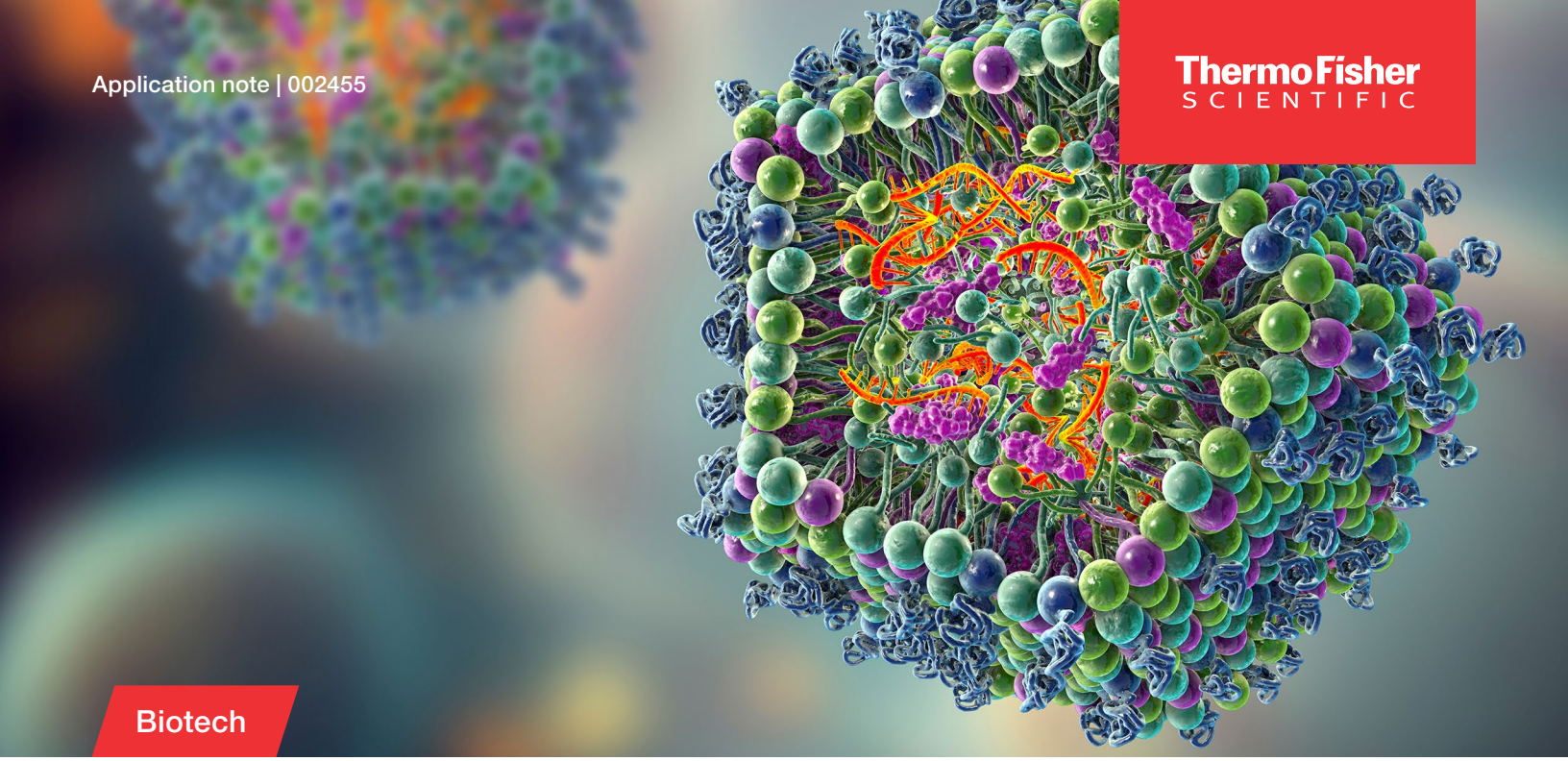
Application note | 002455



Biotech

Confident and sensitive profiling of lipid nanoparticle raw materials and impurity identification using a LC/CAD/HRAM-MS method with inverse gradient

Authors

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Lipid nanoparticles (LNP), PEGylated lipid analysis, pharmaceutical raw materials, impurity identification, structure elucidation, Orbitrap Exploris 120 mass spectrometer, high-resolution accurate mass (HRAM), charged aerosol detection (CAD), Vanquish Inverse Gradient LC system, Compound Discoverer software, Xtract deconvolution

Application benefits

- Confident detection of LNP raw materials and related impurities based on combination of CAD and HRAM-MS
- Benefit of HRAM-MS data to detect impurities co-eluting with the raw material peak
- Demonstrating the facile use of MS^2 fragmentation data and the Thermo Scientific™ Compound Discoverer™ software to elucidate the structure of impurities
- Characterization of a polydisperse PEGylated lipid using HRAM-MS data.

Goal

Demonstrate the capability of the Thermo Scientific™ Vanquish™ Inverse Gradient LC system with Charged Aerosol Detection (CAD) to sensitively detect and quantify raw material impurities, while simultaneously using the Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer to generate high-quality Full MS and MS^2 data to enable their structure elucidation.

Introduction

With their clinical success in COVID-19 vaccine formulations, lipid nanoparticles (LNPs) have moved to the forefront as a delivery vehicle for mRNA vaccines and therapies. The quality and efficacy of these therapies can be directly impacted by the LNP formulation, and as such, its attributes must be monitored carefully. In its regulatory considerations document published in 2021 on the quality, safety, and efficacy of messenger RNA vaccines, the World Health Organization (WHO) stated that "[t]he level of impurities associated with the [LNP] excipients should also be suitably controlled and justified" and their identity and purity to be considered critical quality attributes.[1]

While many different types of lipids have been explored for mRNA delivery, the majority of LNPs are made up of a combination of four lipid classes: (1) a cationic or ionizable lipid, beneficial for delivery efficacy; (2) a phospholipid, to increase stability of the nanoparticle; (3) cholesterol or derivatives thereof, which increase particle stability and biodistribution; and (4) a polyethylene glycol (PEG)-functionalized lipid, which can modulate particle size and increase stability by decreasing particle aggregation and increasing circulation time.[2,3]

Due to their non-chromophoric nature, the individual lipid constituents of LNPs and their respective identity and purity are typically monitored using CAD, which additionally allows for confident quantitation due to its uniform response. However, CAD alone cannot be used to confirm identity of the lipid raw materials or identify associated impurities. Additionally, the WHO stated that because "inclusion of a PEGylated lipid plays a critical role in providing *in vivo* stability and enhancing the cellular interaction of LNPs, adequate controls (for example, of molecular weight and polydispersity) should be in place for the PEGylated lipid."[1]

Here, we show the combined use of CAD and high-resolution accurate-mass mass spectrometry (HRAM-MS) for the detection and structure elucidation of representative LNP raw material impurities, as well as the characterization of the polydispersity of a PEGylated lipid.

