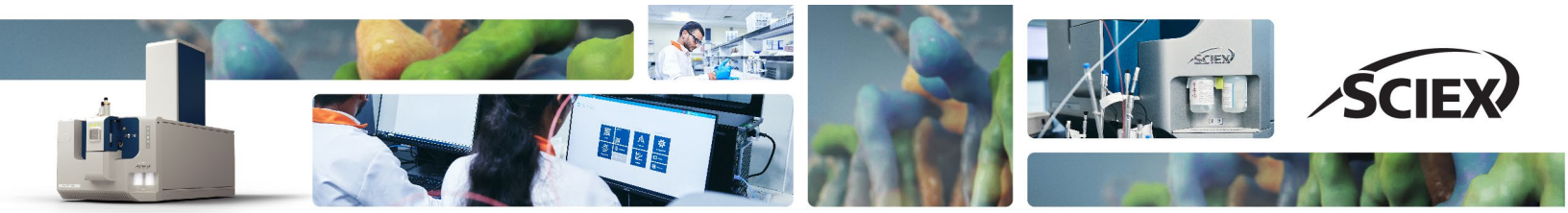

The simultaneous processing of DDA and SWATH data by MS-DIAL software improves coverage for untargeted lipidomics analysis

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In this technical note, data-dependent acquisition (DDA) and data-independent acquisition (DIA; SWATH) experimental data acquired by the ZenoTOF 7600 system were simultaneously processed using MSDIAL software to improve the overall detection and identification of lipid molecular species in biological samples.

Untargeted analysis of lipids presents unique analytical and data processing challenges due to the extensive inter- and intra-class isobaric overlap within the lipidome. Combined with relatively few structurally diagnostic fragment ions generated by collision-induced dissociation (CID), untargeted lipidomics analysis can suffer from misidentification and a high false positive discovery rate using data analysis software. A novel approach was used to leverage electron-activated dissociation (EAD)-based fragmentation using the ZenoTOF 7600 to improve lipid structural characterization, thereby reducing false positive identifications, and combine data from DDA and SWATH experiments to improve the overall coverage of untargeted lipidomics analysis.

Here, human pericyte-derived lipid extracts were analyzed using DDA and SWATH analysis in the positive or negative ion modes using CID- and EAD-based fragmentation techniques. Data were processed using MS-DIAL 5.2 software to identify lipid molecular species.

Key features of lipidomics analysis using the ZenoTOF 7600 system

- The SCIEX ZenoTOF 7600 system offers flexibility in the types of analyses that can be performed, including DDA and SWATH
- Combining DDA and SWATH data during data processing by MS-DIAL software significantly improves the coverage of identified lipid molecular species in biological samples
- Electron-activated dissociation (EAD) produces diagnostic fragments that can help identify the lipid class and the fatty acid composition, determine the fatty acid position, and identify the location of double bonds within the fatty acid

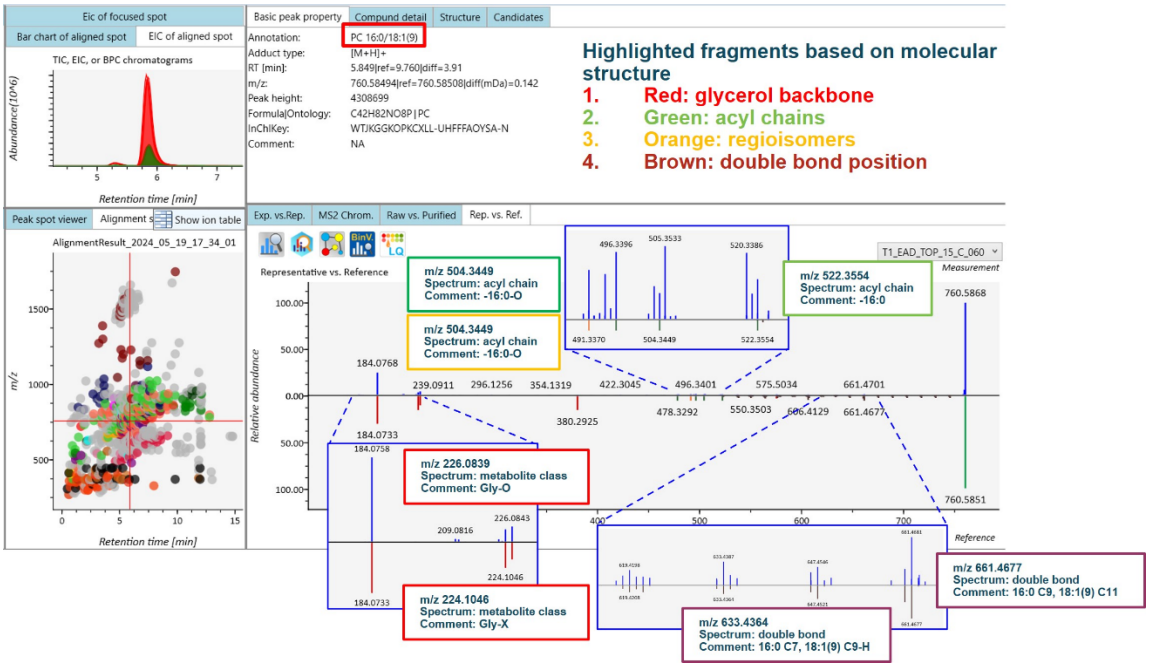

Figure 1. MS-DIAL software user interface for PC (16:0/18:1(9)) identified from positive ion mode DDA data acquired using EAD-based fragmentation. Specific fragments, as indicated, denote structural features of the identified lipid, including the glycerol backbone, palmitic acid (16:0) at the *sn*-1 position, and oleic acid (18:1(9)) at the *sn*-2 position.

Introduction

Brain pericytes are cells proximal to the cerebral microvessel endothelial cells, which form the blood-brain barrier (BBB) [1]. The damage to or absence of pericytes causes significant disruption of the BBB at the integrity level by affecting its permeability to circulating compounds and at the molecular level by disrupting the expression and/or function of important proteins such as P-glycoprotein [2,3]. Other studies have also demonstrated the importance of brain pericytes in lipid metabolism [1,4]. Here, brain pericyte-derived lipid extracts were analyzed for their lipid content using a discovery mass spectrometry approach to explore different data processing approaches using MS-DIAL software.

