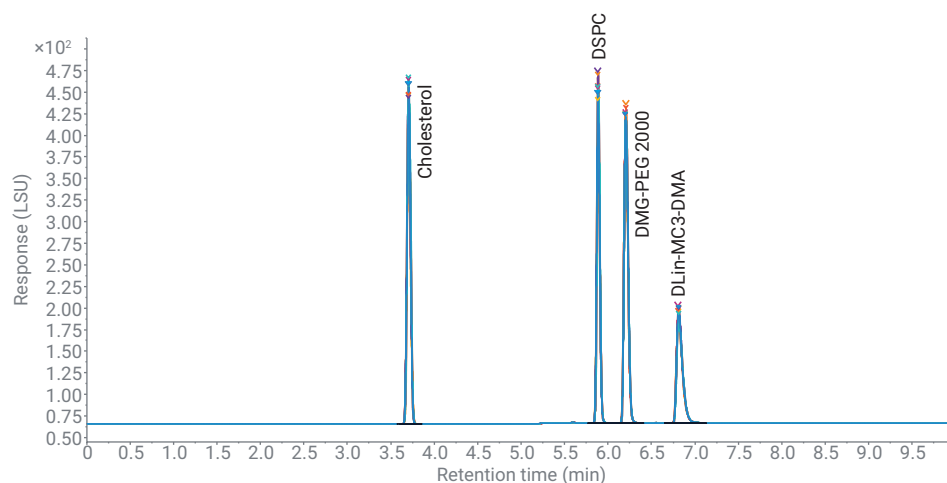


# Analysis of Lipid Nanoparticle Composition

Quaternary method development for highest resolution with evaporative light scattering detection



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## Abstract

Lipid nanoparticles (LNPs) have emerged as promising delivery vehicles for nucleic acids in the pharmaceutical industry. To ensure safety and efficacy of the final drug product, the lipid components need analytical characterization of composition, ratio, and degradation. In this application note, liquid chromatographic method development for the analysis of the lipid components of patisaran (trade name Onpattro) is shown in a quaternary setup. A method-combining methanol (MeOH) and acetonitrile (ACN) resulted in optimal separation of the four LNP components with excellent peak shapes, precision, and sensitivity. The Agilent 1260 Infinity II Prime Bio LC with Agilent 1290 Infinity II ELSD enables universal detection of the lipid components lacking a UV chromophore. In addition, the high dynamic range of the Agilent 1290 Infinity II ELSD allows the detection of all four lipids in the patisaran-like sample.

## Introduction

In recent years, promising opportunities for RNA-based therapy and gene-editing technology have raised interest in LNP research.

LNPs act as safe and efficient delivery vehicles, and combinations of lipids with oligonucleotides have especially shown great success in the pharmaceutical industry. The LNP system enables stable drug loading and enhanced delivery efficiency to the targeted sites of action. In formulated drugs, oligonucleotides such as small interfering RNA (siRNA) or messenger RNA (mRNA) are encapsulated in LNPs to facilitate cellular uptake via endocytosis and delivery into the cytosol.

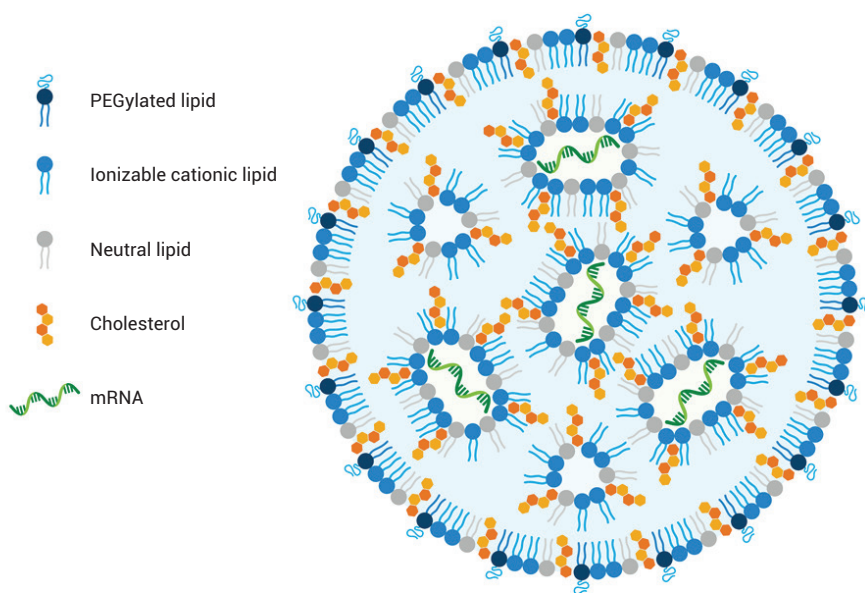
LNPs are spherical vesicles, and are typically composed of four main components (Figure 1):<sup>1,2</sup>