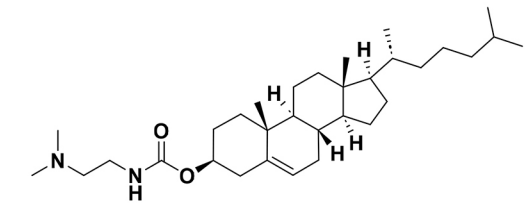
Experimental

List of reagents and consumables used

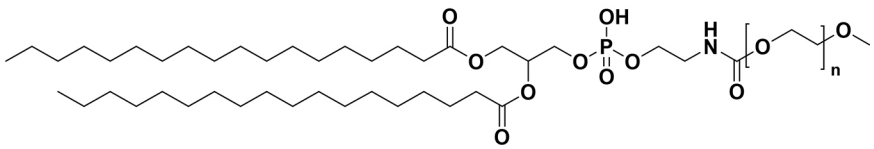
- Thermo Scientific™ Water, UHPLC grade, 1 L (P/N W81)
- Thermo Scientific™ Methanol, UHPLC grade, 1 L (P/N A4581)
- Fisher Chemical™ Isopropanol, Optima™ LC/MS grade, 1 L (P/N A461-1)
- Fisher Chemical™ Ammonium Formate, Optima™ LC/MS grade (P/N A11550)
- Thermo Scientific™ Absolute Ethanol, 200 proof, Molecular Biology grade (P/N T038181000)
- Thermo Scientific™ SureSTART™ Screw Glass Vial, 2 mL, Level 3 (P/N 6PSV9-1PSS)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps, Level 3 (P/N 6PSC9TST)

Sample preparation

Samples of (3β-[N-(N′,N′-dimethylamino-ethane)carbamoyl]-cholesterol (DC-Chol) were obtained from four separate vendors (labeled A-D hereafter) at specified purities ranging from 95% to 98+%. 1,2-Distearoyl-rac-glycero-3-phosphatidylethanolamine-N-methoxy-polyethyleneglycol-2000 (DSPE-mPEG) was obtained from vendor A with a specified purity of 98%. The structures of the two compounds are shown in Figure 1. All samples were obtained as powders and separately dissolved in ethanol and standard solutions prepared at 1 mg/mL for injection.



(3β-[N-(N′,N′-dimethylamino-ethane)carbamoyl]-cholesterol
(DC-Chol)



1,2-distearoyl-rac-glycero-3-phosphatidylethanolamine-N-methoxy-polyethyleneglycol-2000
(DSPE-mPEG)

Figure 1. Chemical structures of the lipids analyzed in this application note

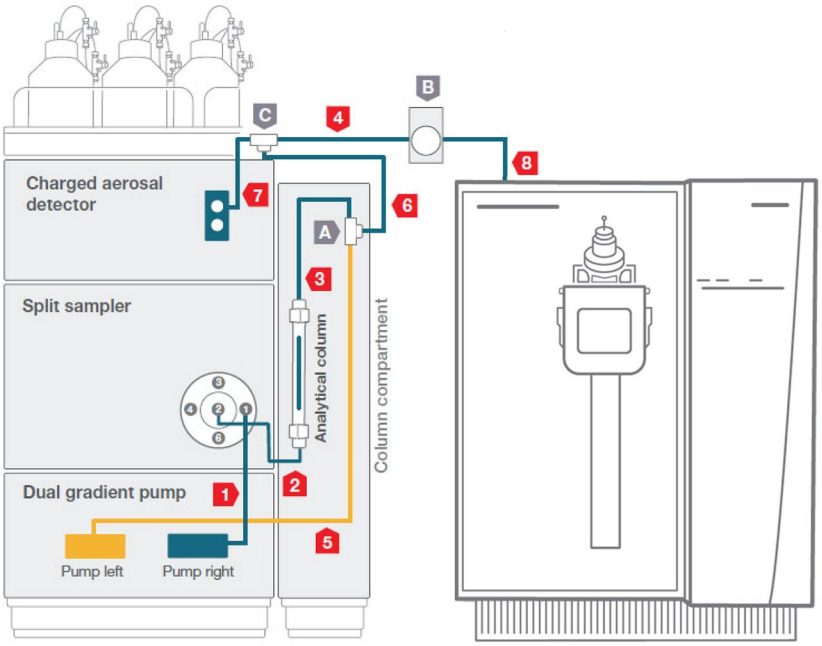
Sample analysis

The LC separation was performed using a Thermo Scientific Vanquish Inverse Gradient LC system, consisting of the following modules:

- Thermo Scientific™ Vanquish™ System Base (P/N VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Dual Pump F (P/N VF-P32-A-01)
- Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-03)
- Thermo Scientific™ Vanquish™ Charged Aerosol Detector H (P/N VH-D20-A)

The merged flow from the analytical and inverse gradient pumps was mixed using a Thermo Scientific™ Viper™ capillary mixer before being split between the CAD and MS. The flow path is detailed in Figure 2.

The MS data was acquired using a Thermo Scientific Orbitrap Exploris 120 mass spectrometer (P/N BRE725531). The LC/CAD/HRAM-MS analysis was carried out using the conditions listed in Tables 1 and 2.



No.	Connection between	Description
1.	Pump right outlet – Injection valve port 1	Viper capillary, ID × L 0.1 × 350 mm, MP35N (P/N 6042.2340)
2.	Injection valve port 2 – Column inlet	Active preheater, 0.1 × 380 mm, MP35N (P/N 6732.0110)
3.	Column outlet – T-piece A	Viper capillary, 0.1 × 250 mm, MP35N (P/N 6042.2330)
4.	Flow splitter C – Divert valve B	Viper capillary, 0.1 × 750 mm, MP35N (P/N 6042.2390)
5.	Pump left outlet – T-piece A	Viper capillary, 0.1 × 950 mm, MP35N (P/N 6042.2395) and nanoViper capillary, 75 µm × 650 mm (P/N 6041.5775) connected by Viper union (P/N 6040.2304)
6.	T-piece A – Flow splitter C	Viper capillary mixer, 25 µL, MP35N (P/N 6042.3020)
7.	Flow splitter C – CAD inlet	Viper capillary, 0.1 × 350 mm, MP35N (P/N 6042.2340)
8.	Divert valve B – MS inlet	Viper capillary, 0.1 × 300 mm, MP35N (P/N 6042.7950)
A.	T-piece	T-piece 500 µm ID (P/N 6263.0035)
B.	Divert valve	Divert valve for Orbitrap Exploris series, 2 position - 6 port, Rheodyne™ MXT715-004 (P/N 00109-99-00046)
C.	Flow splitter	T-Piece 150 µm ID, VICI™ Valco (P/N ZT1XCS6-M)

Figure 2. Inverse gradient setup schematic, with description of labeled flow path components

Table 1. UHPLC method parameters

Parameter	Value
Column	Thermo Scientific™ Hypersil™ GOLD C8, 1.9 µm, 2.1 × 50 mm (P/N 25202-052130)
Mobile phases	A: 5 mM Ammonium formate in 100% H_2O B: 5 mM Ammonium formate in 70% IPA/30% MeOH C: 100% IPA
Flow rate	0.5 mL/min
Column temperature	50 °C (still air mode)
Autosampler temperature	6 °C
Injection volume	1 µL
Needle wash	5 mM Ammonium formate in 70% IPA/30% MeOH, before and after draw
Divert valve timing	Flow to waste from 0–1 min
CAD settings	Evaporation temperature: 35 °C Power function: 1.00 Data collection rate: 5 Hz Filter: 3.6

Table 2. UHPLC gradient conditions

Time (min)*	Analytical gradient (Pump right)		
	%A	%B	%C
0	60	10	30
4	20	30	50
6	10	40	50
7	10	40	50
7.1	60	10	30
12	60	10	30
Time (min)*	**Inverse gradient (Pump left)**		
	%A	%B	%C
0.58	0	30	70
4.58	30	10	60
6.58	50	0	50
7.58	50	0	50
7.68	0	30	70
12.58	0	30	70

*Inverse gradient delay time determined empirically, 0.58 min

Mass spectrometry data from the Orbitrap Exploris 120 mass spectrometer was acquired with a Thermo Scientific™ OptaMax™ NG H-ESI ion source. Untargeted impurity characterization experiments on the lipid samples were carried out using single polarity Top 4 data-dependent MS^2 ($ddMS^2$) experiments. The MS source conditions and important MS experiment parameters are detailed in Tables 3 and 4.

