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Biomarkers and Omics

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High-Throughput Lipid Profiling with SWATH® Acquisition and MS-DIAL

Untargeted Lipidomics Workflow with SWATH® Acquisition on TripleTOF® 6600 System

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Lipids in nature are diverse biomolecules, as the majority of lipids contain different combinations of side chains and head groups. The major bottleneck in untargeted lipidomics analysis is the accurate identification of the lipids in complex biological samples. There is a new interest on accurate identification of the lipids in complex biological samples by matching the MS/MS product ion fragments to a reference MS/MS spectrum or using an in-silico MS/MS library[1]. Data dependent acquisition (DDA) techniques often do not collect MS/MS on all precursors but the data independent approach using SWATH® Acquisition, a data independent acquisition (DIA) method, ensures that product ion spectra is acquired on all detectable compounds in a sample, effectively generating a digitized record of all detectable metabolites in a sample.

Here an untargeted lipidomics workflow is demonstrated which includes: a simple lipid extraction method from 20 μL of mouse plasma, a 15-minute SWATH® acquisition method, and a 1 hour data analysis step using MS-DIAL (**M**ass **S**pectrometry – **D**ata **I**ndependent **A**na**L**ysis). MS-DIAL is an open-source software for the identification and quantification of small molecules and lipids
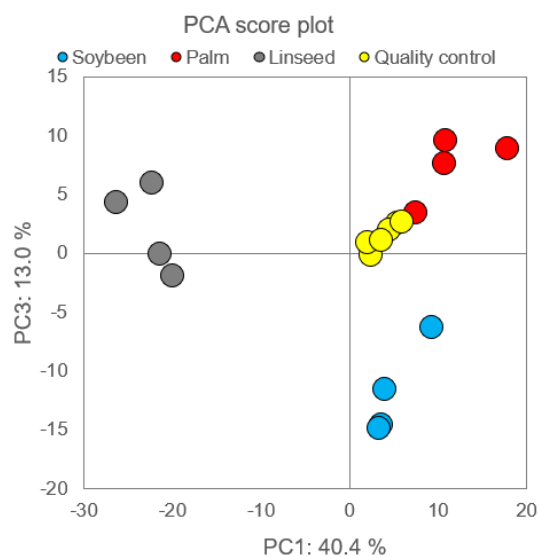

Figure 1. PCA Analysis of all 322 Lipids Identified with MS-DIAL. Here, the PCA score plot shows the clear differentitation between QC samples and other three dietary groups analyzed.

from DIA and DDA-based untargeted LC-MS/MS analysis[2], providing lipids identification by MS/MS confirmation. Using this workflow, 322 lipid molecular species were identified out of 420 spectra matched by MS/MS confirmation from a mouse plasma (3 groups, N = 4) provided by RIKEN Center for Integrated Biomedical Sciences Metabolome Research Team. Quantitative analysis of lipids clearly differentiated the mouse plasma samples from the 3 different dietary groups (Figure 1).

SWATH® Acquisition with MS-Dial Workflow

- Using the data independent SWATH acquisition method, MS/MS spectra is acquired on all detectable species and is used for confident lipid identification

- A waveform separation algorithm (deconvolution)[2] in MS-DIAL is used to deconvolute the blended SWATH acquisition MS/MS spectra to produce simplified spectra for identification

- MS-DIAL uses a database for 61,513 lipid molecular species, with 135,456 product ion spectra corresponding to positive and negative ion mode

- In addition, the "preserved retention time" of lipids under this LC-MS/MS method is used for the improved reliability of identification

Methods

Mouse Plasma Preparation: In this study, 8 weeks old C57BL/6J mice (4 males) were fed for one week with Oriental Yeast diet containing either 4% soybean oil, palm oil or linseed oil. Blood was collected, heparin was added and plasma was extracted for each sample (4 samples collected from 3 dietary groups).

Lipid Extraction: Extraction of lipids was performed by mixing chloroform: methanol: plasma at 100 µL: 200 µL: 20 µL. Here, a pooled quality control (QC) sample was created by mixing 50 µL of the final volume of 320 µL lipid extract from each sample in a separate container. In addition, two blanks (blanks) were prepared that followed the same extraction process. Finally, 150 µL of each sample solution was transferred to a vial for LC-MS analysis, as well as the QC and blank samples. The QC sample was analyzed after each 3 sample injections to monitor the sensitivity of mass spectrometry throughout the analysis. Note: the QC analysis was injected from the same vial for every QC injection. However, when considering sample vaporization etc., it is recommended to have separate aliquoted vials of QCs for large scale analysis.

