Automatic characterization of the lipid nanoparticle ionizable lipid MC3 and its impurities using Molecule Profiler software

Featuring the ZenoTOF 7600 system and Molecule Profiler software

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This technical note demonstrates the comprehensive characterization of impurities in the ionizable lipid (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraene-19-yl-(dimethylamino) butanoate, commonly known as DLin-MC3-DMA (MC3). The electron-activated dissociation (EAD) fragmentation method was implemented on the ZenoTOF 7600 system and the Molecule Profiler software was used for in-depth data analysis (**Figure 1**). EAD provides abundant diagnostic fragments, allowing thorough structural elucidation of singly charged compounds.[1-7] Molecule Profiler software provides data analysis for relative quantification based on TOF MS data. More importantly, it allows structural elucidation with automatic interpretation of MS/MS fragment ions belonging to putative metabolites, facilitating the characterization of ionizable lipids used in lipid nanoparticle (LNP) formulations.

The use of LNPs as drug delivery devices has dramatically increased since the advent of the COVID-19 vaccine and recent gene therapy therapeutics. It has been demonstrated that lipid impurities among the components of the LNP can attenuate the effects of the active pharmaceutical ingredient (API). A recent study reported that N-oxidation of ionizable lipids might lead to covalent modification of ribonucleotides and a loss of mRNA potency.[8] To ensure product quality, detailed and sensitive characterization of the ionizable lipid and its related impurities is necessary. Accurate mass spectrometry (MS) can be used to identify potential impurities by comparing the measured and calculated m/z and isotope patterns. However, structural confirmation leveraging MS/MS facilitates the localization of altered sites in the chemical. Thorough structural elucidation is complex, especially for ionizable lipids with highly symmetrical structures. This necessitates powerful and intuitive processing software to overcome the cumbersome and time-consuming manual interpretation.

Using EAD, the nature and sites of chemical alterations in MC3 that resulted from oxidation were elucidated using structurally diagnostic fragment ions. Molecule Profiler software overcomes the challenges of cumbersome manual interpretation of complex MS/MS spectra and allows for the confident identification and relative quantification of multiple low-abundance impurities at relative abundances as low as 0.05%.

Key features of comprehensive LNP characterization

- Comprehensive structure characterization of MC3 and its impurities was achieved based on informative fragment ions generated by EAD on the ZenoTOF 7600 system
- Automatic metabolite assignments were achieved by the Molecule Profiler software based on highly accurate TOF MS data and thorough interpretation of MS/MS data
- Straightforward relative quantification was achieved based on TOF MS peak areas. Impurities were confidently identified at abundances as low as 0.05%.

