Data processing workflow using MS-DIAL software for untargeted lipidomics data acquired on the ZenoTOF 7600 system

Step-by-step guidelines to use MS-DIAL software, version 5.1 to process discovery lipidomics data

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This technical note demonstrates a robust workflow using MS-DIAL software to process untargeted lipidomics data acquired on the SCIEX ZenoTOF 7600 system. This step-wise guide includes specific software settings to provide the best results using MS-DIAL software. The recent development of electron-activated dissociation (EAD)[1] as a complimentary fragmentation mechanism on the ZenoTOF 7600 system adds an extra dimension to lipid identification that has not been addressed by untargeted lipidomics data processing software. Here, untargeted lipid data were processed using MS-DIAL software and a novel EAD fragmentation-based lipid library was used to identify the unknown lipid species.

Untargeted lipidomics data were collected from *Arabidopsis thaliana* using the SCIEX ZenoTOF 7600 system via data-dependent acquisition (DDA). Using this workflow, 2474 lipid molecular species were characterized out of 3448 spectral matches (Figure 1). MS/MS data confirmed these species, and 2356 additional unknown features were also detected in the plant leaf tissues.

Key features of untargeted lipidomics analysis using the ZenoTOF 7600 system

- The ZenoTOF 7600 system equipped with EAD and the Zeno trap requires specific instrument parameter settings for untargeted lipidomics
- This untargeted lipidomics method leverages the speed and sensitivity of the ZenoTOF 7600 system to maximize throughput while maintaining spectral quality
- MS-DIAL software enables lipidomics data interpretation using either CID- or EAD-based fragmentation

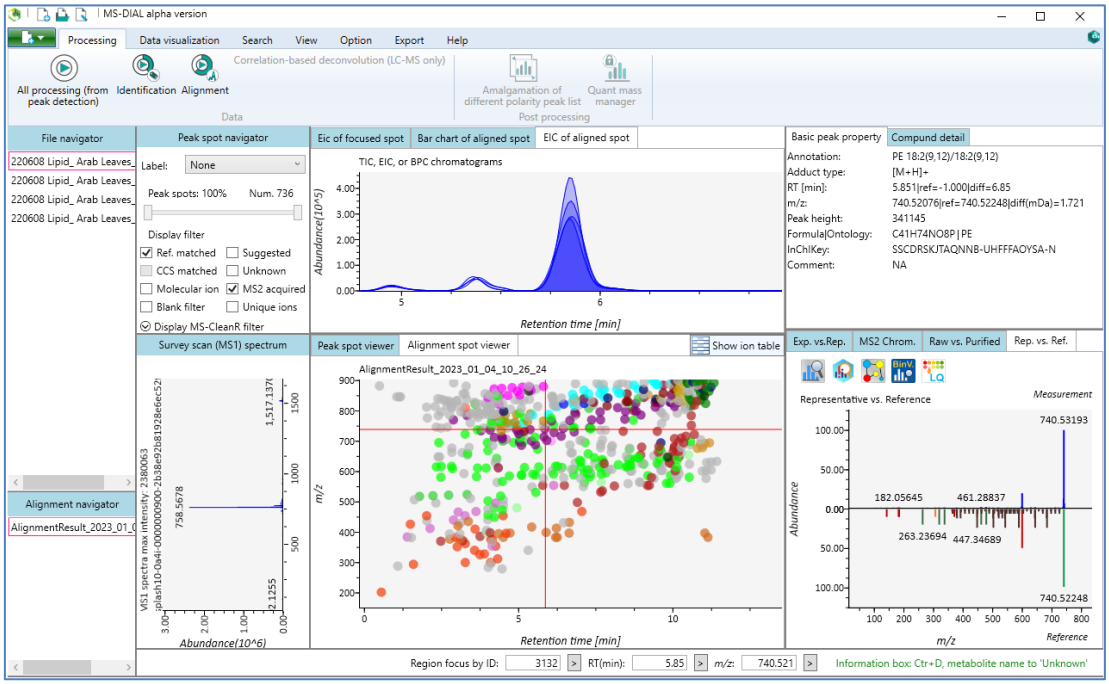

Figure 1. Data processing in MS-DIAL software to identify lipids in plant leaf extract using DDA scan mode. The speed and sensitivity of the ZenoTOF 7600 system enable high-throughput analysis while maintaining high MS/MS spectral quality.

