

# Easy Column Screening for Lipid Nanoparticle Component Analysis

Method development with the Agilent Biocompatible 6-Column Selector Valve and 1260 Infinity II Prime Bio LC System

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## Abstract

The composition of lipid nanoparticles (LNPs) changes with nearly every new RNA-based drug. Over time, this has resulted in many different LNP variations that can be used as delivery vehicles for nucleic acids. The lipid components need to undergo analytical characterization to ensure safety and efficacy of the final drug product. This application note demonstrates a straightforward method for screening columns to identify the ideal hardware conditions that yield the highest level of separation for the lipid components. The combination of the Agilent 1260 Infinity II Prime Bio LC System with the Agilent Biocompatible 6-Column Selector Valve enables optimal method development conditions to find the most suitable column for LNP component analysis.

## Introduction

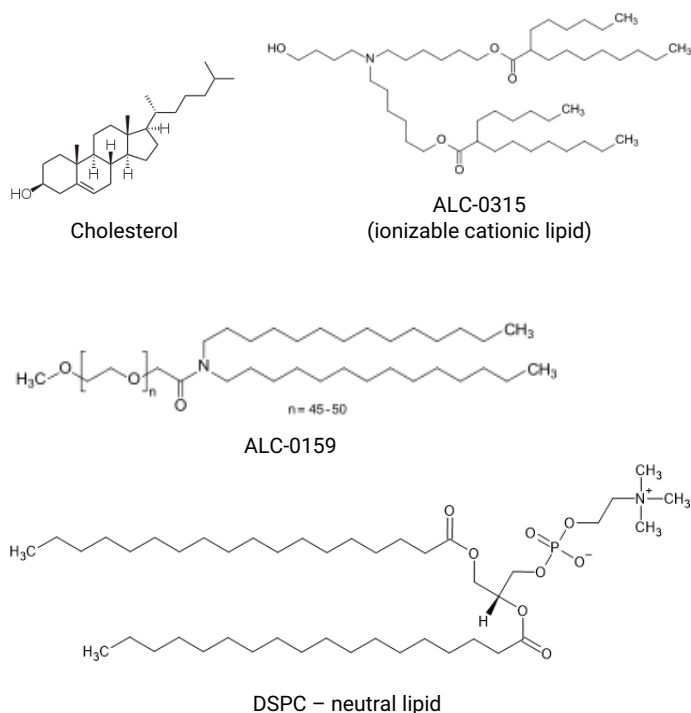
In recent years, promising opportunities for RNA-based drugs have raised interest in LNP research. The LNP system acts as a protective carrier against enzymatic degradation of nucleic acids and enables stable drug loading and enhanced delivery efficiency. Therefore, LNPs are the most widely used delivery vehicles for nucleic acids.<sup>1,2</sup> LNPs are typically composed of four main components<sup>3,4</sup>: cholesterol; a neutral phospholipid (mostly DSPC); and a polyethylene-glycol (PEG)-lipid, which all serve as structural lipids to control the particle size, provide particle stability and blood compatibility, and improve LNP circulation lifetime.<sup>5</sup> The fourth lipid in the composition is an ionizable cationic lipid, which plays an important role in nucleic acid encapsulation and endosome membrane disruption to release the nucleic acid cargo into the cytosol.<sup>6</sup>

The formulation of the LNP composition changes constantly with more RNA-based drugs being released. Furthermore, the design of the ionizable lipids has undergone numerous enhancements to improve potency. This results in various LNP compositions, with proprietary versions for every new RNA-based drug.

The individual lipid components of an LNP system need analytical characterization to ensure safety and efficacy of the complete drug (e.g., composition, identity, and purity).<sup>7</sup> Although the composition consists of only four components, the analytical characterization using high-performance liquid chromatography (HPLC) can be challenging when trying to achieve baseline separation or adequate peak shapes.<sup>8</sup> Typically, LNP analysis for composition or degradation is carried out by reversed-phase high-performance liquid chromatography (RP-HPLC).<sup>9</sup>

There are several RP column phases to be considered for the analysis of LNP composition. To enable quick and effortless column screening, valve solutions need to be part of the instrument setup. The biocompatible version of the 6-Column Selector Valve was used with the 1260 Infinity II Prime Bio LC to enable easy screening of six different column phases. Furthermore, the Agilent 1290 Infinity II Evaporative Light Scattering Detector (ELSD) used in this study is ideal for reproducible and sensitive detection of lipids, as already described in a previous publication.<sup>8</sup>

This application note shows column screening for the analysis of the four components of the Pfizer–BioNTech vaccine (BNT162b2)<sup>3</sup>, shown in Figure 1. The different chemical natures of the four components—including the hydrophobic DSPC, the ionizable lipid, and the PEGylated lipid—make it challenging to achieve symmetrical and sharp peak shapes for all components using one stationary- and mobile-phase combination.



