FIA-HRMS-Based Lipidomics Method: Comparing Measured Lipid Concentration Calculated Using Parent Molecular Ion Abundance Versus Sum of Product Ions Abundance

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INTRODUCTION

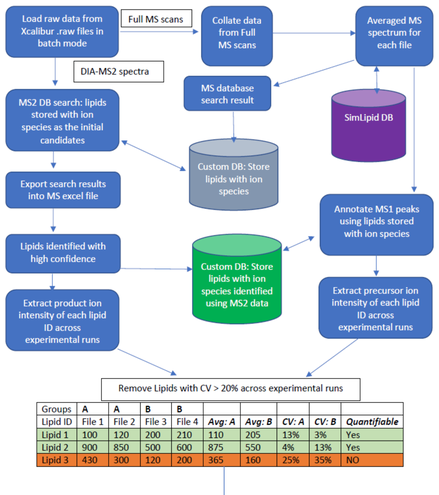
Flow injection analysis (FIA) coupled with high-resolution mass spectrometry (HRMS) instrument-based methods are increasingly becoming recognized as a suitable technique in Lipidomics studies. We present a lipidomic method using FIA coupled to an Orbitrap-based mass spectrometer. Lipid species were measured using data independent acquisition (DIA) based MS/MS data. The concentration of the lipid species present in the samples was measured using the parent ion abundance normalized to the internal standard belonging to the same class and compared to the product ion abundances of the same species.

METHODS

Sample: Lipids were extracted from plasma using MTBE/Methanol extraction in the presence of internal standards (SPLASH LipidoMIX, Avanti Polar Lipids, Alabaster, USA), and the organic phase was resolubilized in the injection solvent.

FIA-MS: Nanomate-based nanoflow injection was coupled to a Q Exactive Plus Orbitrap MS (Thermo Fisher Scientific, MA, USA). Full MS was acquired with Resolution: 140,000, Scan Range: 400-1000. The DIA experiment data was acquired with Resolution: 17,500, Scan Range 100-1000, and Isolation Window: 1 m/z sequentially acquired from 400 to 1000.

Data Analysis: SimLipid® software (PREMIER Biosoft, USA) was used for identification of lipid molecular species.



Groups	A		B						
Lipid ID	File 1	File 2	File 3	File 4	*Avg: A*	*Avg: B*	*CV: A*	*CV: B*	*Quantifiable*
Lipid 1	100	120	200	210	110	205	13%	3%	Yes
Lipid 2	900	850	500	600	875	550	4%	13%	Yes
Lipid 3	430	300	120	200	365	160	25%	35%	NO

Normalize ion abundances of each analyte based on class/subclass specific internal standards				
Lipid ID	*Product Ion Int: A*	*Product Ion Int: B*	*Precursor Ion Int: A*	*Precursor Ion Int: B*
Lipid 1	1	1.5	0.9	1.2
Lipid 2	2.5	2.9	3.2	3.9
Int. Std.	1	1	1	1

Figure 1: Schematic representation of the data analysis workflow using SimLipid software and MS Excel

Microsoft (MS) Excel was used for data normalization, and comparative analysis of results achieved using parent ion abundances and product ion abundances. Schematic representation of the data analysis workflow using SimLipid software and MS Excel is shown in Figure 1.

RESULTS

Raw Data Processing and MS1 Database Search

The raw data (Figure 2(A)) was directly imported as Xcalibur .raw format files into SimLipid software for data processing, identification of probable lipids, and quantitative analysis. While loading the raw data, averaged spectrum (Figure 2(B)) of each data file containing 118 spectra of Full MS1 scans was generated using 5 ppm peak m/z tolerance.

The MS1 database search was performed with mass tolerances of 5 ppm on TG, DAG, PA, PC, PE, PG, PI, PS, Ceramides, Sphingomyelins, Neutral Glycosphingolipids, Steryl Esters, Cholesterols and Derivatives, Oxidized Glycerophospholipids classes. PE and PC lipids with ether- and plasmalogen- substituents were considered. Glycerophospholipids were only considered if containing an even number of carbons on one of its fatty acid chains. A total of 14138 molecular ions belonging to 9866 unique lipid species were obtained from the MS1 database search using a mass tolerance of 5 ppm (Figure 2(C)).