Table 3. MS source conditions

Parameter	Value
Spray voltage	+3,250 V / –3,000 V
Sprayer position	1.5, M/H, center
Vaporizer temperature	300 °C
Ion transfer tube temperature	350 °C
Sheath gas	50 a.u.
Aux gas	10 a.u.
Sweep gas	1 a.u.

Table 4. FullScan - $ddMS^2$ method parameters

Parameter	Value
MS^1 resolution	120,000 @ *m/z* 200
MS^1 mass range	*m/z* 200–2000
RF level, %	70
Easy-IC	Scan-to-Scan
MS^2 isolation window (*m/z*)	1.5
HCD collision energies (Normalized, %)	10, 20, 30 (stepped)
MS^2 resolution	30,000 @ *m/z* 200
Maximum injection time (ms)	Auto
Intensity threshold	1.0e5
Dynamic exclusion	Custom, 8 s, exclude isotopes, 5 ppm mass tolerance

Data analysis and processing software

Thermo Scientific™ Xcalibur™ 4.5 software was used for data acquisition, and Thermo Scientific™ Freestyle™ 1.8 SP2 software was utilized for initial data review. Qualitative and quantitative analysis of the CAD trace data was carried out in Thermo Scientific™ Chromeleon™ 7.3.2 Chromatography Data System (CDS) software. Thermo Scientific™ Compound Discoverer™ 3.3 SP2 software was used for spectral deconvolution and peak detection of the MS data and correlation to the CAD peaks from the analog trace data, as well as compound identification using an adapted version of the default workflow template “Impurity ID w Stats Related and Unknown” without Compound Class scoring. Related compounds were generated using the Expected Compounds node based on the parent structure and the following transformations: dealkylation, dehydration, hydration, oxidation, reduction, methylation, and demethylation, as shown in Figure 3a. Additionally, deconvolution of the mass spectral data for multiply charged species in DSPE-mPEG was carried out using the Xtract algorithm in Freestyle 1.8 SP2 software using the settings shown in Figure 3b.

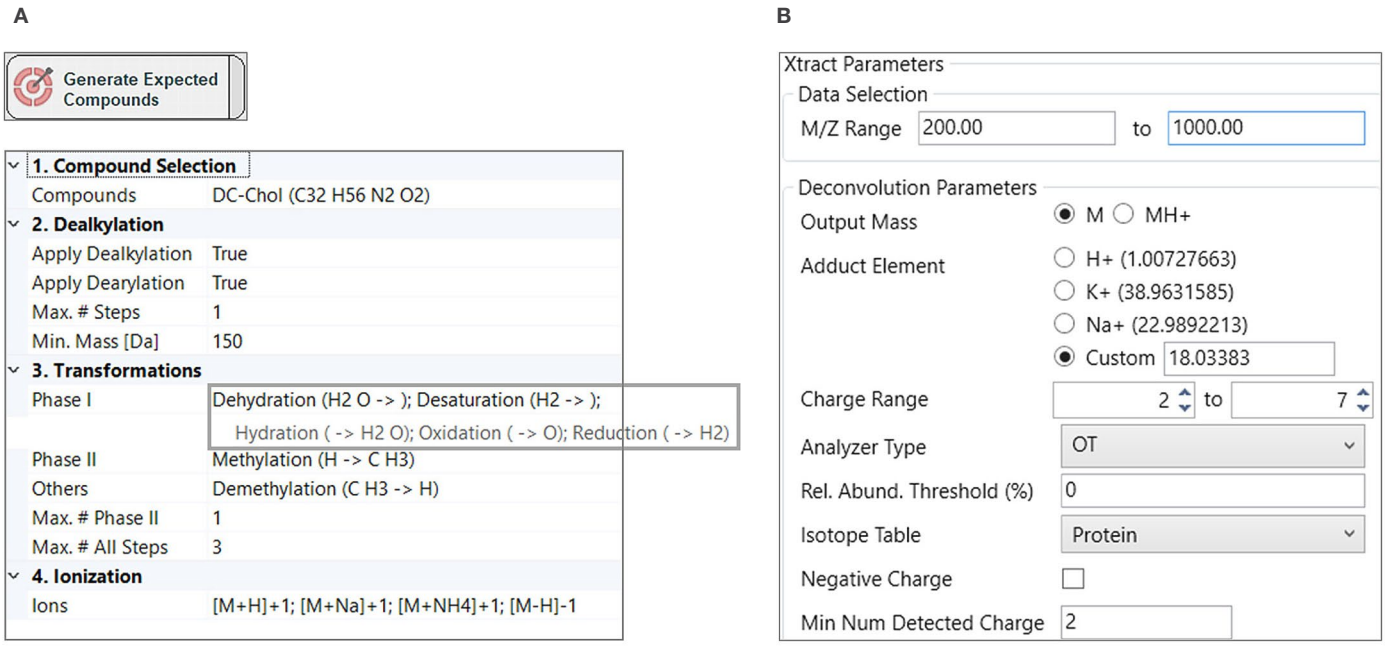


Figure 3. (A) Parameters of the 'Expected Compounds' node used in the Compound Discoverer software and (B) Xtract algorithm settings used in the data analysis of DSPE-mPEG with the Freestyle software

Results and discussion

For separation and impurity profiling by HPLC-CAD of the lipid raw materials investigated here, as well as others, a 7-minute tertiary gradient separation using ammonium formate buffer in water/methanol/isopropanol and a Hypersil GOLD C8 column has been developed previously.[4] This method was adapted to include an inverse gradient for improved uniformity of the CAD response, as detailed elsewhere.[5]

For the instrument and flow path configuration detailed in Figure 2, an inverse gradient offset volume of 290 µL (corresponding to an inverse gradient delay time of 0.58 min) was determined empirically by sending a pulse of isopropanol through the left and then the right pumps and determining the difference in arrival time at the CAD. Additionally, the "maximize %C" option was chosen in the method setup wizard to increase sensitivity of the CAD response, which is greater with higher organic content in the mobile phase.[5]

The resulting CAD and MS total ion chromatograms for the analysis of one of the vendor's materials for both DC-Chol and DSPE-mPEG are shown in Figure 4, together with a UV trace collected separately to show the lack of UV absorption for both lipids.

The raw data from the experiments were processed using the Qualitative workflow in Chromeleon 7.3.2 CDS to detect CAD peaks after automatic background subtraction of a solvent blank injection. Then, unbiased peak detection from the MS data was carried out using the Compound Discoverer software to correlate compounds detected in the MS data to the CAD peaks. In addition, targeted component detection for expected transformation products of the parent lipids could be carried out with the Compound Discoverer software as well. Based on the results of the two, the detected impurities in the different vendors' materials could be identified, as detailed hereafter.

Impurity profiling of DC-Chol

As shown in Figure 5, four impurity peaks (labeled 1–4) could be detected in the CAD data for the DC-Chol samples.