**Figure 1.** Main components of the BNT162b2 vaccine LNP.

## Experimental

### Equipment

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

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option number 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with the Agilent Biocompatible 6-Column Selector Valve (part number 5320-0025) plus Biocompatible Capillary Kit for 6-Column Selector Valve, 0.12 mm id (part number 5005-0070)
- Agilent 1290 Infinity II ELSD (G7102A)

### Software

The software used in this study was Agilent OpenLab CDS software, version 2.6. Later versions also apply.

## Columns

The following Agilent columns were used in this study:

- InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 μm (part number 699675-912)
- AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 μm (part number 799775-944)
- ZORBAX RRHD 300 Å Diphenyl, 2.1 × 50 mm, 1.8 μm (part number 857750-944)
- InfinityLab Poroshell 120 CS-C18, 2.1 × 50 mm, 2.7 μm (part number 679775-942)
- ZORBAX StableBond 300 CN, 4.6 × 50 mm, 3.5 μm (part number 865973-905)
- AdvanceBio Peptide Plus, 2.1 × 50 mm, 2.7 μm (part number 699775-949)

## Chemicals

Agilent InfinityLab ultrapure LC/MS methanol (part number 5191-4497) and Agilent InfinityLab ultrapure LC/MS acetonitrile (part number 5191-4496) was used for all ELSD analyses. 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, U.S.). 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). ALC-0315 (2-hexyl-decanoic acid, 1,1'-[[[4-hydroxybutyl]imino]di-6,1-hexanediy] ester and ALC-0159 (alpha-[2-(ditetradecylamino)-2-oxoethyl]-omega-methoxy-poly(oxy-1,2-ethanediy]) were obtained from Cayman Chemical (MI, U.S.).

Each of the samples were dissolved in methanol at a concentration of 3.89 mM, except ALC-0315, which was dissolved at 7.78 mM. To enable complete dissolution, the tubes were warmed to 35 °C for 3 to 4 minutes before further use. The components of the analyzed samples were used to mimic the LNP used in the BNT162b2 vaccine from Pfizer–BioNTech.<sup>3</sup> For similar peak area, the following mixture was prepared: 50 μL cholesterol (3.89 mM) + 50 μL DSPC (3.89 mM) + 50 μL ALC-0159 (3.89 mM) + 450 μL ALC-0315 (7.78 mM).

## Buffer preparation

Ammonium acetate, 500 mM, at ~pH 7 (no further pH adjustment) was prepared and filtered using a 0.2 μm membrane filter.

