Automated characterization of the lipid nanoparticle ionizable lipid, DODAP, and its degradants using Molecule Profiler software

Featuring the ZenoTOF 7600 system and Molecule Profiler software

Zhichang Yang[1], Sahana Mollah[1], Harini Kaluarachchi[1], Wen Jin[1] and Mays Al-Dulaymi[2]
[1]SCIEX, USA; [2]SCIEX, Canada

This technical note demonstrates the comprehensive characterization of the ionizable lipid 1,2-dioleoyl-3-trimethylammonium propane (DODAP) as it goes through degradation and oxidation. The ZenoTOF 7600 system equipped with electron activated dissociation (EAD) was used for data acquisition. Molecule Profiler software was used for in-depth data analysis. EAD generates abundant diagnostic fragments that are not produced at significant levels by collision induced dissociation (CID)for singly charged lipids but that are important for structural interpretation. Molecule Profiler software provides structural elucidation by automatically interpreting informative EAD-based MS/MS data. This workflow facilitates the understanding of the degradation of ionizable lipids used in lipid nanoparticle (LNP) formulations.

Key features of comprehensive ionizable lipid characterization

- Comprehensive structural characterization of DODAP and its impurities was achieved based on informative fragment ions generated by EAD on the ZenoTOF 7600 system
- Automatic impurity assignments were achieved by the Molecule Profiler software based on highly accurate TOF MS data and thorough interpretation of MS/MS data
- Isomers of the oxidized form of impurities were confidently distinguished with signature ions from EAD, which is challenging with other forms of MS/MS fragmentation
- Potential impurities were comprehensively identified with the new biotransformation list built into the lipid workflow of the Molecule Profiler software