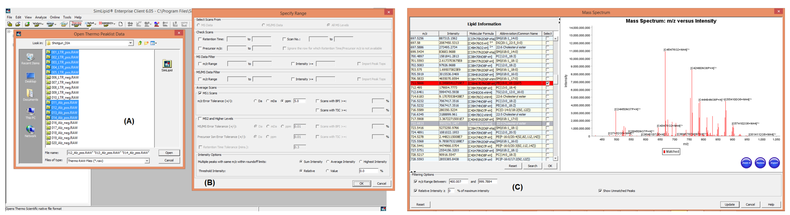


Figure 2: Typical SimLipid software GUI allowing users to (A) import multiple raw files, (B) generate averaged spectra by collating data from full MS1 scans, and (C) MS1 peaks annotated with probable lipid ions.

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Custom Database

Results from the MS1 database search – LipidMaps ID numbers, molecular formula of the parent ions, and their corresponding theoretical m/z values – were exported into a CSV file that can be directly imported as a custom database into SimLipid server database program.

DIA-MS/MS Database Search

DIA-MS/MS spectra were subjected to SimLipid database search on the 14138 molecular ions stored in the custom database using the parameters specified in the Figure 3.

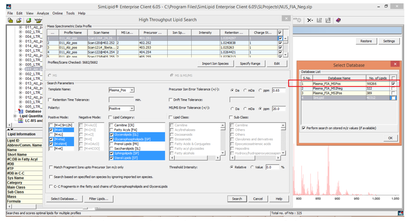

Figure 3: A typical SimLipid GUI showing selection of the custom database for DIA-MS/MS search.

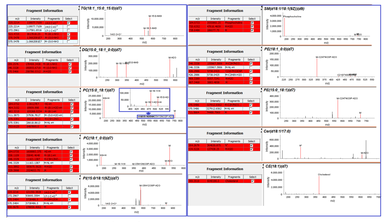

Figure 4: Annotated MS/MS spectra of deuterated TG, DG, PC, LPC, PI, SM, LPE, PE, Cer, and CE lipid species.

Lipid IDs with High Confidence

The raw data from the triplicate analysis was subjected to SimLipid peak detection and peak picking for the preferred list by setting the parameters as shown in Figure 1.

SimLipid search results were exported into MS Excel file. We manually removed unlikely ion species e.g., TG/DG lipids that have three/two unique fatty acid chains but only one/no fatty acid chain resolved by the MS/MS spectra. Lipids from other classes must have their corresponding head group diagnostic ions observed in the MS/MS spectra e.g., peak at 369.3516 m/z for cholesterol esters. Figures 4 shows MS/MS spectra annotated with fragment ions of the deuterated internal standards. A total of 683 lipid ions belonging to 592 unique lipids were identified with high confidence.

Extracting Product Ion Abundance

We use in-built MS Excel formulas to map the total product ions abundances of a lipid species across experimental runs. For example, the MS/MS spectrum annotated with characteristic ions of [TG(18:1(9Z)/15:0/15:0)(d7)+NH4]+ in Figure 4 (top left spectrum) has a total ion abundance of 14517457.5. Table 1 shows the product ion abundances of the lipid molecular ion across the replicates of LTR and Alz.

Quantifiable Lipid Species

Finally, only 330 unique lipid species featuring consistent product ion abundances – coefficient of variation (CV) <20% – across the two plasma samples were saved as a custom database (DB MS2) in SimLipid by storing the identified parent ion species too. The averaged full MS spectra were re-annotated with the newly created DB MS2. A total of 206 peaks were annotated with CV <20% of the precursor ion abundances across replicates of the two plasma samples. The precursor/product ion abundances were normalized to the ion abundances of their respective class-specific internal standards. Table 2 provides a summary of the lipid ions and lipid molecular species that were identified at various steps of the data analysis.