Untargeted (DDA) lipidomics is a commonly used mass spectrometry technique to identify lipids in biological samples [5]. Ideally, this type of experiment generates at least 1 high-quality product ion spectrum for each lipid in a sample, yielding complete coverage of the lipidome. In practical terms, however, coverage is a function of instrument speed and sensitivity, chromatography, extraction efficiency, data processing software, and the availability of reference spectral databases. Coverage is also impacted by the level of structural specificity derived from the data (i.e., from a basic sum composition identification to complete structural characterization). Data acquired from DDA runs are quantitative at the MS1 level but not MS2; consequently, untargeted experiments are limited to reporting and quantifying lipids with sum composition identification.

Data-independent acquisition (DIA; SWATH) experiments offer the promise of qualitative analysis of lipids (i.e., identification) and quantitation at the MS2 level, thereby greatly enhancing the accuracy and specificity of lipid analysis [6]. However, due to the extensive isobaric overlap among lipids, Q1 unit resolution is needed to mitigate the potential for high false positive results, which is not possible with current iterations of SWATH analysis. This need has thus far prevented the use of SWATH as the sole analytical method for reliable lipidomics analysis. However, the combination of DDA and DIA workflows offers the potential for improved coverage, compared to either experiment alone, and it can provide quantitation at the fatty acid identification level from the SWATH data [7].

The ZenoTOF 7600 system is uniquely designed with the speed, sensitivity and specificity to generate discovery lipidomics data with broad coverage. The detector of the ZenoTOF 7600 system can cycle as rapidly as 130 Hz, and the sensitivity has been shown to rival that of a high-end triple quadrupole [8]. The complementary EAD fragmentation mode generates diagnostic fragment ions that enable near-complete structural characterization of lipids in biological samples [9-11]. MS-DIAL software can process both data types and has recently been updated to handle EAD-based fragmentation data [12]. The combination of DDA and SWATH analysis offers the opportunity for excellent coverage, structural specificity and quantitation.

In the experimental results reported here, the combined DDA/SWATH analytical approach was used to identify lipids in human brain pericytes. The data show that MS-DIAL 5.2 software can simultaneously process CID-based DDA and SWATH acquisition data to generate a combined results file that extends coverage beyond either experiment alone and provides quantitative results to facilitate statistical analysis to identify potential biomarkers in a study.

Materials and methods

Sample preparation: Brain pericytes were obtained from Pr. Takashi Kanda (Department of Neurology and Clinical Neuroscience Clinical Neuroscience, Yamaguchi University Graduate School of Medicine of Medicine, Yamaguchi University, Ube, Japan) [13]. The pericytes were resuspended in Accutase reagent, a cell lysis buffer, and centrifuged at 300 × g for 5 min at 4°C. The cell pellet was reconstituted as whole cell lysate or membrane fractions were prepared; both sample types were stored at -80°C before lipid extraction.

Two types of lipid samples were extracted from human pericytes: total cell lipids (labeled TOTAL PERI T1 and T2) and total membrane fraction lipids (labeled MB PERI) via the method of Bligh and Dyer [14]. Lipid extracts were dried under a stream of nitrogen, and the lipids were resuspended in 200 µl IPA/CAN/H_2O (2:1:1, v/v) before analysis by HPLC ESI-MS/MS.

Chromatography: Reversed-phase chromatographic separation was performed using an Exion AD system (SCIEX, Framingham, MA) comprising a binary ultrahigh pressure gradient pump with degasser, a temperature-controlled autosampler, and a column oven. Separation was performed using a Waters Acquity UPLC CSH C18 column (1.7 µm, 130 Å, 100 x 2.1 mm) held at 65 °C. An ACQUITY VanGuard CSH C18 1.7 µm pre-column is recommended to protect the column. For positive ionization mode analysis, mobile phase A was water/acetonitrile/ (40/60, v/v) containing 10 mM ammonium formate, and mobile phase B was propane-2-ol/acetonitrile (90/10, v/v containing 10 mM ammonium formate. For the negative ionization mode analysis, ammonium formate was substituted with ammonium acetate. The flow rate was 0.6 mL/min, with gradient elution starting at 15% B. The gradient profile is shown in **Table 1**. Samples were kept in the autosampler at 4 °C. The injection volume was adjusted for the different experiments and sample types. For DDA and SWATH experiments using the positive ion mode, 5 and 0.5 µL were injected for membrane and total cell lipid extract, respectively. For DDA and SWATH experiments in the negative ion mode, 15 µL total cell lipid extract was injected. For membrane lipid extracts, 3 and 5 µL were injected for DDA and SWATH, respectively.

Table 1 Chromatographic gradient

Time (min)	*Mobile phase A (%)*	*Mobile Phase B (%)*
0	*85*	*15*
2.0	*70*	*30*
2.5	*52*	*48*
11	*18*	*82*
11.15	*1*	*99*
12.0	*1*	*99*
12.1	*85*	*15*
15.0	*85*	*15*

Mass spectrometry: Lipid detection was performed on a SCIEX ZenoTOF 7600 system equipped with an Optiflow Turbo V ion source and an electrospray ionization (ESI) probe. Instrument calibration was maintained using the automated calibrant delivery system (CDS), which calibrated every five samples with an ESI calibration solution. DDA and SWATH experiments were performed using CID-based fragmentation in the positive and negative ion modes. The system was configured for DDA experiments to select the top 50 most abundant ions for fragmentation. Dynamic background subtraction (DBS) with a mass tolerance of 50 mDa was applied to minimize noise and maximize the MS/MS quality. The TOF MS accumulation time was set at 100 ms. For SWATH experiments, 48 variable windows were used. All TOF MS parameters used were maintained as described above for DDA. The TOF MS/MS parameters are shown in **Table 2**.

For EAD-based fragmentation (positive ion mode), the DDA selection criteria were optimized to select the top 15 most abundant ions for fragmentation during each experimental cycle. An electron reaction time of 60 ms, with a 65 ms accumulation time. The system parameter settings are listed in **Table 2**.

Table 2: ZenoTOF 7600 system parameter settings

Parameter	*EAD*	*CID*
Curtain gas (CUR)	*35 psi*	*35 psi*
Ion source gas 1 (GS1)	*50psi*	*50psi*
Ion source gas 2 (GS2)	*70 psi*	*70 psi*
CAD gas (CAD)	*7*	*7*
Source temperature (TEM)	*600 °C*	*600 °C*
Ion spray voltage (IS)	*5500 V*	*5500 V*
Declustering potential (DP)	*50 V*	*50 V*
Electron beam current	*6000 nA*	*N/A*
Electron KE	*12 eV*	*N/A*
Collision energy (CE)	*10 V*	*30 V*
Zeno threshold	*80,000 cps*	*80,000 cps*

Data processing: Data were acquired using SCIEX OS software and processed using MS-DIAL 5.2 software. The results section describes the optimization of the data processing settings in detail.