LC-MS/MS Analysis: The Shimadzu Nexera UHPLC system with Waters ACQUITY CSH C18 2.1 x 100 mm, 1.7 µm column was used for liquid chromatography with a SCIEX TripleTOF® 6600 system. For large-scale analysis, a Waters ACQUITY VanGuard CSH C18 1.7 µm pre-column is recommended to protect the column, replacing it after 300 injections. It is recommended to replace the analytical column after 1000 to 1500 sample injections. The conditions of liquid chromatography are shown in Table 1, the liquid chromatography (LC) method gradient is shown in Table 2, and the mass spectrometer parameters are shown in Table 3.

Table 1. Liquid Chromatography Conditions.

Parameter	Setting
Flowrate	0.6 mL/min
Autosampler temp.	4 ℃
Injection volume	3 mL (positive) 5 mL (negative)
Column temperature	65 ℃

Table 2. LC Gradient.

Time	%B
0	15
2	30
2.5	48
11	82
11.5	99
12	99
12.1	15
15	15

The two prepared blank samples, 12 total samples, 2 vials of QC samples as well as one vial of ultra-pure water as blank buffer were analyzed in both positive and negative polarity mode. Sample injection was performed in order shown in Table 4 with biological samples analyzed in randomized order.

Mobile phase A was 60% acetonitrile with 10mM ammonium formate in 0.1 % formic acid in water. For 200 injections, 1 liter of each mobile phase is required. First, the glass bottle is rinsed with acetonitrile, then 600 mL of acetonitrile, 399 mL HPLC grade water, and 1 mL of formic acid is added. This is mixed well and then 0.63 g of ammonium formate salt is and sonication at room temperature for 10 minutes.

Mobile phase B was 90% isopropanol, 9.8% acetonitrile with 10mM ammonium formate in 0.1 % formic acid in water. Place 1 mL of ultrapure water in a small beaker, add 1 mL of formic acid. Add in 0.63 g of ammonium formate and mix slowly so that the above three are mixed. Rinse a 1 liter glass bottle with acetonitrile, add in the solution prepared in the beaker, then add 900 mL of isopropanol and 98 mL of acetonitrile to 1 L glass bottle. Sonicate at room temperature for 10 minutes.

Do not use mobile phases until ammonium formate is dissolved and bubbles have disappeared.

Lipid Identification

SWATH acquisition data were analyzed by MS-DIAL version 1.98. As a result, a total of 322 lipids were identified by positive and negative mode (Table 5). Lipid identification was achieved using MS/MS confirmation after performing automatic peak detection, waveform processing, and compound identification by MS-DIAL which takes approximately 1 hour. The automatic identification of MS-DIAL is carried out using the highly reliable integration index considering retention time and isotopic ratio in addition to the MS/MS similarity calculation and by dot/product method which is the most frequently used method[2]. MS-DIAL graphical user interface also assists the manual curation for identification results (Figure 2).

Table 3. SWATH Acquisition Method.

Parameter	Setting
MS1 mass range (Positive)	100-1250
MS1 mass range (Negative)	200-1250
SWATH scan range	350-1250
MS/MS mass range	100-1250
TOF MS accumulation time	50 ms
TOF MS/MS accumulation time	10 ms*
Collision energy	45 V
Collision energy spread	15 V
Precursor window	15 Da
Cycle time	700 ms
CUR	35
GS1	60
GS2	60
TEM	350
CE	10
DP	80
ISVF (Positive)	5500
ISVF (Negative)	4500

Note for a more quantitative method, longer MS/MS accumulation times can be used.

Table 4. Order of LC-MS/MS Analysis.

