Utilizing CID and EAD fragmentation for global lipid profiling

High-throughput, untargeted lipidomics using the ZenoTOF 7600 system

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This technical note describes the workflow to interpret untargeted lipidomics data using MS DIAL software. It also highlights the structural elucidation power of the unique fragmentation method, Electron-Activated Dissociation (EAD).The central role lipids play in inter- and intra-cellular communication has driven considerable interest in the use of lipidomics to uncover potential biomarkers of human disease. Electrospray ionization tandem mass spectrometry (ESI LC-MS/MS) is the primary technique used to analyze lipids and has driven powerful advances in this field. The instances in which the collision-induced dissociation (CID) method employed in ESI LC-MS/MS can identify lipid molecular species are limited, especially in untargeted lipidomics analyses. Lipids fragmented using CID do not generate sufficient diagnostic fragments to distinguish lipids among inter- and intra-class isobars and these data do not support full structural characterization. Consequently, lipids are typically identified broadly, rather than specifically (for example, sum composition instead of specific structural annotation).

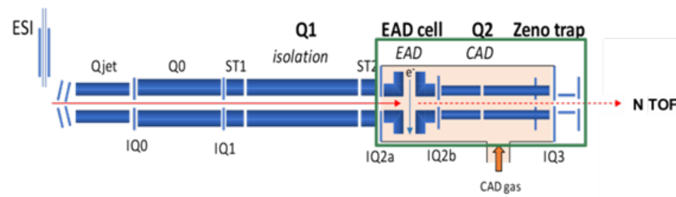

Figure 1. Ion path schematic for the ZenoTOF 7600 system.

Key features of the ZenoTOF 7600 system for lipid analysis

- EAD-based fragmentation provides diagnostic peaks that enable complete structural characterization of lipid molecular species
- The Zeno trap improves the instrument duty cycle to >90%, which improves the instrument sensitivity >10X. This feature improves spectral quality to improve matching and enables the detection of low-abundant lipid species.
- The ZenoTOF 7600 system can acquire CID data at 133 Hz and EAD data at ~30 Hz. These high acquisition speeds enable high-throughput, untargeted analysis of lipids.

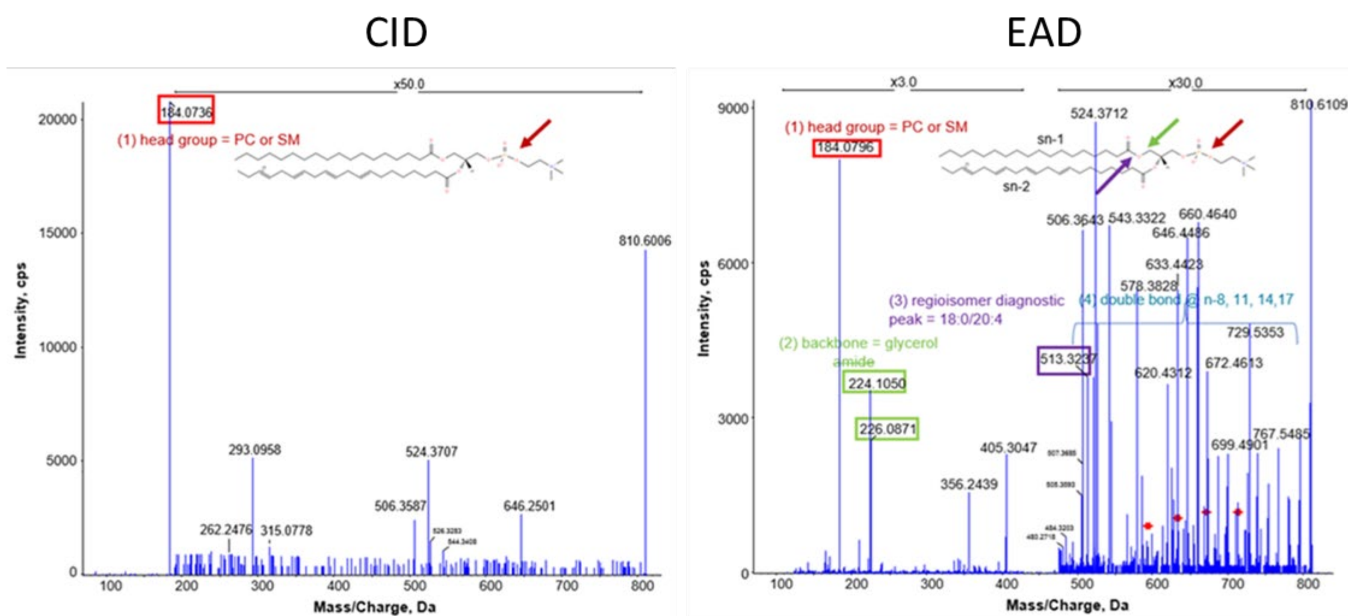

Figure 2. CID vs. EAD product ion spectra. Product ion spectra were obtained for m/z 810 in the positive ion mode. The CID spectrum (left) shows the prominent m/z 184 fragment, consistent with PC or SM. Smaller peaks show lyso PC species from which fatty acid composition can be determined. On the right, the EAD spectrum is full of diagnostic ions, including m/z 224 and 226 that confirm that the molecule is PC. Other ions show fatty acid composition, positional isomers, double bond positions and their respective stereochemistry, as indicated in the figure.

