Systematic determination of lipid structure using electron activated dissociation (EAD)

Characterizing phosphatidylcholine and sphingomyelin lipids using the ZenoTOF 7600 system

Chen Jinmei, Si Dandan, Long Zhimin and Guo Lihai
SCIEX, China

Lipids are one class of important nutrients needed by the human body, as they provide a supply of energy, essential fats and building blocks for cells. The chemical structure of a lipid impacts its biological function. Therefore, detailed structural characterization is necessary to determine the molecular mechanisms by which lipids act in basic biology and disease. This characterization is challenging and often requires many different tools to fully determine the structure.[1] Mass spectrometry (MS) is often used to analyze lipids, however, this approach typically requires complex and lengthy chromatographic separation to fully separate lipid isomers and multiple experiments to yield interpretable structural information. Moreover, the collision-induced dissociation (CID) approach typically used in MS analysis fails to generate sufficient diagnostic fragments to reliably characterize or identify lipids.

The electron activation dissociation (EAD) technology of the ZenoTOF 7600 system can generate unique fragments that can be used to properly identify lipid compounds.[2] This fragmentation

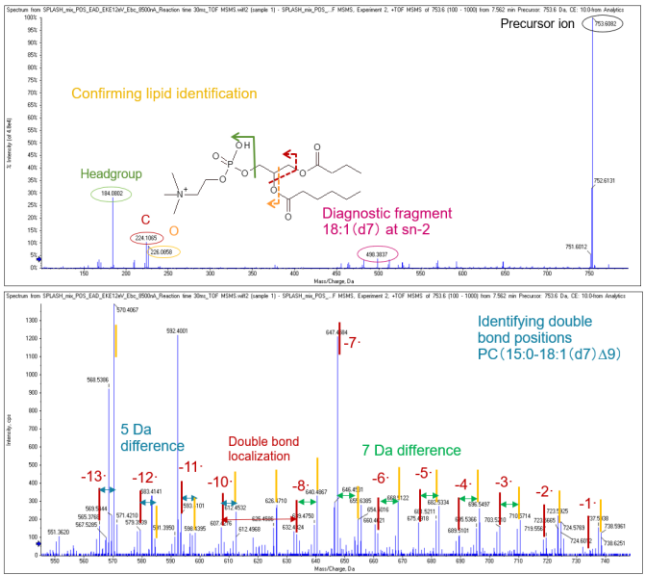

Figure 1. Complete characterization of PC(15:0/18:1(d7)Δ9) using EAD. (Top) Diagnostic fragments to determine headgroup, backbone and localization of acyl chains in EAD MS/MS spectrum. (Bottom) Zoom of higher m/z region of spectrum reveals the position of the double bond on the deuterated standard. Yellow lines indicate the 15:0 fatty acid while the red represent 18:1(d7)Δ9.

mode can provide additional diagnostic fragments to determine lipid class, backbone type and regioisomerism. At the same time, EAD can break acyl chains at each carbon along the fatty acid to determine carbon length and double bond(s) position. Here, EAD using high kinetic energy was used to provide in-depth characterization of different lipid compounds.[3,4]

Key features of EAD and the ZenoTOF 7600 system for lipid analysis

- ZenoTOF 7600 system with both EAD fragmentation and Zeno trap for high sensitivity MS/MS enables in-depth characterization of lipid structure
- EAD produces fragmentation information on a lipid to enable determination of head group, backbone, acyl chain localization, fatty acid chain and double bond position information, in a single MS/MS experiment
- Tunable electron kinetic energy (KE) and electron beam current enable rich and high-quality secondary fragments
- EAD MS/MS enables differentiation between phosphatidylcholine (PC) and sphingomyelin (SM) and complete characterization

Methods

Sample preparation: Avanti SPLASH Lipidomix standards were diluted in mobile phase B (Table 1) and data was acquired on this neat standard injection. Rat plasma was extracted by mixing with isopropyl alcohol in a 1:3 ratio, vortexing and then centrifuging. Supernatant was removed then diluted in mobile phase B for analysis.

