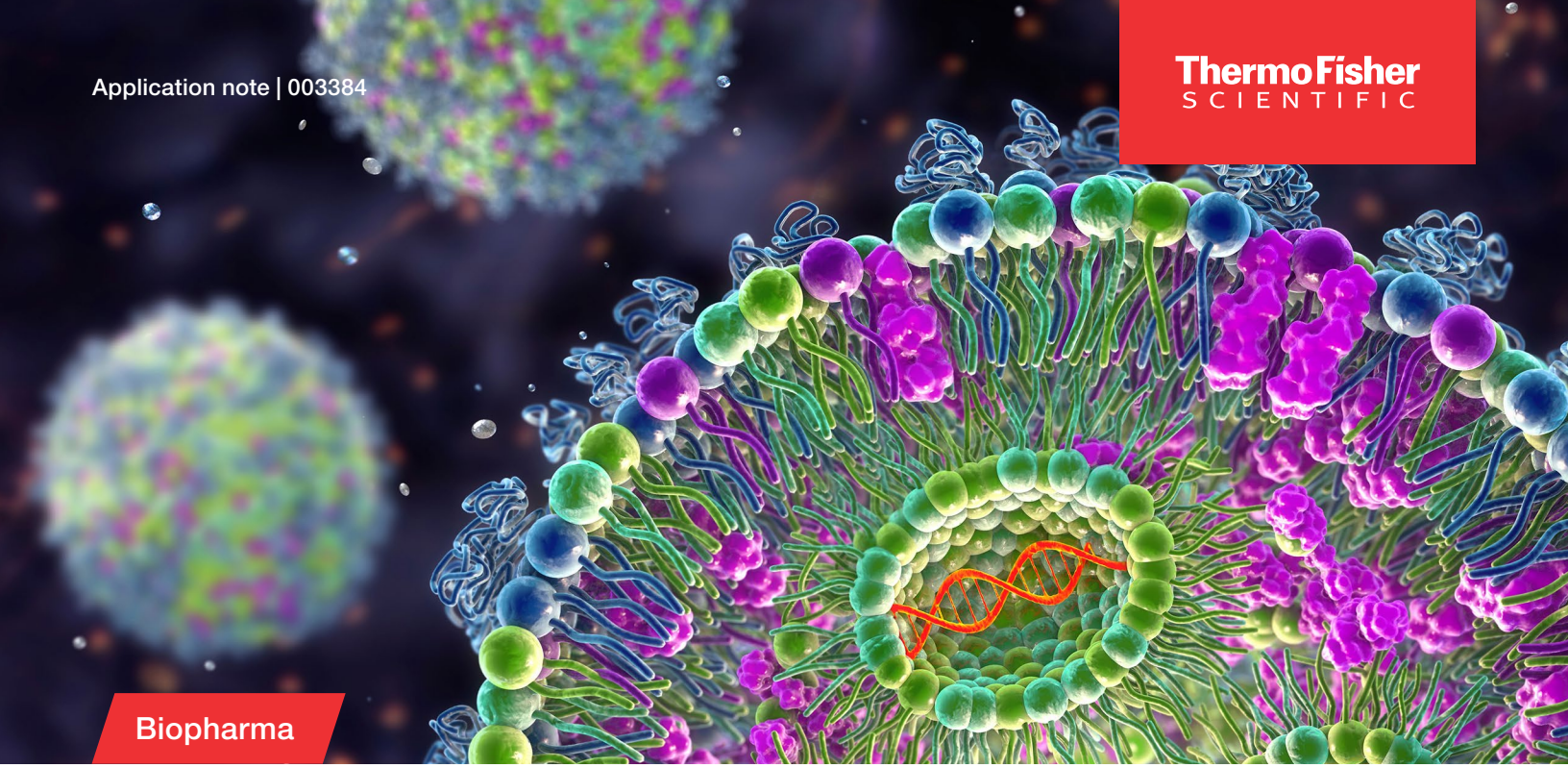
Application note | 003384



Biopharma

Quantifying impurities in cationic lipids raw materials with the inverse gradient method using LC-CAD-MS

Authors

Sissi White[1], Sven Hackbusch[2], Mark Netsch[1], Min Du[1]

[1]Thermo Fisher Scientific, Lexington, MA, USA

[2]Thermo Fisher Scientific, San Jose, CA, USA

Keywords

Cationic lipid, ionizable lipid, lipid nano particle (LNP), Hypersil GOLD C8 column, Charged Aerosol Detector (CAD), single quadrupole mass spectrometer (ISQ)

Application benefits

- Enable the quantification of impurities even in the absence of specific reference standards
- Use a single quadrupole mass spectrometer to match impurities based on their retention time and molecular masses
- Use an inverse gradient with Charged Aerosol Detector (CAD) to enable accurate surrogate quantitation when there is large difference in retention times between the lipid standard and its impurities, even in the absence of impurity standards
- Confirm the unit mass of both the lipid standard and its associated impurities using the correlation of CAD and ISQ channels

Goal

- Provide instructions, methodologies, and recommended techniques to improve quantification of impurities in cationic lipid components
- Use the Thermo Scientific™ Vanquish™ Flex Inverse Gradient UHPLC System coupled to a Thermo Scientific™ Vanquish™ Charged Aerosol Detetor to provide more accurate estimated quantitation of cationic lipid impurities without the need for reference standards

thermo scientific

Introduction

The identity, ratio, and purity of the lipid components in lipid nanoparticle (LNP) formulations are regarded as critical quality attributes that need to be well characterized and routinely monitored during development, manufacturing, QC release, and stability testing to ensure safety and efficacy. For instance, the European Medicines Agency assessment report detailed the attributes in the specification for mRNA-1273 LNP intermediate and finished product release testing to include: lipid identity, lipid content, and lipid impurities by UHPLC-CAD.[1] Cationic lipids play a crucial role in neutralizing negative charges of the phosphate group on the nucleic acids, promoting encapsulation and facilitating cellular uptake in lipid nanoparticle (LNP) systems.[2] Cationic lipids are amphiphilic molecules that possess a hydrophilic region, a hydrophobic region, and a linker structure connecting the two.[2] Given the significance of cationic lipids in LNP nucleic acid formulations, the impurity level of these lipids becomes equally important to ensure product quality. However, it is often difficult to quantify unknown impurities due to lack of individual reference standards.

