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S C I E N T I F I C

UHPLC-CAD

Characterization of lipid nanoparticle (LNP) composition using UHPLC-CAD

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Keywords
Lipid nanoparticle (LNP), lipid analysis, charged aerosol detector (CAD), phospholipid, cationic lipids, Accucore C30 column

Application benefits
- Simple UHPLC method for characterization of the lipid components of LNPs
- Universal response shows relative amounts of each lipid compound in the formulation
- Low limits of detection even for cholesterol
- Wide linear range for all lipid compounds
- Baseline separation of components

Goal
Development of a simple UHPLC method for the characterization of the lipid content of LNP formulations. Implementation of the universal CAD detector to quantify large non-chromophoric lipids present in the LNP structure.

Introduction
Lipid nanoparticles (LNPs) have proven to be a preferred drug delivery system with the successful roll out of the mRNA vaccines during the COVID-19 pandemic. LNP-based therapies include antisense oligonucleotides (ASOs), siRNA therapies, and mRNA vaccines, all of which are growing in popularity. There are over 20 LNP-based therapeutics on the market with a rapidly growing number in development. At least four different lipid types are used in the LNP formulations, the exact composition of which is usually kept as proprietary information. Common constituents are as follows: cholesterol,

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which keeps fluidity in the lipid bilayer; phospholipids, which are also normal constituents of cell membranes; PEGylated lipids, which generate a hydrophilic nature to the outer surface of the LNP; and an ionizable lipid, which is usually cationic and interacts with the negatively charged phosphate backbone of the encapsulated nucleic acid to aid stability. These cationic lipids are crucial components and are usually under patent protection. The composition of the LNP is an important aspect for function and so must be characterized in the formulation. As such, the identification, ratio, and purity of the lipids in the formulation are regarded as critical quality attributes for safety and efficacy. Lipids have no chromophore, so UV detection is not possible during the QC analysis of LNP.

Here we describe a UHPLC-based chromatography method for the lipid component analysis, utilizing the universal detection capabilities of the charged aerosol detector (CAD). This detection mechanism is applicable for virtually any non- or semi-volatile compound. The signal response is uniform for nonvolatile compounds, which makes this detection system ideal for compositional analysis, and the sensitivity is superior to alternative lipid detection systems such as refractive index or evaporative light scattering detectors (ELSD). The column used is a Thermo Scientific™ Accucore™ C30 column, which provides higher shape selectivity for long chain hydrophobic compounds such as lipids. The Thermo Scientific™ Vanquish™ Flex UHPLC system is totally biocompatible and has a metal-free flow path, which will not interact with the metal-chelating phosphate groups present on some of the lipids in the formulation. This ensures the sensitivity and recovery required for robust analysis.

Experimental

Chemicals	Part number
Lipid H, Cayman Chemical	33474
mPEG-DTA-2K, SINOPEG	1849616-42-7
DHA, SINOPEG	2036272-55-4
Cholesterol, Sigma-Aldrich	C1231
DOPE, Avanti polar lipids	850725P
PEG 5000, Avanti polar lipids	880210P
DSPC, Sigma-Aldrich	816944
Trifluoroacetic acid (TFA), Optima™ LC/MS grade, Fisher Chemical™	A11650
Formic acid (FA), Optima™ LC/MS grade, Fisher Chemical™	A11710X1AMP
Ethyl alcohol (EtOH), 200 proof, 99.5+%, Thermo Scientific™	61519-0010
Water, Optima™ LC/MS grade, Fisher Chemical™	W64
Isopropanol (IPA), Optima™ LC/MS grade, Fisher Chemical™	A461-4
Acetonitrile (ACN), Optima™ LC/MS grade, Fisher Chemical™	A955-4
Methanol, Optima™ LC/MS grade, Fisher Chemical™	A456-4
Autosampler inert vials and inserts, Thermo Scientific™ Chromacol™ GOLD-grade	13-622-351

Instrumentation	Part number
Vanquish Flex Binary system consisting of:	
Vanquish System Base	VH-S01-A
Vanquish Binary Pump F	VF-P10-A-01
Vanquish Sampler F	VF-A10-A
Vanquish Column Compartment H	VH-C10-A-02
Vanquish Charged Aerosol Detector H	VH-D20-A

Sample preparation

- It is recommended to use glass pipets to transfer lipids/LNPs in organic solvents.