Results

DDA and SWATH Analysis of brain pericyte lipid extracts

The analysis of lipids using the DDA scan mode was optimized through an iterative process to identify the best instrument parameter settings and precursor ion selection criteria settings, the latter of which is termed IDA criteria. For CID-based experiments, a top-50 approach was used to maximize coverage. In contrast, for EAD-based experiments, a top 15 approach was used to accommodate the increased reaction time needed for efficient electron-based fragmentation (65 ms). **Figure 2** shows an example total ion chromatogram (TIC) for 3 samples analyzed by DDA using CID-based fragmentation. SWATH experiments were also run to analyze the brain pericyte extracts (**Figure 2**). Using a variable window approach (48 windows), these data were acquired using the same instrument parameter settings used for the DDA experiments.

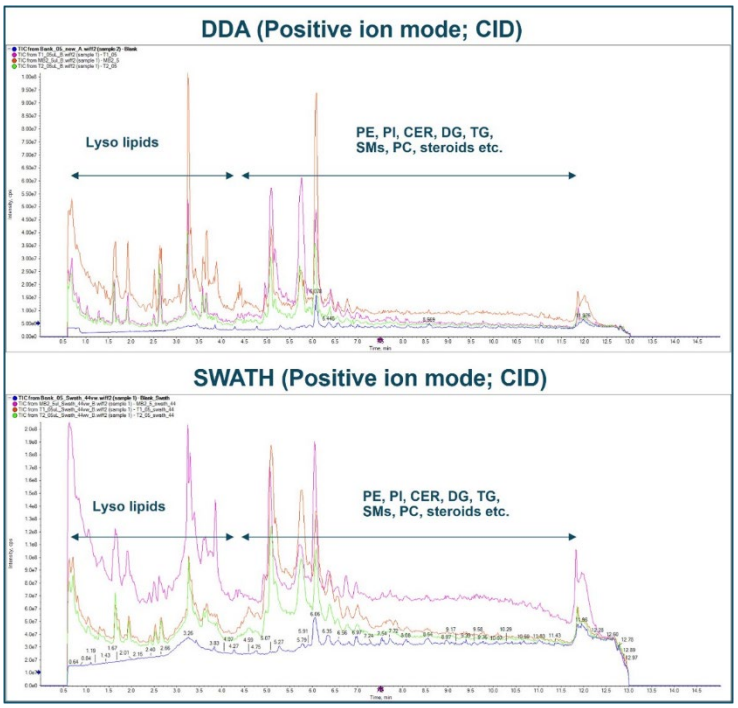

Figure 2. TIC for DDA and SWATH experiments performed in the positive ion mode using CID-based fragmentation. A reverse phase chromatographic strategy was employed to separate lipids based on their hydrophobicity. Although an effective strategy, care must be taken with data processing to avoid false positives attributed to intra-class lipid isobars. Note that the two experiment types are closely aligned regarding feature retention times. Each experiment was also run in the negative ion mode (not shown).

The optimization of MS-DIAL 5.2 software parameter settings for DDA and SWATH data analysis

The data processing parameters of the MS-DIAL 5.2 software series are similar to those of the 4.9 series, as detailed in a previous technical note (15). However, additional features in version 5.2 enable the processing of the data acquired here. In version 5.2, there is an *in silico-generated* EAD spectral database, and it is now possible to simultaneously process DDA and SWATH data. Additionally, several new annotations cut-off scores have been implemented, and MS-DIAL 5.2 software can align identified lipids according to their "match score."

The correct data processing parameters for MS-DIAL 5.2 software are essential to generating the best quality results. Consequently, a step-by-step procedure with user interface examples is presented here that details how to simultaneously use this software to process DDA and SWATH metabolomics data.

Raw measurement files: Raw data files are selected from the SCIEX OS project folder **(Figure 3)**. When using data acquired on the ZenoTOF 7600 system, it is recommended that Wiff2 files be used with MS-DIAL software. Type, class ID, and the acquisition method should be entered for each data file. The Class ID of the samples can be changed after processing; however, the "Blank" sample should be selected here initially to enable the "blank filtering" feature.

Measurement parameters: Measurement parameters should be selected as shown in the example in **Figure 4**. The data type should be centroid for MS1 and MS/MS. If EAD-DDA analyses are to be included in the data processing, click EIEO from the "collision type" tab.

Peak detection: In the peak detection tab, set the minimum peak height to 100 cps for DDA experiments **(Figure 5)**. This setting gives the highest number of compounds while minimizing false positives during optimization. However, it is recommended that the setting be