Methods

Sample preparation: *Arabidopsis thaliana* seeds were placed on 42 mm Jiffy-7 pellets (Garden City Plastics, Australia) and vernalized at 4°C for 3 days. Following cold treatment, trays were placed in a growth chamber under a 16 h light/8 h dark regime at 22°C and 50% relative humidity with a daytime light intensity of 100-120 microeinsteins (µE) at the plant level. The rosettes were harvested at 14 and 24 days, submerged in liquid nitrogen and stored at -80°C until lipid extraction.

Lipid extraction: The plant material was homogenized by cryo-milling (Precellys 24, Bertin Technologies) with 400 µL of 2-propanol containing 0.01% butylated hydroxy toluene (BHT). Cryo-milling was performed at 6100 rpm and 10°C for 2 45-sec intervals interleaved with a 30-sec pause. Next, samples were incubated at 75°C for 15 min under constant shaking and then centrifuged at 15,700 x g for 10 min. Samples were then cooled to room temperature and 1.2 mL of a mixture containing 30 parts chloroform, 41.5 parts methanol and 3.5 parts water by volume was added to each sample. The samples were incubated at 25°C for 24 h under constant shaking and centrifuged at 300 x g for 10 min. Finally, the supernatant was separated and dried in a vacuum concentrator. The dried lipid extracts were re-suspended in 200 µL of 1:1 (v/v), butanol/methanol with 10mM ammonium acetate and subjected to LC-MS analysis, as reported.[2,3]

Chromatography: Prepared lipid extracts were placed in the autosampler set to 12°C and 5 µL of each was injected onto a Phenomenex Kinetex C18 column (2.6 µm, 100 Å, 100 x 2.1 mm) held at 55°C. Separation was performed using a SCIEX ExionLC system. Elution was performed using the gradient and solvents described in Table 1. The total runtime was 15 min.

Mass spectrometry:

Lipids were analyzed using a ZenoTOF 7600 system with a DuoSpray Turbo V ion source and an electrospray ionization (ESI) probe. The automated calibrant delivery system (CDS) calibrated the instrument every 5 samples using ESI calibration solution. Analysis was performed using a DDA scan (shown as IDA in the software) in the positive ion mode. The maximum candidate ions parameter was set to top 10 and dynamic background subtraction (DBS) was applied with a mass tolerance of 50 mDa. A TOF MS accumulation time of 250 ms and a collision energy (CE) of 10 V with a 50 ms accumulation time were used for TOF MS/MS acquisitions. Other instrument parameter settings used are shown in Table 2.

Data processing: All data were analyzed using MS-DIAL software, version 5.1 with a lipid library containing CID- and EAD-based MS/MS fragments to improve the identification of lipid molecular species.[4,5]

Table 1. LC gradient. The flow rate was 0.4 mL/min.

Time (min)	%B
0	*10*
2.7	*45*
2.8	*53*
9.0	*65*
9.1	*89*
11.0	*92*
11.1	*100*
11.9	*100*
12.0	*10*
15.0	*10*

Mobile phase A: 20% isopropanol and 30% acetonitrile with 1.0mM sodium acetate in water
Mobile phase B: 90% isopropanol and 9% acetonitrile with 10mM ammonium acetate in water

Table 2. Instrument parameter settings.