In this work, an inverse gradient method is described for quantifying the impurities of the raw materials by using a reference standard as single calibrant. Charged aerosol detection is a mass sensitive detection technique with near universal response for all non-volatile lipids, provided that the eluent composition (%gradient) remains constant.[3] The final method enables accurate measurements with a wide dynamic range, reducing the need for extensive sample preparation. To demonstrate the method's utility, it was applied to three cationic lipids commonly found in LNP formulation: R-DOTAP, DLin-KC2-DMA, and ALC-0315.

The chromatography system used was a Vanquish Flex Inverse Gradient UHPLC System with integrated biocompatibility to provide inverse gradient and resolution for valuable biomolecules. The column used was a Thermo Scientific™ Hypersil GOLD™ C8 column, which was chosen for its outstanding selectivity and excellent peak shape for the analyzed lipids. A monoisotopic mass conformation of the detected impurities in these raw materials was further performed using a Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Spectrometer. An inverse gradient method, including wizard-guided steps for method creation and report generation, is employed using Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software. Figure 1 shows the summary of the inverse gradient method layout.

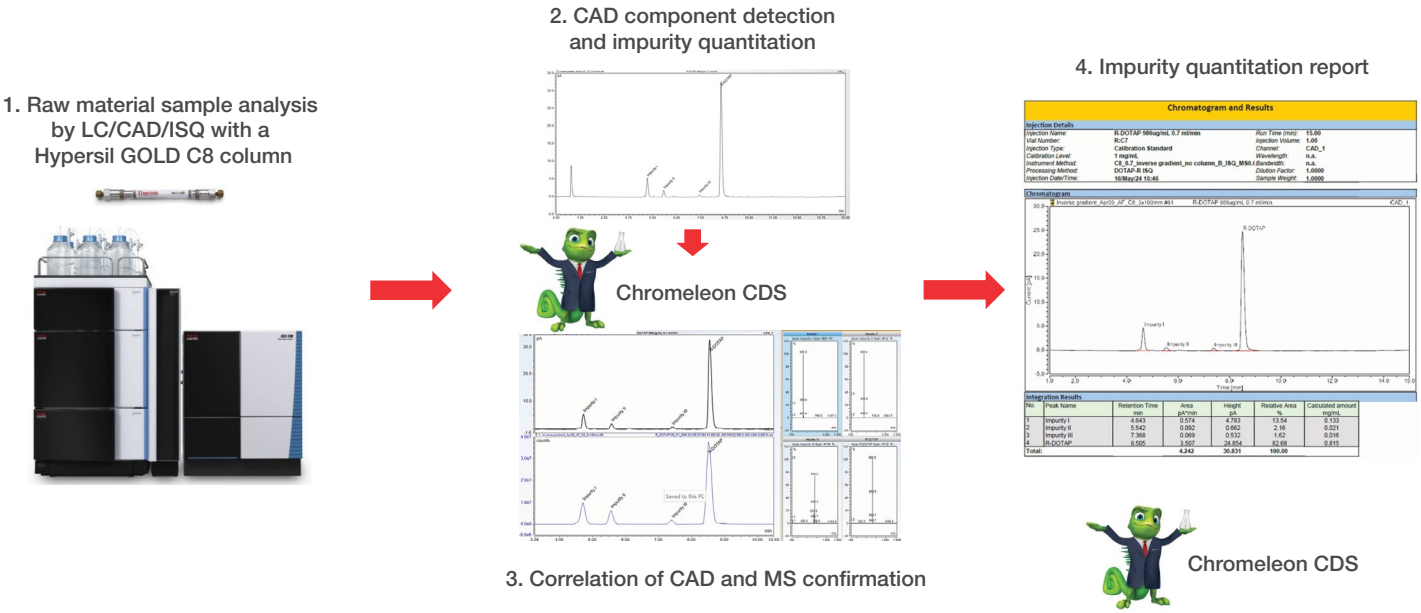


Figure 1. Summary of the inverse gradient method layout and data processing workflow within Chromeleon CDS

Experimental

Chemicals

- Fisher Scientific™ Water, Optima™ LC/MS grade (P/N W64)
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A461-4)
- Fisher Scientific™ Methanol, Optima™ LC/MS grade (P/N A456-4)
- Ammonium formate 10 M in H_2O, BioUltra, Sigma-Aldrich (P/N 78314)

Sample handling

- Fisher Scientific™ Fisherbrand™ Ultrasonic Cleaner (P/N FS60D)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-151)
- Fisher Scientific™ Fisherbrand™ Disposable Controlled Drop Pipets (P/N 13-678-30)
- Fisher Scientific™ Fisherbrand™ Pipets (P/N 13-678-25D)
- Thermo Scientific™ SureSTART™ 2 mL GOLD-Grade Glass Screw and Crimp Top Autosampler Vials (P/N 6PSV9-1PG)

Sample preparation

Each lipid was individually dissolved in 100% methanol and vortexed. The final concentration of each lipid was approximately 1 mg/mL.