- Cholesterol
- A neutral phospholipid (mostly DSPC)
- A polyethylene-glycol (PEG)-lipid
- An ionizable cationic lipid (often proprietary)

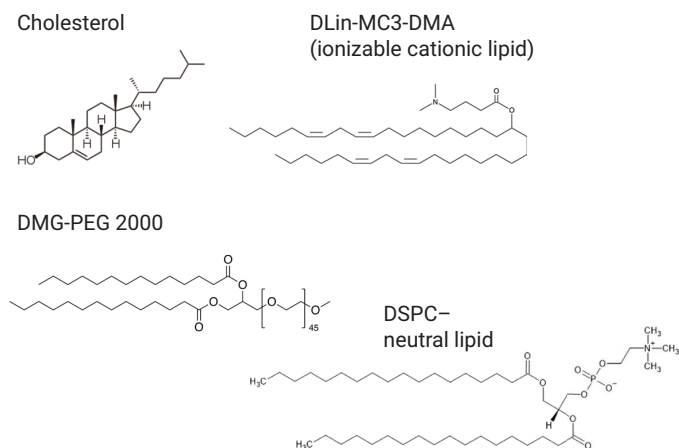
The primary purpose of the structural lipids DSPC, cholesterol, and the PEG-lipid is to control the particle size, provide particle stability and blood compatibility, and in addition, improve LNP circulation lifetime.<sup>3</sup> The PEG-lipid, the least abundant lipid in the LNP formulation, also acts as a steric barrier to prevent aggregation during storage. Ionizable lipids, the second generation of cationic lipids, are pH-dependent. At low pH, their positively charged ionizable amine groups enable these lipids to interact (for example, with the anionic RNA used in the loading process of LNP-RNA formation). They are virtually uncharged at physiological pH in the bloodstream, minimizing toxicity.

The first LNP-encapsulated RNA drug approved by the FDA and the EMA is patisiran (trade name Onpattro), which is an LNP formulation of siRNA.<sup>4</sup> It contains DLin-MC3-DMA as ionizable cationic lipid, DSPC, cholesterol, and

DMG-PEG-2000 (see Figure 2). The regulatory approval of Onpattro paved the way for the development of many nucleic acid-based therapies, enabled by nanoparticle delivery.



**Figure 1.** Schematic representation of mRNA or siRNA-LNP structure inspired by Schoenmakers *et al.*<sup>1</sup> and Evers *et al.*<sup>2</sup>.



**Figure 2.** Main components of the Onpattro Patisiran LNP.

In addition to the analysis of the loaded RNA portion of the LNPs, the lipid components need analytical characterization to ensure safety and efficacy of the complete drug. In the process of drug and formulation design, extensive analysis is required for *in vivo* performance and quality control of the finished drug product.<sup>5</sup> Regulatory specifications include tests for physical parameters such as siRNA encapsulation or particle size, as well as assays for the individual lipid components (e.g. composition, identity, and purity).<sup>5</sup>

Before analysis, liposomes and LNPs are usually disrupted by dilution with organic solvents such as methanol or isopropanol.

Typically, LNP analysis for composition or degradation is carried out by reverse-phase high performance liquid chromatography (RP-HPLC).<sup>6</sup>

Following chromatographic separation, evaporative light scattering detection (ELSD) is an ideal detection technique for molecules such as lipids, which lack a UV chromophore. The Agilent 1290 Infinity II ELSD is ideal for reproducible and sensitive detection of lipids.<sup>7</sup> In addition to its universal detection capabilities, a huge advantage is the flexibility to allow the use of gradients (in contrast to refractive index detection (RID)).

This application note shows UHPLC method development for the analysis of the four components of the LNP composition of Onpattro. The different chemical nature of the four components (including the hydrophobic DSPC, the ionizable lipid, and the PEGylated lipid) makes it very challenging to achieve symmetrical and sharp peak shapes for all components within one stationary and mobile phase combination. The use of the Agilent 1260 Infinity II Prime Bio LC system provides the flexibility of a quaternary pump, and enables easy method development to test different solvent and buffer combinations.