File Name	Scan Name	Matched Ions	Matched Ion Abundance (Sum)
001_LTR_pos	Scan562	14:0 C=O+_225.2209(164391.0), 17:1 C=O+_272.2958(165315.1406), M-18:1_523.4714(4795392.5), M-15:0_570.5466(1.3802956E7)	18928048.77
002_LTR_pos	Scan562	14:0 C=O+_225.2214(119977.7109), 17:1 C=O+_272.2961(1175818.516), M-18:1_523.4725(3617818.25), M-15:0_570.5478(1.0662081E7)	14517457.5
003_LTR_pos	Scan562	14:0 C=O+_225.221(139723.0156), 17:1 C=O+_272.2959(149279.875), M-18:1_523.4717(4499379.5), M-15:0_570.5469(1.3345439E7)	18133822.55
004_LTR_pos	Scan562	14:0 C=O+_225.2208(140597.0), 17:1 C=O+_272.2956(121403.4219), M-18:1_523.4708(3316333.75), M-15:0_570.546(9959466.0)	13537802.54
005_LTR_pos	Scan562	14:0 C=O+_225.2213(125254.0469), 17:1 C=O+_272.296(139520.625), M-18:1_523.4721(3910206.75), M-15:0_570.5474(1.1615424E7)	15790402.31
011_Alz_pos	Scan562	14:0 C=O+_225.2215(108610.75), 17:1 C=O+_272.2966(117496.7734), M-18:1_523.4725(3562666.75), M-15:0_570.5477(1.0525509E7)	14314281.75
012_Alz_pos	Scan562	14:0 C=O+_225.2204(87213.4688), 17:1 C=O+_272.2952(89490.9766), M-18:1_523.4711(2899138.75), M-15:0_570.5463(8555618.0)	11631465.34
013_Alz_pos	Scan562	14:0 C=O+_225.2208(127992.1406), 17:1 C=O+_272.2955(161082.4844), M-18:1_523.4715(4281151.0), M-15:0_570.5466(1.2684512E7)	17254741.67
014_Alz_pos	Scan562	14:0 C=O+_225.2211(126722.6094), 17:1 C=O+_272.2962(137954.75), M-18:1_523.4718(3554142.75), M-15:0_570.5471(1.0176076E7), M-H2O_794.7608(6288.2163)	14001181.56
Average Abundance of TG(18:1(9Z)/15:0/15:0)(d7)[rac] in LTR			16181506.74
Average Abundance of TG(18:1(9Z)/15:0/15:0)(d7)[rac] in Alz			14300417.58
LTR: Coefficient of Variation			14%
Alz: Coefficient of Variation			16%

Table 1: Product ion abundance of [TG(18:1(9Z)/15:0/15:0) (d7)+NH4]+ across the experimental runs extracted from typical SimLipid result file. The column "Matched Ions" follows the reporting nomenclature: <Fragment Name>_<Observed m/z>(<Observed Abundance>).

Groups	MS1: # Lipid Ions	MS1: # Unique Lipids	MS2: # Lipid Ions	MS2: # Unique Lipids	# Lipids with CV< 20%	# Unique isomeric groups (e.g., TG 40:2)	# Lipids* with CV< 20%
TG	7719	4576	256	239	150	43	44
PC	1075	806	93	93	65	55	54
PE	816	721	18	18	6	6	6
DG	775	434	19	15	5	5	5
PG	676	629	0	0	3	3	3
PS	666	629	6	6	2	2	1
PI	621	578	35	35	1	1	1
PA	580	544	2	2	0	0	0
Chol & Der	209	188	0	0	0	0	0
PC P-	170	122	11	11	9	9	6
GlcCer	124	56	0	0	0	0	0
PC O-	117	100	37	37	23	23	23
Cer	113	61	4	4	1	1	0
CE	73	69	42	28	15	15	15
PE P-	71	68	11	11	1	1	0
SM	66	65	95	42	32	31	31
LPE	62	48	14	13	1	1	1
PE O-	61	57	9	9	1	1	1
LPC	39	28	18	17	9	9	9
LPG	25	24	0	0	0	0	0
LPA	22	21	0	0	0	0	0
LPS	18	17	0	0	0	0	0
LPI	17	16	4	4	0	0	0
Oxidized PE	15	5	5	5	5	5	5
Oxidized PC	8	4	4	3	1	1	1
	14138	9866	683	592	330	212	206

Table 2: A summary of the number of lipid ions annotated using MS1 and MS2 data. * The lipid molecular species that were annotated on the averaged MS1 spectra using the 330 lipid species were identified with high confidence based on MS/MS data, and extracted product ion intensity across the replicates of two plasma samples have CV<20%.

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Comparative Analysis

Out of the 206 lipids for which we compared the normalized precursor versus product ion abundances (Figures 5(A)-5(C)), 169 lipids showed similar patterns of change in relative concentrations calculated using normalized precursor/product ion abundances between the study groups i.e., if a lipid species showed increase/decrease in the normalized precursor ion abundances from the sample "LTR" to "Alz", the normalized product ion abundances also showed the same pattern. Figures 6 shows the 37 lipids that exhibit different patterns.

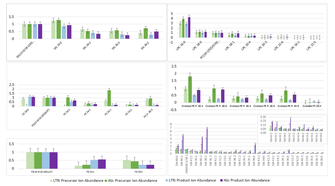

Figure 5(A): Bar charts showing the normalized precursor/product ion abundances of DG, LPC, PE, PR-O, Oxidized-PE/PC, PG, and SM lipids. Inset in the chart of SM lipids displays the zoom in region of low abundant SM lipids.

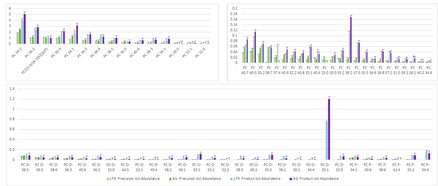

Figure 5(B): Bar charts showing the normalized precursor/product ion abundances of PC, PC O-, and PC P- lipids.

Figure 5(C): Bar charts showing the normalized precursor/product ion abundances of TG lipids: The upper charts showed the concentrations of the isomeric groups, and the lower charts show the concentrations of the TG lipids with known fatty acyl composition.

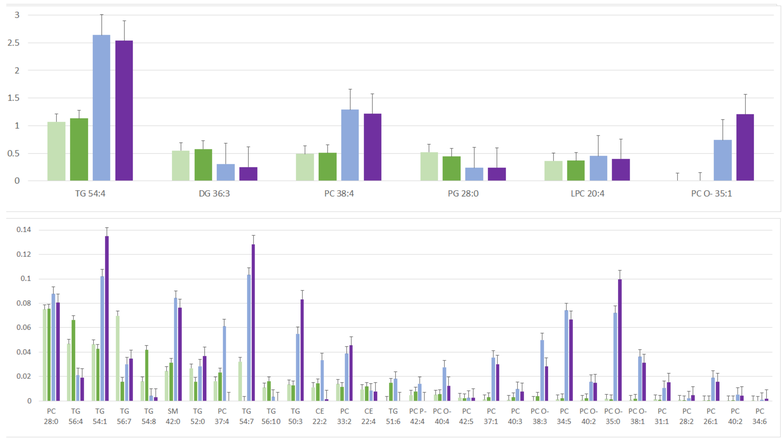

Figure 6: Bar charts showing lipid species that exhibit different patterns of change in relative concentrations calculated using normalized precursor/product ion abundances between the study groups (Upper chart: normalized ion abundances >=0.16, and Lower chart: normalized ion abundances <0.16)

CONCLUSION

We have developed a high throughput lipidomic workflow for nanoflow injection and data independent acquisition, identification and relative quantitation. We investigated to check whether the product ion abundances of lipids could be utilized as a measure of quantifying their concentrations present in the samples. Comparative analysis showed strong agreement in the pattern of change in relative concentrations calculated using normalized precursor/product ion abundances between the study groups. The question whether normalized product ion abundances from using FIA-MS based workflows can deconvolute the concentrations of isobaric lipid molecules is still left unanswered. One possible way could be comparing the normalized precursor/product ion abundances against LC-MS peak areas.