Lipid	Formula weight	Concentration (mg/mL)
R-DOTAP	698.5	0.99
DLin-KC2-DMA	642.1	1.00
ALC-0315	766.3	1.00

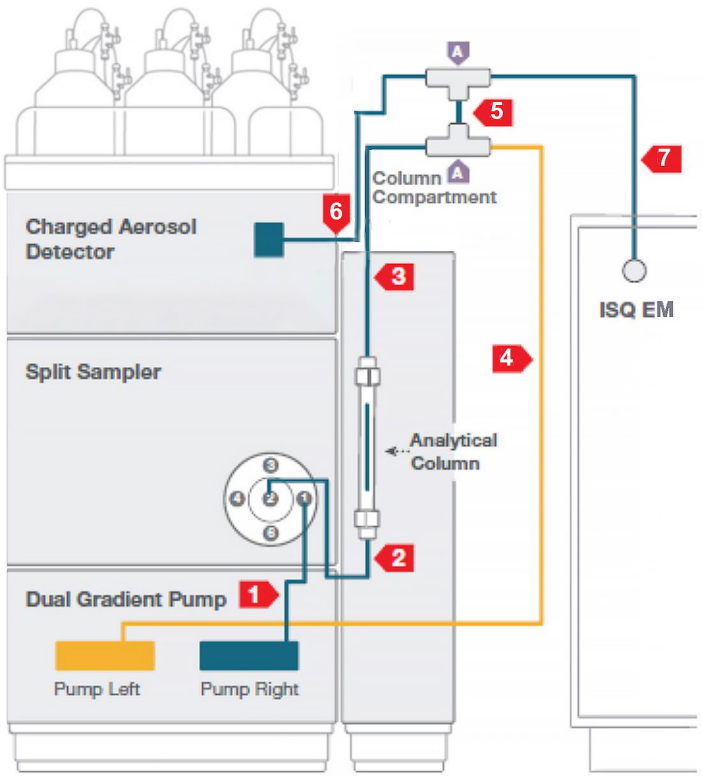
Liquid chromatography

Instrumentation

Vanquish Flex Inverse Gradient UHPLC system consisting of:

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

Inverse gradient system setup



No.	Connection between	Description
1	Pump right outlet – Injection valve port 1	Thermo Scientific™ Viper™ capillary, ID × L 0.10 × 350 mm, MP35N, P/N 6042.2340
2	Injection valve left port 2 – Column inlet	Vanquish Active pre-heater, 0.10 × 380 mm, MP35N, P/N 6732.0110
3	Column outlet – T-piece	Viper capillary, ID × L 0.10 × 550 mm, MP35N, P/N 6042.2360
4	Pump left outlet – T-piece	Viper capillary, ID × L 0.10 × 950 mm, MP35N, P/N 6042.2395
5	T-piece – T-piece	Viper capillary mixer, 25 µL, MP35N, P/N 6042.3020
6	T-piece – Charged Aerosol Detector inlet	Viper capillary, ID × L 0.10 × 550 mm, MP35N, P/N 6042.2360
7	T-piece – MS inlet	Viper capillary, ID × L 0.10 × 350mm, MP35N, P/N 6042.2340
No.	**Additional part**	**Description**
A	T-piece	Standard 500 µm ID, P/N 6263.0035

Figure 2. Schematic diagram of inverse gradient setup

Mobile phase preparation

For mobile phase A, 500 µL ammonium formate (10 M) was added into 500 mL water and 500 mL acetonitrile. For mobile phase B, 500 µL was added into 1000 mL methanol. Mobile phase A and B were degassed for 5 min using an ultrasonic bath. In the conventional gradient (CG) method, the baseline may exhibit baseline drift if the mobile phase is not degassed, but baseline drift was not observed when using the inverse gradient (IG) method. To ensure a fair comparison, the sonication procedure was performed for both methods.

Chromatographic conditions

Table 1. Chromatographic conditions

Parameter	Value
Column	Hypersil GOLD C8 column, 3.0 × 100 mm, 3 µm, P/N 25203-103030
Mobile phases	A: 5 mM ammonium formate in 50% acetonitrile/50% water B: 5 mM ammonium formate in 100% methanol
Analytical gradient	Time (min) % A % B 0.0 70 30 1.0 50 50 6.0 10 90 10.0 1 99 12.0 1 99 12.1 70 30 15.0 70 30
Inverse gradient	Time (min) % A % B 0.829 1 99 1.829 21 79 6.829 61 39 10.829 70 30 12.829 70 30 12.929 1 99 15.0 1 99
Flow rate	0.7 mL/min for both analytical and inverse gradients
Column temperature	50 °C
Autosampler temperature	8 °C
Autosampler wash solvent	Mobile phase B
Injection volume	1 µL
CAD detector settings	Power function: 1.0 Evaporator temperature: 35 °C Data rate: 10 Hz Filter: 5.0 s

The CG method used the analytical gradient only and the IG method used both the analytical gradient and inverse gradient.

Mass spectrometry

Instrumentation

ISQ EM Single Quadrupole Mass Spectrometer (P/N ISQEM-ESI)

MS conditions

Table 2. Instrument and scan settings for the mass spectrometer

Parameter	Value
Vaporizer temperature	338
Ion transfer tube temperature	300
Source voltage	+3,000 V
Sheath gas	56.9 psig
Auxiliary gas	6.5 psig
Sweep gas pressure	0.5 psig
Scan range	100–1,300

Software

Chromeleon CDS version 7.2.10 was used for data acquisition and data processing. The Vanquish Inverse Gradient UHPLC system was configured in the Fluidic Configuration wizard from the top menu of the instruments page. After entering the column dimensions, the void volume was automatically calculated. This instrument method was created by opening the Instrument Method wizard and selecting “inverse gradient”. The inverse gradient was automatically generated after the analytical gradient was entered and the calculation mode (Keep solvent composition) was selected. The inverse gradient offset (579.95 µL) was more than the calculated column void volume (445.3 µL) since the method also included default sample loop and default tubing volume in addition to the column volume (refer to Installation Guide Doc. No 4820.3616).