## Experimental

### Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger (G7116-60071)
- Agilent 1290 Infinity II ELSD (G7102A)

### Software

Agilent OpenLab CDS Version 2.6 or later versions

### Column

InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 μm (part number 699675-912)

### Chemicals

Agilent InfinityLab ultrapure LC/MS methanol (5191-4497) and Agilent InfinityLab ultrapure LC/MS acetonitrile (5191-4496) was used for all ELSD analyses. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 μm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Ammonium acetate was obtained from Sigma-Aldrich (Steinheim, Germany).

### Samples

Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (18:0 PC or DSPC) were obtained from Merck (Darmstadt, Germany). DLin-MC3-DMA (4-(dimethylamino)-butanoic acid, (10Z,13Z)-1-(9Z,12Z)-9,12-octadecadien-1-yl-10,13-nonadecadien-1-yl ester) was obtained from Cayman Chemical (MI, USA). 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) was obtained from Avanti Polar Lipids (AL, USA).

The samples were dissolved in two different concentrations. To enable complete dissolution, the tubes were warmed to 35 °C for 3 to 4 minutes before further use.

The mixed sample for method development was composed in 1,220 μL MeOH to enable approximately similar peak heights and areas for all components, as described in Table 1.

The components of the sample mimicking the Onpattro LNP component ratio were mixed as follows: The lipids were dissolved in MeOH to a concentration of 3.89 mM. Cholesterol, DLin-MC3-DMA, DMG-PEG, and DSPC were dissolved in a ratio of 38.5:50:1.5:10 from the 3.89 mM equimolar concentrations.

From this mix, a dilution series was prepared, ranging from 972.5 μM down to 0.44 μM for linearity analyses in 1:3 serial dilution steps (4.8625 nmol to 2.22 pmol for 5 μL injections).

**Table 1.** Sample composition for method development.

Lipid	Molecular Weight [Da]	Concentration [mM]	Mass [μg]
DMG-PEG 2000	2,509.20 (average molecular weight due to polydispersity of PEG)	0.0984	300
DLin-MC3-DMA	642.09	0.6380	500
Cholesterol	386.67	0.1060	50
DSPC	790.15	0.1670	150

## Buffer preparation

500 mM ammonium acetate (~pH 7, no further pH adjustment) was prepared and filtered using a 0.2 µm membrane filter. For the binary method, the ammonium acetate stock solution was diluted to 100 mM with water, and 100 mL of this buffer was mixed with either 900 mL of methanol (Channel A) or 900 mL of acetonitrile (Channel B).

## Results and discussion

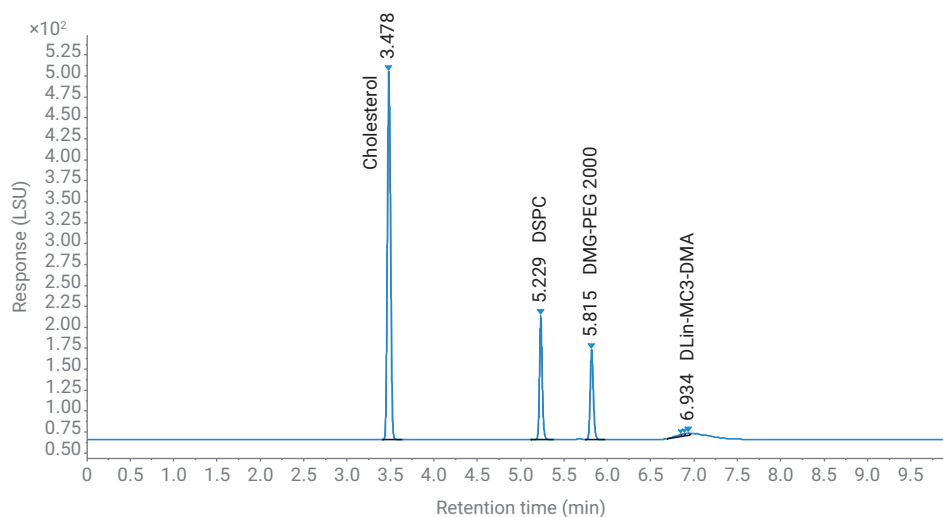
An initial binary gradient setup using water + 10 mM ammonium acetate (Channel A) and MeOH + 10 mM ammonium acetate (Channel B)<sup>7</sup> for the analysis of components (running a gradient from 82 to 100% B in 5 minutes with a 5-minute hold) showed the following result, as displayed in Figure 3. All other parameters were the same as described in the Experimental section. The first three neutral lipid peaks showed a good peak shape and resolution. The last ionizable lipid DLin-MC3-DMA eluted in a broad (and therefore shallow) peak. Method development options such as changing temperature or gradient slope did not improve the peak shape of the ionizable lipid. Therefore, changing the solvent combination was the central point of intervention. Further experiments using water and ACN (both with 0.1% formic acid) instead of water and MeOH, did not result in good resolution or peak shape (data not shown).