The identification of the compounds related to the impurity peaks was primarily facilitated by the expected compound workflow in the Compound Discoverer software. This approach, which is described in more detail in a previous application note, is based on targeted detection of impurities generated by common reactions from the expected parent compound, such as desaturation, demethylation, and others.[6]

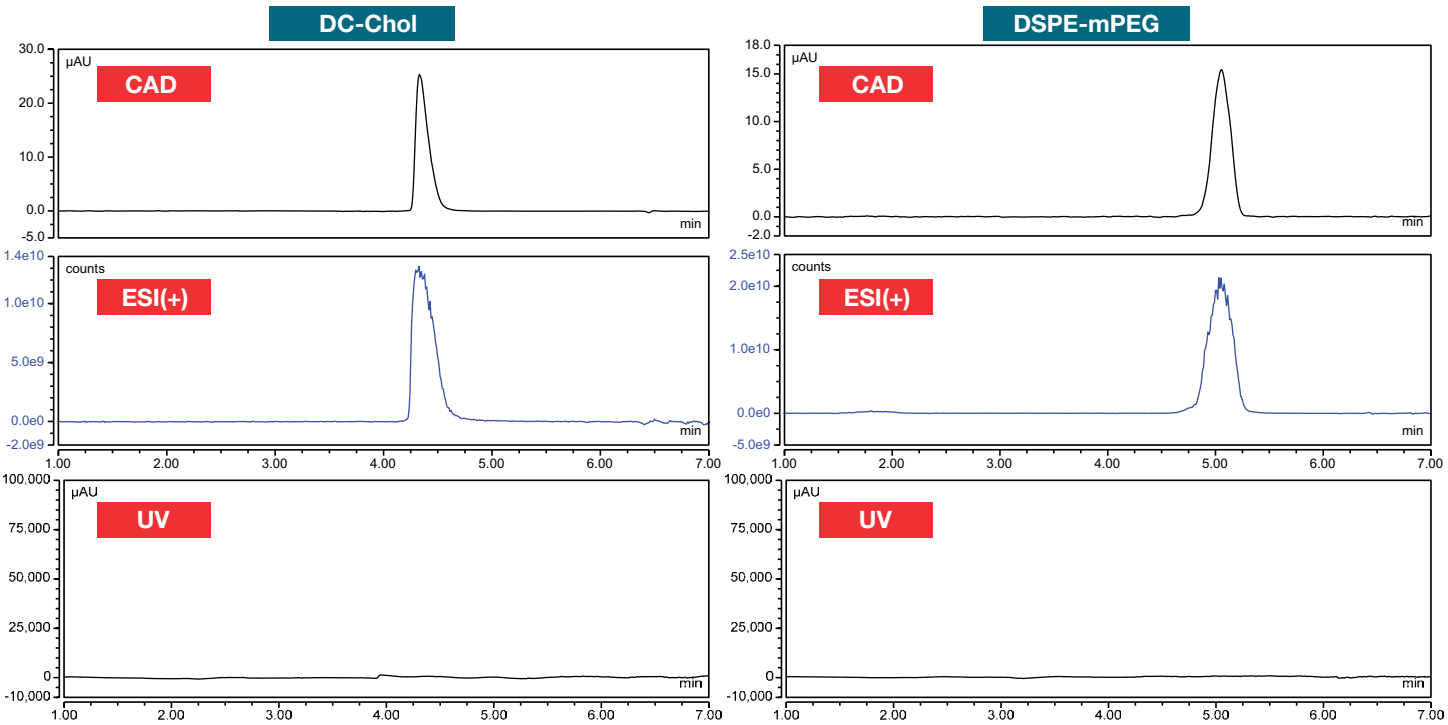


Figure 4. Representative LC/CAD/HRAM-MS chromatograms of DC-Chol and DSPE-mPEG from vendor A at 1 mg/mL concentration showing the correlation between CAD and MS peaks, and lack of signal in LC/UV analysis

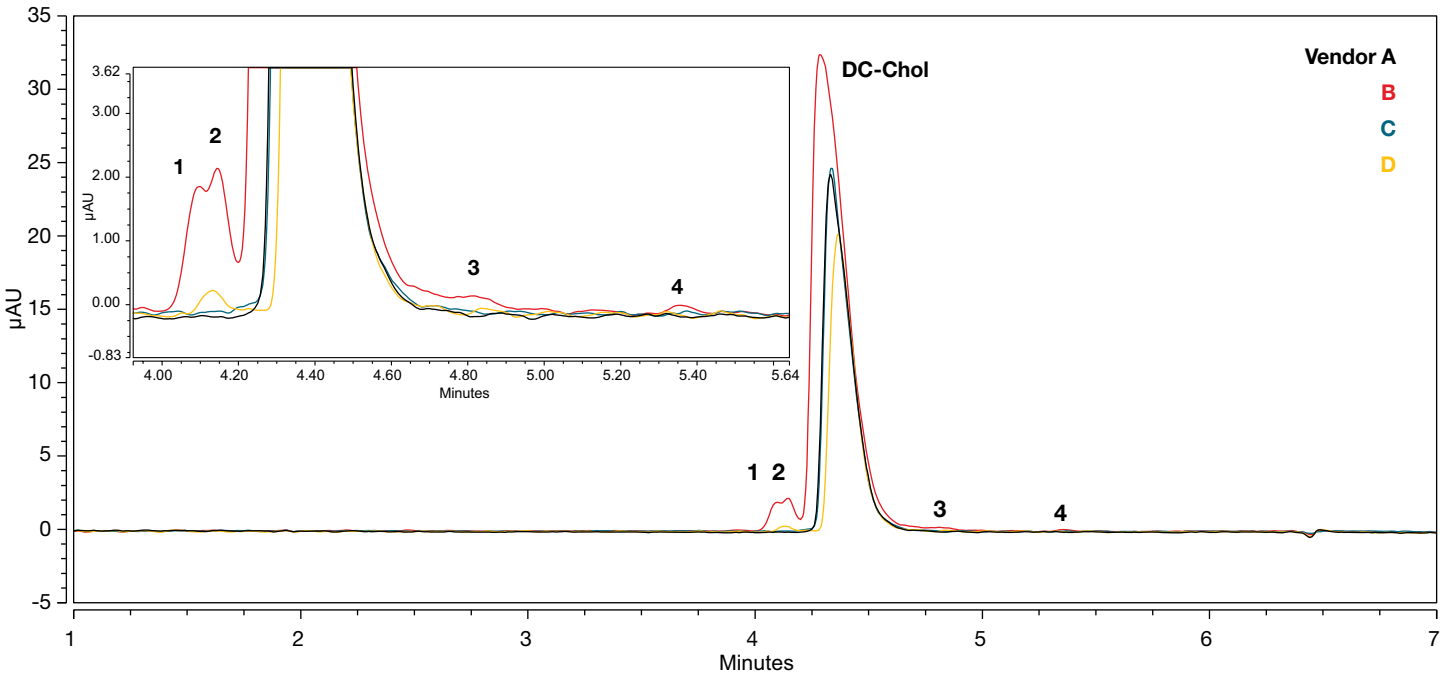
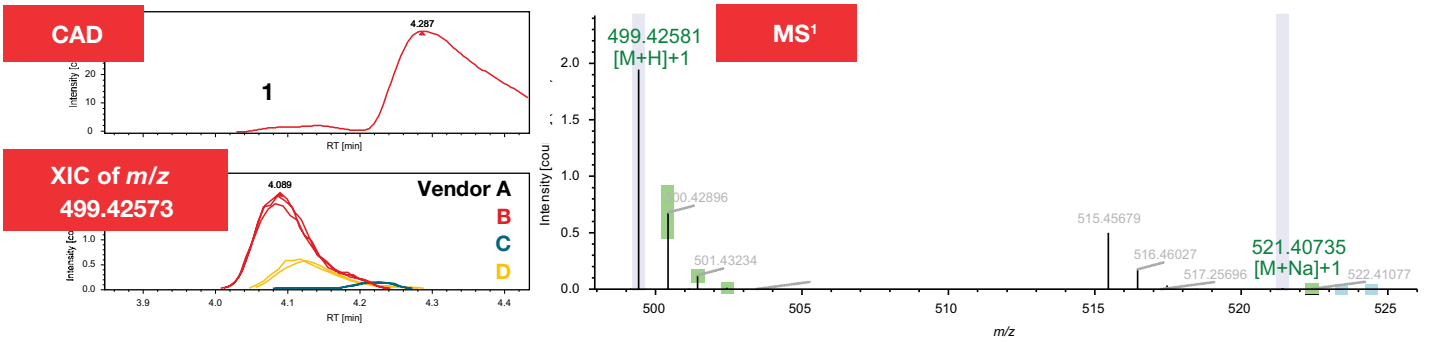


