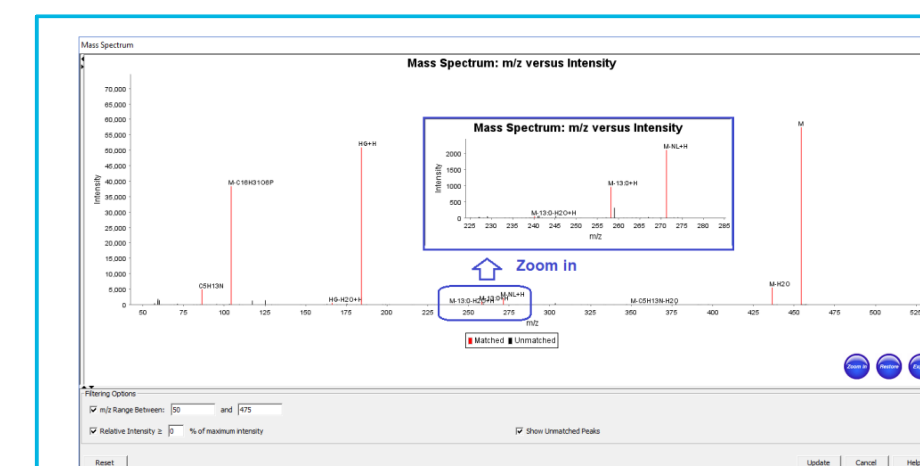


Figure 4. Typical SimLipid software GUI showing MS/MS spectrum annotated with fragment ions of LysoPC (13:0/0:0). Zoom in spectrum showing the ions corresponding to neutral loss of PC (a) head group, (b) the fatty acid chain13:0, and (c) loss of water molecule from (b)

The un-normalized raw data with only a scalar normalization using cell count was subjected to multivariate analysis but showed no grouping of samples based on treatment. Figure 5 (right) shows poor correlation in the PCA plot performed on the Log2 transformed and cell count corrected data.

Normalization was next done using internal standards spiked into the sample, covering the common lipid classes. Peaks were normalized using the criteria of "closest retention time." The peaks within a retention time window closest to an internal standard representing a lipid class were normalized to the internal standard response.

This worked well but still suffered from the possibility that certain lipids might elute close to an internal standard and not belong to that class of the internal standard.

The next method of normalization was based on first identifying and annotating the lipids with SimLipid software. The annotated lipids belonging to a certain lipid class were then normalized to the intensity of the internal standard for that lipid class. Unidentified compounds and molecules of a lipid class with no matching internal standard were normalized using the criteria of a choice between "closest retention time" or "average of all internal standards response."

This normalization procedure also works well with flow injection analysis where there is no retention time information. Differential statistics was performed on the internal standard normalized data. Compounds that were detected by the differential statistics clearly separated the study groups in the multivariate analysis (PCA, PLSDA) and in the hierarchical clustering analysis performed. Lipid expression profiling data is indicated as a Heat Map.

Multivariate and clustering analysis that was performed clearly indicated that the replicate samples for the same treatment group are highly correlated after performing lipid class-based ISTD Normalization but not after log2 transformation and/or cell count correction.

Figure 6 (right) shows high correlation after normalization in both the PCA plot as well as in hierarchical cluster analysis.