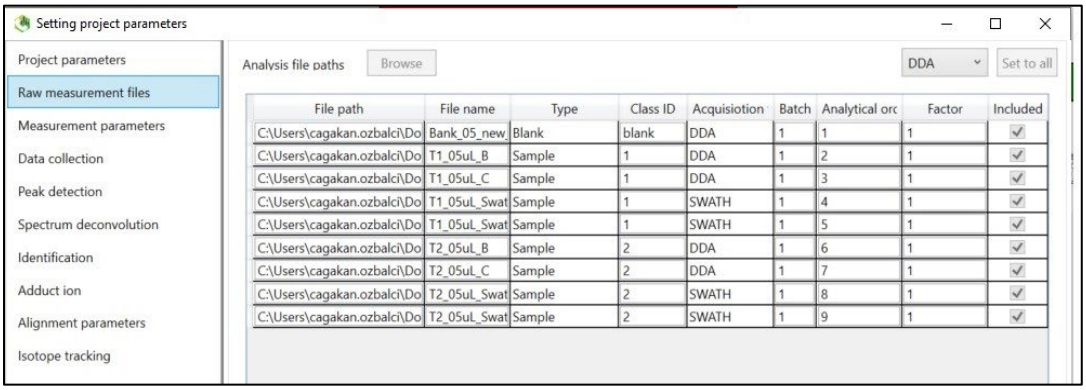

Figure 3. Raw Measurement files

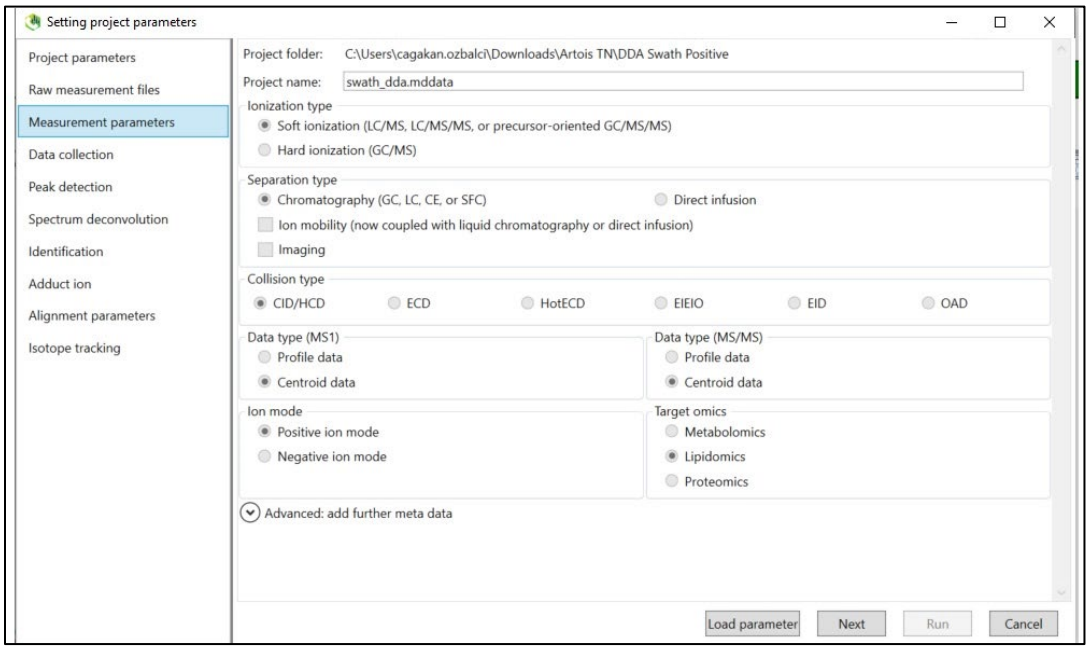

Figure 4. Measurement parameters

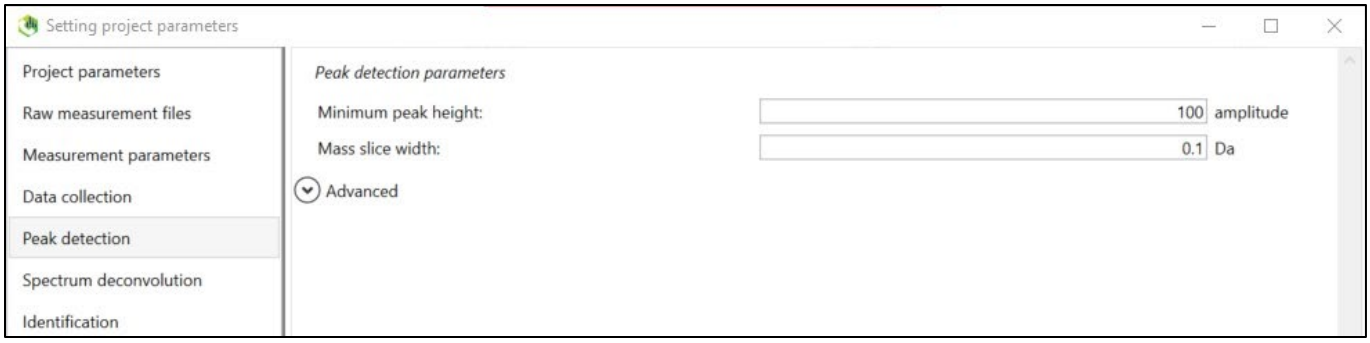

Figure 5. Peak detection

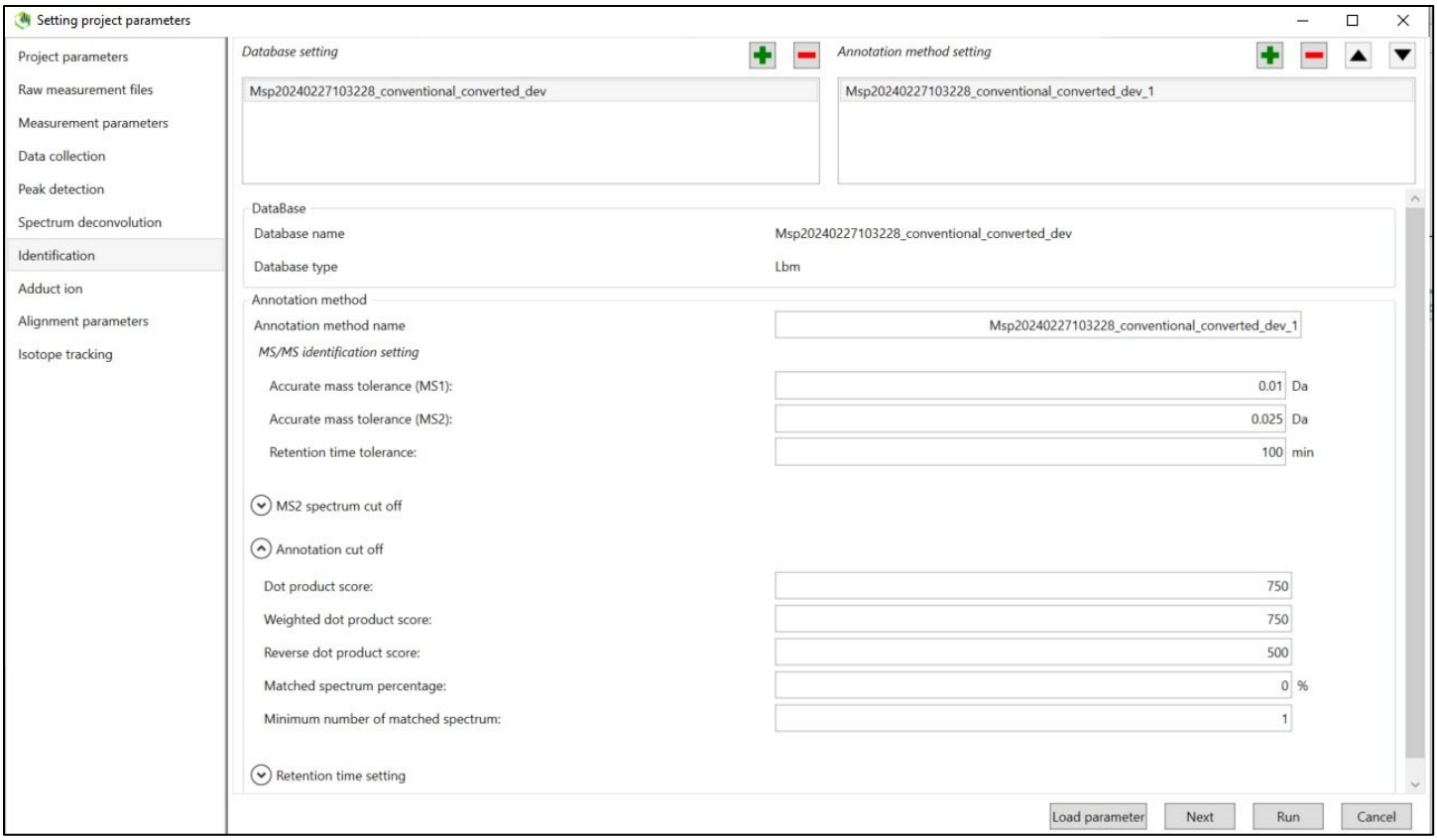

Figure 6. Identification

1000 cps for SWATH data analysis to reduce the time required for data processing.