Figure 5. Overlay of the background-subtracted CAD traces for the DC-Chol raw material samples from vendors A–D, with inlaid detail of detected impurity peaks

Impurity **1** was found to have a composition change of "$-H_2$", characteristic of a desaturation. As highlighted in Figure 6, the automated fragment ion search (FISh) annotation of the MS^2 spectrum based on *in silico* prediction of fragments from the parent molecule allowed localization of the desaturation to the cholesterol moiety. In comparison to the parent compound, a shift of 2 Da was observed for the main cholesterol fragment at *m/z* 367, while the DC fragment was unchanged at *m/z* 133.



Parent Compound	Dealkylated	Transformations	Composition Change	Formula	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]
DC-Chol		Desaturation	-(H2)	C32 H54 N2 O2	-0.15	498.41845	499.42573	4.096

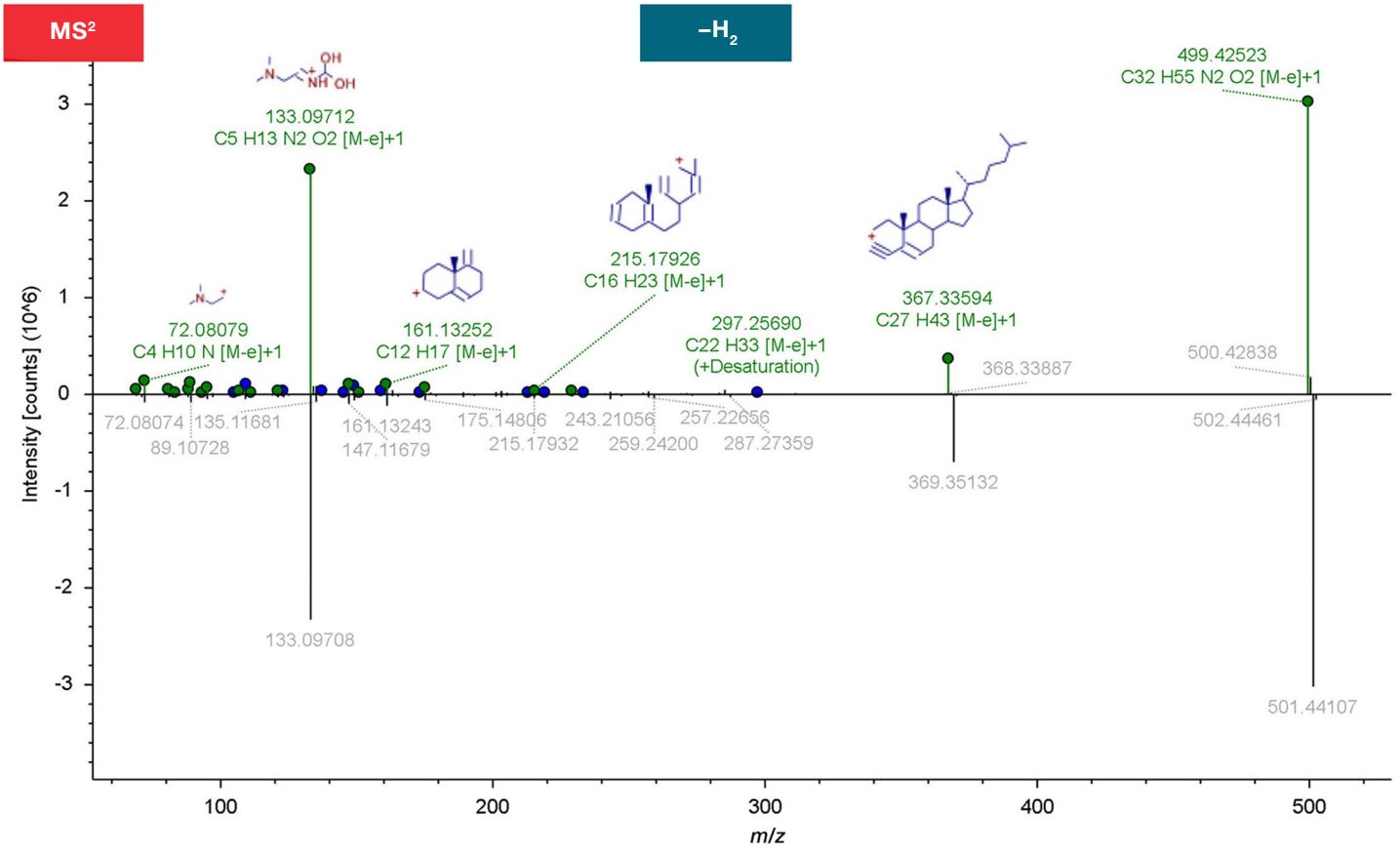


Figure 6. Correlation of CAD impurity peak 1 to the MS component for the desaturation transformation in Compound Discoverer 3.3 software, with the shifted fragment at *m/z* 367 compared to the parent compound allowing the localization of the additional double bond to the cholesterol moiety

In addition to impurity **1**, impurity **4** was also found to result from a desaturation. However, as detailed in Figure 7, the fragmentation spectrum differed due to the difference in transformation sites. Based on the shifted fragment ion at *m/z* 131 and the unchanged cholesterol fragment at *m/z* 369, the additional double bond of impurity **4** was determined to be located on the cationic DC moiety.

Beyond the capability to determine structural information, the other significant benefit of HRAM-MS data for the impurity profiling of LNP raw materials can be seen in the analysis of impurity peak **2**. The correlation of the CAD peak to compounds detected with Compound Discoverer software revealed the existence of three co-eluting impurities, as highlighted in Figure 8. Specifically, impurities matching both methylation and demethylation, as well as the addition of a chloromethyl group (found using the untargeted peak detection workflow) could be detected in the material from vendor B. In all three cases, the MS^2 spectra allowed localization of the modification to the DC moiety.