## Methods

**Table 1.** Chromatographic conditions.

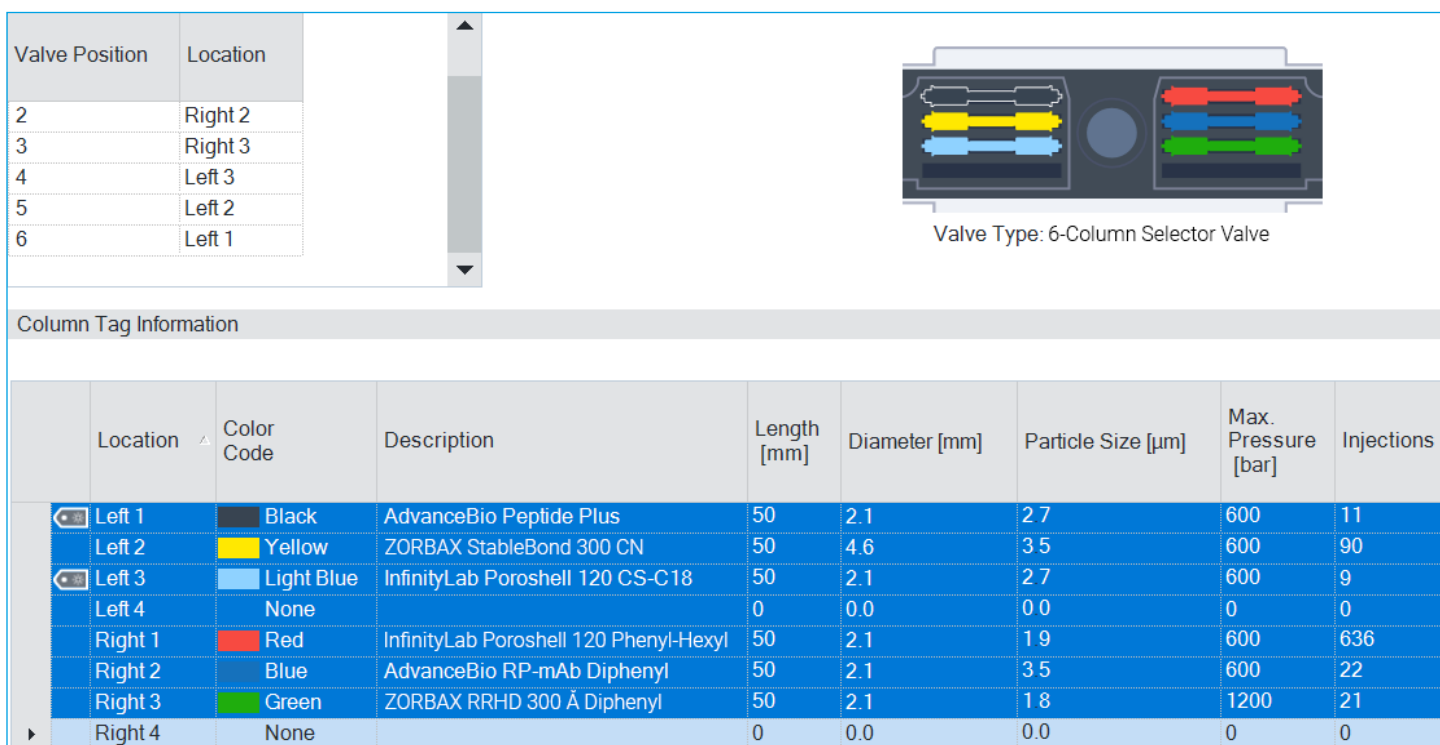
Parameter	Value				
Solvent	A: Methanol (MeOH) B: Acetonitrile (ACN) C: 500 mM ammonium acetate D: H <sub>2</sub> O				
Flow Rate	0.4 mL/min				
Quaternary Gradient General 1	Time (min)	A (%)	B (%)	C (%)	D (%)
	0	70	0	2	28
	3	70	0	2	28
	5	90	0	2	8
Stop time: 10 min Post time: 5 min					
Quaternary Gradient General 2	Time (min)	A (%)	B (%)	C (%)	D (%)
	0	50	0	2	48
	4	90	0	2	8
	7	0	90	2	8
Post time: 5 min					
Gradient Optimized for Agilent ZORBAX RRHD 300 Å Diphenyl	Time (min)	A (%)	B (%)	C (%)	D (%)
	0	60	0	2	38
	3	90	0	2	8
	5	0	90	2	8
Stop time: 10 min Post time: 5 min					
Gradient Optimized for Agilent InfinityLab Poroshell 120 Phenyl-Hexyl	Time (min)	A (%)	B (%)	C (%)	D (%)
	0	90	0	2	8
	3	90	0	2	8
	5	10	90	0	0
Stop time: 10 min Post time: 5 min					
Detection ELSD	Evaporator temperature: 40 °C Nebulizer temperature: 40 °C Gas flow rate: 1.6 SLM Data rate: 80 Hz Smoothing: 10 (1 s)				
Injection	Injection volume: 2 μL Sample temperature: 25 °C Needle wash: 3 s with 50% isopropanol in H <sub>2</sub> O				