Identification: The settings for lipid identification should be selected with care as these parameters have the most significant impact on the accuracy of the data processing (**Figure 6**). The conventional CID library is automatically selected in the "annotation method setting" if CID-based fragmentation is used in the experiment. However, when EAD-based fragmentation data are used, it must be specifically selected. The mass tolerance settings in **Figure 6 are** the parameters for CID-based identification and are the same for EAD data (not shown). Optimized "annotation cut-off" settings are also shown, and their impact on the results is discussed here:

- The "Reverse dot score" parameter most impacts the balance between the number of lipids identified and their match score. Through a series of iterative data processing steps, the setting of 500 gave the best results.
- The "Dot product score" and "Weighted product score" were found to have less significant effects on the match score numbers; both were set to 750.

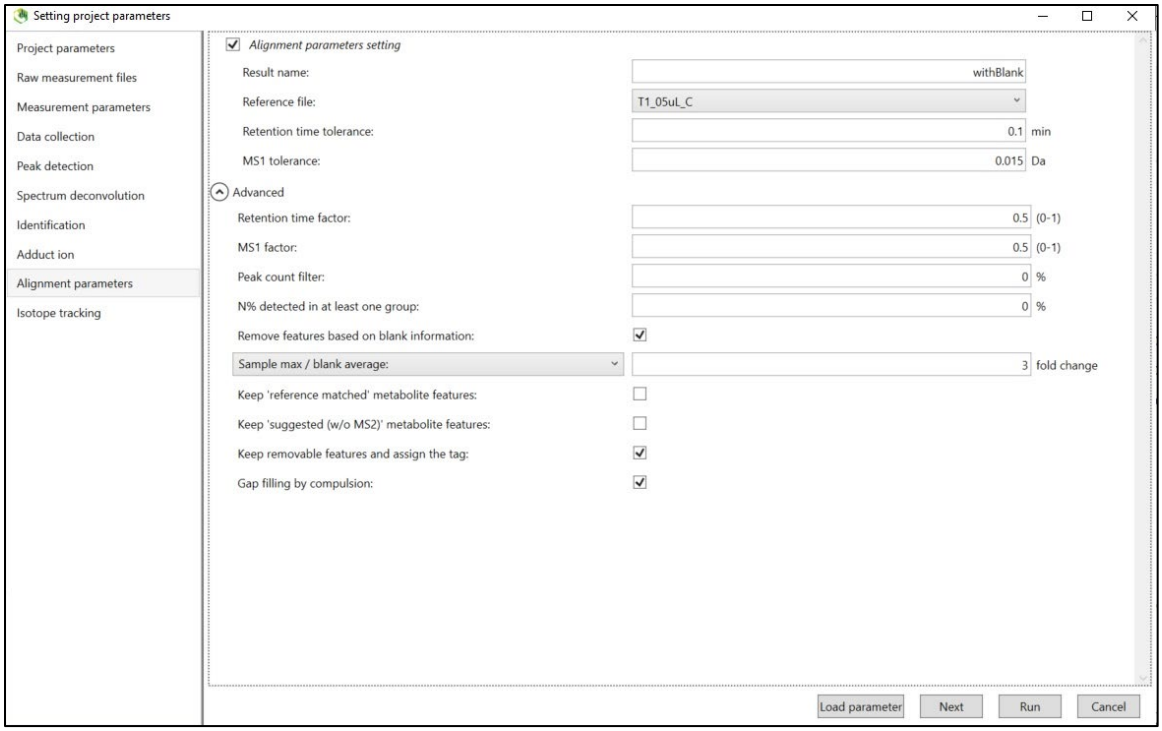

Figure 7. Alignment parameters

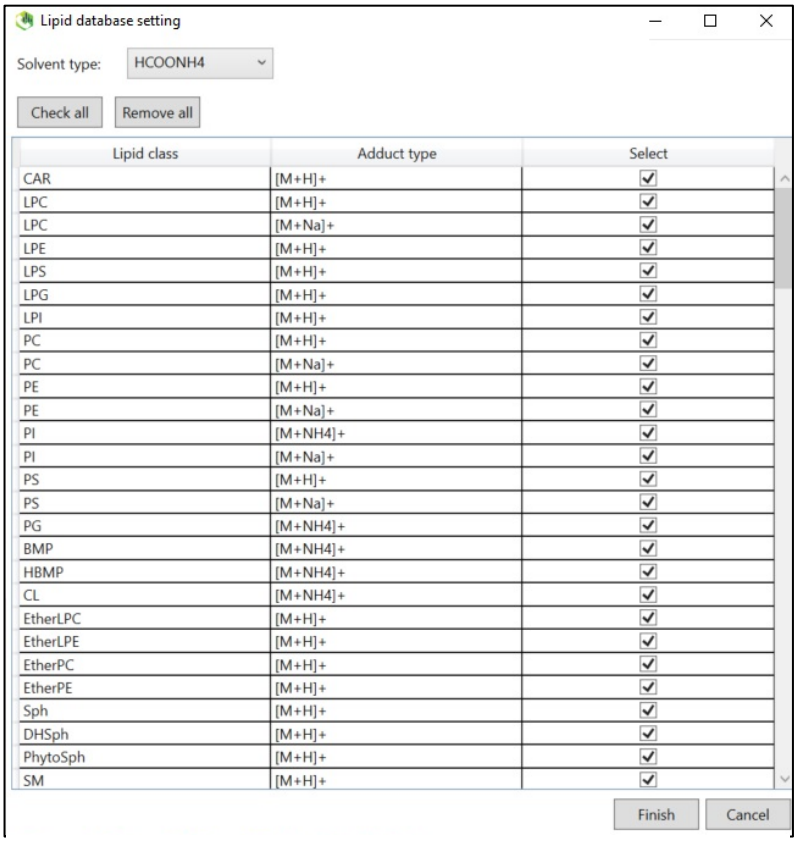

Figure 8. Lipid database

- The "Matched spectrum percentage" and the "Minimum number of match spectrum" parameters did not significantly impact the results. Their settings were set to 0 and 1, respectively.