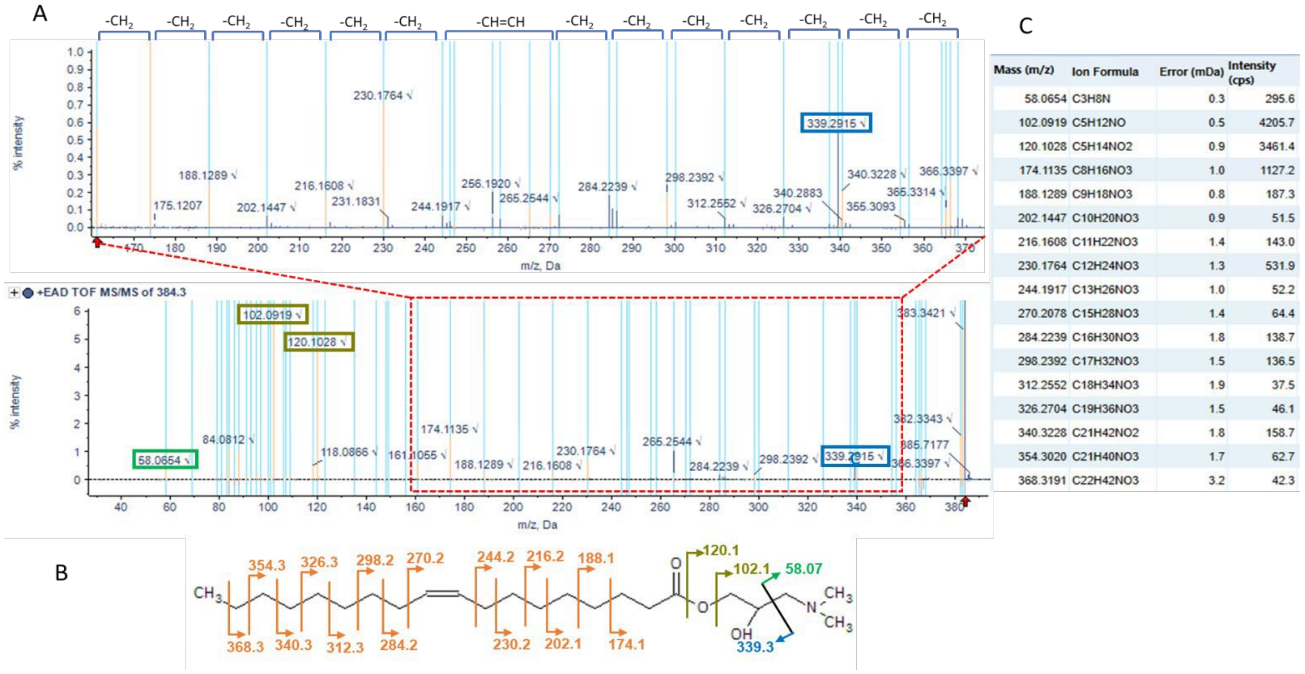


Figure 1. Workflow showing the structural characterization of DODAP degradants by Molecule Profiler software. Panel A shows an EAD MS/MS spectrum of m/z 384.3 showing comprehensive fragmentation for high confidence identification and structural elucidation. When a peak is assigned to a proposed structure or formula, the peak gets highlighted in light blue. Panel B shows the structure assigned to the spectrum in panel A. Panel C shows the selected fragments list corresponding to the assigned structure in panel B.

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 used in 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.[1] It is still unknown if the oxidized form of lipid degradants can also form adducts to mRNA and reduce downstream translation efficiency. Thus, it is important to have a workflow to thoroughly characterize the structure of ionizable lipids and their related impurities and degradants.

Informative MS/MS spectra facilitate structural confirmation and localization of altered sites in the molecule. However, the manual interpretation of complex MS/MS data is complicated and time-consuming, especially for ionizable lipids with highly symmetrical structures. This necessitates powerful and intuitive processing software to overcome the cumbersome and time-consuming manual interpretation.

In this technical note, the original and oxidized forms of DODAP and its degradants were structurally characterized using EAD and the Molecule Profiler software. Molecule Profiler software overcomes the challenges of cumbersome manual interpretation of complex MS/MS spectra and allows for the confident distinction of closely eluted oxidized products of DODAP degradant.

Methods

Sample preparation: A stock solution of DODAP (0.5 mg/mL) was diluted 1:5 in mobile phase A, which contained 15% water, 30% acetonitrile and 55% methanol with 10mM ammonium acetate.

DODAP was oxidized by treating a sample with 1% H_2O_2 and incubating at room temperature for 3 days. DODAP was degraded by leaving samples at 8°C for 5 to 8 days for degradation.

Chromatography: A 2 µL sample of diluted DODAP (0.1 mg/mL) was injected into an Agilent LC system equipped with a reversed-phase column (Acquity UPLC Peptide BEH C18, 1.7 µm, 2.1 x 150 mm). The column oven was set to 70°C. A total runtime of 27 min was used with a flow rate of 0.4 mL/min.

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*

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 quantitation were performed using the Molecule Profiler software modules of SCIEX OS software. The lipid workflow with a built-in biotransformation list was applied in the settings for processing parameters. The parameters were set to default.

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	*70 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	*5*	
Intensity threshold	*5,000 cps*	
Inclusion list	*Intensity threshold 1000 cps (see Table 3)*	
Exclusion list	*Active (common background ions)*	
Start mass	*300 m/z*	*30 m/z*
Stop mass	*1,000 m/z*	*1,000 m/z*
Electron KE	*N/A*	*10 eV*
Electron beam current	*N/A*	*8000 nA*
ETC	*N/A*	*100*
Reaction time	*N/A*	*30 ms*
Zeno trap	*N/A*	*ZOD with a 20,000 cps threshold*
Accumulation time	*0.1 s*	*0.095 s*
Time bins to sum	*6*	*6*

Table 3. Inclusion list for the MS/MS method.