As a basis to enable solvent combination method development, the quaternary Agilent 1260 Infinity II Prime Bio LC system was used. With water, ACN, MeOH, and 500 mM ammonium acetate supplied separately in each of the four channels, different solvent combinations were tested with a continuous delivery of 2% ammonium acetate from channel D to enable a constant concentration of 10 mM buffered solution.

## Method

**Table 2.** Chromatographic conditions.

Parameter	Value				
Solvent	A: Water B: ACN C: MeOH D: 500 mM Ammonium Acetate				
Flow Rate	0.4 mL/min				
Quaternary Gradient Final	Time	Channel A %	Channel B %	Channel C %	Channel D %
	0 minutes	16	0	82	2
	3 minutes	8	0	90	2
	5 minutes	8	90	0	2
	Stop time: 10 minutes Post-time: 5 minutes				
Quaternary Gradient Final Short	Time	Channel A %	Channel B %	Channel C %	Channel D %
	0 minutes	8	0	90	2
	3 minutes	8	90	0	2
	Stop time: 5 minutes Post-time: 5 minutes				
Gradient Binary	Time	Channel A %	Channel B %		
	0 minutes	90% Methanol, 10% H <sub>2</sub> O, 10 mM ammonium acetate	0	90% Acetonitrile, 10% H <sub>2</sub> O, 10 mM ammonium acetate	
	4 minutes	100	0	100	
	Stop time: 7 minutes Post-time: 5 minutes				
Column Temperature	30 °C				
Detection ELSD	Evaporator Temperature: 40 °C Nebulizer Temperature: 40 °C Gas Flow Rate: 1.6 SLM Data Rate: 80 Hz Smoothing: 10 (1.0 second)				
Injection	Injection volume: 2 and 5 µL Sample temperature: 25 °C Needle wash: 3 seconds with 50 % Isopropanol in H <sub>2</sub> O				



**Figure 3.** Binary gradient with water and MeOH, with 10 mM ammonium acetate for the analysis of the four LNP components.

### Gradient using MeOH

Using MeOH in the quaternary setup, it was not possible to elute all four lipids (Figure 4), as presumably 100% of MeOH in a longer hold would be needed for complete elution. In the quaternary setup, a maximum of 90% MeOH was possible, while still including the buffer functionality of ammonium acetate and some water to prevent crystallization. Figure 4 shows the results for the ternary separation from 80 to 90% MeOH. Only two peaks were eluted from the column, even with a 5-minute 90% hold of MeOH. In the next experiments, ACN was used as a stronger eluent to test for complete elution of all four lipid components.

### Gradient using ACN

Figure 5 shows the results of a ternary gradient using ACN as eluent. The gradient was set to 50 to 90% B in 5 minutes, with a 5-minute hold. With ACN, all four peaks could be completely eluted. However, the peak shapes of three lipids were suboptimal, with only cholesterol (first peak) showing a Gaussian peak shape.

Further method development to enable faster elution and enhanced resolution was started by using higher organic starting conditions and shallower gradients. Although faster elution was achieved, flatter gradients did not show an improvement in resolution of the first two peaks. See Figures 6A to 6C.