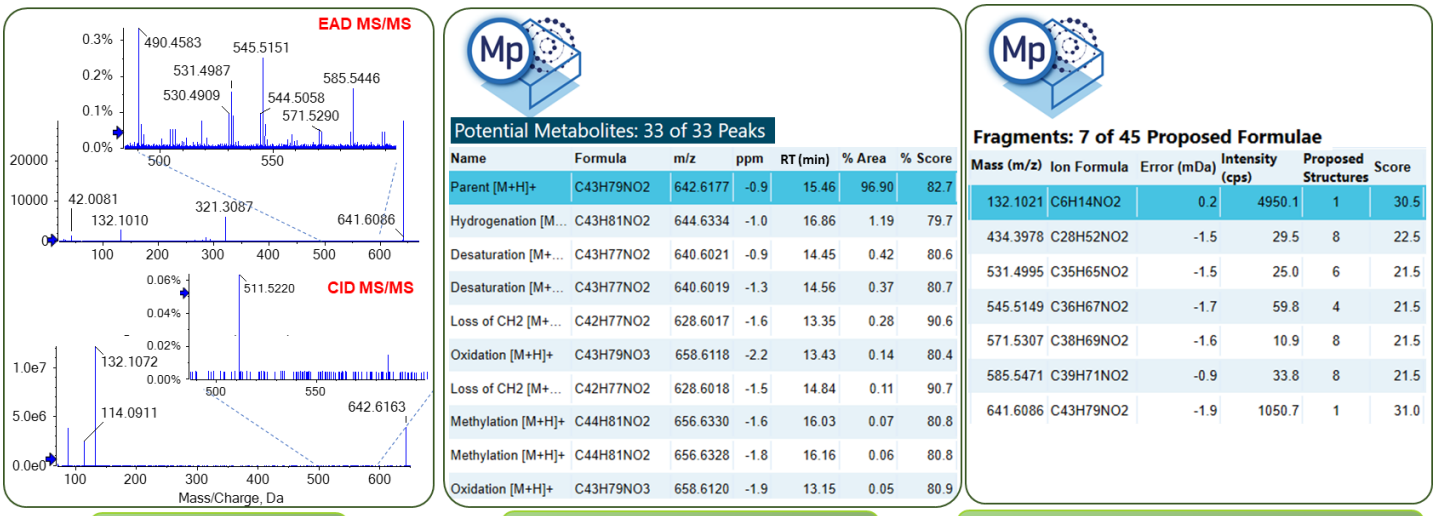


Figure 1. Workflow for the relative quantification and structural elucidation of MC3 and impurities using EAD-based MS/MS. EAD outperforms collision-induced dissociation (CID) in providing quality fragment ions for the structural confirmation of MC3.

Methods

Sample preparation: A stock solution of MC3 (2 mg/mL) was diluted 1:10 in mobile phase A, which contained 15% water, 30% acetonitrile and 55% methanol with 10mM ammonium acetate.

Chromatography: A 2 µL sample of the diluted MC3 (0.2 mg/mL) was injected into an ExionLC AD system equipped with a reversed-phase column (C18, 1.7 µm, 2.1 × 150 mm). The column oven was set to 70°C. A total runtime of 27 min was used with a flow rate of 0.5 mL/min. Mobile phase A is described above and mobile phase B was 60:40, acetonitrile/methanol with 10mM ammonium acetate. The chromatographic conditions used are described in **Table 1**.

Mass spectrometry: Data were acquired using SCIEX OS software on the ZenoTOF 7600 system in positive polarity. Data were collected from a single injection, using a combination of data-dependent acquisition (DDA) and a targeted approach that implemented an inclusion list. Relevant MS parameters for the EAD method are described in **Tables 2** and **3**.

Data processing*:* Structural elucidation and relative quantification were performed using the Molecule Profiler software modules of SCIEX OS software. A self-built biotransformation list was integrated into the processing parameters (**Table 5**). The maximum C-C bond to break was set to 1 and the number of EAD fragment peaks selected for the assignment was set to 100 under MS/MS parameters. The rest of the parameters were set to default.

Table 1. LC conditions.

Time (min)	%A	%B
Initial	*100*	*0*
2.0	*100*	*0*
11	*0*	*100*
21	*0*	*100*
21.1	*100*	*0*
27	*100*	*0*

Table 2. TOF MS and EAD MS/MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF MS*	*DDA*
Polarity	*Positive*	
Gas 1	*60 psi*	
Gas 2	*80 psi*	
Curtain gas	*35 psi*	
Source temperature	*450°C*	
Ion spray voltage	*5500 V*	
Declustering potential	*60 V*	
Collision energy	*10 V*	*12 V*
CAD gas	*7*	
Workflow	*Small molecule*	
Maximum candidate ion	*2*	
Intensity threshold	*10,000 cps*	
Exclusion time	*5 s after 3 occurrences*	
Inclusion list	*Intensity threshold 1000 cps (see Table 3)*	
Exclusion list	*Active (common background ions)*	
Start mass	*300 m/z*	*20 m/z*
Stop mass	*1,000 m/z*	*1,000 m/z*
Electron KE	*N/A*	*16 eV*
Electron beam current	*N/A*	*5000 nA*
ETC	*N/A*	*100*
Reaction time	*N/A*	*30 ms*
Zeno trap	*N/A*	*ON*
Accumulation time	*0.1 s*	*0.605 s*
Time bins to sum	*6*	*6*

Table 4. Inclusion list for the MS/MS method.