Compound	Name	m/z
[M-2H+H]+	Desaturation	646.5769
[M+H]+	Parent	648.5925
[M+2H+H]+	Saturation	650.6082
[M+O+H]+	Oxidation	664.5875
[M+2O+H]+	Di-oxidation	680.5824
[M-CH_2+H]+	Loss of methyl	634.5769
[M-C_2H_4+H]+	Loss of 2 methyl	620.5612
[M+H_2O+H]+	Hydroxylation	666.6031

Detection of DODAP and low abundance impurities

DODAP is an ionizable cationic lipid with low cytotoxicity and high transfection efficiency. It is widely used to encapsulate bioactive molecules used to treat disease, including mRNA, siRNA and plasmid DNA. It is reported that when combined with a helper lipid (DOPE), DODAP shows efficient delivery of the target gene in vivo and high gene expression in the spleen.[2-4] The structure of DODAP contains an amine group. The amine is on a head group bonded via an ester linkage to 2 identical alkyl chains containing double bonds. A preparation of DODAP, its degradants and its oxidized form were subjected to reversed-phase LC-MS analysis using the ZenoTOF 7600 system. Chromatographic separation showed a main peak (DODAP) at 15.5 min (Figure 2). Several degradation products were present in the untreated DODAP sample, with a main degradation peak eluting at 3.15 min. This result indicates that DODAP is easily degradable. Additionally, this result highlights the importance of characterizing the intact and degraded structures in both the original and oxidized forms when exploring potential mRNA adducts of DODAP in an LNP formulation.

The Molecule Profiler software was used to identify DODAP impurities. Figure 3 shows results for the subset of impurities 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 main peak. Excellent mass accuracy was achieved due to the high resolution of the ZenoTOF 7600 system. The monoisotopic peak for most of the impurities had mass accuracy within 2 ppm of mass error of the expected value. The Molecule Profiler software performed thorough data analysis for all impurities, including the species present with low abundance.

Name	Formula	m/z	ppm	R.T. (min)	Peak Area	% Area
Parent [M+H]+	C41H77NO4	648.5931	0.9	15.01	6.27E+08	70.22
Loss of C18H32O [M+H]+	C23H45NO3	384.3477	1.1	3.14	2.26E+08	25.32
Loss of C18H32O [M+H]+	C23H45NO3	384.3473	0.1	3.58	2.30E+07	2.58
Oxidation [M+H]+	C41H77NO5	664.5875	0.1	12.35	5.28E+06	0.59
Desaturation to Alkene [M+...	C41H75NO4	646.5768	-0.2	14.15	3.10E+06	0.35
Bis-Methylation [M+H]+	C43H81NO4	676.6239	0.0	16.17	1.56E+06	0.17
Methylation [M+H]+	C42H79NO4	662.6083	0.2	15.57	6.43E+05	0.07
Loss of C18H32O2+Ketone...	C23H43NO3	382.3313	-0.8	2.44	5.86E+05	0.07
Methylation [M+H]+	C42H79NO4	662.6074	-1.1	11.64	5.42E+05	0.06
Loss of C18H32O [M+H]+	C23H45NO3	384.3467	-1.4	5.15	4.45E+05	0.05
Oxidation [M+H]+	C41H77NO5	664.5874	-0.1	12.62	3.68E+05	0.04

Figure 3. DODAP and the top 10 of 49 identified putative impurities, sorted by %area.