- It is recommended to use glass inserts, vials, and bottles to store lipids and LNP samples.

- All lipids/LNPs were dissolved in 100% ethanol.

- Two different formulations are tested.

FA method

Column	Accucore C30, 3.0 × 100 mm, 2.6 µm, P/N 27826-103030	
Mobile phase A	0.1% FA in 50% ACN, 50% water	
Mobile phase B	0.1% FA in 60% IPA, 30% ACN, 10% water	
	Time (min)	A	B
	0.0	25	75
	1.0	25	75
Gradient	9.0	0	100
	10.0	0	100
	10.1	25	75
	12	25	75
Flow rate	0.9 mL/min	
Autosampler temperature	15 °C	
Column temperature	50 °C	
Post column cooler	40 °C	
Injection volume	1 µL	
Injection wash solvent	Mobile phase B	

CAD settings

Power function	1.0
Evaporator temperature	35 °C
Gas resolution mode	Analytical
Data rate	2 Hz
Filter	3.6

TFA method

Mobile phase A	0.1% TFA in 50% ACN, 50% water	
Mobile phase B	0.1% TFA in 70% IPA, 25% ACN, 5% water	
	Time (min)	A	B
	0.0	35	65
	1.0	35	65
Gradient	12.0	0	100
	13.0	0	100
	13.1	35	65
	16	35	65

Other conditions are the same as the FA method.

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software 7.2.10 ES was used for data acquisition and analysis.

Results and discussion

The individual lipid components of the LNP are quite different in their composition. They all still need to be separated in the chromatography.

A cationic lipid is required to interact with the phosphate backbone of the oligonucleotide to be encapsulated. An example of the structure of 4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl) bis(2-hexyldecanoate (DHA), which is used in LNP formulations.
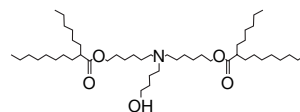

A phospholipid, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), is a naturally occurring phosphatidylcholine found in cell membranes.
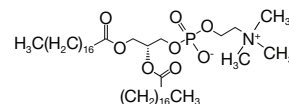
Cholesterol is also naturally occurring and found in cell membranes to add fluidity.
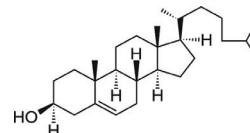
A synthetic PEGylated lipid is used to give the LNP surface hydrophilicity and low immunogenicity. Methoxypoly(ethylene glycol) ditetradecylacetamide (mPEG-DTA-2K) has been used in LNP formulations.
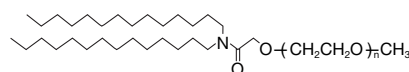
Long chain lipids are well separated on columns with a C30 functional group. The long hydrocarbon C30 chain lines up better with longer lipids to give good shape selectivity and impurity detection of these lipids. The Accucore columns are packed with solid core silica particles that give high separation power without high backpressures; this is important when using the more viscous eluents such as IPA. The Accucore columns have also been found to give extremely good recoveries for hydrophobic lipids. For these reasons, the Accucore C30 column was selected for this work.

The CAD detector gives close to a universal response for each lipid type in this formulation. This attribute makes the CAD ideal for compositional analysis of lipid formulations. The sensitivity is also greater than found with ELSD or refractive index detectors.[1] This is an attribute which aids in the detection of any low-level impurities in the formulation, which could come from any of the

lipid components used in the LNP. Figure 1 shows the separation and compositional analysis of selected LNP lipids from formulation #1 injected at the same concentration. Baseline separation and detection of each lipid is obtained within 10 minutes.