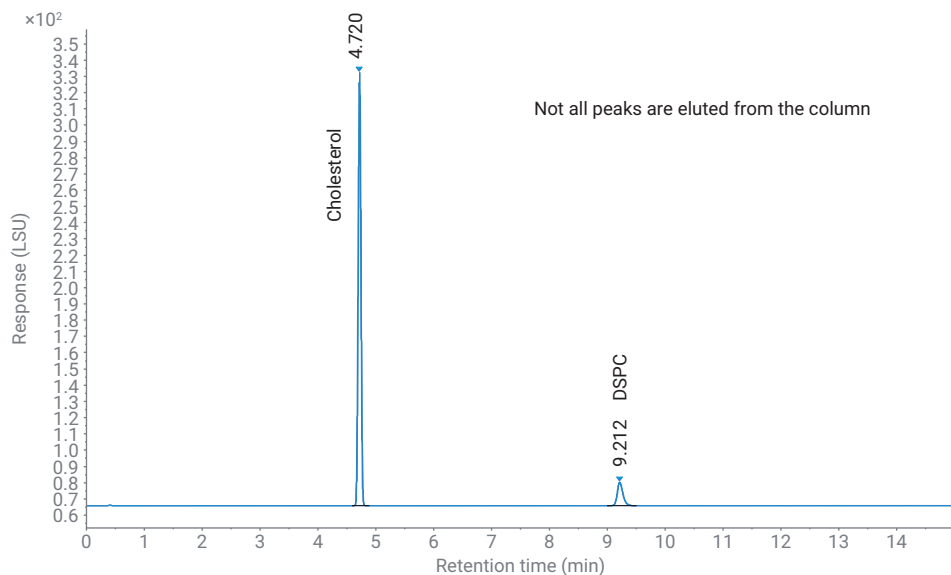


Figure 4. Ternary gradient from 80 to 90% MeOH in 5 minutes with a 5-minute hold.

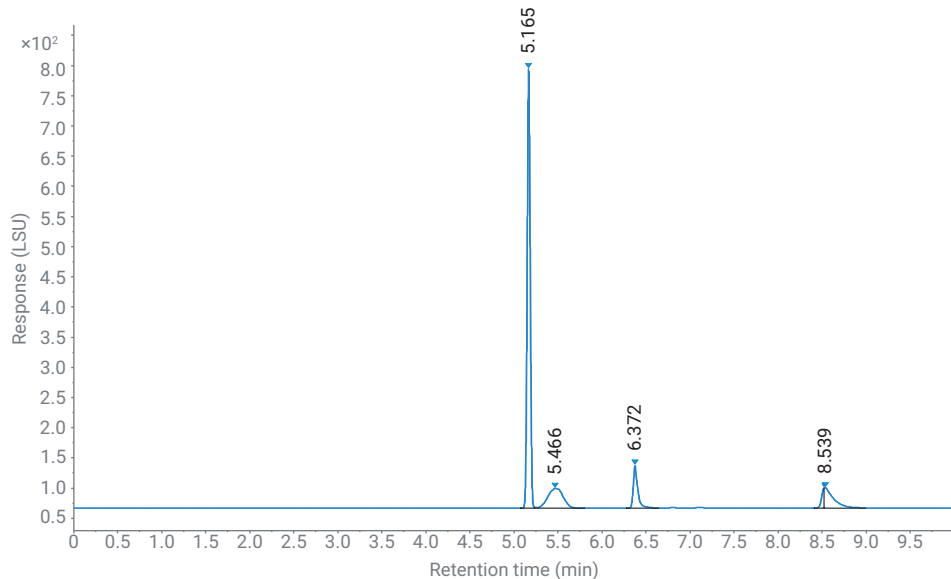
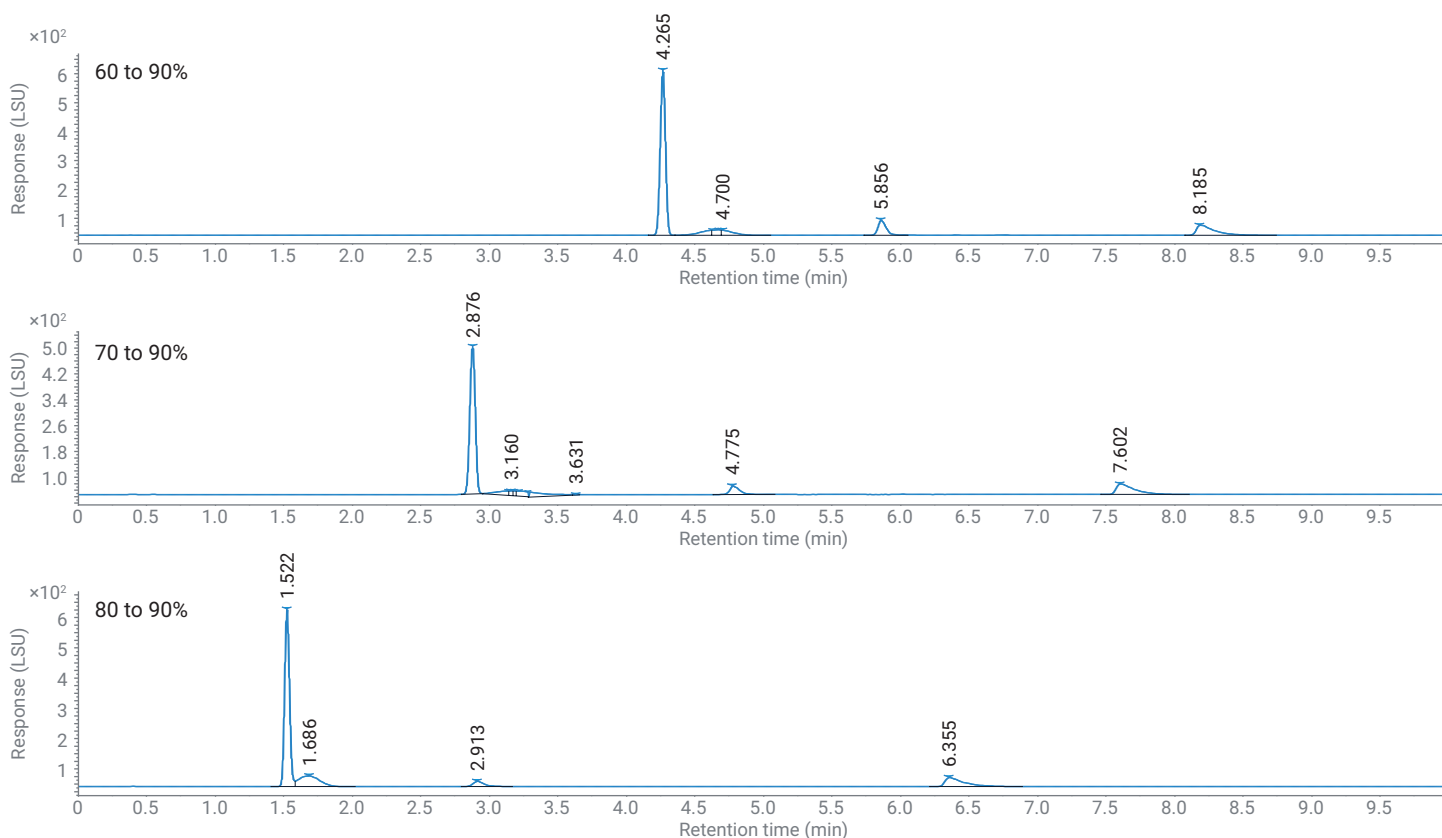