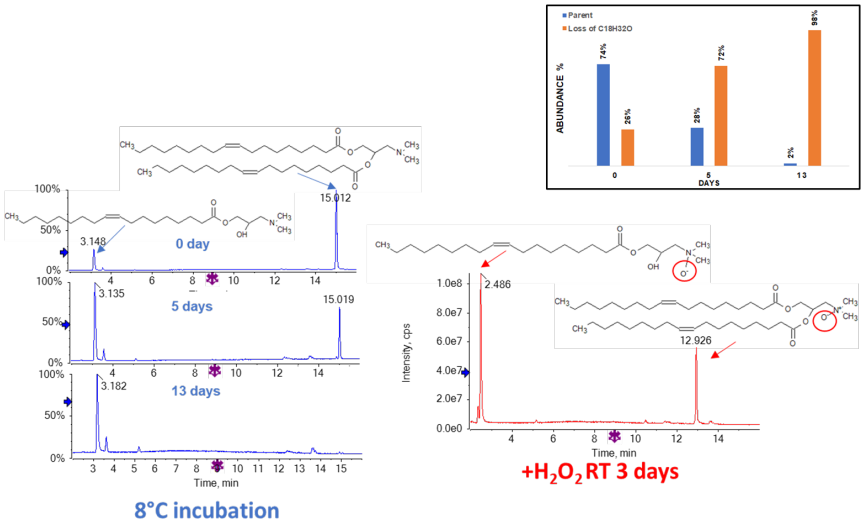
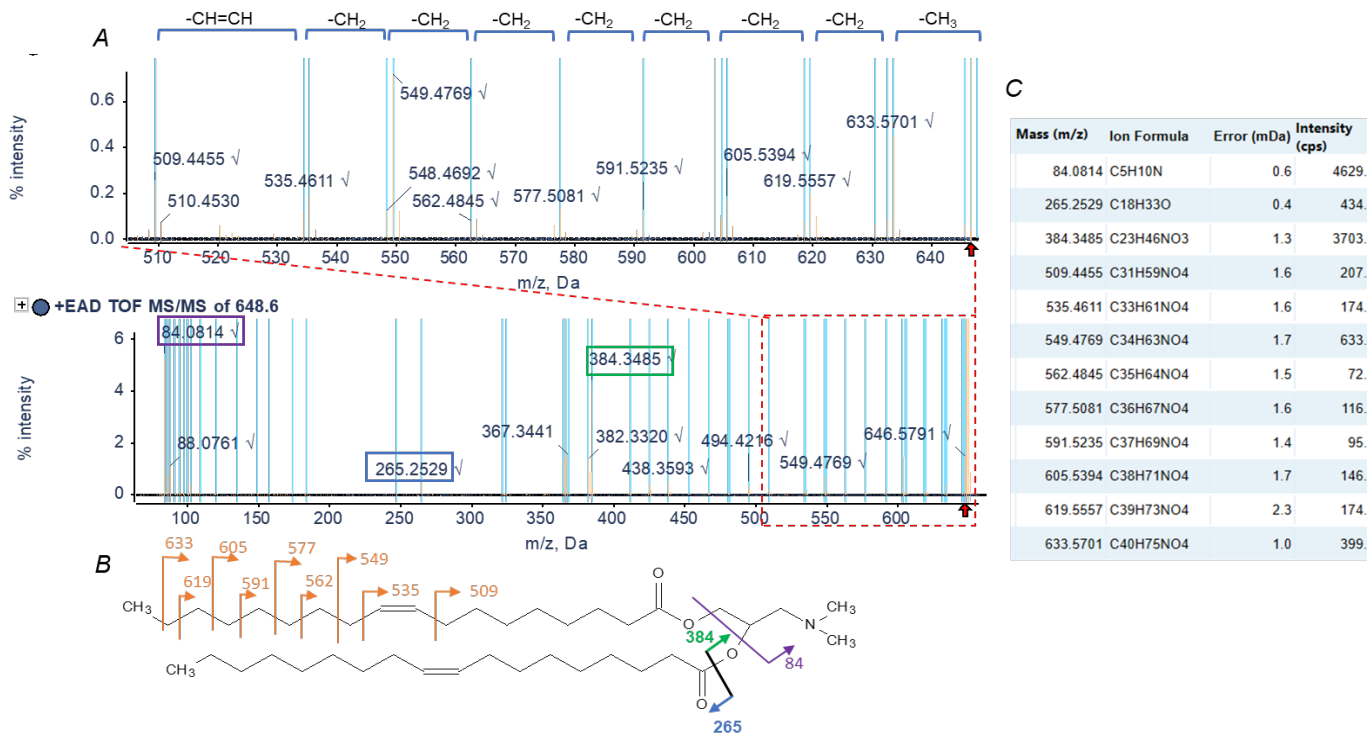


Figure 2. Degradation of DODAP. Samples of DODAP were degraded by incubating them at 8°C for 5 days (left) and were oxidized by treatment with H_2O_2 at room temperature for 3 days (right). The bar plot shows the increase in abundance of the main impurity over time, becoming the main peak at day 13.