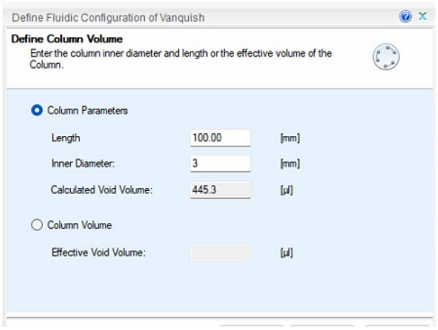


Figure 3. Fluidic Configuration wizard

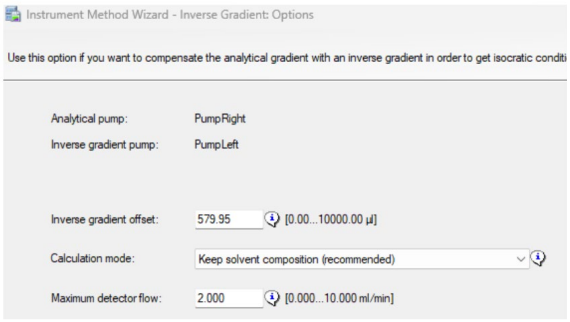


Figure 4. Instrument Method Editor wizard

Results and discussion

Peak resolution between impurities and the main peak is crucial for accurate quantification. In previous work, isopropanol (IPA) was used to elute hydrophobic lipids, such as PEG-lipid and phospholipid. This work focused on cationic lipids that have a higher hydrophilicity so they elute earlier. Therefore, acetonitrile was added in the mobile phase A and methanol was used in the mobile phase B to enhance peak shape and resolution. Peak areas were determined separately for the inverse gradient and conventional gradient methods and the results were compared to assess any differences or variations between the two gradient conditions. Three commercially available lipid standards were chosen, and triplicate injections were made for each standard. Every injection was checked individually to verify automatic peak integration performed by Chromeleon CDS.

The inverse gradient system consistently quantifies any non-volatile and semi-volatile compounds using universal CAD. All standards and impurities need to be confirmed to be non-volatile before this testing. Best practices for confirming non-volatile substances were detailed in Application Note 001342.

R-DOTAP analysis

1,2-Dioleoyl-3(R)-trimethylammoniumpropane (R-DOTAP) is a cationic lipid and is considered the more immunologically active isomer of DOTAP. R-DOTAP has been previously recognized for its capacity to elicit anti-tumor immunity.[2]

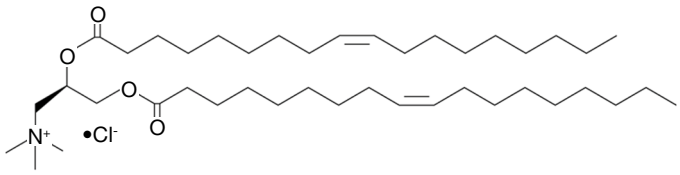


Figure 5. Structure of R-DOTAP

Three impurities peaks were observed for R-DOTAP (Figure 6). The mass of R-DOTAP and all associated impurities were confirmed using MS spectra data. Because impurity I peak eluted much earlier (3.9 min) than the main peak, the calculated amount using the inverse gradient method was found to be 36% higher than that using the conventional gradient method (Table 3). On the other hand, the difference in calculated amount between the inverse gradient and conventional gradient methods was only 5% for impurity III peak, which eluted closest to the main peak.