## Results and discussion

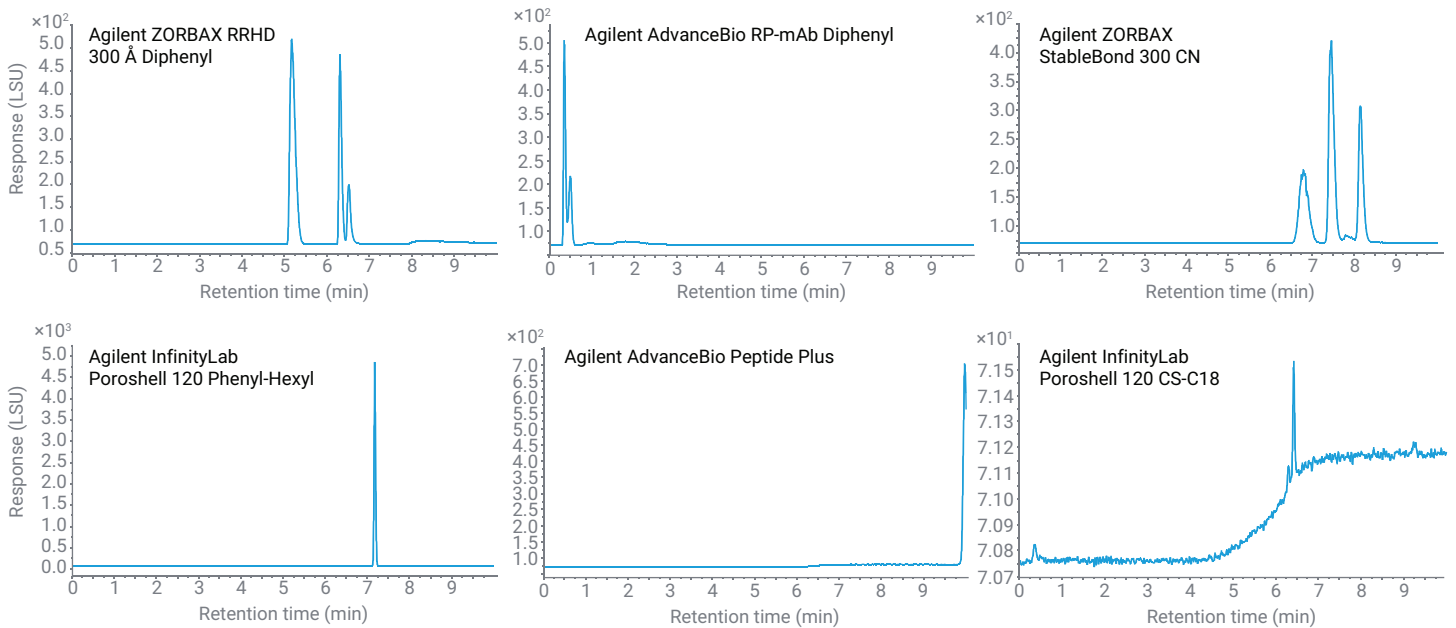
The preselected six columns were installed in the 1290 Infinity II Multicolumn Thermostat using the Biocompatible 6-Column Selector Valve plus Biocompatible Capillary Kit for the 6-Column Selector Valve, 0.12 mm id, also including six Agilent Quick-Connect Biocompatible Heat Exchangers, standard flow. Figure 2 shows the column setup displayed in the method driver of the 1290 Infinity II Multicolumn Thermostat in OpenLab CDS version 2.6. The color coding in the scheme and the table help to gain full visibility and a clear view of the column positions.

To screen all six columns in the first round, a general gradient from 70 to 90% MeOH was employed with a continuous delivery of 2% ammonium acetate of 500 mM ammonium acetate stock solution to enable a constant concentration of 10 mM buffered solution. Figure 3 shows the chromatograms

of all six columns for the separation of the used LNP sample mix comprising the four lipid components of the BNT162b2 vaccine. The separation on the ZORBAX RRHD 300 Å Diphenyl column looked already promising, so the column was used for further method development. The ZORBAX StableBond 300 CN also looked promising; however, the peak shape was not ideal for all peaks. For the AdvanceBio RP-mAb Diphenyl, the elution conditions were already too strong, so everything eluted at the beginning of the chromatogram. For the InfinityLab Poroshell 120 Phenyl-Hexyl, the AdvanceBio Peptide Plus, and the InfinityLab Poroshell 120 CS-C18, the elution conditions were not strong enough; so, in the second round, a stronger eluent (ACN) was employed. All columns except the ZORBAX RRHD 300 Å Diphenyl were moved to the second round using a second general gradient, this time using a gradient from 50 to 90% MeOH in 4 minutes, then to 90% ACN from 4 to 7 minutes.