From the optimization process for identification, the results suggest that lipids with a "match score" lower than 1.8 need increased scrutiny since those "identified" lipids are more likely to be categorized as a false positive or as an unidentified but detected lipid species, which are generally annotated "RIKEN P-VS1 ID…" MS-DIAL software detects these features but does not identify them, and they are color-coded as grey, with their ontology labeled "unknown." In contrast, lipids identified with a "match score" higher than 2.0 were accepted with better confidence. Consequently, annotation cut-off scores were optimized by adjusting parameters to minimize the number of annotations with a match score lower than 1.8 and maximize the number of lipids identified with a match score greater than 2.0. EAD-derived data generated "match scores" above 3.0 for some lipids, which is not possible using CID-based fragmentation. A match score above 3.0 reflects the potential of nearly complete lipid characterization, including specifying the *sn*-1/*sn*-2 acyl chain positions and the localization of double bonds within the acyl chains. The stereochemistry of the fatty acid double bonds is not characterized in MS-DIAL software, but it can be determined from EAD data as described (16).

Alignment parameters: For the "Reference file," choose the most representative sample, a QC, or the most concentrated sample to drive the data alignment. Click "Advanced," as shown in **Figure 7**, and choose the blank filtering option. There are a few options available in this section. For instance, any feature in the blank sample with an intensity of more than 33% of the sample with the highest intensity of this feature will be removed if the user clicks the "blank filter" option during data review after the processing is done. This feature significantly improves the class separations during visual statistical analysis such as PCA and heatmap analyses. For detailed information regarding this feature, users can refer to Section 2-3-6 in the MS-DIAL tutorial [MS-DIAL tutorial | mtbinfo.github.io]

Lipid database: The lipid database settings should be set based on the modifiers present in the LC mobile phases. Ammonium formate and ammonium acetate are the most commonly used modifiers to generate ammonium adducts of neutral lipids in the positive ion mode and formate or acetate adducts of some phospholipids in the negative ion mode. If the DG lipids are selected in the identification tab (configure lipid class) with Na^+ adduct, many false positive DGs are matched with the low "match score" at the wrong retention times. Unclicking the DG with Na+ adduct from the identification settings is recommended.

Amplitude: This parameter is the minimum ion count (height) to be considered for analysis; it filters out the smaller peaks. The smaller you set the threshold, the more hits you will have; however, there will also be more low-confident features detected. However, it is important to keep it around 100 if your lipids of interest are in low abundance.

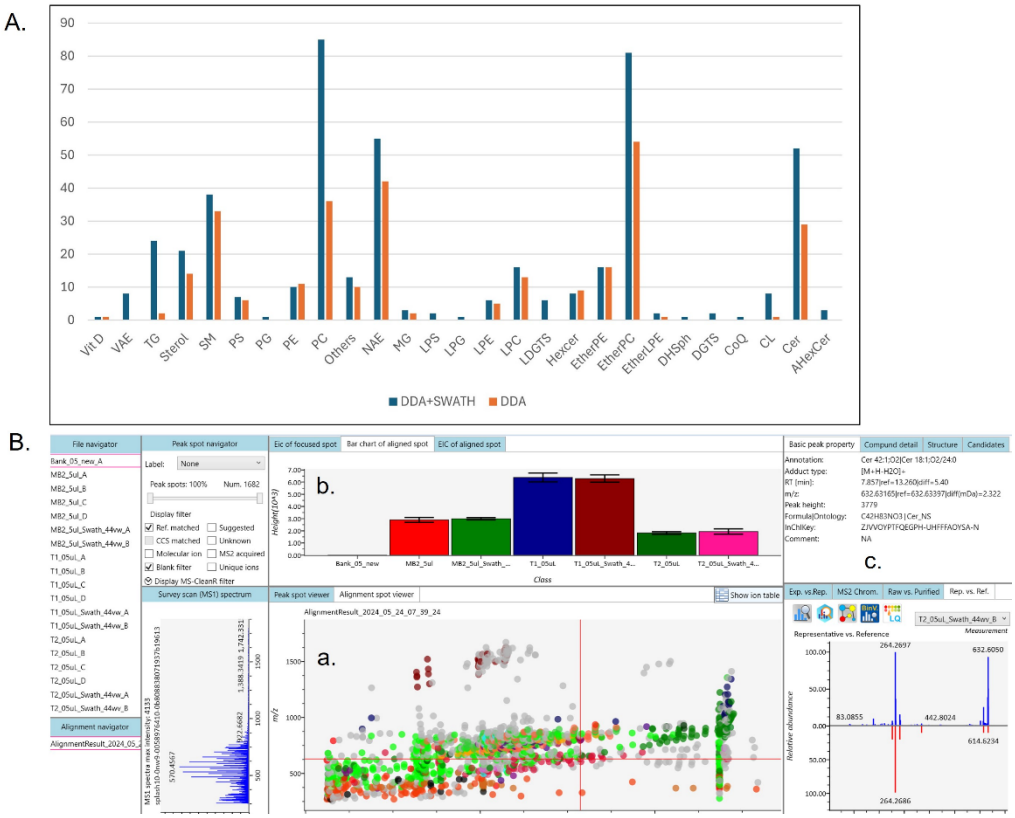

Figure 9. A. Lipids identified using MS-DIAL 5.2 software to process DDA data (Orange) or DDA and SWATH data (acquired separately but processed together). Data indicate improved lipid identification when data from both scan types are combined for processing. **B**. Example of MS-DIAL user interface showing (**a**) coverage, (**b**) quantitative reproducibility, and (**c**) library matching.

MS-DIAL Results from DDA and SWATH data processing

Eight samples for DDA, DDA+SWATH and EAD analyses were processed for this study to compare the method outcomes. When DDA and DDA+SWATH are compared, significantly more lipids are detected with the combined DDA+SWATH analysis (**Figure 9**). Comparing the number of lipids that passed the blank filtering with a "match score" above 2.0 resulted in 92 more lipids identified with the DDA+SWATH hybrid acquisition method. As mentioned above, if DGs with Na+ adduct are selected during data processing, MS-DIAL generates many false positive DGs, as evidenced by their incorrect retention times, due to the accurate mass similarity of some features. To avoid this problem, sodium adducts were not considered. Consequently, the number of annotated lipids with a "match score" of 1.8 significantly decreased, and the annotation numbers over a "match score" of 2.0 remained the same. Lastly, data acquired using EAD-based fragmentation enabled the identification of 20 lipids with a "match score" of 3. Most of these lipids were identified with near complete structural conformation with a better "match score" fitting than lipids identified using CID-based fragmentation.