Chromatography: An ExionLC system with a Phenomenex Kinetex column (C18, 2.6 µm, 100x3.0 mm; P/N 00F-4462-Y0) was used to perform the separation. The total run time was 20 min. A 2.0 µL injection was used. Column temperature was held at 50°C. Table 1 shows the chromatographic gradient used.

Table 1. Chromatographic gradient.

Time (min)	Flow (µL/min)	B Conc (%)	B Curve
0.5	*300*	*25.0*	*0*
13	*300*	*98.0*	*0*
17	300	*98.0*	*0*
17.1	*300*	*25.0*	*0*
20	*300*	*25.0*	*0*

Mobile phase A: 1:1:1, water/acetonitrile/methanol with 5 mM ammonium acetate
Mobile phase B: Isopropanol with 5 mM ammonium acetate

Table 2. MS conditions for the ZenoTOF 7600 system.

Parameter	Value
CUR	*35*
CAD	*10 (medium)*
TEM	*550*
DP	*50*
ISV	*5500*
GS1	*55*
GS2	*60*
CID CE	*45±20*
EAD CE	*10*
KE	*10-12*
Electron beam current	*8500*

Mass spectrometry: Samples were analyzed on the ZenoTOF 7600 system using information dependent acquisition (IDA) in the positive ion mode. Table 2 lists the conditions for analysis, including EAD parameters.

Structural confirmation of phosphatidylcholine standard

CID is the most widely used dissociation when studying lipids such as phosphatidylcholine (PC). However, for proper identification, this lipid class is often analyzed in both positive and negative ion mode. The positive ion mode provides information about the headgroup, while the negative ion mode provides information about the fatty acid chains. This joint verification can confirm the class and fatty acid composition of the lipid, but that is the limit of information from CID MS/MS.

EAD fragmentation generates more informative fragment ions and therefore enables a deeper characterization of the compound. As a result, it is possible to discern the position of the fatty acid chains and the double bond position Figure 1 shows a standard for PC(15:0/18:1(d7)Δ9). The headgroup is characterized by the canonical loss of m/z 184.0802 and the glycerol backbone is identified by 2 fragments at m/z 224.1065 and 226.0858. The diagnostic ion m/z 498.3837 determines that the sn-2 fatty acid chain is FFA18:1(d7). The fragmentation efficiencies along the acyl chain are low, especially around the double bond (Figure 1, bottom). However the MS/MS sensitivity afforded by the Zeno trap enables interrogation. The position where the double bond appears is the difference in m/z of 12 (C=C) rather than 14 (CH_2 loss) and the ladder of peaks are highlighted with red arrows. Figure 1 therefore indicates that a double bond is present at C9 position.

Confirmation of the structure of phosphatidylcholine in plasma

For characterization of a lipid in matrix, rat plasma was extracted and evaluated. Figure 2 (left panel) shows the EAD MS/MS spectrum for a lipid detected in plasma with a precursor ion at m/z 758.5675. This compound was identified as a PC based on the fragments detected at m/z 184.0816, m/z 224.1067 and m/z 226.0860.[5] However without further information, this lipid would need to be reported as PC(34:2), with multiple combinations for fatty acid chain lengths, their localization, and degrees of desaturation occurring at one or both fatty acids, ie. PC(18:1_16:1), PC(16 :1_18:1), PC(18:2_16:0), PC(18:0_16:2), etc. Detecting the diagnostic ion m/z 489.3263 confirms the localization of fatty acid 18:2 at the sn-2 position, which therefore identifies the lipid as PC(16:0/18:2).