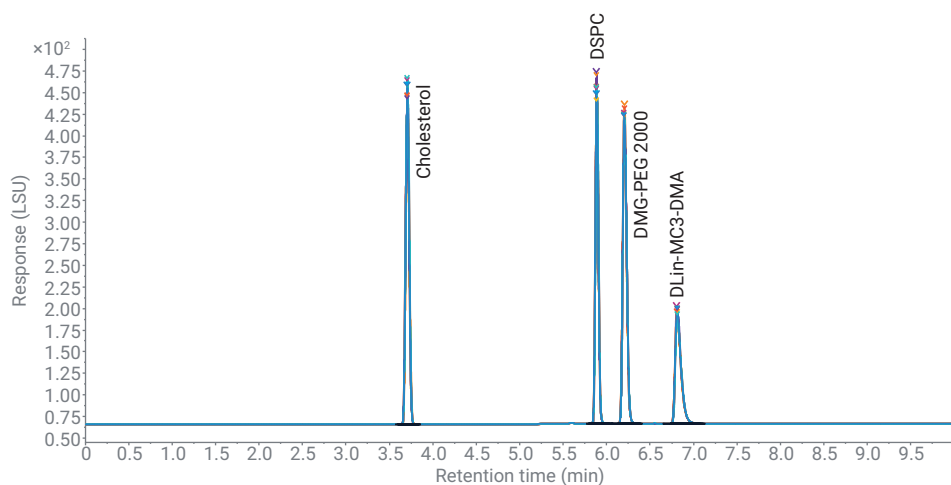
Figure 5. Ternary gradient from 50 to 90% B (ACN) in 5 minutes with a 5-minute hold.



**Figure 6.** Different gradient slopes for the elution from the four LNP components using ACN.

### Quaternary gradient combining MeOH and ACN

To enable complete elution with improved peak shapes, a quaternary gradient combining ACN with MeOH was evaluated, see Table 2 (experimental conditions). The quaternary gradient starting from 82% MeOH over 90% MeOH to 90% ACN resulted in baseline chromatographic separation with excellent peak shapes for all LNP components, even the ionizable lipid. An overlay of seven subsequent runs also showed excellent precision of retention time (RT) and good area precision (Figure 7 and Table 3).