Parameter	Setting
Source temperature	*250°C*
Curtain gas	*35 psi*
Gas 1	*25 psi*
Gas 2	*25 psi*
Declustering potential	*80 V*
Ionization voltage	*5500 V*
TOF MS	*100-1600 m/z*
TOF MS/MS	*100-1200 m/z*
Fragment mode	*EAD*
Electron KE (eV)	*10*

MS-DIAL data processing guidelines

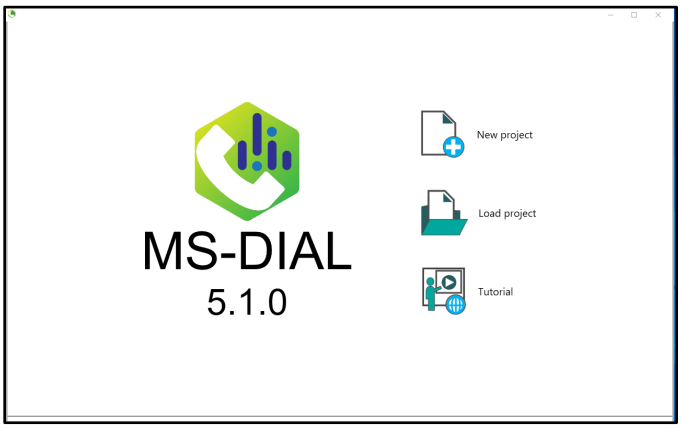

Figure 2 MS-DIAL data processing set up. *The MS-DIAL interface to set up projects for processing LCMS data*

Starting a project: MS-DIAL software, version 5.1, is an open-source, free software to process untargeted metabolomics and lipidomics data from mass spectrometry. The free software can be downloaded, as detailed on the MS-DIAL software website: CompMS | MS-DIAL (riken.jp). Once the software is installed, a new project can be created by clicking "New project," as shown in Figure 2. Then, raw data files (*.wiff and *.wiffscan) can be selected, and the project will be managed as a *.MTD file. Select "Wiff 2 files" to process and open the raw data files without converting them to another format. Each step is guided and navigated with the "next" button.

Measurement parameter settings: To set up measurement parameters for LC/MS data processing, select "soft ionization" and set the separation type to "chromatography," as shown in Figure 3. To process DDA data, set the MS method type to "conventional LC/MS or data-dependent MS/MS." To process SWATH data-independent acquisition (DIA) data, select "SWATH-MS or conventional All-ions method." To process EAD data acquired on the ZenoTOF 7600 system, set the collision type to EIEIO to enable the use of the library containing the *in-silico* EAD fragment database.

Data collection settings: To set up data collection, the MS1 and MS2 tolerances should be set to the default 0.01 Da and 0.0025 Da values, respectively. The retention time and mass range parameters can be updated in the advanced setup section, as indicated in Figure 4.

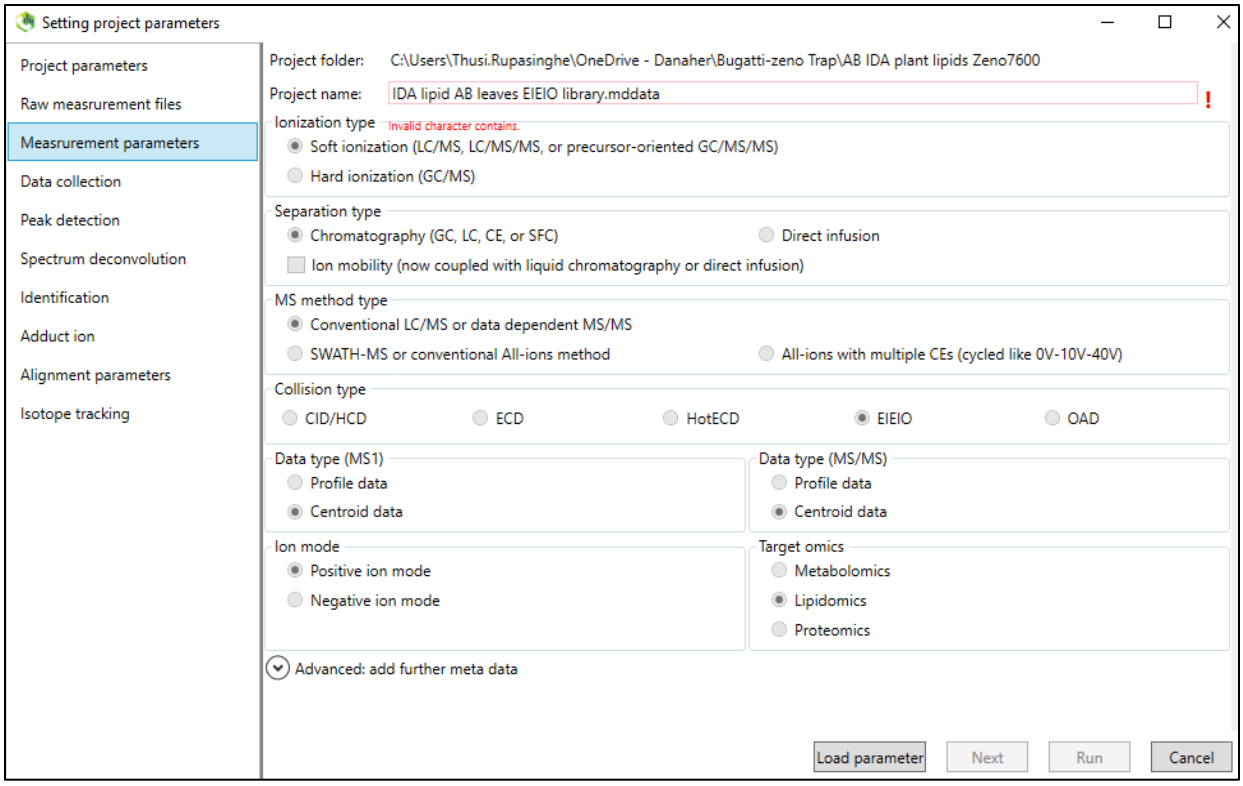

Figure 3. MS-DIAL software interface to set project parameters.