The ZenoTOF 7600 system is capable of data-dependent workflows that leverage a hybrid collision cell that contains a Zeno trap and an electron activated dissociation (EAD) cell (Figure 1). The Zeno trap improves instrument duty cycle to >90% and the EAD cell provides complementary fragmentation to enable complete structural characterization of lipids[1-3]. Electron kinetic energies can be precisely tuned from 0-25 eV without chemical transfer reagents. Consequently, EAD can fragment singly charged ions in both positive and negative ion modes. The EAD cell contains a high density of electrons, allowing rapid reaction rates to keep up with fast chromatographic separations, which makes the ZenoTOF 7600 system ideal for high-throughput lipidomics.

EAD-based fragmentation provides a >15-fold increase in the number of fragment ions produced, compared to CID fragmentation. Many of the fragments produced by EAD are structurally diagnostic and therefore support the full structural characterization of a lipid molecular species. Here, the impact of the highly sensitive Zeno MS/MS on the detection of lipids from extracted plasma samples was explored using a single injection, data-dependent acquisition (DDA) workflow using CID- and EAD-based fragmentation methods. MS-DIAL software was used to identify lipids form the DDA data (Figure 2). Chromatographic separations were significantly accelerated and the impact on identification rates was evaluated.

Methods

Sample preparation: NIST 1950 human plasma and Sprague Dawley and Zucker rat plasmas were used. To volumes of plasma ranging from 1 to 20 µL, 100 µL of ice-cold chloroform was added and vortexed for 10 seconds. After 1 hour of incubation on ice, 200 µL of ice-cold methanol containing Lipidyzer internal standards was added and vortexed for 10 seconds. After 2 hours of incubation on ice, the solvent tube was centrifuged at 2000 x g for 10 minutes at 4°C. Then, 100 µL of the supernatant was transferred for analysis.

HPLC conditions: LC separation was performed on a Phenomenex Luna Omega Polar C18, 100 x 2.1 mm column. The gradient separation used is described in Table 1. The flow rate was maintained at 0.4 mL/min for all the gradients used in the study. A 3 µL injection volume was used.

MS/MS conditions: A ZenoTOF 7600 system with a Turbo V ion source and electrospray ionization (ESI) was used. The samples were analyzed in data-dependent mode, using DDA with CID or EAD, with and without Zeno trap activation. The TOF MS scan was performed between m/z 70–1750. A top 45 method, in which the MS/MS was scanned between m/z 70–1250, was used.

Data processing: All data were processed using MS-DIAL software[4], version 4.8. Lipid structures and MS/MS annotations were achieved with 3 levels of confirmation: reference-matched, suggested and unknown. For reference-matched lipid annotation, peaks were matched with reference libraries of MS/MS spectra curated from authenticated lipid standards. Suggested lipids were annotated without MS/MS spectra. The unknown features were not further identified (Figure 3).

Results

The impact of different gradient lengths on the number of putatively identified lipid species in a 20 µL sample of extracted plasma was investigated. LC gradients with runtimes of 25, 19, 15.5, 12.5 and 10.5 minutes were tested. Based on the 25-minute runtime, the 4 shortened gradients had the same starting mobile phase composition and maintained a gradient proportional to the total runtime.

The Zeno CID DDA analysis in positive ion mode indicated that less than 3% of annotations were lost when the gradient was shortened from 25 minutes to 12.5 minutes. Further, only 12% of annotations were lost when the gradient was shortened to 10.5 minutes (Table 1). In negative ion mode, the annotations improved by up to 13% with a faster gradient of 12.5 minutes (Table 2). From these results, the optimal gradient for positive ion mode analysis was 15.5 minutes when comparing reference-matched lipids. For negative ion mode, the best LC gradient had a 12.5-minute runtime. Note that the DDA criteria and MS/MS accumulation times were not optimized for each gradient length.

Table 1. The number of lipid species putatively identified in positive ion mode using CID DDA with the Zeno trap enabled.