**Figure 7.** Quaternary gradient using MeOH as well as ACN – Overlay of seven subsequent runs.

**Table 3.** Figure 7 peak RSD information.

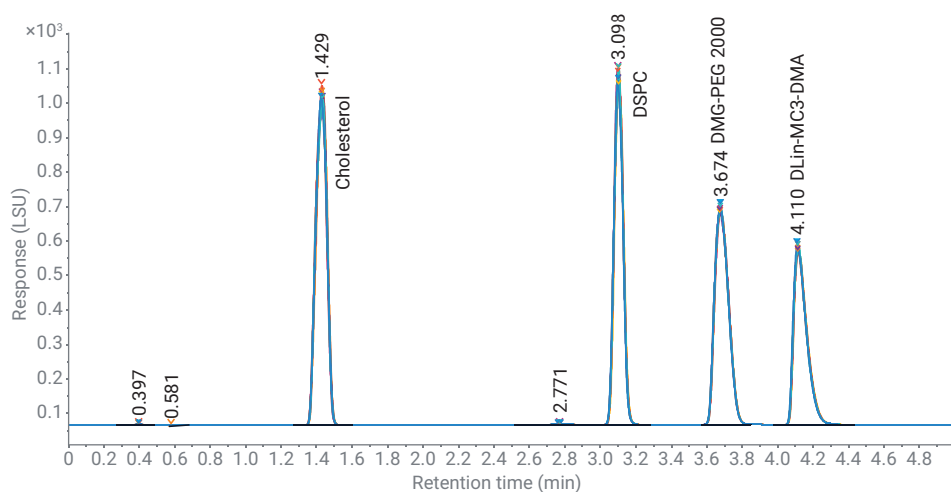
Peak	RSD RT (%)	RSD Area (%)
Cholesterol	0.1	1.875
DSPC	0.054	3.236
DMG-PEG 2000	0.055	1.172
DLin-MC3-DMA	0.061	0.998

Using this method, an equimolar mixture of all four lipids was analyzed on the column. These were diluted in series 1:3, ranging from 4.8625 nmol down to 2.22 pmol. Excellent limits of detection (LOD) were found between 0.46 and 8.1 pmol on column for a signal-to-noise (S/N) ratio of 3. Limits of quantification (LOQ) were found between 1.6 and 27 pmol on column for S/N = 10. The noise was calculated P2P for all peaks. All correlation curves showed excellent values for a quadratic curve model, with coefficients of determination ( $R^2$ ) over 0.999, except for DSPC with 0.995 (Table 4).

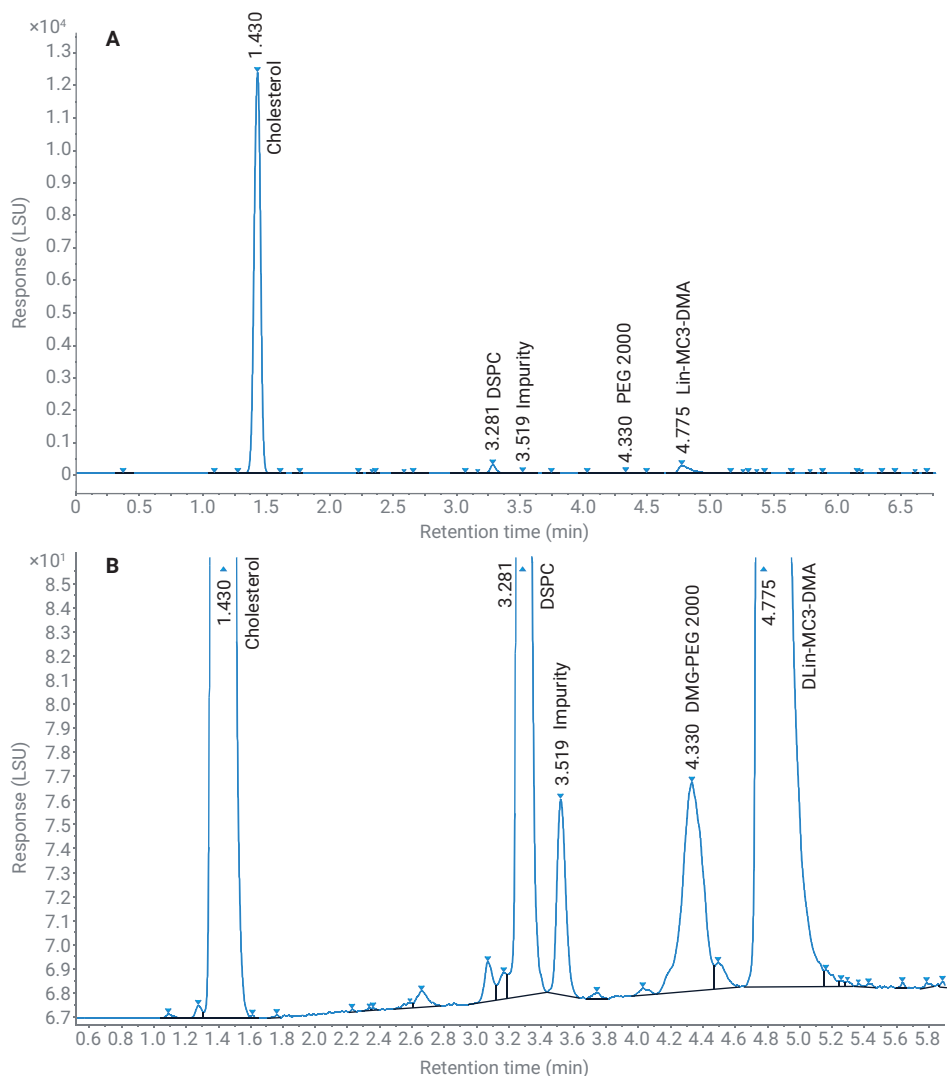
**Table 4.** LOD, LOQ, and correlation for the LNP components.