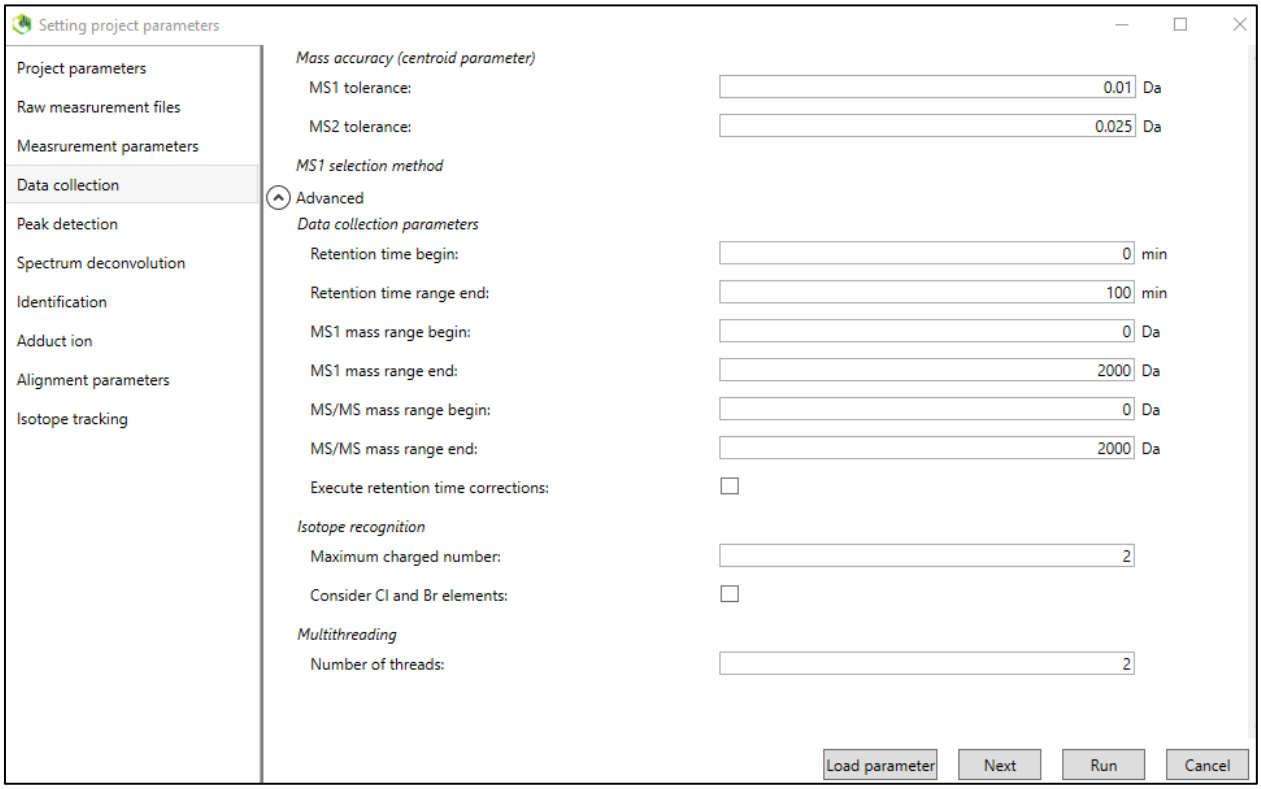

Figure 4. MS-DIAL software interface to set data collection parameters.

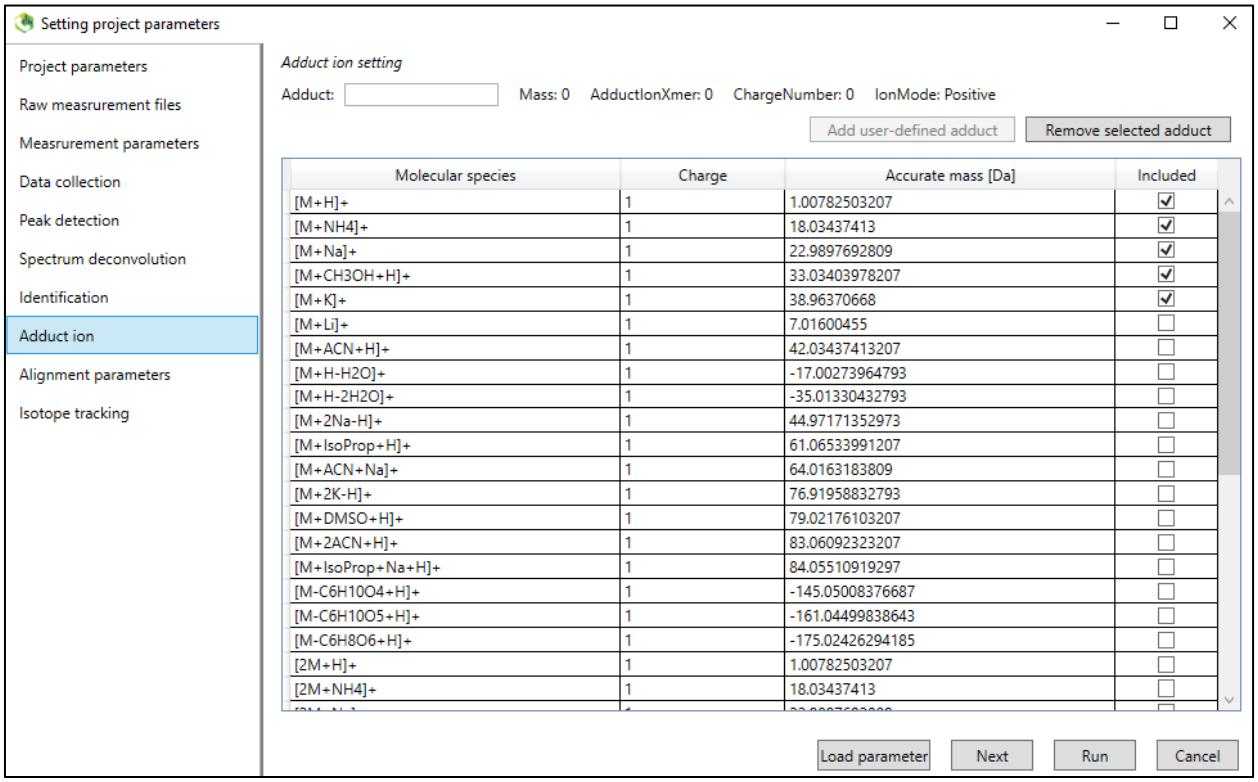

Figure 5. MS-DIAL software interface to select adduct ions. The MS-DIAL software interface for selecting possible adduct ions depends on the LC mobile phase used in the experiment.