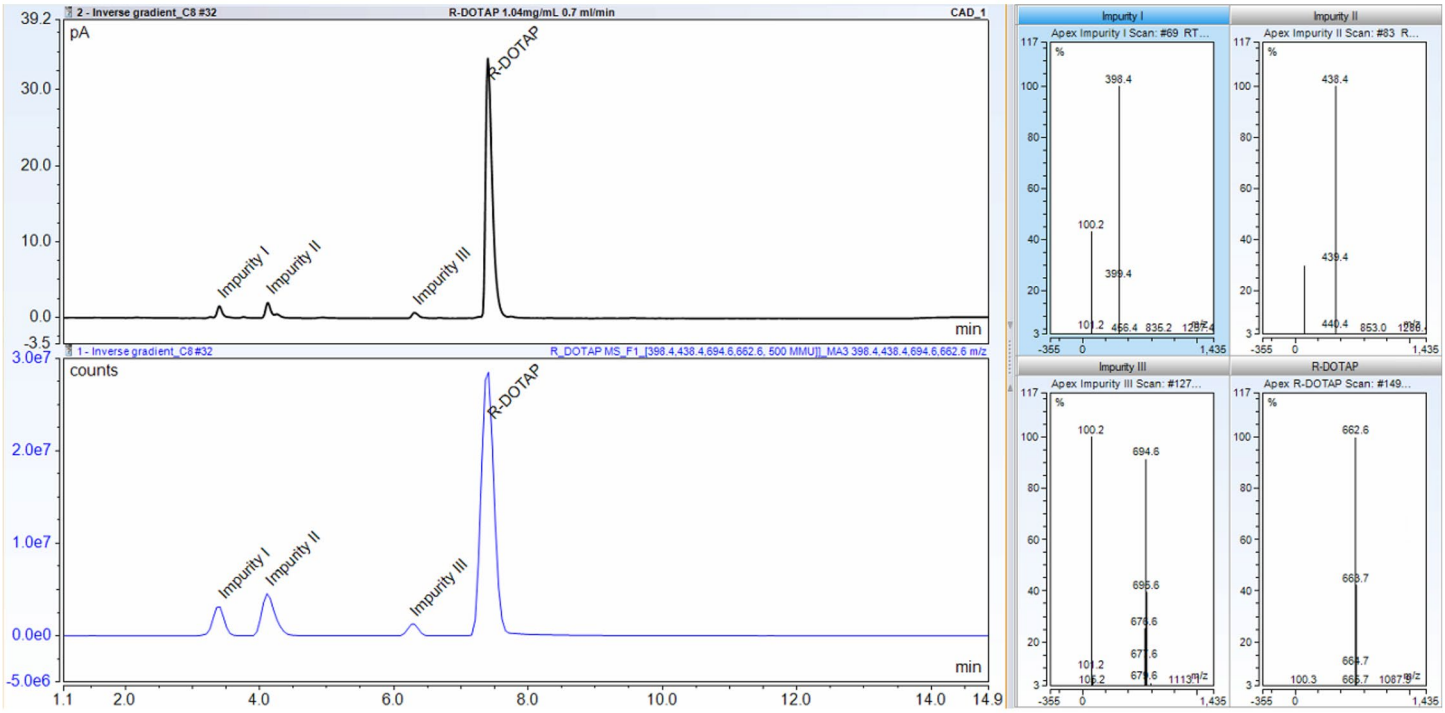


Figure 6. Separation of R-DOTAP in CAD channel and extracted MS channel with mass spectra corresponding to the labeled peaks. All mass spectra of the impurities and standards can be displayed when clicking the SmartLink icon.

Table 3. DOTAP-R results based on CAD signal

	Retention time (min)	Relative area % with IG	Relative area % with CG	Calculated amount with IG (mg/mL)	Calculated amount with CG (mg/mL)	% Difference of calculated amount	Delta RT (min)	*m/z*
DOTAP-R Impurity I	4.6	12.86	9.99	0.136	0.100	36%	-3.9	398.4
DOTAP-R Impurity II	5.5	2.36	2.06	0.024	0.021	18%	-3.0	438.4
DOTAP-R Impurity III	7.4	1.70	1.83	0.018	0.019	5%	-1.1	694.6
DOTAP-R	8.5	83.19	85.29	0.834	0.853	2%	-	662.6

Delta RT = retention time (impurity peak) - retention time (main peak)

In this method, data acquisition was performed using a mass range of 100–1,300 (Figure 7), as all selected standards and their impurities fell within this range. However, the ISQ EM mass spectrometer offers a wide mass range of 10–2,000. This capability allows for the monitoring of higher mass impurities or degradants, enhancing the comprehensive analysis of the sample. Based on the mass spectra analysis, four ions corresponding to three impurities and the main peak were identified, providing confirmation that there was no coelution observed.

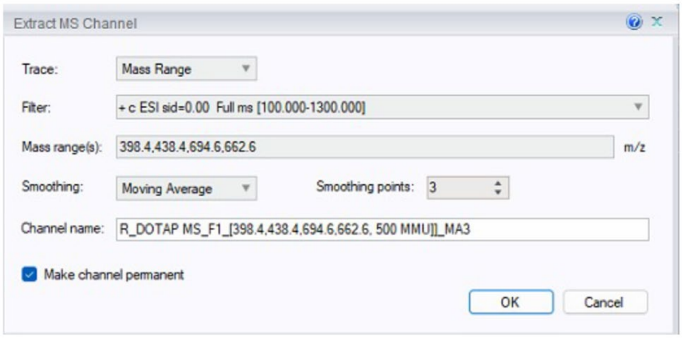


Figure 7. Extract MS channel panel. The extracted MS channel can be obtained by entering *m/z* values of the impurities and standards in the mass range section.

DLin-KC2-DMA analysis

N,N-dimethyl-2,2-di-(9Z,12Z)-9,12-octadecadien-1-yl-1,3-dioxolane-4-ethanamine (DLin-KC2-DMA) is an ionizable lipid for siRNA delivery. LNP containing DLin-KC2-DMA and androgen receptor siRNA effectively reduce tumor androgen receptor protein levels.

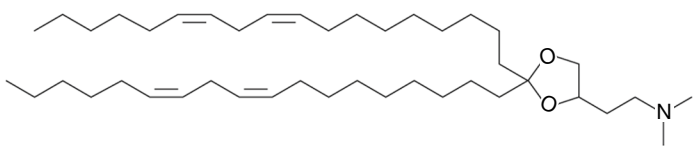


Figure 8. Structure of DLin-KC2-DMA

Using the same method, one impurity was observed right next to the main peak DLin-KC2-DMA (Figure 9). In earlier work, the impurity peak tended to co-elute or exhibit a shoulder peak with the main peak using isopropanol as the eluent due to similar molecular masses. From the mass spectra, it can be deduced that the impurity was a saturated modification of the parent ion. Using acetonitrile instead of isopropanol helped to improve the peak shape and made this impurity peak fully resolved. The SmartPeaks feature was used to facilitate the desired integration.

The calculated percentage impurity was 7% different compared to the conventional gradient. Since the impurity eluted later than the main peak, the conventional gradient overestimated the impurity amount.