Mass (m/z)	Ion Formula	Error (mDa)	Intensity (cps)
84.0814	C5H10N	0.6	4629.
265.2529	C18H33O	0.4	434.
384.3485	C23H46NO3	1.3	3703.
509.4455	C31H59NO4	1.6	207.
535.4611	C33H61NO4	1.6	174.
549.4769	C34H63NO4	1.7	633.
562.4845	C35H64NO4	1.5	72.
577.5081	C36H67NO4	1.6	116.
591.5235	C37H69NO4	1.4	95.
605.5394	C38H71NO4	1.7	146.
619.5557	C39H73NO4	2.3	174.
633.5701	C40H75NO4	1.0	399.

Figure 4. Structural characterization of DODAP product by Molecule Profiler software. Panel A shows a comprehensive EAD MS/MS spectrum of the main product at m/z 648.6. When a peak is assigned to a proposed structure or formula, the peak is highlighted in light blue. Panel B shows the structure assigned to the spectrum and panel C shows the selected fragments list corresponding to the highlighted structure in panel B.

The m/z observed by TOF MS for the singly charged DODAP matched the theoretical m/z of DODAP within 1 ppm. This m/z was selected for fragmentation using EAD. Figure 4 shows an overview of the interpretation of the structure of DODAP 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 DODAP. The partial fragment structure corresponding to the selected formula is indicated in Figure 4B. Ions at m/z 384 and 265 indicate ester bond breakage. A series of fragment ions identified indicate methyl group loss from one end of the alkyl chain. Ions at m/z 535 and 509 confirmed the localization of the double bond.

The impurity with loss of $C_{18}H_{32}O$ had a high abundance (25%, Figure 2) in the original DODAP sample, indicating that this ionizable lipid is easily degraded. A detailed structural characterization of this impurity is shown in Figure 1. Figure 1A shows a zoomed-in view of the EAD-based MS/MS spectrum. Ions 270, 258 and 244 confirm the localization of double bonds among a series of fragment ions originating from the loss of a methyl group from the alkyl chain. Ion 58 represents the head group, which is crucial for localizing possible N-oxidation.

Structural elucidation of oxidized impurities of DODAP

Characterization of N-oxidation of ionizable lipids is important as it can lead to covalent modification of ribonucleotides and a loss of mRNA potency.[1] For this characterization work, oxidation was induced by treating DODAP with 1% H_2O_2. EAD LC-MS/MS was used to characterize the treated DODAP. A detailed structural characterization of the oxidized DODAP by the Molecule Profiler software is shown in Figure 5. Ions at m/z 551 and 525 confirm the localization of double bonds. Ions at m/z 398, 603 and 104 definitively confirm the oxidation on the N.

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 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 in the design of new synthetic lipids.