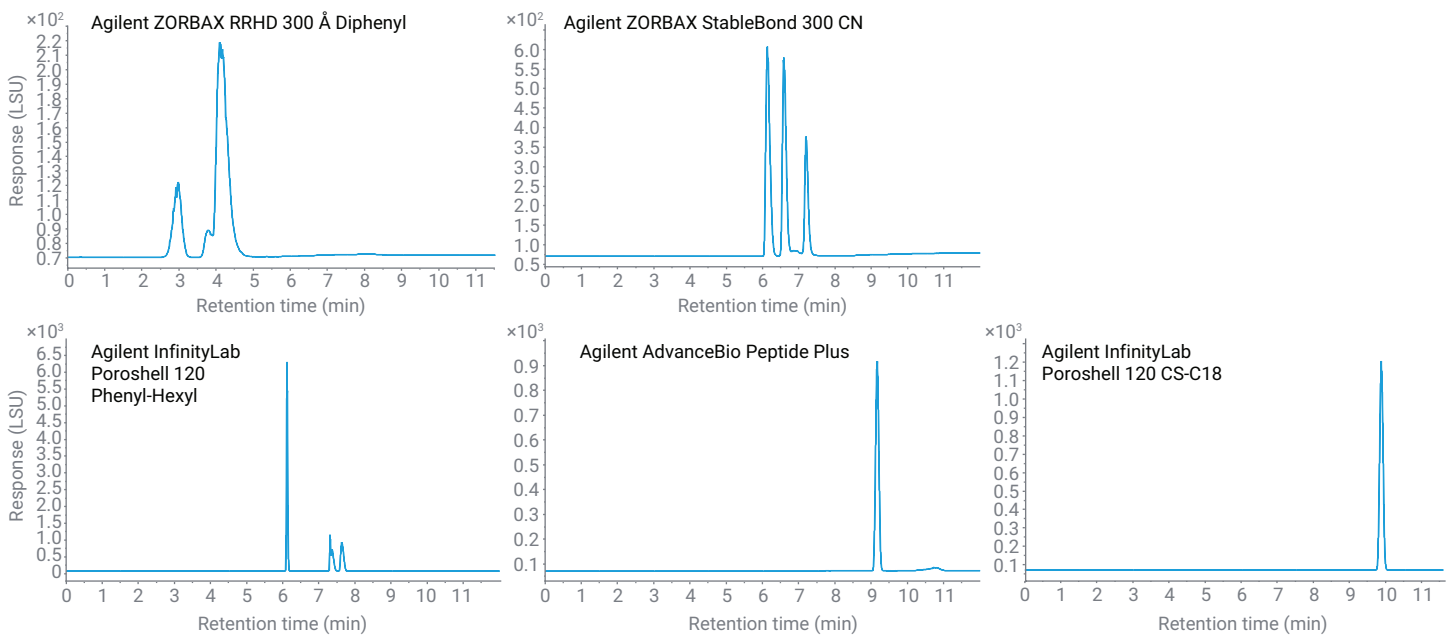
**Figure 2.** Setup of six different columns with the Agilent Biocompatible 6-Column Selector Valve, displayed in the method driver of the Agilent 1290 Infinity II Multicolumn Thermostat.



**Figure 3.** Column screening first round—general gradient 1.

The results of the second round using general gradient 2, which used ACN, are displayed in Figure 4. The separation on the AdvanceBio RP-mAb Diphenyl was not very promising, so this column did not move to further method development. The same is true for both the AdvanceBio Peptide Plus and the InfinityLab Poroshell 120 CS-C18 column. For both columns, the elution strength of the used methods was not

sufficient, so both columns would not be used in further method development. In contrast, the separation on both the ZORBAX StableBond 300 Å CN and the InfinityLab Poroshell 120 Phenyl-Hexyl looked promising, with already good separation of three peaks and good peak shape. Both columns were employed for further method optimization.



**Figure 4.** Column screening second round—general gradient 2.

### Optimization for the ZORBAX RRHD 300 Å Diphenyl

The start point for further method optimization was general gradient 1, with a gradient from 70 to 90% MeOH. With this gradient, three of four components were clearly separated with good peak shape. The PEGylated lipid, ALC-1059, was visible as small hump at the end of the chromatogram. To improve the peak shape for ALC-1059, ACN was added to the gradient (Figure 5). Additional method development by adjusting gradient parameters did not further improve the peak shape of ALC-1059, so the final method was found as a gradient from 60 to 90% MeOH in 3 minutes, going to 90% ACN from 3 to 5 minutes (see the "Experimental" section).