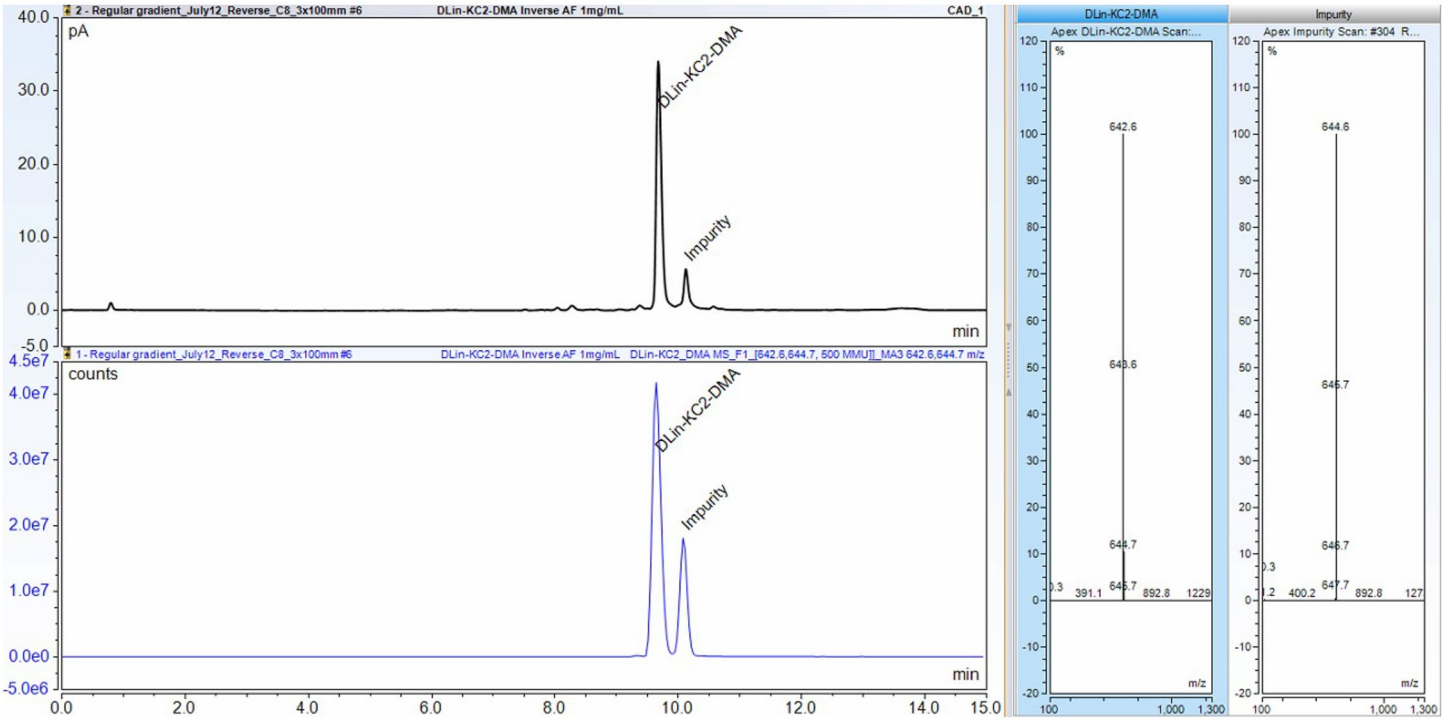


Figure 9. Separation of DLin-KC2-DMA in CAD and extracted MS channels with corresponding mass spectra

Table 4. DLin-KC2-DMA results

	Retention time (min)	Relative area % with IG	Relative area % with CG	Calculated amount with IG (mg/mL)	Calculated amount with CG (mg/mL)	% Difference of calculated amount	Delta RT (min)	*m/z*
DLin-KC2-DMA	9.8	86.23	84.02	0.862	0.840	3%	-	642.6
DLin-KC2-DMA Impurity	10.3	13.77	14.75	0.138	0.148	7%	+0.5	644.6

Delta RT = retention time (impurity peak) - retention time (main peak)

ALC-0315 analysis

2-hexyl-decanoic acid, 1,1'-[[(4-hydroxybutyl)imino]di-6,1-hexanediyl] ester (ALC-0315) is an ionizable cationic amino lipid. Formulations containing ALC-0315 have been used in the development of LNPs for the delivery of mRNA-based vaccines.

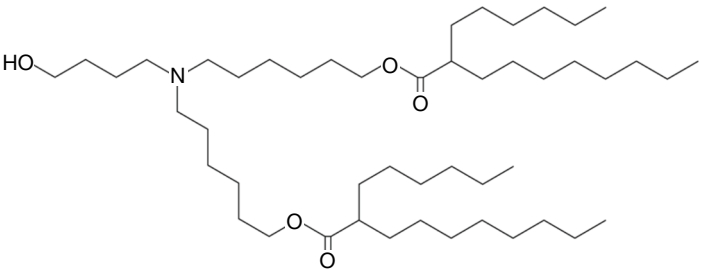


Figure 10. Structure of ALC-0315

In the analysis of ALC-0315, one main impurity peak could be detected, eluting at 5.9 min, which is 2.7 min away from the ALC-0315 peak (Table 5, 8.6 min). The impurity peak had a similar retention time difference as the impurity II peak of R-DOTAP (2.7 vs. 3.0 min), so the percentage of impurity difference was very similar between them (18% vs. 20%). Based on the mass spectra analysis, two ions corresponding to one impurity and the main peak were identified, and no co-elution was observed (Figure 11).