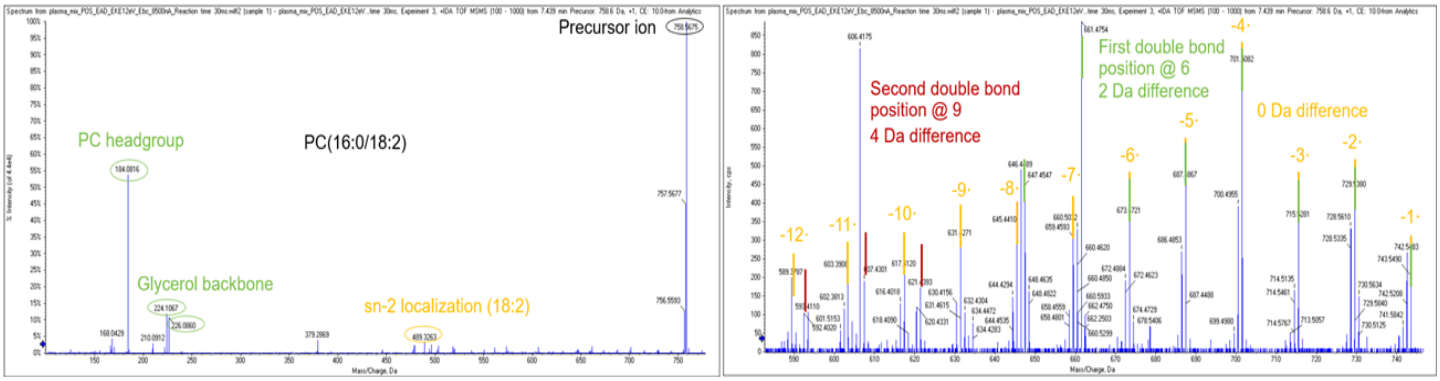

Figure 2: Characterization of PC(16:0/18:2(Δ6,9)). A PC molecule was identified in a plasma matrix. The diagnostic fragments in the left panel identified the lipid as a PC and revealed that the fatty acid 18:2 was located at the sn-2 position. The right panel identifies the localization of both double bonds on the 18:2 chain.

A magnified view of the acyl chain fragmentation is shown in the right panel of Figure 2. Calculating the difference between the fragments reveals that double bonds are present at the C6 and C9 positions. This information completes the characterization of this lipid compound as PC(16:0/18:2 (Δ6,9)).

Structural confirmation of sphingomyelin standard

When using CID fragmentation, the head group fragments of sphingomyelin and phosphatidylcholine are the same. It can be seen in Figure 3 that both types of lipids will provide a characteristic choline headgroup fragment of m/z 184.0769 and m/z 184.0739, and it is impossible to directly confirm which lipid these fragments belong. Often, researchers will infer SM and PC identity based on odd and even precursor m/z values, respectively. However, this approach is unreliable due to isobaric overlap and can result in errors. For example, a PC can be falsely identified or sometimes overreported, as it will appear as the C13 isotope of SM if proper deconvolution is not performed.

The use of EAD fragmentation generates distinctive fragments that allow lipid species to be properly identified. The sphingosine backbone of SM produces 2 peaks at m/z 225.1015 and m/z 253.1081.[6] This is pattern is unique to SM and unlike the C-O doublet that characterizes the glycerol backbone of PC (Figure 1). Figure 4 illustrates the full characterization of the SM(d18:1/18:1(d9)) standard. The left panel shows the full spectrum for the SM standard, including fragments indicative of the amide-linked backbone, specifically sphingosine, along with the localization fragments for the sn-1 and sn-2 chains. Magnified on the right panel indicates the presence of a double bond at the C9 position on the sn-2 chain.

Characterization of endogenous sphingomyelin in plasma

To evaluate capabilities of characterization of an endogenous SM extracted plasma was analyzed. The lipid shown in Figure 5 can be identified as a SM by the fragments at m/z 184.0838, m/z 225.1015 and m/z 253.1075, but this lipid could be SM(d16:0_18:1), SM(d16:1_18:0), SM(d18:0_16:1),