Compound	*m/z*
[MC3 -2H + H]$^+$	*640.6027*
[MC3 + H]$^+$	*642.6184*
[MC3 + 2H + H]$^+$	*644.6340*
[MC3 + O + H]$^+$	*658.6133*
[MC3 + 2O + H]$^+$	*674.6082*
[MC3 – CH_2 + H]$^+$	*628.6027*
[MC3 + H_2O + H]$^+$	*660.6289*

Table 5. Biotransformation used in data processing parameters setting .

Name	*Mass shift*	*Description*
Bis-demethylation	*-28.0313*	*CH3RCH3 to R*
Demethylation & desatureation	*-16.0314*	*-CH2 & -H2*
Demethylation	*-14.0157*	*R-CH3 to R-H*
Demethylation and hydrogenation	*-12.0000*	*-CH2 & +2H*
Bis-disaturation	*-4.0313*	*2 double bond formation*
Desaturation	*-2.0157*	*R_1CH2-$CH2R_2$ to R_1CHCHR_2*
Hydrogenation	*2.0157*	*+2H*
Di-hydrogenation	*4.0313*	*+2H2*
Methylation	*14.0157*	*R-H to R-CH3*
Oxidation	*15.9949*	*+O*
Bis-methylation	*28.0313*	*+2 CH2*
Di-oxidation	*31.9898*	*+2O*
Tri-methylation	*42.0470*	*+3 CH2*

Detection of MC3 and low abundance impurities

MC3 is the ionizable lipid used in LNP formulations for the therapeutic siRNA, patisiran. Like other ionizable lipids, its structure contains a tertiary amine. The amine is on a 3-carbon head group that is bonded via an ester linkage to 2 identical alkyl chains, each containing double bonds at C6 and C9. A preparation of MC3 was subjected to reversed-phase LC-MS analysis using the ZenoTOF 7600 system. Chromatographic separation showed a main peak (MC3) at 15.5 min (**Figure 2**). The extracted ion chromatogram (XIC) also showed several low abundance impurity peaks at relative intensities as low as 0.05%

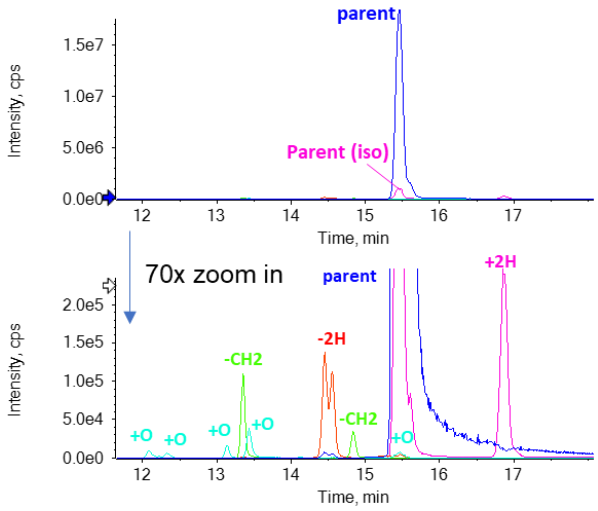


Figure 2. XIC of intact MC3 and low abundance impurities. The top panel shows the normalized XIC of all compounds. The MC3 parent peak is the base peak of the chromatogram. The bottom panel shows the same chromatogram with a 70x zoom of the y-axis to better visualize the peaks corresponding to the low abundance impurities.

of the MC3 peak, demonstrating the wide interscan dynamic range of the ZenoTOF 7600 system for lipid impurity analysis.