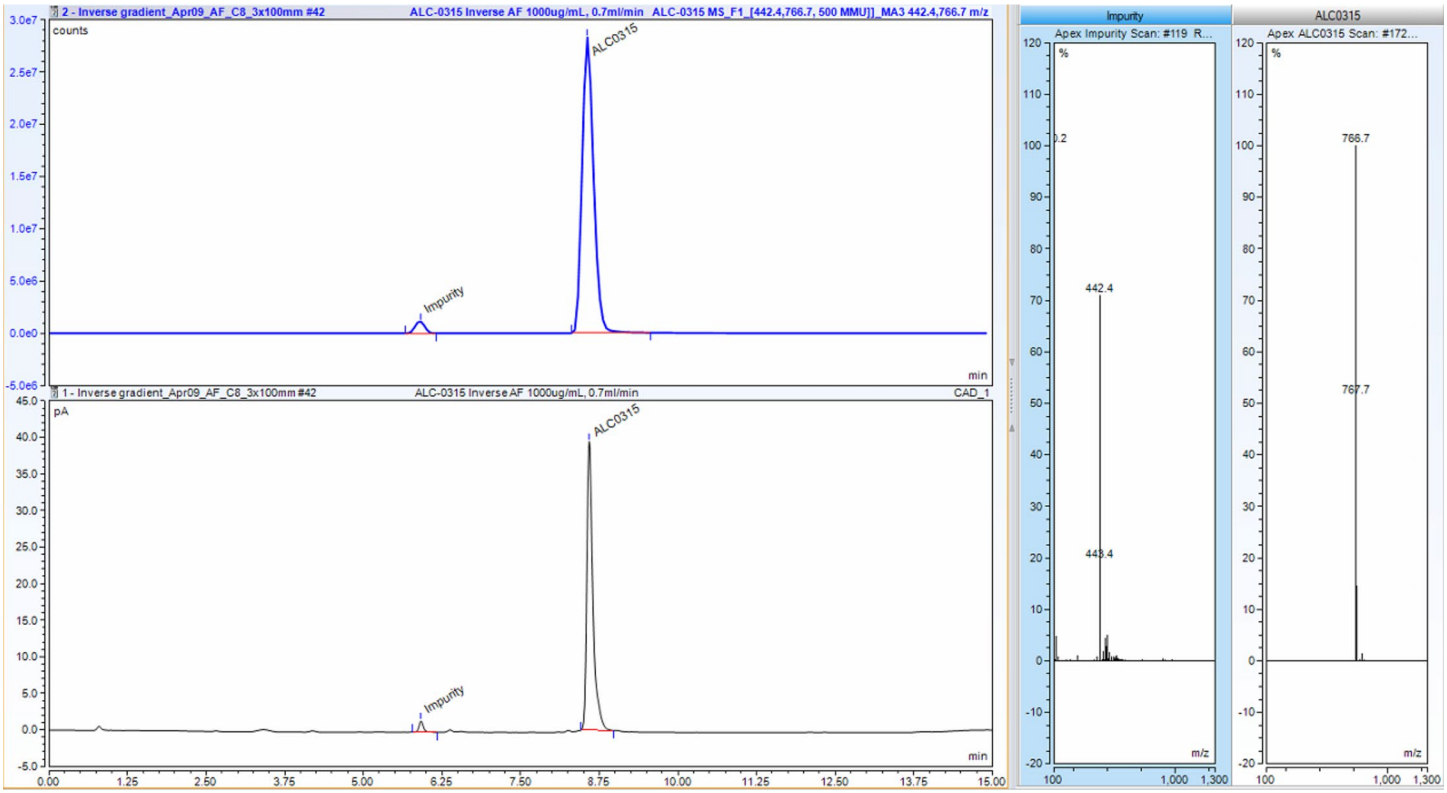


Figure 11. Separation of ALC0315 in the CAD and extracted MS channels with corresponding mass spectra

Table 5. ALC-0315 results

	Retention time (min)	Relative area % with IG	Relative area % with CG	Calculated amount with IG (mg/mL)	Calculated amount with CG (mg/mL)	% Difference of calculated amount	Delta RT (min)	*m/z*
ALC-0315 impurity	5.9	2.65	2.17	0.026	0.022	20%	-2.9	442.4
ALC-0315	8.6	96.36	97.28	0.964	0.974	-1%	-	766.7

Delta RT = retention time (impurity peak) - retention time (main peak)

As seen in the data summarized in Table 6, the % impurity difference between the amount determined using a conventional gradient method and the more accurate inverse gradient method tends to increase dramatically when the retention time of an impurity peak is far away from the main peak. In contrast, the % impurity difference tends to decrease by smaller variations when the impurity peak is close to the main peak. This observation suggests that the use of an inverse gradient can significantly reduce the error in surrogate estimation of impurities in lipid raw materials that elute far away from the parent compound. This would reduce the tendency to over- or underestimate potentially detrimental impurities. This may be attributed to the fact that the inverse gradient method compensates for the change in mobile phase composition across the gradient elution, which would otherwise negatively impact CAD response for earlier eluting compounds.[4]

The default template was used in the report designer section. Peak retention time, area, and notably the calculated amount are displayed on the Chromatogram and Results page (Figure 12). In the Integration Results section, the calculated amount was a customized field that involved a simple calculation using % relative peak area. This calculation allowed for the determination of the amount of impurities based on its relative peak area compared to the total peak area.

Table 6. Summary of the calculated amount difference for the impurities of three lipid components based on the use of inverse or conventional gradient methods

	Calculated amount with IG (mg/mL)	Calculated amount with CG (mg/mL)	% Difference of calculated amount	Delta RT (min)
DOTAP-R Impurity I	0.136	0.100	36%	-3.9
DOTAP-R Impurity II	0.024	0.021	18%	-3.0
DOTAP-R Impurity III	0.018	0.019	5%	-1.1
ALC-0315 Impurity	0.026	0.022	20%	-2.9
DLin-KC2_DMA Impurity	0.160	0.175	-9%	+0.5