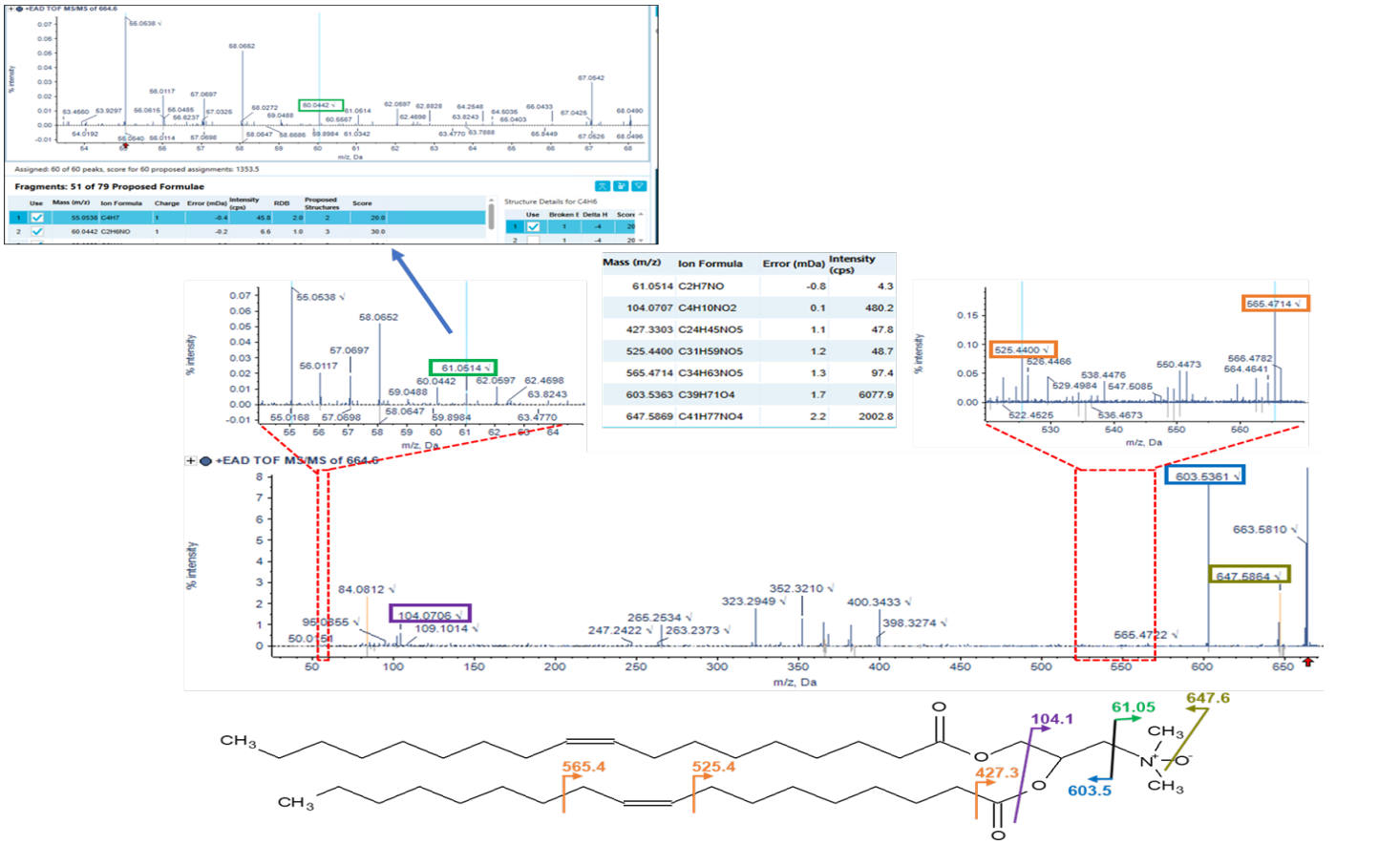


Figure 5. Structural characterization of N-oxidized DODAP by Molecule Profiler software. Panel A shows the EAD MS/MS spectrum of oxidized DODAP at m/z 664.6. When a peak was assigned to a proposed structure or formula, the peak was highlighted in light blue. Panel B shows the structure assigned to the spectrum. Panel C shows the diagnostic ions that confirm the structure in panel B. The inset shows a low abundance peak at m/z 60.04 that corresponds to the $NO(CH_3)_2$ head group.

Conclusions

- Confident identification of DODAP, its oxidized form and its degradants was achieved with excellent mass accuracy using information-rich MS/MS data generated by EAD on the ZenoTOF 7600 system
- The process of automatically identifying, thoroughly determining the structure and relatively quantifying DODAP and its related impurities was expedited using Molecule Profiler software. This software efficiently annotated TOF MS/MS spectra by relying on suggested structures. Moreover, it automated the assignment of structures in EAD MS/MS data for DODAP and its impurities
- 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 DODAP

References

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