All limits of detection were found to be around 10 µg/mL, which was targeted as the lower end of the calibration range. This is especially true for cholesterol (2.6 µg/mL), which is known to yield a diminished response with ELSD. The CAD, in contrast, gives a similar response for each lipid class. The detection is almost linear from 10 to 220 µg/mL. For CAD, it is possible to use either PFV optimization or nonlinear fits. Here a nonlinear fit (quadratic fit) was used in detection up to 220 µg/mL. The calibration curves are shown in Figure 2. The carryover from each injection was negligible using this workflow (not shown).
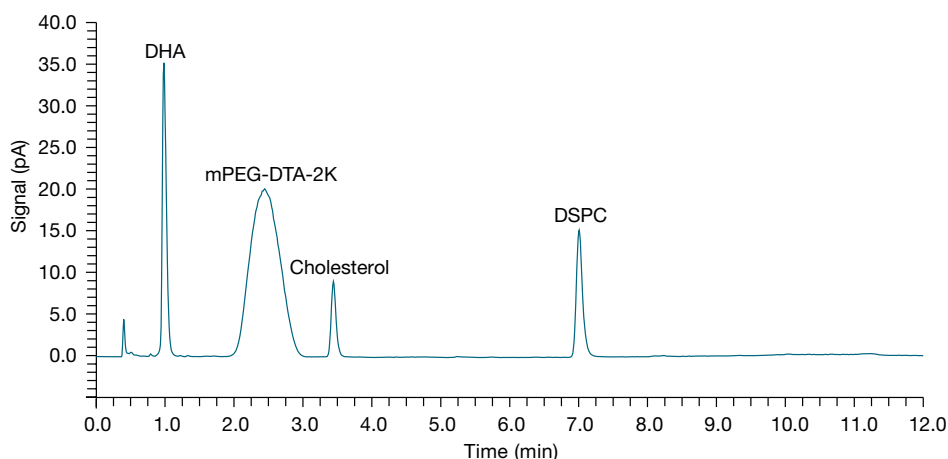

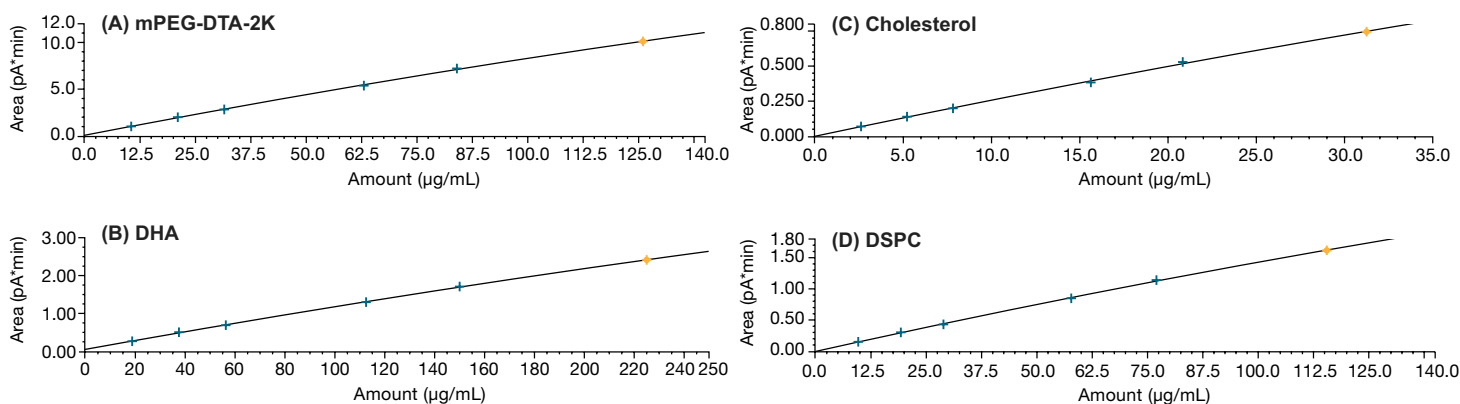Figure 1. Separation of LNP formulation #1 using the FA method

Figure 2. Calibration curves for the individual lipid components

All lipid components are separated well with good quadratic calibration curves for accurate quantitation. The calibration curves for all lipids show a calibration coefficient of 0.999 (Table 1).

In the overlay of all calibration standards, all peaks show good resolution and reproducibility using FA (Figure 3).

Figure 4 shows the separation of LNP formulation #1 using TFA. The elution order of mPEG-DTA-2K and DHA has changed. The sensitivity of the CAD allows small impurities to be observed in the baseline around the main peaks. Again, baseline separation can be achieved in 10 minutes.