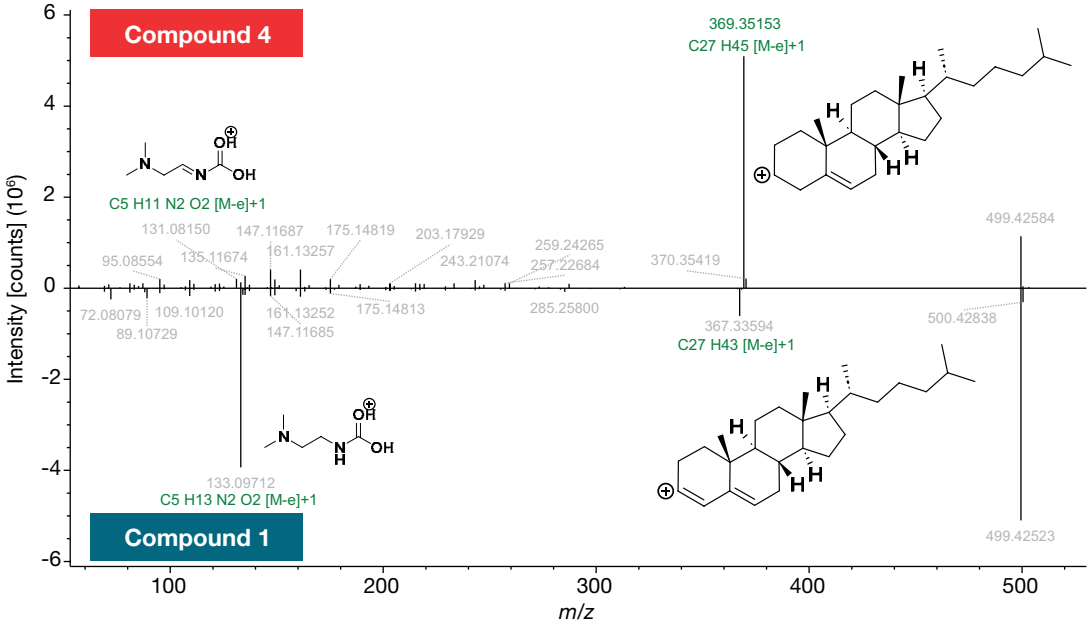


Figure 7. Differences in fragmentation spectra of isomeric impurities 1 and 4 allowing the determination of the respective transformation sites

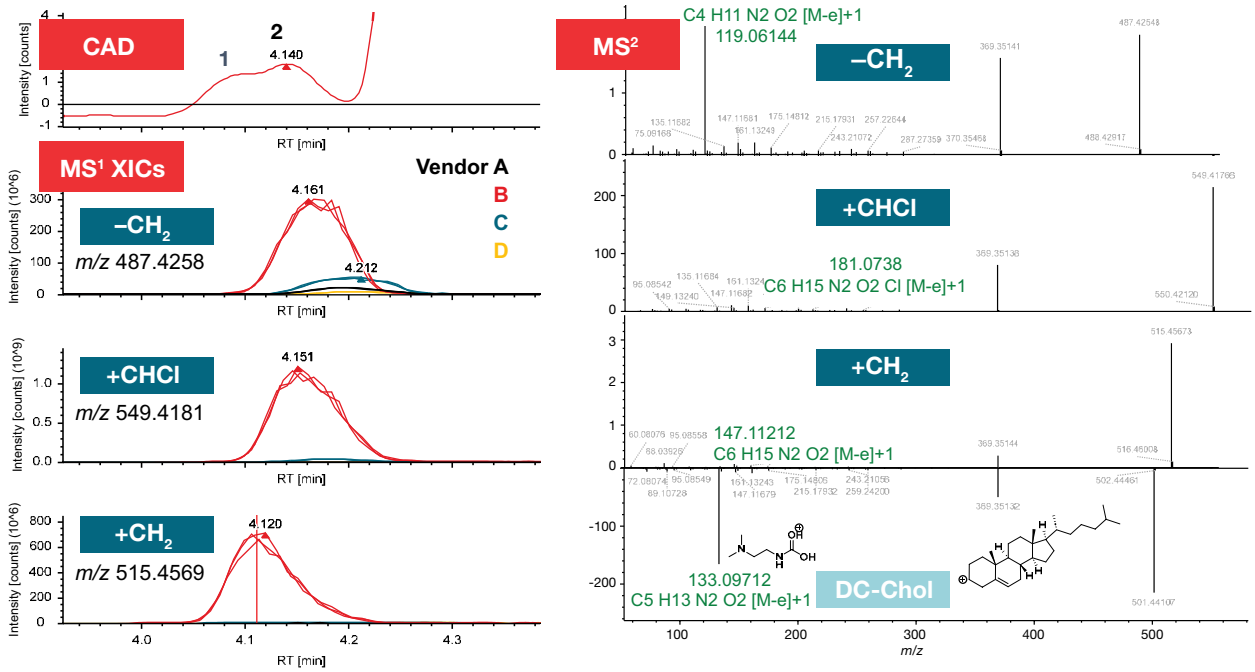


Figure 8. MS data correlated to impurity peak 2 in the CAD data reveals three co-eluting impurities with the MS^2 spectra indicating the respective transformations on the DC group

Investigation of impurity **3** revealed it to also correspond to a "+CH2" modification, with the modification found to be localized to the DC moiety, based on its MS^2 spectrum. However, the MS^2 spectrum differed from the isomeric impurity **2c**, as shown in Figure 9. While both spectra included the transformation shifted fragment at *m/z* 147, the detail view at the bottom of the figure reveals differences in lower *m/z* fragments, which can be explained by the different methylation position on either the quaternary amine for **2c** or the carbamoyl nitrogen for **3**.

In addition to the above impurities, which were detected in the CAD traces, the MS data allowed for the detection and identification of impurities that were co-eluting with the parent compound, and were thus obscured in the CAD trace, as shown in Figure 10. Namely, both saturation and desaturation modifications as well as an oxidized analog of DC-Chol could be detected. Using the MS^2 spectra, shown on the right of Figure 8, the site of modification could be determined for all three compounds, as seen from the mass-shifted fragments highlighted in blue. The complete results of the impurity profiling for DC-Chol are summarized in Table 5.