SWATH and DDA experiments can be performed in either the positive or negative ion mode. CID-based analysis should be done in the negative ion mode to identify phospholipids at the fatty acid level by generating fragments diagnostic of the esterified fatty acids. **Figure 10** shows the match of experimental data acquired in the negative ion mode (blue peaks) to the library spectrum when processed using MS-DIAL 5.2 software. Because the library spectra were created *in silico*, the peak intensities will not necessarily match up, but the presence of the fragments is typically sufficient to identify lipids.

Figure 11 compares the quality of DDA vs. SWATH data acquired in the positive ion mode. There is a perception that SWATH data has more background noise than DDA, which can affect library matching. As shown, data acquired by SWATH analysis are comparable to those acquired by DDA using the same sample and HPLC ESI-MS/MS hardware configuration. The spectra on the right and left panels are shown with an expanded y-axis in the center panel that confirms similar background noise levels. The inability to use SWATH as the sole means of analyzing lipids, which is possible with DDA experiments, is the limitation of the SWATH window size (currently a minimum of 3 Da). The extensive isobaric overlap among lipids makes it imperative to have unit resolution in the Q1 dimension of analysis. This enables greater specificity at the precursor ion level and helps to mitigate false positive identification. Here, the SWATH data were acquired independently of the DDA data. However, the combined processing of these data improves the overall results while maintaining a lower false positive rate than would result with SWATH data alone. Overall, the data show that MS-DIAL software can process both SWATH and DDA data, but the combination enhances the coverage and the confidence in identification.

Near-complete structural characterization of lipids using EAD-based fragmentation

A significant challenge in lipidomics has been the complete structural characterization of lipids from untargeted data. This is likely a

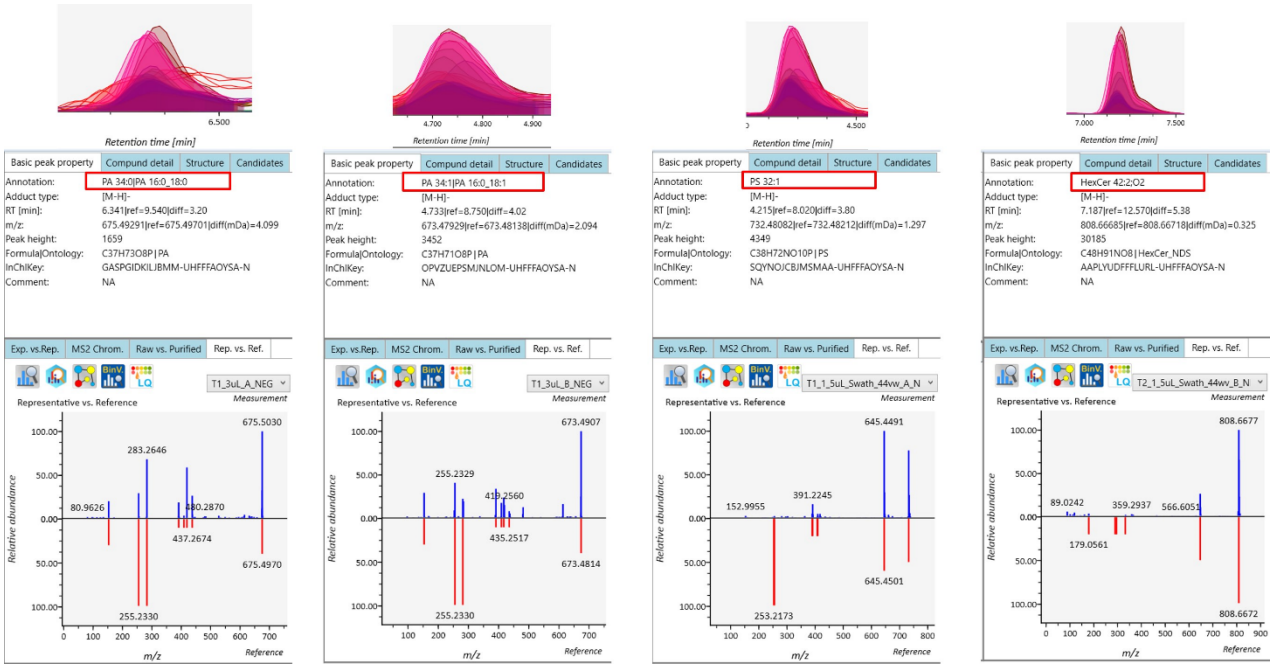

Figure 10. MS-DIAL 5.2 analysis of SWATH and DDA data acquired in the negative ion mode. 4 lipid molecular species across 3 class are shown. The matching results for each compound show a strong correlation between the experimentally derived spectra (blue) with the *in* silico-generated library spectra (red). The small graphs atop the matching results for each compound show the retention time alignment among all injections.

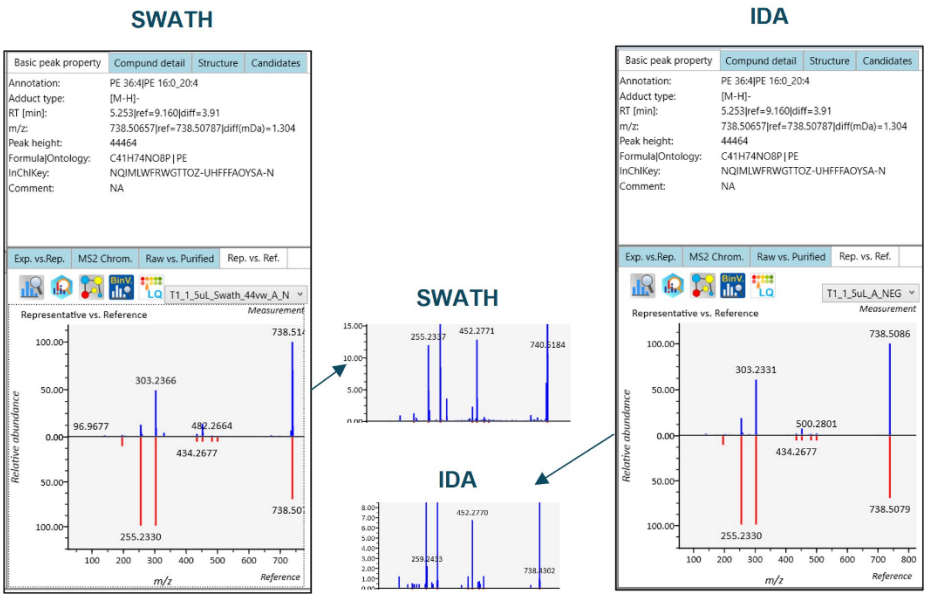

Figure 11. Comparison of MS/MS data acquired by SWATH and DDA scan modes. The MS/MS spectrum for CER 18:1/24:0 using a DDA (labeled IDA; right) experiment (blue peaks) shows excellent matching with the library spectrum (red peaks). When compared to the SWATH data (left), a similar correlation is obtained. Notably, there is very little noise in the SWATH-acquired spectrum, which enables a strong match.