### Optimization for the ZORBAX StableBond 300 CN

Although the initial separation with the second general gradient looked promising for the ZORBAX StableBond 300 CN (see Figure 4), it was not possible to improve the separation further. Additional method development regarding solvent optimization might be required, but was not pursued.

### Optimization for the InfinityLab Poroshell 120 Phenyl-Hexyl

The start point for further method optimization was general gradient 2, which had a gradient from 50 to 90% MeOH in 4 minutes, increasing to 90% ACN from 4 to 7 minutes. With this gradient, two of the four components were not baseline separated, so the gradient was adjusted to include an initial 3-minute isocratic hold at 90% MeOH, increasing to 90% ACN from 3 to 5 minutes (see the "Experimental" section). With this optimized gradient, baseline separation with excellent peak shapes was achieved. For the screened columns, this was the best result achieved. Further studies for this column and solvent combination on reproducibility, linearity, and sensitivity for similar conditions can be found in a previous application note.<sup>8</sup>

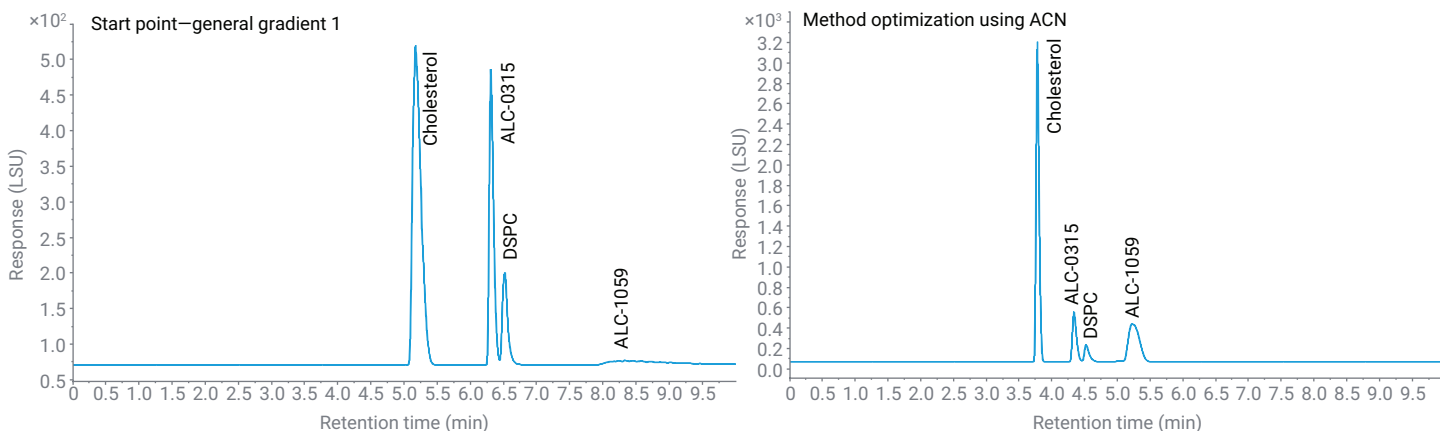


Figure 5. Method optimization for the Agilent ZORBAX RRHD 300 Å Diphenyl column.