Total runtime (min)	Reference-matched	Suggested	Unknowns	Total features
25	*478*	*955*	*4461*	*5894*
19	*484*	*832*	*4244*	*5560*
15.5	*475*	*892*	*4391*	*5758*
12.5	*433*	*915*	*4595*	*5943*
10.5	*406*	*909*	*4562*	*5877*

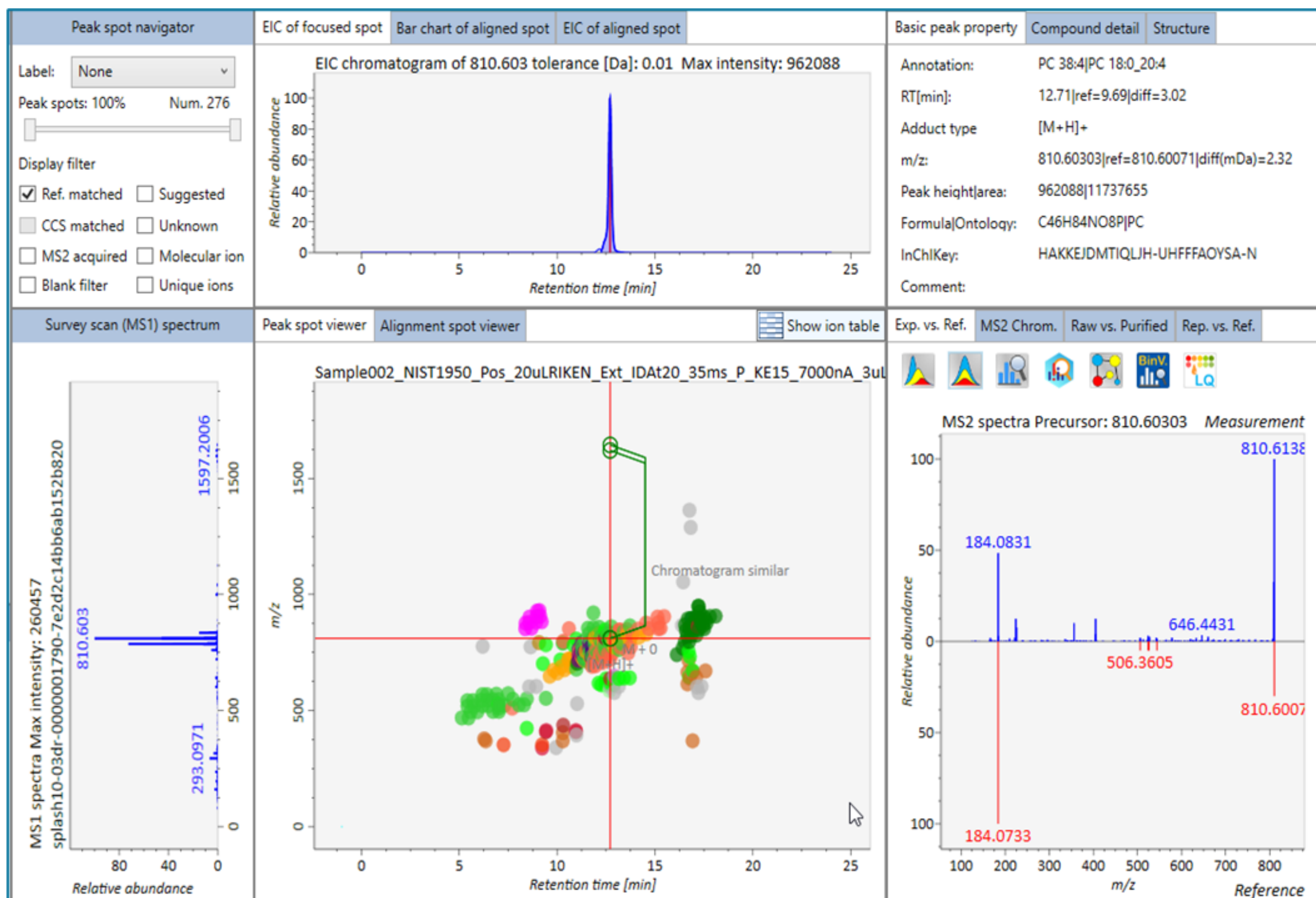

Figure 3. Sample data output from MS-DIAL software, version 4.8. In this example, the EAD and CID MS/MS spectra of m/z 810 were compared to the spectral library in MS-DIAL software, version 4.8. The data strongly support the identification of this molecule as PC 18:0/20:4 (Z8,Z11,Z14,Z17).

Table 2. The number of lipid species putatively identified in negative ion mode using CID DDA with the Zeno trap enabled.

Total runtime (min)	Reference-matched	Suggested	Unknowns	Total features
25	255	1195	5802	7252
19	260	1143	5427	6830
15.5	269	1128	5413	6810
12.5	265	1368	5980	7613
10.5	276	1292	6190	7758

The Zeno trap performance was evaluated using various plasma loads in both positive and negative ion modes by toggling the Zeno trap on and off during lipid analysis. A 25-minute gradient was used for both positive and negative ion mode experiments. The Zeno trap significantly increased MS/MS sensitivity and spectral quality, which resulted in better detection of lower-level fragment ions in the spectra. The putative identifications of lipids in plasma samples are shown in Tables 3 and 4 for positive and negative ion modes, respectively.