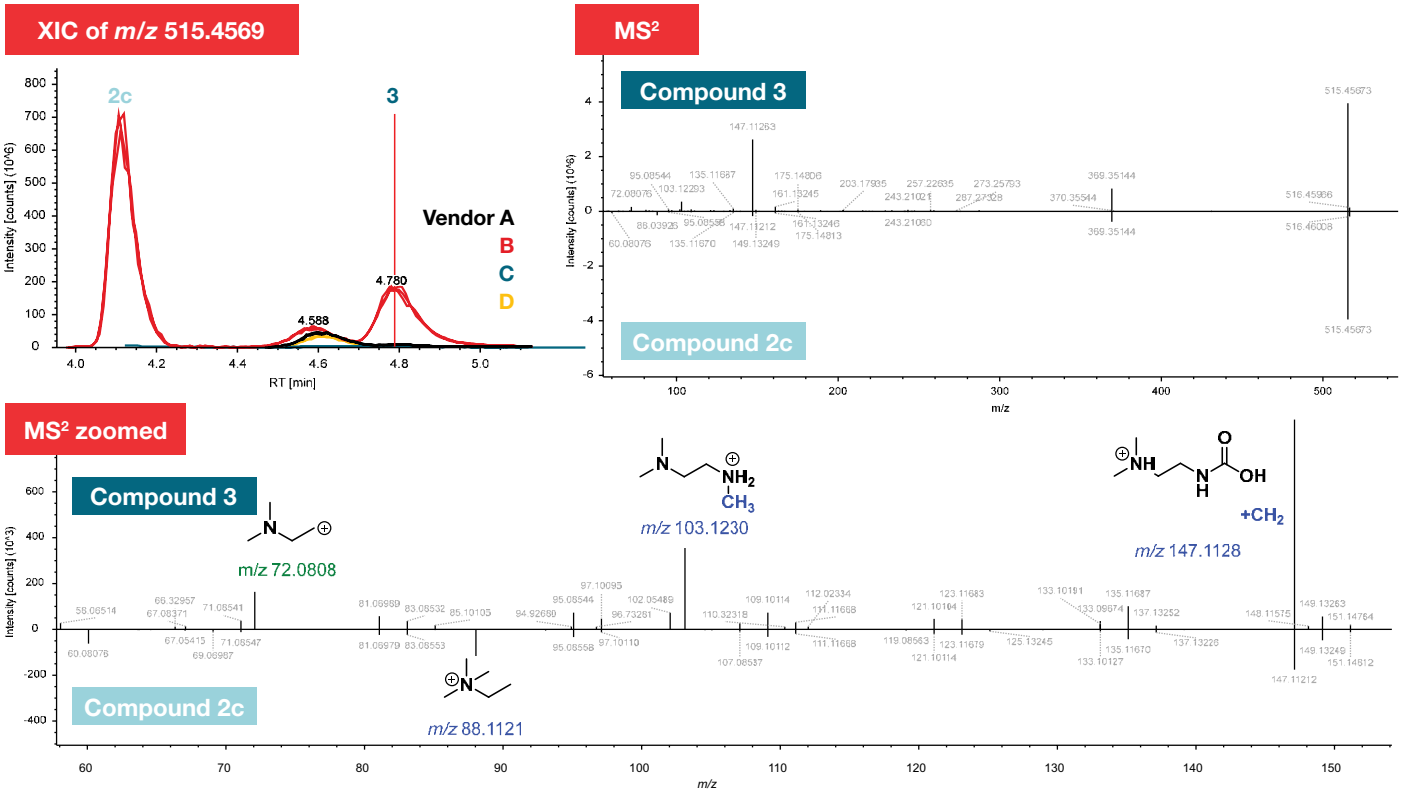


Figure 9. Comparison of isomeric impurities 3 and 2c based on their XIC and fragmentation spectra, indicating their difference in methylation sites based on unique low *m/z* fragment ions

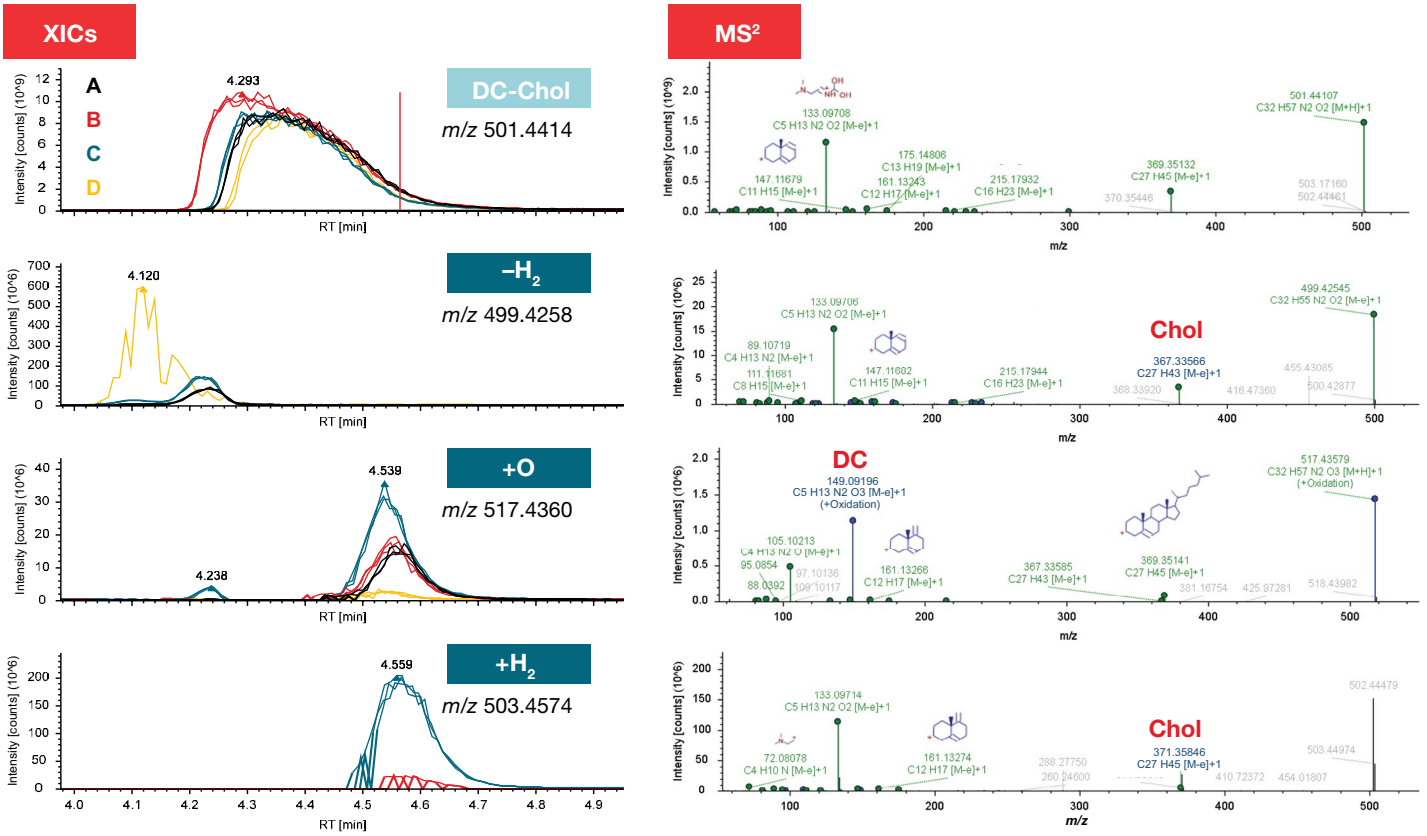


Figure 10. Detection of closely eluting trace impurities of DC-Chol from the MS data that were not detected in the CAD trace due to masking by the dominant main compound, and elucidation of the modification site based on the fragmentation spectra with transformation-shifted fragments highlighted in blue

Table 5. Summary of the impurity profiling results for the analyzed DC-Chol samples from vendors A–D

CAD peak #	RT (min)	MS compound			%Total CAD peak area by vendor			
		m/z	Mass accuracy (ppm)	Identity	A	B	C	D
1	4.08	499.4258	–0.15	$-H_2$ (Chol)	0.0	1.9	0.0	0.0
2	4.14	487.4258	–0.09	$-CH_2$ (DC)	0.0	2.2	0.0	1.0
		549.4180	–0.15	+CHCl (DC)				
		515.4569	–0.46	$+CH_2$ (DC)				
DC-Chol	4.34	501.4414	–0.24	DC-Chol	100.0	95.7	100.0	99.0
3	4.78	515.4569	–0.46	$+CH_2$ (DC)	0.0	0.1	0.0	0.0
4	5.35	499.4258	+0.03	$-H_2$ (DC)	0.0	0.1	0.0	0.0
-	4.23	499.4258	–0.03	$-H_2$ (Chol)	-	-	-	-
	4.55	517.4360	–0.65	+O (DC)				
	4.57	503.4574	+0.52	$+H_2$ (Chol)				
				Vendor specified purity of raw material	98+%	95+%	98+%	98+%

Characterization of DSPE-mPEG

As detailed by others, PEGylated lipids serve a critical role in the LNP formulation to modulate particle size and polydispersity, as well as stability.[3] However, from an analytical development and CMC standpoint, this class of lipids creates unique challenges, in part due to the variability in the PEG chain length. The polydispersity of the PEG moiety itself and its average molecular weight can potentially vary between different vendors and lots. As shown in Figure 11, the DSPE-mPEG sample analyzed here did not contain any significant impurities based on the CAD measurement.