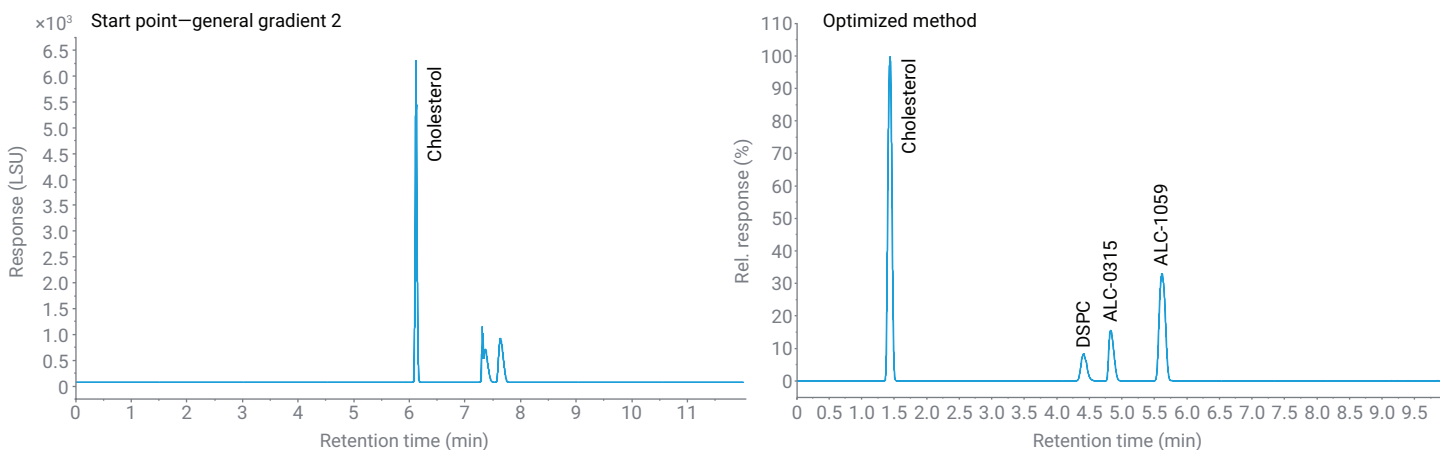


Figure 6. Method optimization for the Agilent InfinityLab Poroshell 120 Phenyl-Hexyl column.

## Conclusion

The Agilent 1260 Infinity II Prime Bio LC with the Agilent Biocompatible 6-Column Selector Valve has been shown to be ideal for convenient column screening to find the optimal hardware conditions for LNP component analysis. From the six screened columns, the Agilent ZORBAX RRHD 300 Å Diphenyl and InfinityLab Poroshell 120 Phenyl-Hexyl columns showed the best baseline separation of the four employed lipids, mimicking the BNT162b2 vaccine from Pfizer–BioNTech. From these two, the InfinityLab Poroshell 120 Phenyl-Hexyl showed superior performance regarding baseline separation as well as peak shape. For the analysis of LNP components, the combination of the 1260 Infinity II Prime Bio LC with the InfinityLab Poroshell 120 Phenyl-Hexyl column can be highly recommended.

## References

1. Pardi, N. *et al.* Expression Kinetics of Nucleoside-modified mRNA Delivered in Lipid Nanoparticles to Mice by Various Routes. *J. Control. Release* **2015**, *217*, 345–351. DOI: 10.1016/j.jconrel.2015.08.007
2. Wang, Y. *et al.* Delivery of Oligonucleotides with Lipid Nanoparticles. *Advanced Drug Delivery Review*. **2015**, *87*, 68–80. DOI: 10.1016/j.addr.2015.02.007
3. Schoenmaker, L. *et al.* mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *Int. J. Pharm.* **2021**, *601*, 120586. DOI: 10.1016/j.ijpharm.2021.120586
4. Evers, M. J. W. *et al.* State-of-the-Art Design and Rapid-Mixing Production Techniques of Lipid Nanoparticles for Nucleic Acid Delivery. *Small Methods* **2018**, *2*(9). DOI: 10.1002/smt.201700375
5. Kim, J. *et al.* Self-assembled mRNA vaccines. *Advanced Drug Delivery Reviews*. **2021**, *170*, 83–112. DOI: 10.1016/j.addr.2020.12.014
6. Albertsen, C.H. *et al.* The Role of Lipid Components in Lipid Nanoparticles for Vaccines and Gene Therapy. *Advanced Drug Delivery Reviews* **2022**, *188*, 114416. DOI: 10.1016/j.addr.2022.114416
7. Center for Drug Evaluation and Research, Food and Drug Administration. NDA 210922 ONPATTRO (patisiran) Lipid Complex Injection, *Addendum to Drug Product Quality Review*, November **2018**. [https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2018/210922Orig1s000ChemR.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210922Orig1s000ChemR.pdf)
8. Schneider S. Analysis of Lipid Nanoparticle Composition. *Agilent Technologies application note*, publication number 5994-4709EN, **2022**.
9. Fan, Y.; Marioli, M.; Zhang K. Analytical Characterization of Liposomes and Other Lipid Nanoparticles for Drug Delivery. *J. Pharm. Biomed. Anal.* **2021**, *192*, 113642. DOI: 10.1016/j.jpba.2020.113642

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DE13117191

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Printed in the USA, April 25, 2023  
5994-6051EN