Peak detection settings: In the peak detection window, set the minimum peak height to 500 amplitudes. This setting can be adjusted depending on the background noise level in the chromatogram.

Identification: Lipid species identification is performed using MSDIAL-TandemMassSpecralAtlas-V68 lipid-pos Msp. This database contains the EIEIO fragment library derived from EAD-based fragmentation and can enable near-complete structural characterization.[5] Multiple databases with different formats, such as "Msp," "Lbm," "Text," "Fasta," "Eieio Lipid," and "OadLipid," can be added.

Adduct ion and alignment parameters: The adduct ion is an important parameter to set in the data processing method, and it depends on the composition of the mobile phases used during data acquisition (Fig. 5). Retention time tolerances can be set to the default value of 0.05 Da, provided the LC gradient is highly reproducible. However, this tolerance should be changed to reflect the retention time variation in the data. The MS1 tolerance should be set to 0.015 Da.

Isotopic tracking settings: If deuterated analyte homologs are used as internal standards, they can be identified in the isotopic tracking settings window.

Once all parameters are set up for data processing, select "Run" to process the data. Once MS-DIAL software has processed the data, results can be exported using the "Export" and "Alignment results" functions. Users can select the type of raw data to export, such as peak height or peak area, as shown in Figure 6.

Lipidomic profiling results

Plant tissue lipid data collected using DDA in the positive mode were processed using MS-DIAL software, version 5.1. Lipids were annotated using the internal lipid database in MS-DIAL software with MS1 accurate mass tolerance set to 0.01 Da and MS2 exact mass tolerance set to 0.05 Da.[3,4] The lipids that had MS/MS spectral similarity to the reference spectra and that eluted at the predicted retention times were used to generate the final lipid profile (Table 3). A total of 2474 lipid molecular species were annotated using MS-DIAL software. These results are based on the reproducibility of 4 biological replicates with a %CV <30% with the correct RT for each lipid species identified. After