Peak	Lipid	LOD [pmol]	LOQ [pmol]	$R^2$
1	Cholesterol	8.1	27	0.99913
2	DSPC	2.3	7.8	0.99524
3	DMG-PEG 2000	0.46	1.6	0.99988
4	DLin-MC3-DMA	5.31	17.7	0.99963

Further method development to achieve shorter methods, while maintaining excellent peak shapes and resolution, led to a second variation of the MeOH/ACN solvent combination. The linear MeOH gradient step from 82 to 90% MeOH was omitted, and a gradient from 90% MeOH to 90% ACN in 3 minutes with 2-minute hold (Table 2) was used. Figure 8 shows the separation of the four LNP components using the short gradient. Table 5 lists the RSD values for Figure 8 peaks. Excellent resolution, peak shapes, and reproducibility for all lipid components was obtained using this short method. The area precision could be improved by injecting 5  $\mu$ L instead of 2  $\mu$ L (long method). The short method has the further advantage of being feasible to run on a binary pump, which can be beneficial for LC/MS and other analyses.



**Figure 8.** Short quaternary gradient from 90% MeOH to 90% ACN – Overlay of seven subsequent runs.



**Figure 9.** Analysis of the patisiran-like LNP sample (A), zoomed in version (B).

**Table 5.** Figure 8 peak RSD information.

Peak	RSD RT (%)	RSD Area (%)
Cholesterol	0.148	0.428
DSPC	0.090	1.807
DMG-PEG 2000	0.040	0.788
DLin-MC3-DMA	0.032	0.759

The quaternary method was transferred to a binary method, and applied to a sample mix of the four LNP components mimicking the LNP ratio of patisiran<sup>1</sup> with DMG-PEG 2,000, DLin-MC3-DMA, Cholesterol, and DSPC in a ratio of 1.5:50:38.5:10. This shows a need to achieve detection and quantification over a wide dynamic range, as shown in Figure 9.

## Conclusion

The quaternary setup used with the 1260 Infinity II Prime Bio LC has been shown to be ideal for solvent method development. The InfinityLab Poroshell 120 Phenyl-Hexyl chemistry provides superior flow dynamics with its superficially porous base particle, and a slightly lower hydrophobicity from traditional C18 when interacting with the large hydrophobic lipids found in LNPs. Along with the column, the solvent combination has proven to be the most critical factor in influencing peak shape and resolution. Within one organic solvent setup using only MeOH or ACN as a strong mobile phase, either incomplete elution of the lipid components (MeOH) or suboptimal peak shape and resolution were observed for all four peaks (ACN). With the quaternary setup, a high-resolution method using both organic solvents MeOH and ACN was developed.

The developed methods enabled the complete elution of all four LNP components with excellent peak shape and high resolution in a highly precise method for RT and area. In addition to excellent quadratic correlation with high coefficients of determination, the high dynamic range of the 1290 Infinity II ELSD enabled the detection and quantification of all four lipid components in the original ratio of the patisiran sample. The 1260 Infinity II Prime Bio LC, in combination with the 1290 Infinity II ELSD can therefore be highly recommended for the analysis of LNP components.

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RA44620.0930902778

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Printed in the USA, July 27, 2022  
5994-4709EN