Injection	Sample
1	Water
2	Blank-1
3	Blank-2
4	QC-1
5	QC-2
6	Sample-1
7	Sample-2
8	Sample-3
9	QC-3
10	Sample-4
11	Sample-5
12	Sample-6
13	QC-4
14	Sample-7
15	Sample-8
16	Sample-9
17	QC-5
18	Sample-10
19	Sammple-11
20	Sample-12
21	QC-6

Using QC Samples

Standardization of quantitative values was also carried out by the LOESS (LOcally WEighted Scatter-plot Smoother) Cubic spline method using quantitative values of pooled quality controls (QCs) [4]. The quantitative drift of MS signal for each compound is computed using the assumption that the quantitative value of QC measured at a rate of 1 per every 3-5 sample, a common method used for large-scale cohort studies and implemented in MS-DIAL[3]. Each quantitative value is expressed as a relative value when the quantitative value of QC is set to 1. The merit of this method is that it compensates for drift in the MS quantitation value for each analysis, but also it can integrate positive ion mode data and negative ion mode data (as it is already standardized based on QC).
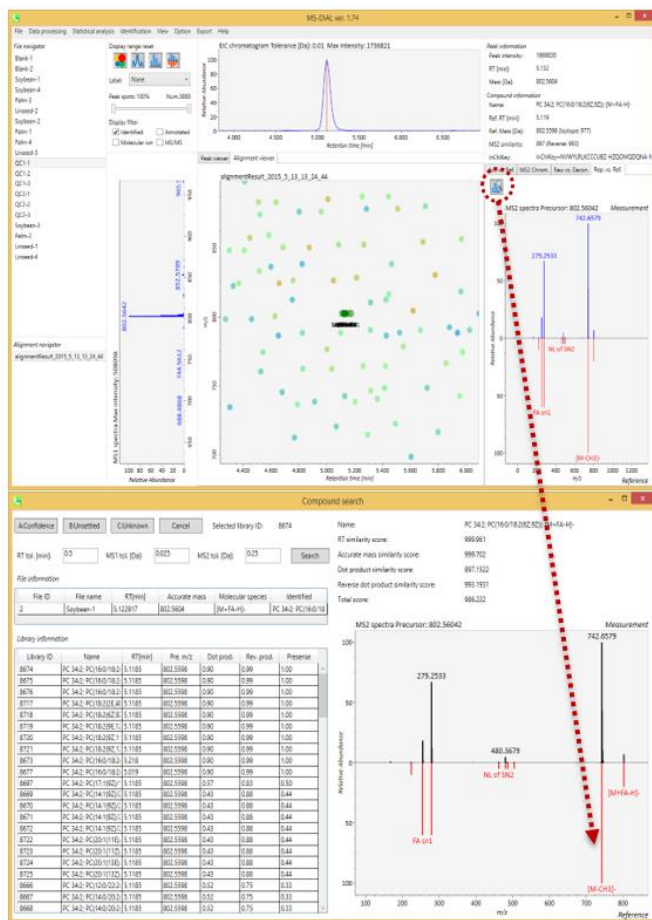
Figure 2. MS-DIAL Identification and Confirmation Workflow. MS-DIAL main page provides alignment and identification results. The lower right figure shows the confirmation of time, m/z and MS/MS spectra for a certain spot with the library data. By clicking the magnifying glass mark, a list of candidate compounds for this identification is displayed, and the specification is such that the user can correct the identification result if needed.

Furthermore, availability of a QC sample of the same quality can enable integration of data across months and years. The mean or median value of the original QC ion abundances can be multiplied to the normalized values so that the ion abundance information for each metabolite is considered for data interpretation.

Quantitative Lipid Results

Results from principal component analysis (PCA) on the identified 420 lipid spectra clearly distinguishes the differences in phenotype reflecting the dietary condition (Figure 1). PCA Analysis highlighted that 55% of the data was explainable by the biological effect of the mice fed by different diets.

Table 5. Lipid Species Identified from SWATH Acquisition data from MS-Dial. The SWATH acquisition data analysis by the MS-DIAL program identified a total of 322 unique lipids in positive mode and negative mode.

Lipid Class	Positive Modes	Negative Mode
Acylcarnitine (AC)	6	0
Free fatty acid (FFA)	0	17
LysoPC	32	26
LysoPE	2	9
Phosphatidylcholine (PC)	69	43
Phosphatidylethanolamine (PE)	15	22
Phosphatidylglycerol (PG)	0	1
Phosphatidylinositol (PI)	0	8
PlasmenylPC	17	7
PlasmenylPE	9	15
Sphingomyelin (SM)	5	0
Diacylglycerol (DAG)	12	0
Triacylglycerol (TAG)	96	0
Cholesteryl ester (CE)	9	0
Total	272	148

List of identified lipids shown in Table 6 indicates 18: 3 (α-linolenic acid), 20: 5 (eicosapentaenoic acid, EPA), 22: 6 (docosahexaenoic acid, DHA) derived from ω3 series fatty acids, are constituent fatty acids in mice with the linseed oil containing diet. The phospholipid and triacylglycerols also increased in mouse plasma samples fed with linseed lambda oils rich in ω3 fatty acids.