Table 1. Calibration results

| Peak no.	Peak name	Ret. time (min)	Cal. type	Eval. type	Number of points	Rel. std. dev. %	Coeff. of determination	C0 (offset)	C1 (slope)	C2 (curve)
1	DHA	0.983	Quad, WithOffset	Area	6	1.3619	0.99978	0.0501	0.0119	0.0000
2	mPEG-DTA-2K	2.442	Quad, WithOffset	Area	6	1.8382	0.99962	0.0634	0.0918	-0.0001
3	Cholestrol	3.442	Quad, WithOffset	Area	6	2.9175	0.9991	-0.0004	0.0266	-0.0001
4	DSPC	7.008	Quad, WithOffset	Area	6	1.7888	0.99966	-0.0038	0.0158	0.0000
Maximum						2.9175	0.99978			
Minimum						1.3619	0.9991			
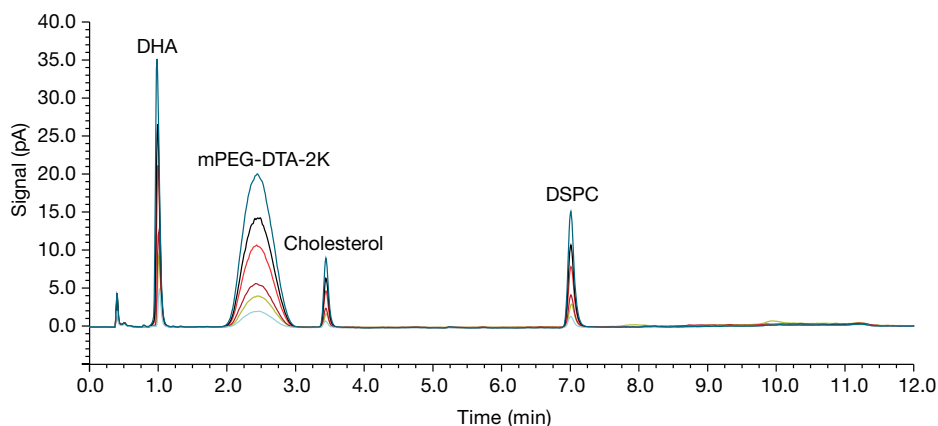
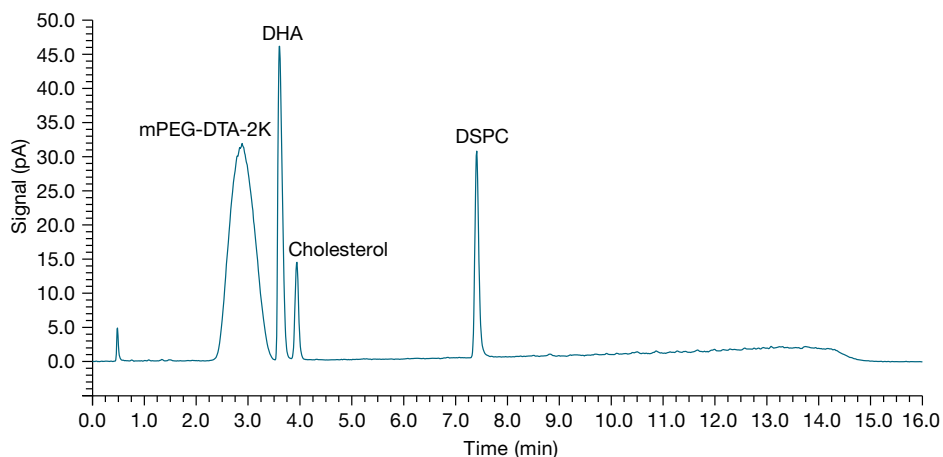Figure 3. Overlaid chromatograms of calibration standards using the FA method

Figure 4. Separation of LNP formulation #1 using the TFA method

In comparing TFA and FA methods using the lowest concentration standard from the calibration plot, the TFA method showed higher sensitivity than the FA method but the FA method showed better separation (Figure 5).