When the Zeno trap was enabled, there was a significant increase in putative identifications in both positive and negative ion modes. When the Zeno trap was turned on, 30% more annotations were achieved in positive ion mode than when the Zeno trap was turned off. A 92% annotation gain was obtained in negative ion mode at 20 µL. When 10 µL of plasma was loaded for analysis in negative ion mode, a 140% increase in annotations was achieved. Overall, using the Zeno trap in positive ion mode resulted in an average 33% increase in annotations. In comparison, its use in negative ion mode resulted in an average gain of 113%.

The analytical reproducibility was established across the 3 lowest plasma loads tested: 2, 3 and 5 µL. Each plasma volume was extracted in duplicate and injected with triplicate technical replicates. More than 500 lipids were annotated for a 20 µL injection of the NIST 1950 human plasma. From these annotations, a subset of high-, mid- and low-abundant lipid species were selected for precision assessment. These included PC 34:2, SM 32:1;2O, LPE 20:4, Hex2Cer 34:1;2O and Cer

42:1;2O, with abundances ranging from 1.4e3 to 1.5e6 cps (Table 5).

The raw peak areas of these species were analyzed without internal standard normalization (Table 5). Across 3 plasma volumes for the high-abundant PC 34:2, the %CV was less than 2% for 6 injections, as expected. Maintaining low %CV values for low-abundant species is often challenging. For the 3 lowest abundant species, LPE 20:4, Hex2Cer 34:1;2O and Cer 42:1;2O, the %CV of peak area across all plasma volumes was less than 6%.

At the lowest extracted plasma volume, the %CV values were slightly greater than 5% for the low-abundant species, with

Table 3. The number of lipid species putatively identified in positive ion mode using a 25-minute gradient.

Plasma load (µL)	Zeno trap on/off	Putative lipid annotations*	% Gain with the Zeno trap enabled
3	On	278	40
3	Off	198	--
5	On	338	30
5	Off	260	--
10	On	413	31
10	Off	316	--
20	On	515	31
20	Off	392	--

**Putative lipid annotations are reported only for reference-matched annotations*

Table 4. The number of lipid species putatively identified in negative ion mode using a 25-minute gradient.

Plasma load (µL)	Zeno trap on/off	Putative lipid annotations*	% Gain with the Zeno trap enabled
3	On	117	100
3	Off	49	--
5	On	135	121
5	Off	61	--
10	On	161	140
10	Off	67	--
20	On	223	92
20	Off	116	--

**Putative lipid annotations are reported only for reference-matched annotations*

Table 5. Raw peak area precision at low plasma loads using a 25-minute gradient in positive ion mode.

Extracted plasma volume (µL)	LPE 20:4 (%CV)	Hex2Cer 34:1;2 (%CV)	Cer 42:1;2 (%CV)	SM 32:1;2 (%CV)	PC 34:2 (%CV)
2	5.68	5.13	5.66	2.72	1.57
3	3.6	3.39	3.00	0.49	1.21
5	1.33	0.53	7.64	0.73	1.37

average areas of approximately 1.3e3 cps (data not shown). For the 5 µL extraction plasma volume, the %CV value for each lipid species was less than 1.50%, except Cer 42:1;2, which had a %CV value of 7.6%.

Using the optimized gradient conditions from the previous experiments, data acquisition was repeated on the plasma samples using the Zeno EAD DDA workflow. Global analysis of these data files will be used to characterize the impact of using CID vs. EAD fragmentation for untargeted lipidomics. A preliminary manual exploration of the EAD data from selected lipids was performed and revealed that the EAD data contained richer MS/MS information compared to the complementary CID spectrum. Figure 1 compares the CID and EAD spectra of the PC 38:4 species, analyzed in positive ion mode.

Whereas the information gathered from the CID spectrum could narrow the identification of the lipid species (PC or SM), the information collected from the EAD spectrum was used to confidently identify the specific lipid molecular species. The EAD spectrum (Figure 1, right) contained the fragment ions m/z 224.10 and 226.08, which confirmed the PC species. Other ions in the spectrum enabled the determination of the *sn*-position to determine the 18:0/20:4 structure and identify double bonds at 8Z, 11Z, 14Z and 17Z.

Conclusions

- Increased MS/MS sensitivity with Zeno trap activation significantly improved lipid detection and enabled accelerated method runtimes
- Zeno DDA with EAD is a complementary workflow to CID that can be used when higher quality fragmentation data are needed for complete structural identification of the lipid molecular species
- MS-DIAL software, version 4.8 is an effective tool to help identify lipids in untargeted DDA experiments using either CID or EAD MS/MS data

References

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