The Molecule Profiler software was used to identify the MC3 impurities. **Figure 3** shows results for the subset of impurities that were automatically identified by the Molecule Profiler software. In addition to the mass accuracy of each impurity, the relative abundance of each impurity was determined based on TOF MS peak areas compared to the MC3 main peak. Excellent mass accuracy was achieved due to the high resolution of the ZenoTOF 7600 system. The majority of the impurities had mass accuracy of the monoisotopic peak within 3 ppm of mass error of

Name	Formula	m/z	ppm	RT (min)	% Area	% Score
Parent	C43H79NO2	642.6177	-0.9	15.46	96.90	82.7
Hydrogenation	C43H81NO2	644.6334	-1.0	16.86	1.19	79.7
Desaturation	C43H77NO2	640.6021	-0.9	14.45	0.42	80.6
Desaturation	C43H77NO2	640.6019	-1.3	14.56	0.37	80.7
Loss of CH2	C42H77NO2	628.6017	-1.6	13.35	0.28	90.6
Oxidation	C43H79NO3	658.6118	-2.2	13.43	0.14	80.4
Loss of CH2	C42H77NO2	628.6018	-1.5	14.84	0.11	90.7
Methylation	C44H81NO2	656.6330	-1.6	16.03	0.07	80.8
Methylation	C44H81NO2	656.6328	-1.8	16.16	0.06	80.8
Oxidation	C43H79NO3	658.6120	-1.9	13.15	0.05	80.9
Oxidation	C43H79NO3	658.6120	-1.9	15.46	0.05	81.0

Figure 3. MC3 and the top 10 of 33 identified putative impurities sorted by %area.

the expected value. The Molecule Profiler software performed thorough data analysis for all impurities, including the low-abundance species. Species with relative abundances less than 0.1% were assigned a structure with high confidence.

The m/z observed by TOF MS for the singly charged MC3 matched the theoretical m/z of MC3 within 1 ppm. This m/z was selected for fragmentation using EAD. **Figure 4** shows an overview of the interpretation of the structure of MC3 using Molecule Profiler software. **Figure 4A** shows a zoomed-in view of the EAD-based MS/MS spectrum. The blue highlights on the fragment ions indicate assignments of fragment ions to a proposed structure or an ion formula, which are listed in **Figure 4C**. EAD provides many structurally informative fragment ions for the structural interpretation of MC3. If multiple proposed structures are possible, there are options to navigate across the proposed structures in the "structure details for selected ion formula" window. The partial fragment structure corresponding to the selected formula is highlighted in **Figure 4B** to allow direct visualization of the proposed structures. Finally, **Figure 4D** lists all other possible structures matching the MS/MS spectrum in order from the highest to lowest matching score.

Structural elucidation of oxidized impurities of MC3

N-oxidation of ionizable lipids can lead to covalent modification of ribonucleotides and a loss of mRNA potency.[8] The Molecule Profiler software identified several oxidized isomers as low-abundance impurities, shown in **Figure 5**. The identification list aligns with the peaks observed in the XIC at m/z = 658 (**Figure 2**, aqua trace, annotated with +O). A detailed analysis by Molecule Profiler software on 2 of the oxidized impurities corresponding to peaks at retention times (RT) 13.15 min and 13.43 min (**Figure 6A**) are presented below

Despite the relatively low abundance of these 2 impurities (0.14% and 0.05% reported from the Molecule Profiler software), high spectral quality was observed using EAD, which can be attributed to the use of the Zeno trap.

The first peak (**Figure 6A**, RT = 13.15 min) was assigned to a structure with oxidation on the alky chain on the C6/C31 double bond. Signature ions observed at m/z = 559, 574 and 586 were assigned to the structure with oxygen incorporated on the C6/C31 double bond by the Molecule Profiler software. The structure of oxidized MC3 is shown in Figure 6C. The parts of the