On the other hand, 80% of the constituent fatty acids of palm oil are composed of 16: 0 (palmitic acid) and 18: 2 (linoleic acid), and when looking at the lipid profile of mouse plasma with palm oil as the diet, phospholipid and triacylglycerol with these two fatty acids as constituents are increased. In addition, the main constituent fatty acid of soybean oil was 18: 2 (linoleic acid), which also increased in the plasma of mice fed with soybean oil.

Because the position information of the double bond position and the acyl group cannot be determined in this method, A and B were annotated by the elution order (Table 6). The "P-" is to indicate that it is a plasmalogen type.

Table 6. List of Lipid Molecules That Have Changed Significantly in Mouse Plasma Given Each Diet.

Linseed Oil	Palm Oil	Soybean oil
18:3 Cholesteryl ester	DAG(16:0/18:1/0:0)	DAG(18:2/20:1/0:0)
20:5 Cholesteryl ester	DAG(18:1/18:0/0:0)	lysoPC 18:2
DAG(18:2/18:3/0:0)	DAG(18:1/18:1/0:0)	lysoPC 20:4
FA 18:3	PC(16:0/16:1)	PC(18:0/20:4)
FA 20:5	PC(16:0/17:1)	PE(P-20:0/18:2)
lysoPC 18:3-A	PC(16:0/18:1)	PE(P-20:0/20:4)
lysoPC 18:3-B	PC(17:0/18:1)	
lysoPC 20:5-A	PC(18:0/18:1)	
lysoPC 20:5-B	PE(16:0/18:1)	
lysoPC 22:5	PE(18:0/18:1)	
PC(14:0/20:5)	PI(18:0/20:3)	
PC(16:0/20:5)	TAG(14:0/16:0/16:0)	
PC(16:1/18:3)	TAG(14:0/16:0/16:1)	
PC(16:1/20:5)	TAG(16:0/16:0/16:0)	
PC(18:0/20:5)	TAG(16:0/16:0/18:1)	
PC(18:2/18:3)	TAG(16:0/16:0/20:2)	
PC(18:3/18:3)	TAG(16:0/16:1/16:1)	
PC(18:3/20:5)	TAG(16:0/16:1/17:0)	
PC(20:5/22:6)	TAG(16:0/16:1/17:1)	
PC(22:6/18:3)	TAG(16:0/16:1/18:1)	
PE(16:0/20:5)	TAG(16:0/17:0/18:1)	
PE(16:1/20:5)	TAG(16:0/18:0/16:0)	
PE(18:0/20:5)	TAG(16:0/18:0/18:1)	
PE(18:1/20:5)	TAG(16:0/18:1/20:1)	
PE(P-16:0/20:5)	TAG(17:0/17:1/17:1)	
PE(P-16:0/20:5)	TAG(18:0/18:1/20:4)	
PE(P-18:0/20:5)	TAG(18:1/18:1/18:1)	
PE(P-18:0/20:5)	TAG(18:1/18:1/20:1)	
PE(P-20:0/20:5)		
TAG(16:0/18:2/18:3)		
TAG(16:0/18:3/18:3)		
TAG(16:0/18:3/20:5)		
TAG(16:0/20:5/20:5)		
TAG(16:1/18:2/22:6)		
TAG(16:1/18:3/18:3)		
TAG(16:1/18:3/20:5)		
TAG(16:1/20:5/20:5)		
TAG(16:2/20:3/22:5)		

Table 6. Continued.

Linseed Oil	Palm Oil	Soybean oil
TAG(18:1/18:3/18:3)		
TAG(18:1/20:5/22:6)		
TAG(18:2/18:3/18:3)		
TAG(18:2/18:3/20:5)		
TAG(18:2/20:5/20:5)		
TAG(18:3/18:3/18:3)		
TAG(18:3/18:3/20:5)		
TAG(18:3/20:5/20:5)		

Conclusions

The SWATH acquisition with MS-DIAL lipid identification workflow introduced here provides identification of structural isomers and binding information of organic elements. Out of 420 matched lipid spectra, 322 unique lipids were identified excluding all duplicates such as those identified in both in positive and negative modes, and those detected with different adduct ions. Quantitation of identified lipid species clearly differentiates the three sample groups with the different dietary oil supplements.

References

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