Using the Xtract algorithm in the Freestyle 1.8 software, the multiply charged ammonium adducts of the DSPE-mPEG peaks at 5.05 min could be deconvoluted to generate the monoisotopic mass distribution shown in Figure 12, which revealed the material to have a Gaussian distribution of compounds with differing amounts of PEG units. As illustrated in the figure for the most abundant variant of DSPE-mPEG with 44 PEG units, excellent accuracies could be achieved in their mass measurement using the Orbitrap Exploris 120 MS. Additionally, the DSPE substructure could be confirmed with the MS^2 spectrum that revealed the characteristic distearoyl glyceride ion at *m/z* 607 (Figure 12C).

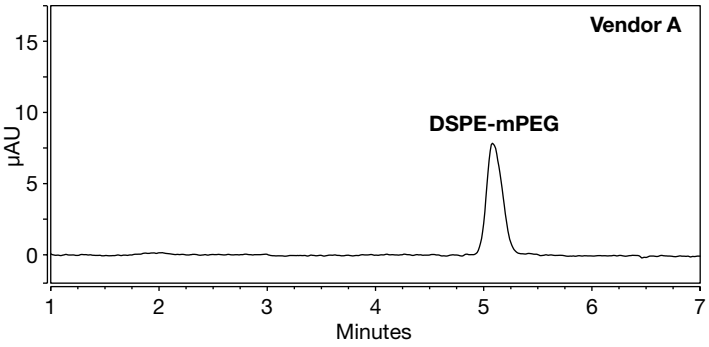


Figure 11. Background-subtracted CAD trace for the DSPE-mPEG raw material sample from vendor A, showing the main component eluting at 5.05 min

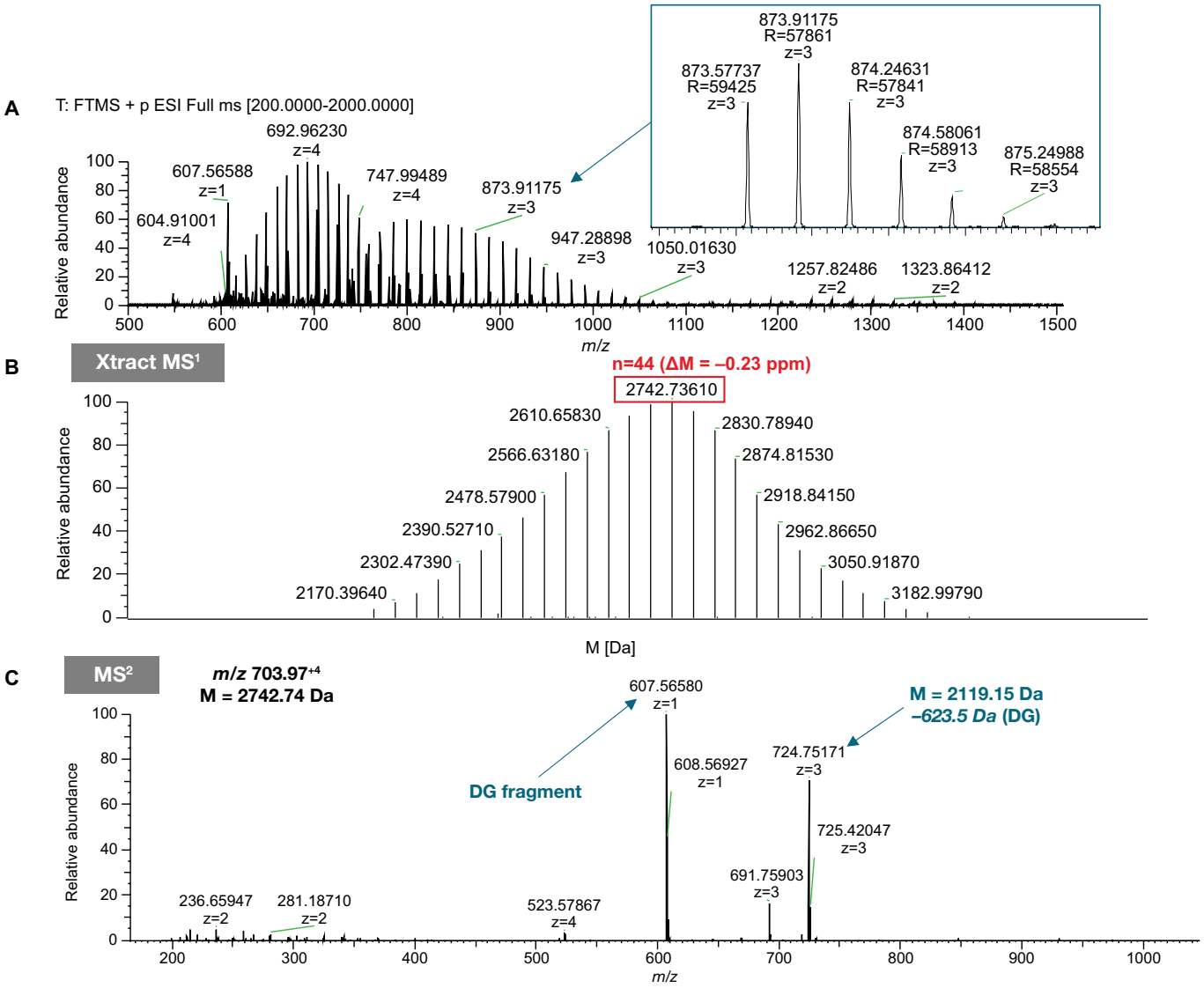


Figure 12. (A) Averaged mass spectrum of DSPE-mPEG from 4.8–5.4 min for vendor A, (B) deconvoluted mass spectrum showing the different polydispersity of the PEG chain lengths, and (C) evidence of the distearoyl glyceride in the fragmentation spectrum of the +4 ion of the most abundant species at MW 2742.74 Da

Conclusions

As demonstrated in this application note, different vendors' raw materials can vary significantly in their impurity profiles. As such, the impurity profiling and identification for lipid raw materials is an essential part of LNP formulation development.

- The combination of the Vanquish Inverse Gradient LC system with CAD and Orbitrap HRAM-MS allows for confident detection of LNP raw material and related impurities for quantitation and identification.
- The utility of Orbitrap HRAM-MS to distinguish and identify co-eluting impurities was demonstrated.
- Differences in the structure of isomeric impurities could only be revealed from MS^2 fragmentation data, which enabled their structure elucidation with tools available in Compound Discoverer software.
- The detection of the different molecular species of DSPE-mPEG as multiply charged adducted ions allowed the determination of their molecular masses and revealed the polydispersity of the PEGylated lipid material.
- Investigation of the fragmentation spectra allowed the identification of likely modifications for all impurities and, importantly, allowed the localization of their transformation site to trace their origin.

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