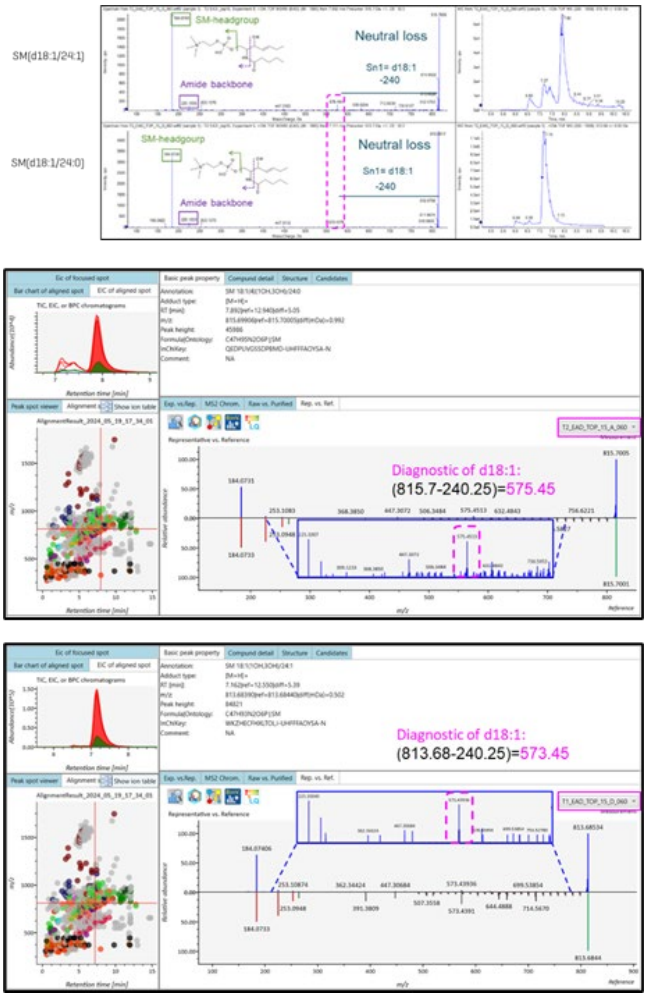

Figure 12. Structural characterization of sphingomyelin molecular species by MS-DIAL 5.2 software. SM(d18:1/24:0) and SM(d18:1/24:0) were identified from DDA data acquired using EAD-based fragmentation. **Top panel**: The sphingoid base-specific fragment at *m/z* 225 identified these molecules as sphingomyelins. **Middle and bottom panels:** Diagnostic fragments identifying the sphingoid backbone as d18:1.

significant reason for the palpable shift toward targeted lipid analysis.

A significant challenge in lipidomics has been the complete structural characterization of lipids from untargeted data. This is likely a significant reason for the palpable shift toward targeted lipid analysis

in lipidomics. Shotgun lipidomics, the precursor to DDA and SWATH analysis, can only identify lipids at the sum composition level, which is insufficient for adequate metabolic studies. Advances in mass spectrometry technology, methodology, and software have enabled the evolution of analysis to where it is today. However, the dearth of structurally diagnostic fragment ions generated by CID-based fragmentation has still limited the field. EAD shows promise as a fragmentation technique to generate sufficient structural information to completely characterize lipids analyzed by discovery workflows such as DDA and SWATH [11]. **Figure 1** shows the MS-DIAL results from EAD-based DDA data derived from pericyte lipid extracts. In this example, PC 16:0/18:1(9) was identified using the features highlighted in the figure. The lipid class is confirmed by a doublet of peaks at *m/z* 224 and 226 (red), which are diagnostic for the PC class of lipids. Peaks for palmitic acid (16:0) are detected (green), as is the diagnostic peak for its position at the *sn*-1 carbon of the glycerol backbone (orange). The double bond position within oleic acid (18:1) is identified from the information highlighted in brown.

Figure 12 shows a similar information set that identifies two sphingomyelin molecular species: SM(d18:1/24:0) and SM(d18:1/24:1). The sphingoid base-diagnostic ion m/z 225 is present in both spectra, which enables distinction from the PC class of lipids. More in-depth characterization of the sphingoid backbone is shown in the middle and bottom panels, as indicated. This level of structural characterization from data acquired in a single DDA experiment is unprecedented. Previous reports have shown structural characterization of lipids [8-11]; however, the EAD-based MS/MS spectra were processed manually. Here, MS-DIAL 5.2 software was used to interpret the EAD-based data in an automated fashion.

The evolution of technology has driven the growth of lipidomics research. From the humble beginnings of thin-layer chromatography and GC analysis to the current tools of mass spectrometry and alternative means of molecular fragmentation, lipidomics is an important field in understanding human biology. As the technology grows, so does the volume of data. A simple software workflow is presented here that enables the automation of lipidomics data interpretation. MS-DIAL 5.2 software can process all types of data acquired using SCIEX instrumentation, and novel software features can leverage the unique information generated by different workflows, such as SWATH and DDA, and unique fragmentation modalities.

Conclusions

1. **Automated processing of DDA data requires software parameter optimization. The information herein provides a starting point to use MS-DIAL 5.2 software to interpret discovery lipidomics data acquired on the ZenoTOF 7600 system**
2. **Presented here is a proof-of-concept framework for future studies to understand the role of lipids in the function of the blood-brain barrier and its role in health and disease**
3. **The combined processing of DDA and SWATH data provides deeper coverage of the pericyte lipidome**
4. **MS-DIAL 5.2 software can process discovery data generated using EAD-based fragmentation on the ZenoTOF 7600 system and provide automated, near-complete lipid structural characterization**

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