The separation of LNP formulation #2 containing four different lipids using the TFA method is shown in Figure 6. Some impurities are present before and after the PEG 5000 peak. Baseline separation and detection of each lipid is obtained within 5 minutes. The FA separation with LNP formulation #2 did not provide the required resolution (not shown).
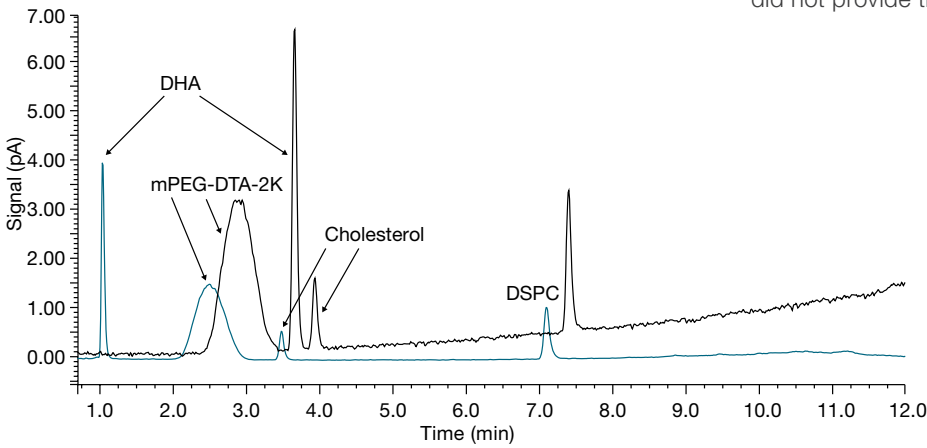

Figure 5. Comparison of separation of LNP formulation #1 using the TFA and FA methods.
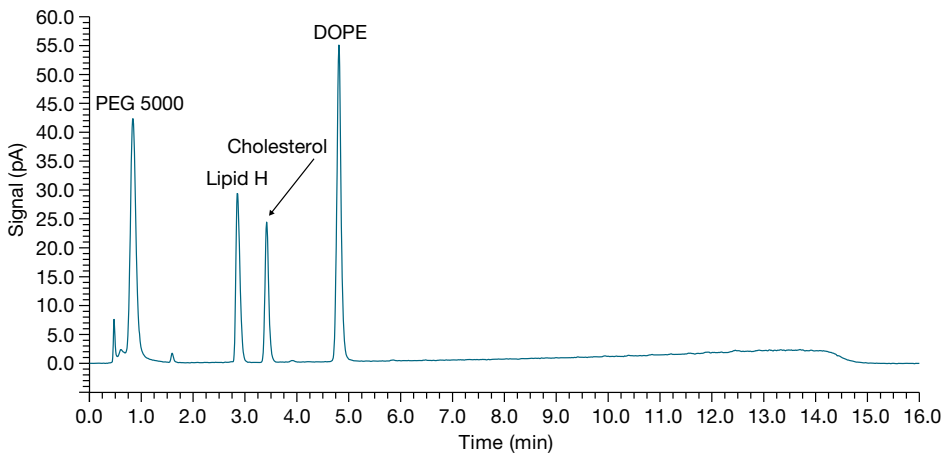Black Trace: TFA method; Blue Trace: FA method.

Figure 6. Separation of LNP formulation #2 using the TFA method. PEG5000: 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt).
Lipid H: 8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-octylnonyl ester.
Cholesterol: Plant-derived. DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine.

Conclusion

- Two fast, robust methods for the characterization of the lipid components in the lipid nanoparticles used in oligonucleotide therapeutics have been described. Formulation #1 has better separation using the FA method, whereas formulation #2 has better separation using the TFA method.

- Separation of several components from each LNP class was achieved on the Accucore C30 column in 10 minutes.

- The CAD has low limits of detection for all the components, including cholesterol. The high sensitivity of the CAD allows any trace impurities to be monitored.

- The calibration curves for all lipids show a calibration coefficient of better than 0.999.

References

1. Kinsey, C.; Lu, T.; Deiss, A.; Vuolo, K.; Klein, L.; Rustandi, R.R.; Loughney, J.W. Determination of lipid content and stability in lipid nanoparticles using ultra high-performance liquid chromatography in combination with a Corona Charged Aerosol Detector, *Electrophoresis* **2021**, 1–24. https://doi.org/10.1002/elps.202100244

2. Tracy, M.; Hillbeck, D. Thermo Scientific Application Note 20663 – Comparative analysis of cooking oils using a solid core HPLC column. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN20663-Comparative-Cooking-Oils-HPLC-EN.pdf

3. Criscuolo, A.; Zeller, M.; Cook, K.; Angelidou, G.; Fedorova, M. Rational selection of reverse phase columns for high throughput LC–MS. *Chemistry and Physics of Lipids* **2019**, *221*, 120-127.

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