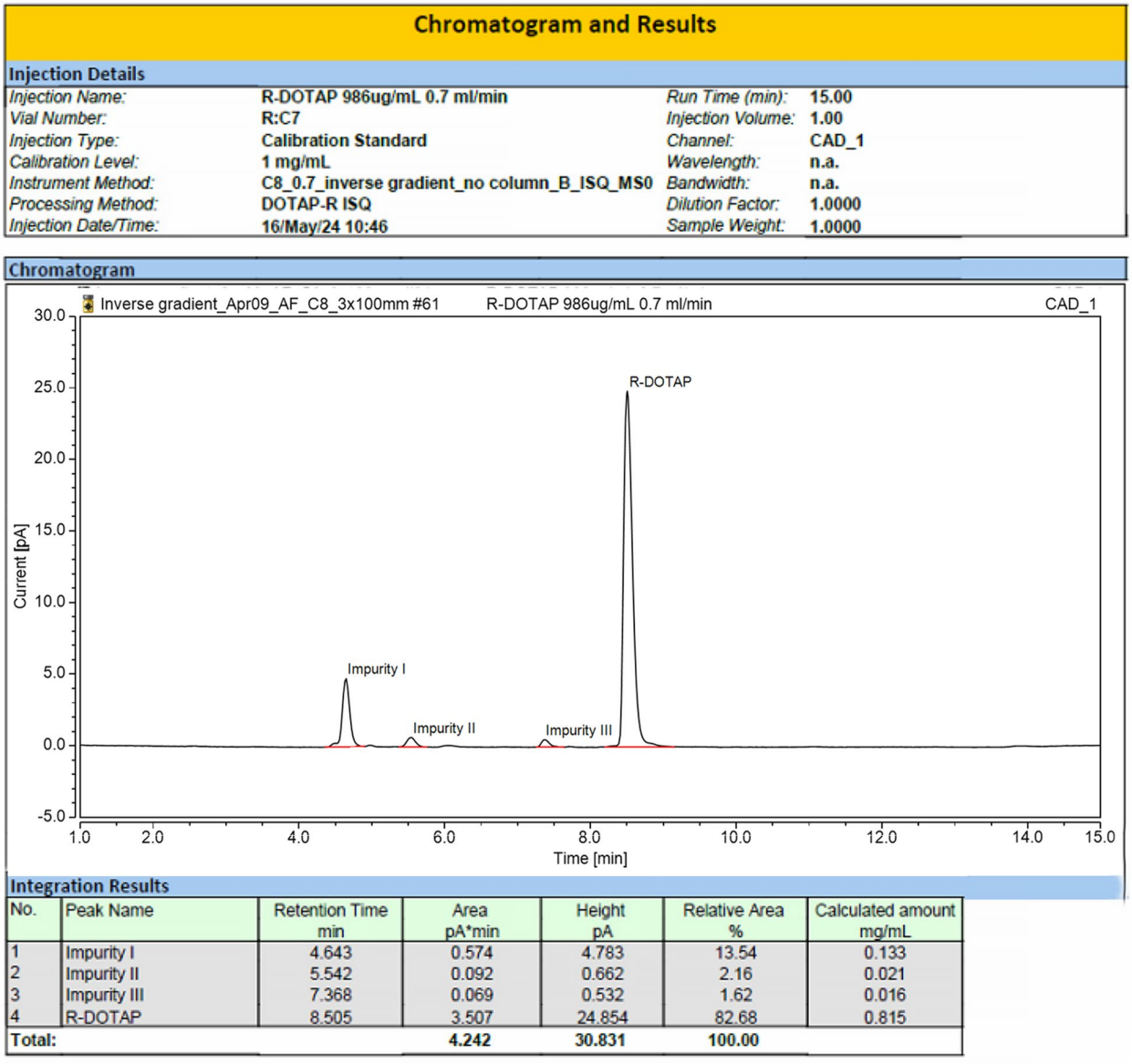


Figure 12. Report for chromatogram and results

Conclusion

In this study, we have developed an inverse gradient quantification method for cationic lipids impurity analysis when impurity reference standards are not available. For further identification of lipid impurities, Application Note 002455[5] can be referenced.

- The impurity amount can be automatically estimated using another peak or peak group within the same run for this surrogate quantitation in Chromeleon CDS.
- Both inverse gradient configuration and method can be automatically generated via the wizard.
- When there is a significant difference in retention times between the lipid standard and its impurities, using an inverse gradient provides more accurate estimated quantitation than a conventional gradient method.
- The use of the ISQ EM MS in conjunction with CAD allowed for confirmation of the *m/z* of the lipid raw material as well as determination of the *m/z* for the unknown impurities.

References

1. Committee for Medicinal Products for Human Use (CHMP) 2020. Assessment report: Comirnaty, COVID-19 mRNA vaccine (nucleoside-modified). https://www.ema.europa.eu/en/documents/assessment-report/comirnaty-epar-public-assessment-report_en.pdf
2. Henson, H.T. R-DOTAP Cationic Lipid Nanoparticles Outperform Squalene-Based Adjuvant Systems in Elicitation of CD4 T Cells after Recombinant Influenza Hemagglutinin Vaccination, Viruses **2023**, *15*(2), 538.
3. Crafts, C. et al, Thermo Fisher Scientific Application Note 72490. Improving the quantification of Unknown Impurity Analysis Using Dual-Gradient HPLC with Charged Aerosol Detection. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-72490-lc-unknown-impurities-an72490-en.pdf
4. Lovejoy, K. et al, Thermo Fisher Scientific Application Note 001630. A reliable UHPLC/UV/CAD/MS multidetector method for routine quantification and library matching of extractables and leachables in pharmaceutical-grade plastics. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-001630-hplc-vanquish-duo-extractables-leachables-cad-single-quad-an001630-na-en.pdf
5. Hackbusch, S. et al, Thermo Fisher Scientific Application Note 002455. Confident and sensitive profiling of lipid nanoparticle raw materials and impurity identification using a LC/CAD/HRAM-MS method with inverse gradient. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-002455-bt-lc-cad-hram-ms-lipid-nanoparticle-an002455-na-en.pdf

Learn more at **thermofisher.com/vaccines**

AN003384-EN 1124S