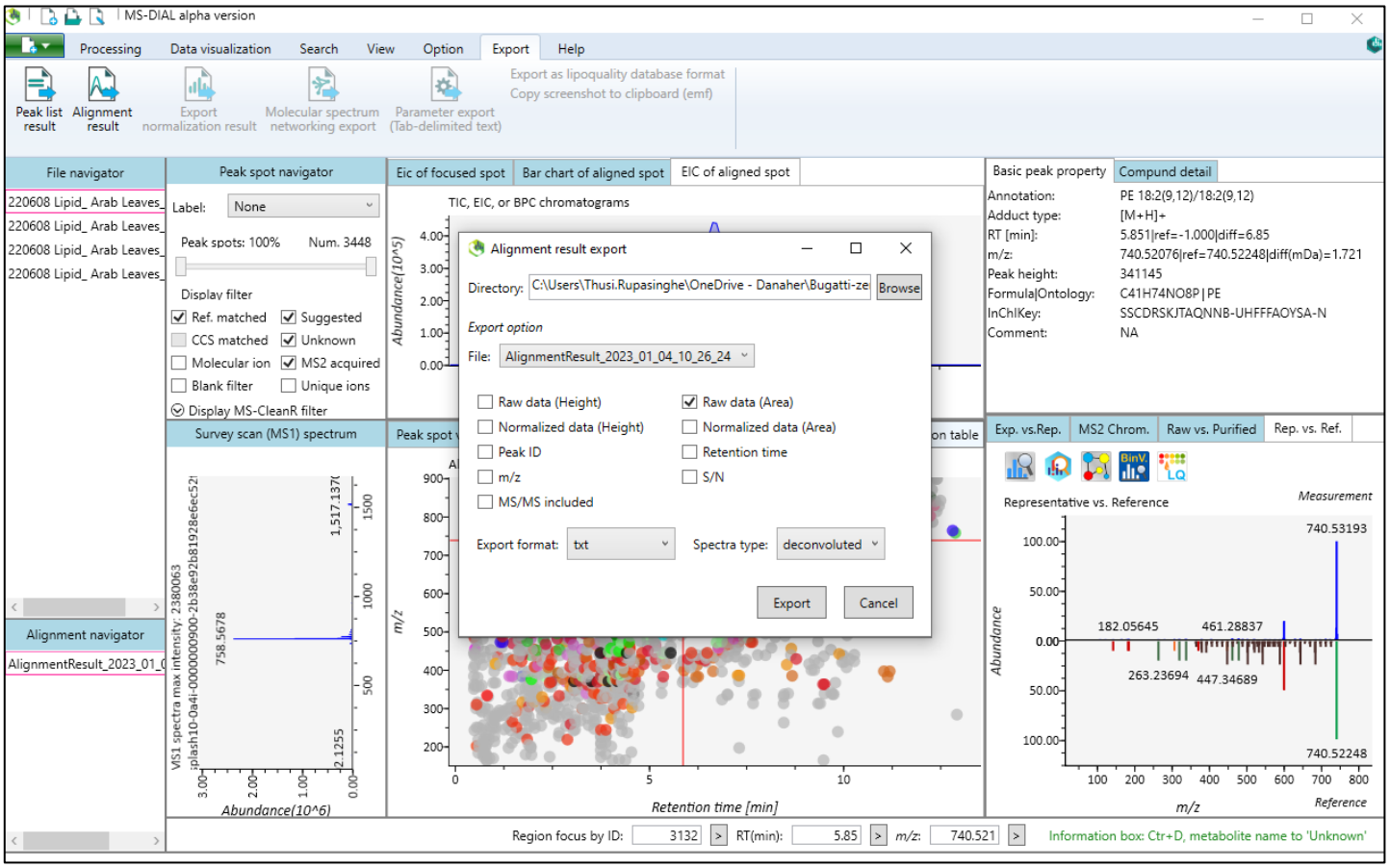

Figure 6. MS-DIAL software interface to align data export for further analysis. Lipid data can be exported in a variety of file formats, including *.txt.

Table 3. Lipid species were identified using DDA data from the MS-DIAL software database with EAD fragments. The analysis of DDA data by MS-DIAL software identified 927 unique lipids in the positive ion mode.

Lipid class	# of lipids identified (Reference matched)	# of lipids identified (Suggested)
Acylated sterol glucoside (ASG)	*10*	*21*
Ceramide (Cer)	*66*	*150*
Diacylglycerol (DG)	*40*	*35*
Triacylglycerol (TG)	*20*	*283*
LysoPC (LPC)	*4*	*6*
LysoPE (LPE)	*3*	*0*
Phosphatidylcholine (PC)	*3*	*40*
Phosphatidylethanolamine (PE)	*35*	*4*
Phosphatidylglycerol (PG)	*12*	*0*
Phosphatidylinositol (PI)	*14*	*2*
Phosphatidylserine (PS)	*19*	*0*
Digalactosyldiacylglycerol (DGDG)	*23*	*15*
Monogalactosyldiacylglycerol (MGDG)	*2*	*60*
Hex ceramides (HexCer)	*21*	*10*

the lipidomics data were post-processed, the number of uniquely identified lipid species across the different lipid classes was reduced to 927, covering 14 lipid classes out of 3448 features (Table 3). The graphical user interface in MS-DIAL software provides the number of peak spots identified using either reference matched or suggested, as shown in Figure 1.

Conclusions

- Source parameters, such as probe position, drying and nebulizing gases and the electrospray voltage, must be optimized for untargeted lipidomics experiments based on the flow rate and the sample matrix composition
- Parameter settings, such as collision energy and accumulation, must be adjusted to accommodate diverse lipids
- The ZenoTOF 7600 system is ideal for untargeted lipidomics due to its speed, sensitivity and the complementary EAD fragmentation method
- MS-DIAL software, version 5.1, can process both CID- and EAD-based fragmentation data
- EAD-based fragmentation data provides diagnostic information that enables near-complete structural characterization of lipid molecular species

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