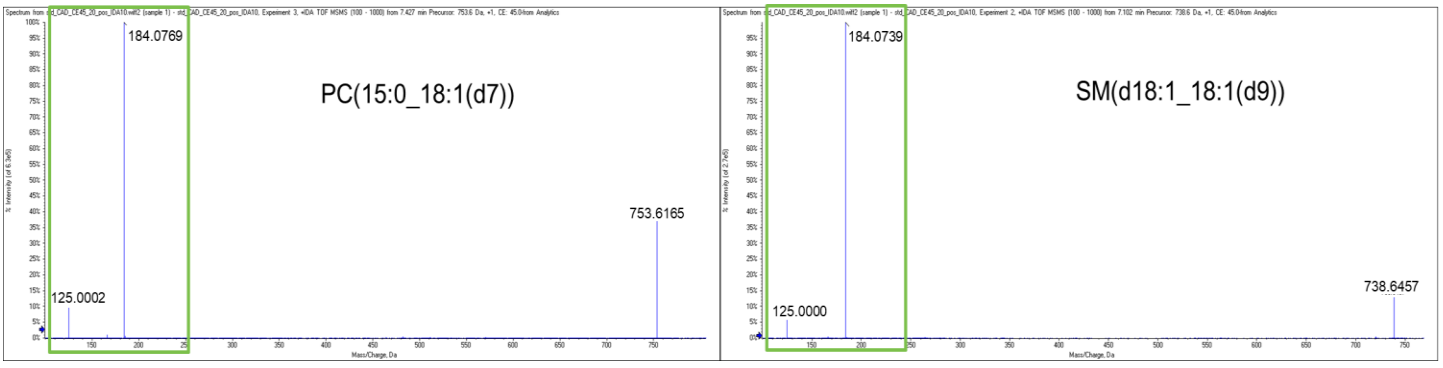

Figure 3. CID MS/MS of sphingomyelins and phosphatidylcholines. Diagnostic fragment ions at the low mass end identify the PC and SM headgroup at *m/z* 184. No other unique fragments are found to distinguish between these lipids.

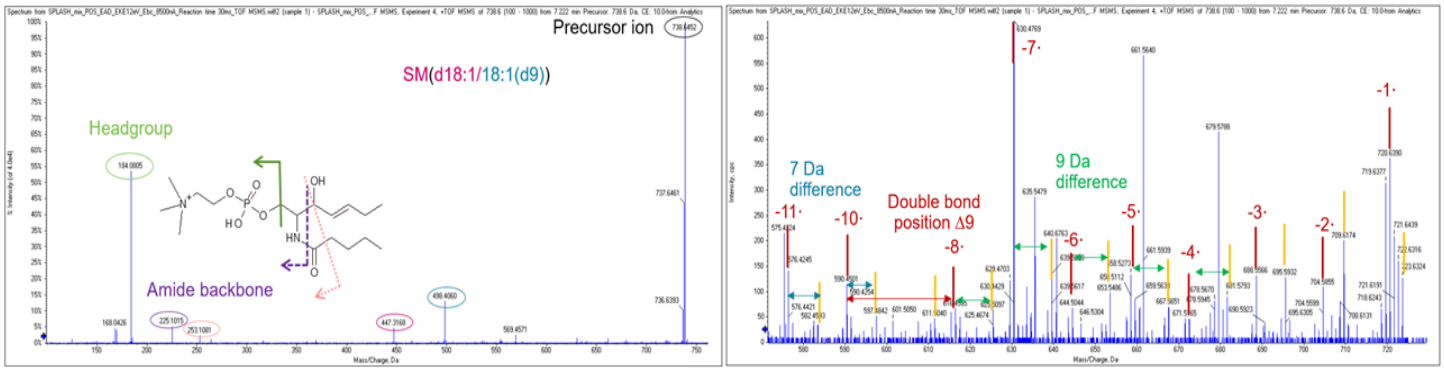

Figure 4. Characterization of SM(d18:1/18:1(d9)) standard. EAD fragmentation generates diagnostic fragments for the sphingomyelin standard that indicate the presence of the amide-linked backbone. These fragments enable differentiation from PC. The sphingosine loss of d18:1 and the sn-2 loss of 18:1(d9) are indicated by fragments at m/z 447.3168 and m/z 498.4060, respectively (left panel). The double bond was located at the C9 position of the sn-2 chain (right panel).