Results and Discussion

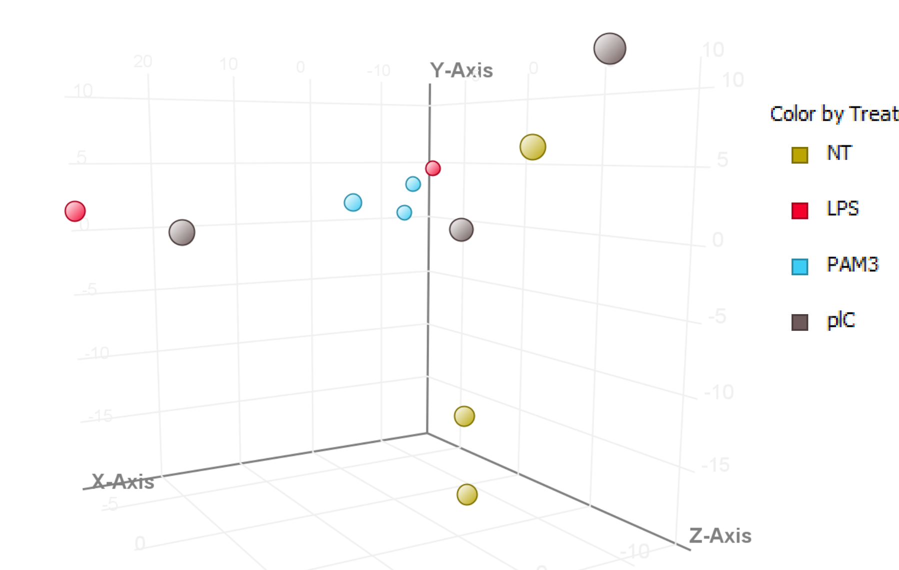
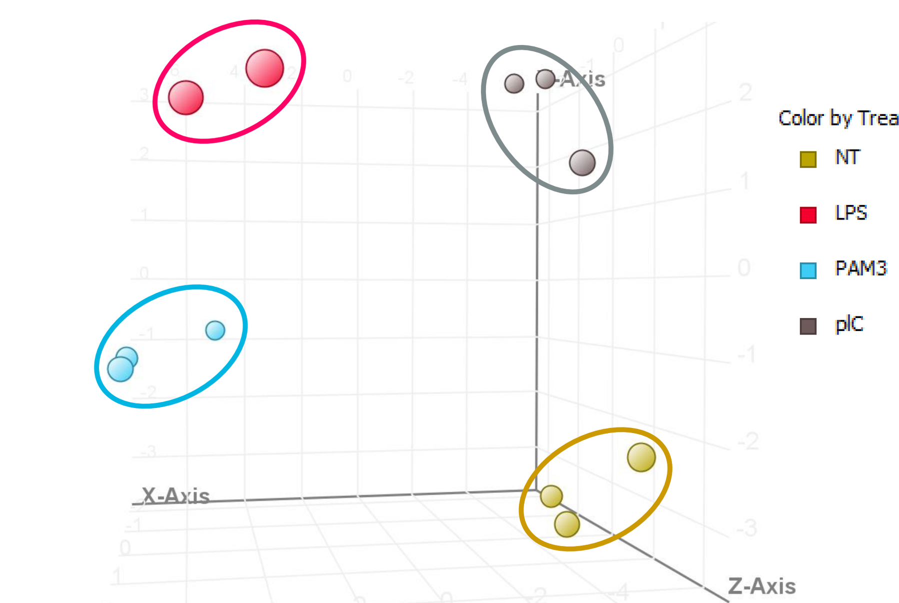
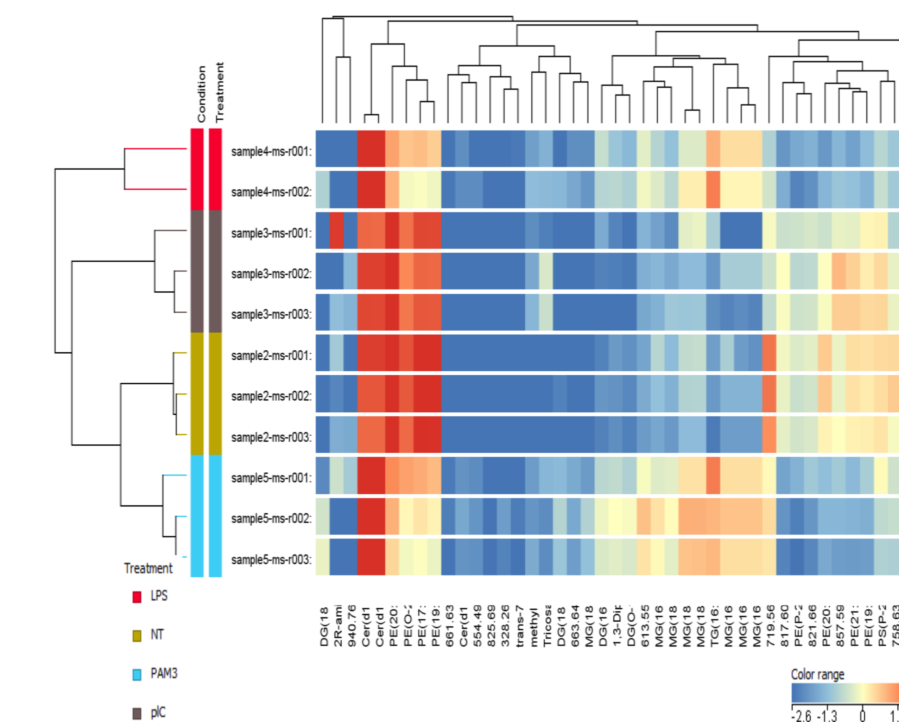


Figure 5. Replicate samples for the same treatment are not well correlated in the Principal Component Analysis performed on the Log2 transformed and cell count corrected lipid profiling data. Figure 6. below shows high correlation after normalization both in Principal Component Analysis and in Hierarchical Clustering Analysis





Retention Time	Intensity	Chemical Composition	Abbreviation common Name	IS Name	IS Conc	IS Intensity	Lipid Class	Normalized Intensity	Relative Quantity
11.404	27632	C38H76NO8P	PC(14:0/16:0)	PC(12:0/13	2	393060	PC	0.070	0.141
11.408	253688			PC(12:0/13	2	393060		0.645	1.291
11.409	112938	C42H82NO8P	PC(16:0/18:1(9Z))	PC(12:0/13	2	393060	PC	0.287	0.575
11.411	257339			PC(12:0/13	2	393060		0.655	1.309
11.412	2021782	C40H80NO8P	PC(16:0/16:0)	PC(12:0/13	2	393060	PC	5.144	10.287
11.413	1293			PC(12:0/13	2	393060		0.003	0.007
11.416	1035311	C38H76NO8P	PC(14:0/16:0)	PC(12:0/13	2	393060	PC	2.634	5.268
11.423	1319			PC(12:0/13	2	393060		0.003	0.007
11.424	1250			PC(12:0/13	2	393060		0.003	0.006
11.426	3835989	C44H84NO8P	PC(18:1(10Z)/18:1(10Z	PC(12:0/13	2	393060	PC	9.759	19.519
11.426	1205			PC(12:0/13	2	393060		0.003	0.006
11.428	1203			PC(12:0/13	2	393060		0.003	0.006
11.428	204596			PC(12:0/13	2	393060		0.521	1.041
11.432	543535			PC(12:0/13	2	393060		1.383	2.766
11.432	2669434	C40H78NO8P	PC(16:0/16:1(9Z))	PC(12:0/13	2	393060	PC	6.791	13.583

Table 2. Example of a Quantitation Report for Lipids belonging to the PC Lipid Class from SimLipid Report Output

Conclusions

We describe an integrated comprehensive workflow for high-throughput profiling, quantitation and multivariate statistical analysis of lipids in complex biological samples. SFC is the analytical method of choice for the profiling of complex lipids with a wide range of polarity and concentrations in a single analytical run.

Statistical analysis with normalized data generated using class-specific internal standards works well for differential analysis, fold changes and the estimation of concentrations of lipids with diverse polarity in the sample.

References

Mass Spectrometry Based Lipidomics: An Overview of Technological Platforms, Harald C. Köfeler,Alexander Fauland, Gerald N. Rechberger, and Martin Trötzmüller, *Metabolites* 2012, *2*(1), 19-38

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