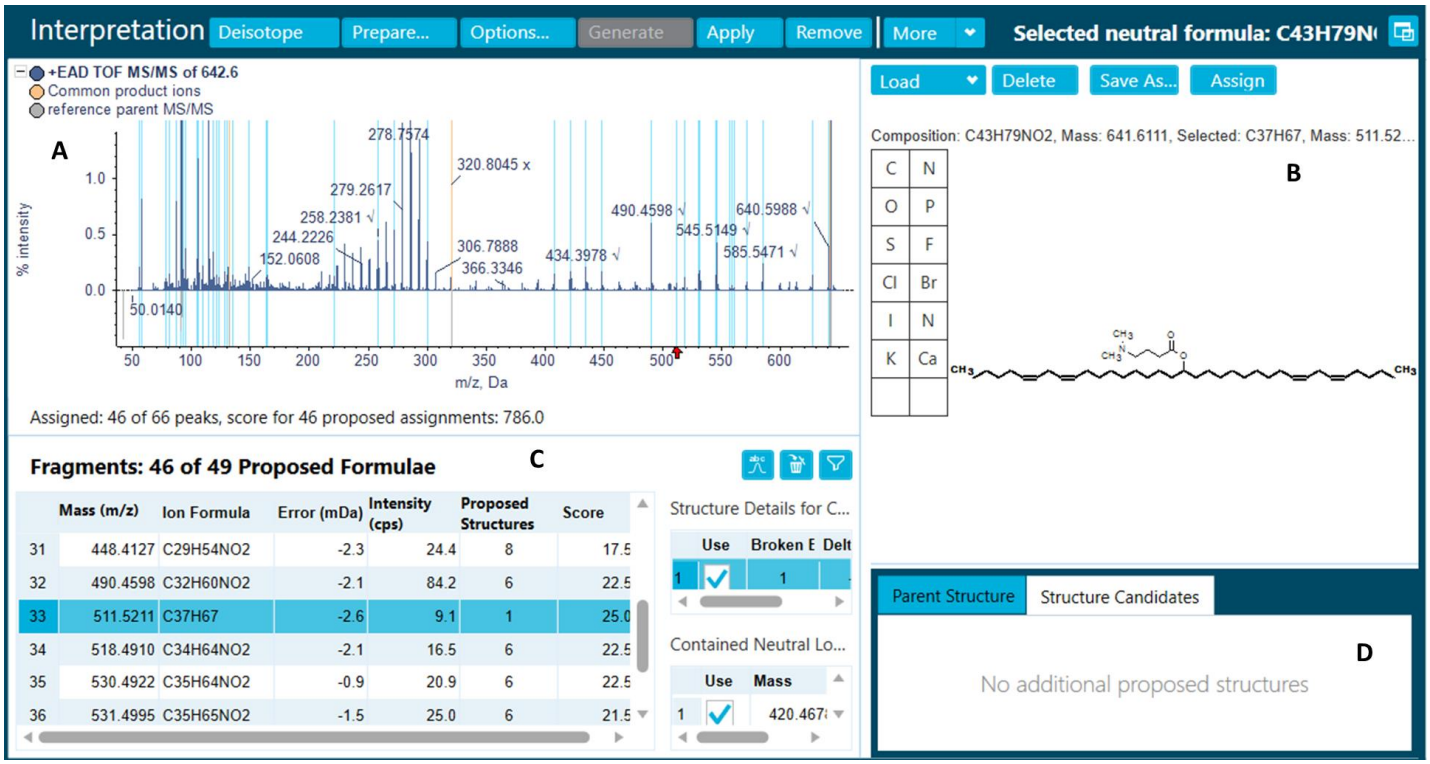


Figure 4. Overview of the spectrum interpretation by the Molecule Profiler software. Panel A) The spectrum under investigation. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. Panel B) The structure assigned to the spectrum. The structure is highlighted in bold (for example, the alkyl chain) when a fragment peak is selected in the fragments list (m/z = 511.5211 selected, panel C). Panel C) The fragments list that corresponds to the highlighted peak in panel A. Panel D) Other possible structures that match the MS/MS spectrum in order from the highest to lowest matching score.

Name	Formula	m/z	ppm	RT (min)	% Area	% Score
Oxidation	C43H79NO3	658.6122	-1.7	12.07	0.04	81.3
Oxidation	C43H79NO3	658.6120	-1.9	12.22	0.01	81.7
Oxidation	C43H79NO3	658.6120	-1.9	13.15	0.05	80.9
Oxidation	C43H79NO3	658.6118	-2.2	13.43	0.14	80.4
Oxidation	C43H79NO3	658.6120	-1.9	15.46	0.05	81.0

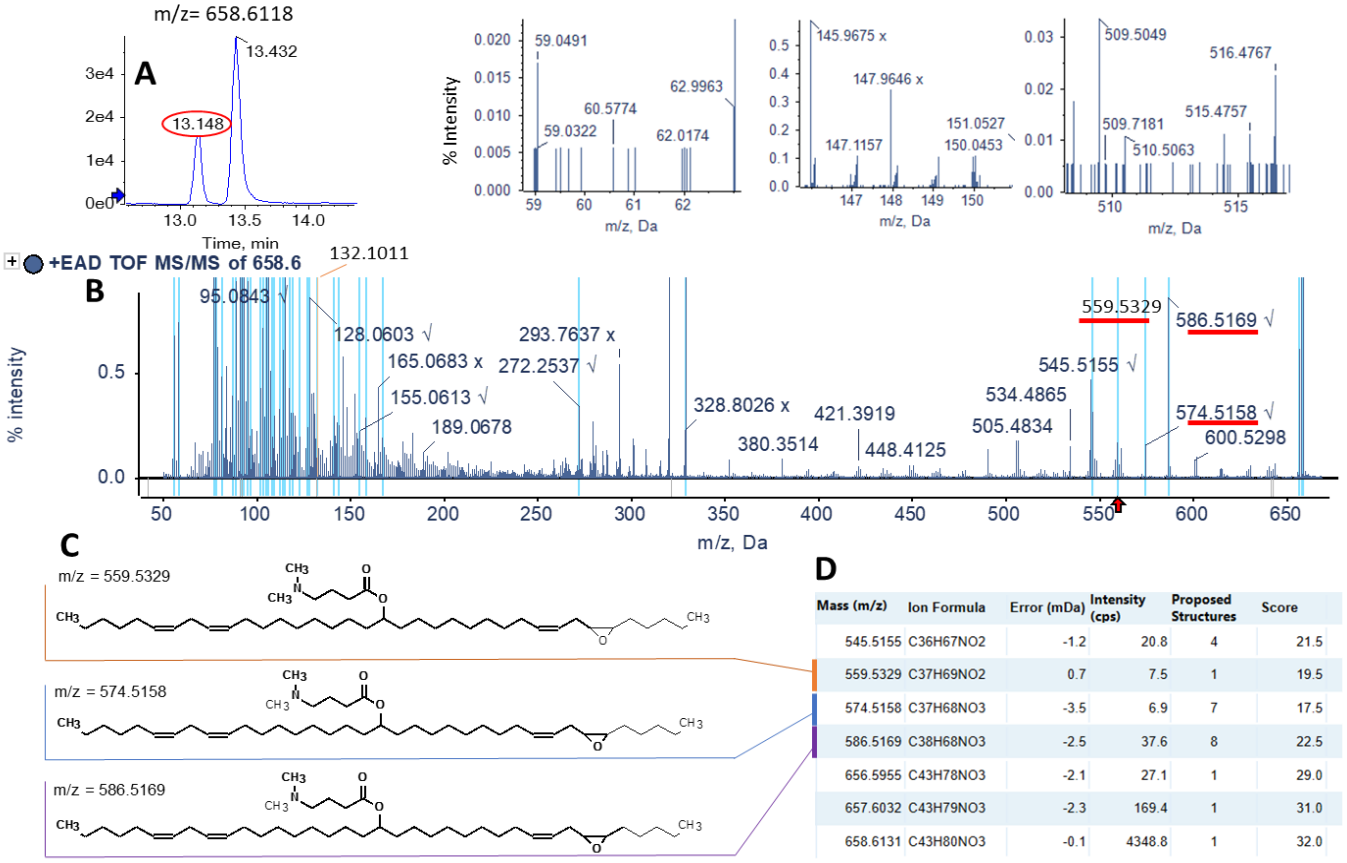
Figure 5. Oxidized impurities identified by the Molecule Profiler software sorted by retention time.

structures highlighted in bold correspond to the fragment ions at m/z 559.5, 574.5, and 586.5 that support the oxygen incorporation on the C6 double bond. The absence of peaks at m/z = 148.1, 61.05, and 511.5 (inset above **Figure 6B**), used as signature ions for the N-oxidation described below, further confirmed that the added oxygen was not in the head group.

The second peak (**Figure 7A,** RT = 13.43 min) was assigned a structure with oxidation on the nitrogen of the head group.

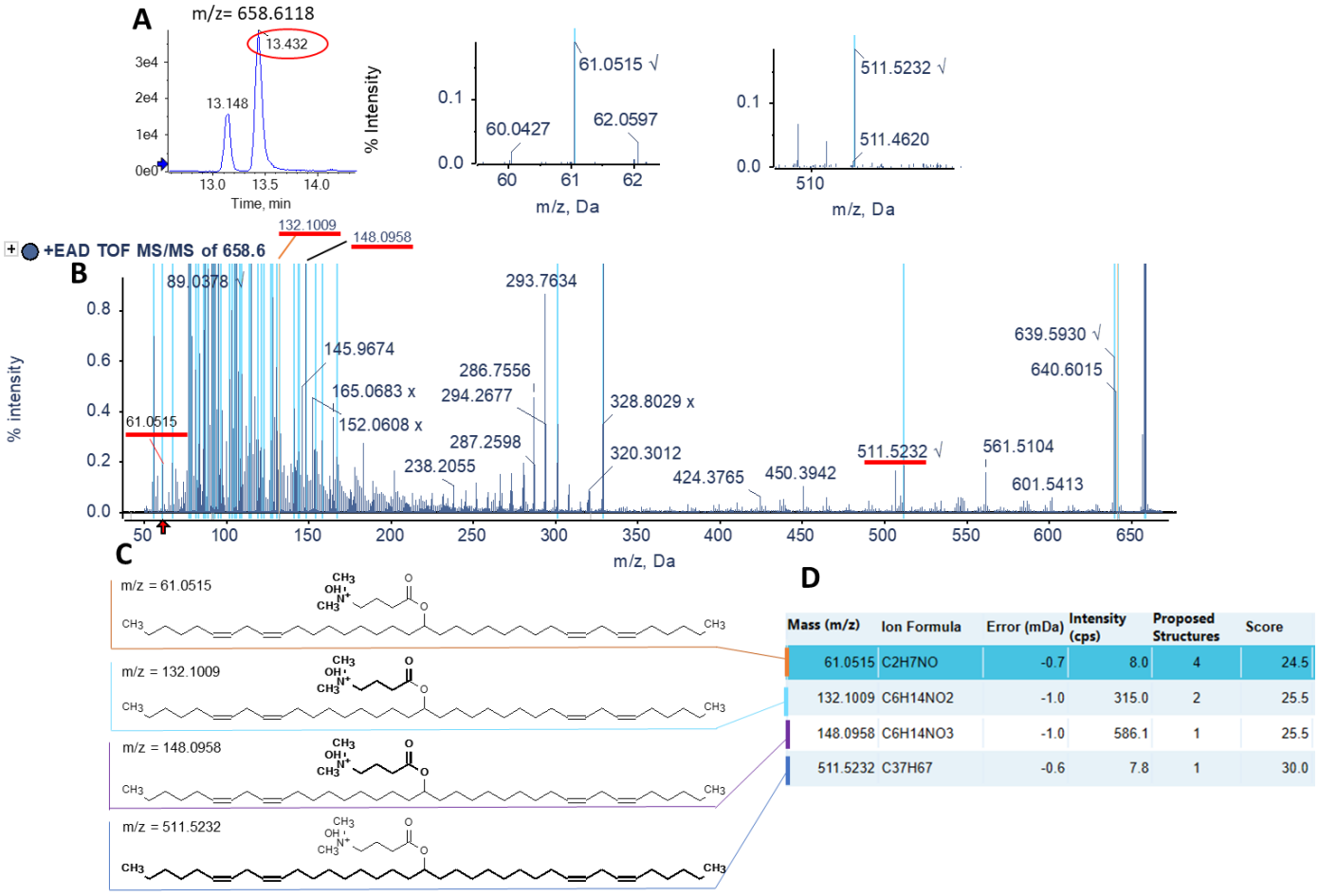
Signature ions peaks at m/z = 148.1 and 61.05 (insets above **Figure 7B**) were assigned to the structure with oxygen incorporated on the nitrogen of the head group. The peak at m/z 511.5232 indicates the alkyl chain was not oxidized. The structures of interest corresponding to the signature ions are highlighted in bold in **Figure 7C**.

This workflow leverages the speed, sensitivity and broad dynamic range of the ZenoTOF 7600 system and the capability of the Molecule Profiler software to perform automatic spectrum interpretation and structural elucidation. The workflow provides a complete solution for ionizable lipid characterization and can streamline the analysis and quality control of LNP formulations and their individual components. The data generated from this workflow can be further used to determine the drug efficacy and safety of formulated LNPs. Additionally, they can aid the rational design of new synthetic lipids.



Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)	Proposed Structures	Score
545.5155	C36H67NO2	-1.2	20.8	4	21.5
559.5329	C37H69NO2	0.7	7.5	1	19.5
574.5158	C37H68NO3	-3.5	6.9	7	17.5
586.5169	C38H68NO3	-2.5	37.6	8	22.5
656.5955	C43H78NO3	-2.1	27.1	1	29.0
657.6032	C43H79NO3	-2.3	169.4	1	31.0
658.6131	C43H80NO3	-0.1	4348.8	1	32.0

Figure 6. Characterization of the first peak of oxidized impurities. Panel A) The XIC at m/z = 658.6118. The impurity that eluted at 13.15 min was analyzed in detail. Panel B) The EAD-based MS/MS spectrum of the impurity. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. The insets above panel B are zoomed in on the spectrum at m/z 61, 148 and 511. Panel C) The structures of MC3 with oxidation on the alkyl chain at the C6/C31 double bond. The structure highlighted in bold corresponds to the signature ions for structural elucidation. Panel D) Part of the fragment ions corresponding to the assigned peaks in panel B.



Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)	Proposed Structures	Score
61.0515	C2H7NO	-0.7	8.0	4	24.5
132.1009	C6H14NO2	-1.0	315.0	2	25.5
148.0958	C6H14NO3	-1.0	586.1	1	25.5
511.5232	C37H67	-0.6	7.8	1	30.0

Figure 7. Characterization of the second peak of oxidized impurities. Panel A) The XIC at m/z = 658.6118. The impurity that eluted at 13.43 min was analyzed in detail. Panel B) The EAD-based MS/MS spectrum of the impurity. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. The insets above panel B are zoomed in on the spectrum at m/z 61 and 511. Panel C) The structures of MC3 with oxidation on the head group nitrogen. The structure highlighted in bold corresponds to the signature ions for structural elucidation. Panel D) Part of the fragment ions corresponding to the assigned peaks in panel B.

Conclusions

- Confident identification of low abundace impurities present in the MC3 sample was achieved with excellent mass accuracy and information-rich MS/MS generated by EAD on the ZenoTOF 7600 system
- Automatic identification, thorough structural elucidation and relative quantification of MC3 and related impurities were accelerated by the automatic annotation of TOF MS/MS spectra based on proposed structures in Molecule Profiler software
- The ZenoTOF 7600 system and Molecule Profiler software provided improved risk assessment of formulated LNPs through explicit structural elucidation and site-specific localization of oxygen incorporation into impurities derived from cationic lipids such as MC3

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