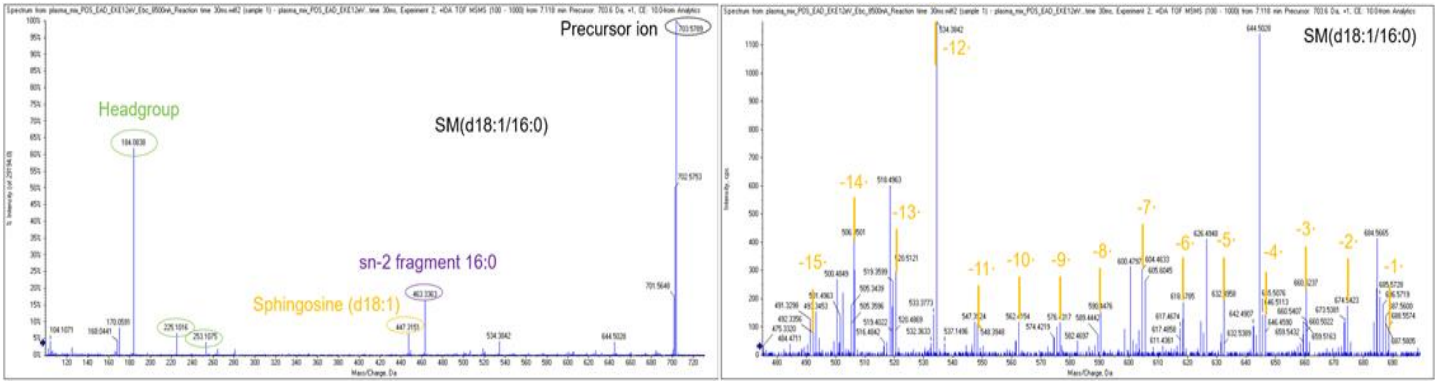

Figure 5. Endogenous characterization of SM(d18:1/16:0). Extracted plasma was investigated to characterize an endogenous SM molecule. The left panel shows the unique sphingosine chain (d18:1), sn-2 (16:0) and the amide backbone fragments. The right panel indicates that the 16:0 acyl chain fragment is fully saturated.

SM(d18:1_16:0), etc. The diagnostic ions produced at m/z 447.3151 and m/z 463.3363, shown in the left panel of Figure 5, reveal that the sphingosine chain is d18:1 and the sn-2 fatty acid chain is 16:0. Calculating the m/z difference between the fragments in the right panel confirmed that the sn-2 fatty acid chain does not have any double bonds. The lipid compound detected in plasma was therefore identified as SM(d18:1/16:0).

Conclusions

The use of electron activated dissociation (EAD) fragmentation on the ZenoTOF 7600 system generates rich lipid structural information, which enables complete structural identification. The fragmentation spectra generated with EAD enabled the differentiation of PC and SM lipid standards compared to conventional CID. The Zeno MS/MS functionality provides significant MS/MS sensitivity gains that enables localization of the double bond(s). This approach successfully generated data that were used to identify phosphatidylcholine (PC) and sphingomyelin (SM) lipids in complex plasma samples.

- Headgroup information obtained by CID is the same for PC and SM lipids. EAD generates unique fragments from their glycerol and amide backbones, respectively, which can be used to distinguish between common isomers.
- Size and location of the acyl chains can be determined from key fragments
- Distance between peaks of fragmentation ladders allows for the localization of double bonds

References

1. High complexity of the lipidome. SCIEX community post, RUO-MKT-11-13093-A.
2. Complete structural elucidation of lipids in a single experiment using electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-13050-B.
3. Baba T, Campbell JL, et al. Development of a Branched Radio-Frequency Ion Trap for Electron Based Dissociation and Related Applications. *Mass Spectrometry, 2017, 6(1):A0058.*
4. Baba T, Campbell JL, et al. Electron Capture Dissociation in a Branched Radio-Frequency Ion Trap. *Analytical Chemistry, 2015, 87(1):785-792.*
5. Campbell JL, Baba T. (2015) Near-complete structural characterization of phosphatidylcholines using electron impact excitation of ions from organics *Anal. Chem.* **87**, 5837−5845.
6. Baba T, et al. (2016) In-depth sphingomyelin characterization using electron impact excitation of ions from organics and mass spectrometry *J Lipid Res*. **57(11)**:858-867.

SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices