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Applikationsbericht

Optimized ELSD Workflow for Improved Detection of Lipid Nanoparticle Components

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Abstract

Gene and nucleic acid-based therapeutics have received notable attention in recent years. In order for these therapeutics to reach their desired target, a suitable delivery vehicle is necessary to maintain stability and bioavailability. Lipid nanoparticles (LNPs) have been proven to be highly effective delivery vehicles. Careful analysis of LNP related species is critical to ensuring their safety and efficacy through the development and manufacturing process. In these workflows, evaporative light scattering (ELS) is effective as a “universal” detection technique. Optimization of detector response to increase sensitivity in terms of limit-of-detection (LOD) can increase confidence in the analysis of LNPs and their associated impurities. This study shows the systematic optimization of an LNP workflow utilizing ELSD from the solvent in which samples are reconstituted to the parameters used for ELS detection. The final optimized method yields on average a five-fold improvement in signal-to-noise ratio for each of the four lipid components tested without increasing analysis time or sample consumption. These results demonstrate how taking the time to mindfully optimize a workflow can substantially improve the analysis of LNPs.

Benefits

- Easy-to-use ACQUITY™ ELSD enables detection of lipid nanoparticle components for routine monitoring

workflows

- Systematic optimization of detection parameters leads to increased sensitivity for lipid analyses
- Simple modification to mobile phase composition increases resolution and detection of polydisperse compounds without sacrificing analytical throughput

Introduction

With the onset of the COVID-19 global pandemic, there has been elevated focus on mRNA-based vaccines and other gene and nucleic acid-based therapeutics. To ensure targeted delivery of the mRNA to cells, a delivery vehicle is necessary for encapsulation to increase the stability and bioavailability of the mRNA. LNPs have been developed as highly effective delivery vehicles for mRNA and have enabled the successful development of vaccines to combat COVID-19.[1] These lipid shells are comprised of four types of lipids. The primary driver of potency is the ionizable lipid, which binds to the negatively-charged mRNA and stabilizes the LNP.[2] This ionizable lipid accounts for approximately 50% of LNP composition. The other three lipids include cholesterol (CHO), which provides fluidity to the lipid shell, a phospholipid such as distearoylphosphatidylcholine (DSPC), which acts as a helper lipid, and a PEGylated lipid (PEG) that generates the hydrophilic micelle outer surface.[2] The exact percentages of these remaining lipids are specific to the individual therapeutic and are generally regarded as intellectual property. Ensuring that the LNPs are prepared with the correct ratios of different lipid components, as well as assessing the purity and quality of the raw materials used to prepare the LNPs, are critical steps to ensuring their safety and efficacy as delivery vehicles.

There are several platforms available for carrying out LNP analytical workflows from development to manufacturing. For upstream workflows such as discovery and characterization, liquid chromatography (LC) coupled to mass spectrometry is typically employed. For downstream processes, including monitoring and manufacturing workflows, optical methods such as ultraviolet (UV) or fluorescence detection are used with LC separation. However, these detection methods require analyte molecules to contain chromophores, which is not always the case for lipids. Alternatively, evaporative light scattering detection (ELSD), often referred to as a “universal” detection technique, offers detection of non-volatile and semi-volatile compounds making this detector a logical choice for analysis of lipid nanoparticles in downstream workflows. The ACQUITY ELS Detector contains an optimized drift tube, nebulizer, and gas flow to minimize dispersion for optimal UPLC™ System performance.

Furthermore, this detector has a compact footprint and easily integrates into existing UPLC System configurations, including those with optical detection.

The general mechanism for ELS detection is shown in the schematic in Figure 1. As sample elutes from the column and enters the detector, it mixes with nitrogen carrier gas and is nebulized into an aerosol of droplets. The larger droplets move to the outer walls of the nebulization chamber and are diverted to waste, while smaller droplets are carried by the nitrogen gas to the drift tube. While in the drift tube, the volatile mobile phase is evaporated, leading to the production of particles that then pass through a light path. The particles are then detected based on the amount of light scattered, which is related to the amount of compound. Mindful optimization of each of the parameters affecting this process is critical as it can impact the formation of droplet particles and affect the resulting detection. Here, we systematically evaluated and optimized ELSD parameters for the analysis of LNP components in a routine monitoring workflow.

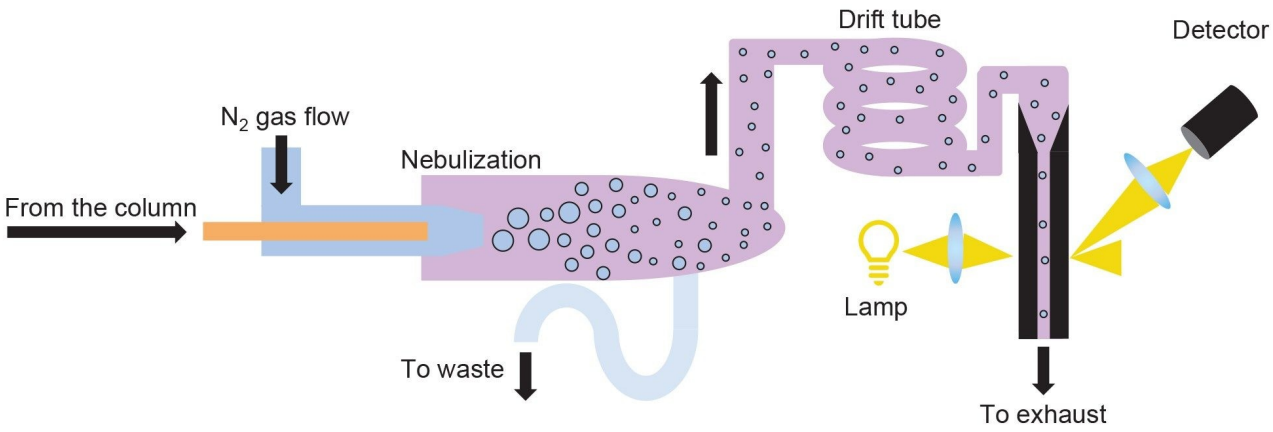


Figure 1. Overview of mechanism for ELS detection.

Experimental

All lipids in this study were used exclusively for research and demonstration purposes and were purchased from the following vendors: cholesterol and DSPC from Sigma-Aldrich; DMG-PEG 2000 from Avanti Lipid; SM-102 from BroadPharm.

Sample Description

Stocks of each lipid were prepared in methanol at 1 mg/mL. Samples were diluted to the appropriate concentration at 90/10 methanol/water (v/v).

LC Conditions

LC system:	ACQUITY Quaternary Solvent Manager System
Detection:	ACQUITY UPLC Evaporative Light Scattering
Gas flow pressure:	20.0 psi
Nebulizer mode:	Heating
Power level:	80%
Drift tube temp.:	48 °C
Vials:	TruView™ Max Recovery Vials, (p/n: 186005662CV)
Vial caps:	Polyethylene Septumless Screw Cap, (p/n: 186004169)
Column(s):	ACQUITY Premier CSH™ Phenyl-Hexyl Column, 1.7 µm, 2.1 mm x 50 mm (p/n: 186009474)
Column temp.:	50 °C
Sample temp.:	8 °C
Injection volume:	3 µL
Flow rate:	0.400 mL/min

Mobile phase A:	0.1 % formic acid (v/v) in water (LCMS grade)
Mobile phase B:	0.1 % formic acid/25% isopropyl alcohol/75% acetonitrile (v/v/v) (LCMS grade)

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.400	40.0	60.0	Initial
6.00	0.400	0.0	100.0	6
8.00	0.400	0.0	100.0	6
8.50	0.400	40.0	60.0	6
12.00	0.400	40.0	60.0	6

Data Management

Chromatography software:	Empower™ 3, FR5

Results and Discussion

In LNP workflows, sensitive analysis of raw materials for impurities and accurate quantitation of compositional ratios are essential to ensuring the safety and efficacy of the resulting LNP delivery vehicle. Paying careful attention to the effect of each parameter and tailoring workflows to the needs of specific analyses is critical during the method development phase to ensure assays are performing optimally. Here, we discuss the effects of various parameters on the resulting ELS detection of LNP components.

Sample Preparation

When preparing lipid samples for analysis on any instrument, considerations should be made for the composition of the solvent the sample is reconstituted in for injection. To maximize the detection of each lipid, different solvent compositions containing 40–100% methanol were systematically tested. The resulting peak areas for each component were plotted as a function of methanol composition, as shown in Figure 2. While each component varied in response at different solvent compositions, generally, higher methanol composition led to greater analyte response, with 90% methanol yielding the highest response on average. This finding is consistent with previously published data[3] and aligns with expectations given that the lipid structures evaluated, with the exception of cholesterol, consist of a polar head group and two hydrophobic tails, making them primarily hydrophobic. Therefore, 90/10 methanol/water was used as the sample solvent for injection of all lipid samples in this study.

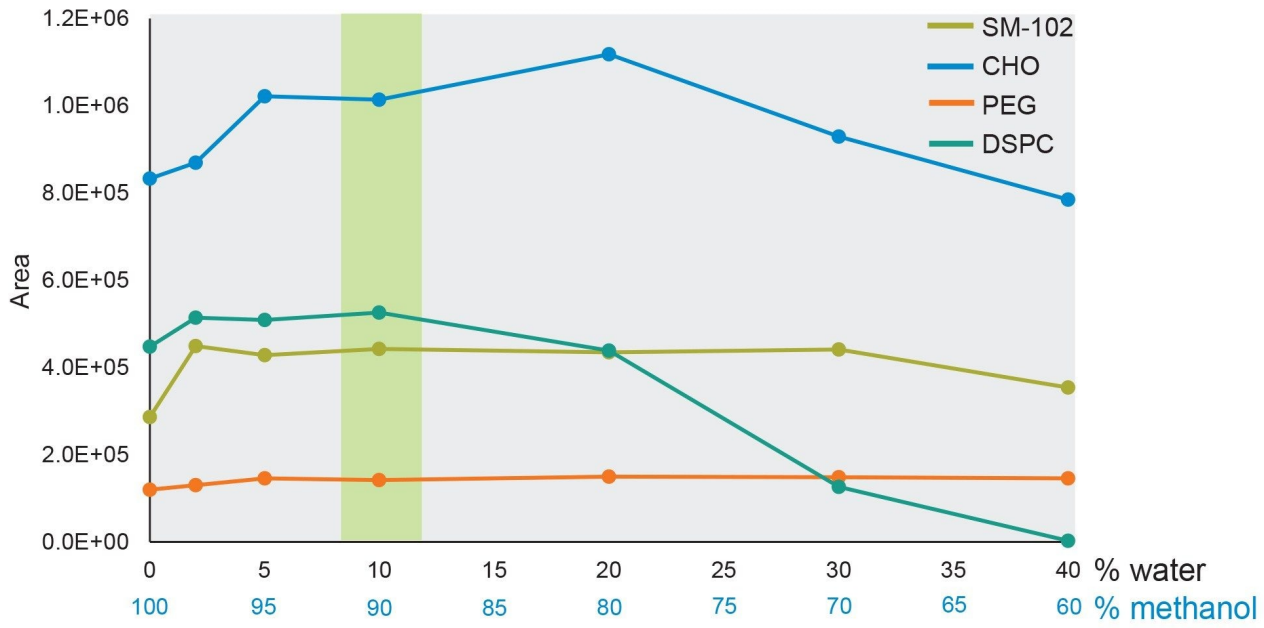


Figure 2. The effect of sample solvent composition on resulting peak areas of each lipid nanoparticle component. Each line indicates a different lipid component, including an ionizable lipid (SM-102), cholesterol (CHO), PEGylated lipid DMG-PEG 2000 (PEG), and distearoylphospatidylcholine (DSPC).

LC Separation

To ensure accurate quantification of lipid nanoparticle components, peaks that are sufficiently resolved and can

be detected reliably at low concentration levels are highly desirable. The first step in optimizing an LC separation is to perform a column screening of the analyte mixture using a panel of appropriate columns to determine which format and stationary phase offers the best performance based on the application needs. This step has been discussed in a previous Waters application note, in which the ACQUITY Premier CSH Phenyl Hexyl Column was demonstrated to yield the best separation between the four lipid nanoparticle components.[4]

Building on the previous study's findings, column length and separation time were evaluated with respect to theoretical plates and gradient as a means to further improve the resolution between peaks, most notably between the CHO and PEG peaks. To evaluate these aspects, two different column lengths were tested, including 50 mm and 100 mm. An LNP mixture was run on each column at three different gradients, including a 6-minute (steepest), 10-minute, and 12-minute (shallowest) gradient. Figure 3 shows the resulting chromatogram for each of the separations. As expected, the peak-to-peak resolution increases with shallower gradients, as depicted left to right. The peak-to-peak resolution increases as well with increased column length, as can be seen when comparing the top row and bottom row of Figure 3, with the exception of the 6-minute gradient, which did not allow sufficient time for compounds to elute from the 100 mm column. In both cases, increased peak broadening was observed for the PEG peak as shown in the insets of Figure 3, possibly due to a combination of sample heterogeneity and increased longitudinal diffusion. This peak broadening causes PEG to become more challenging to detect and lowers the overall sensitivity of the analysis.

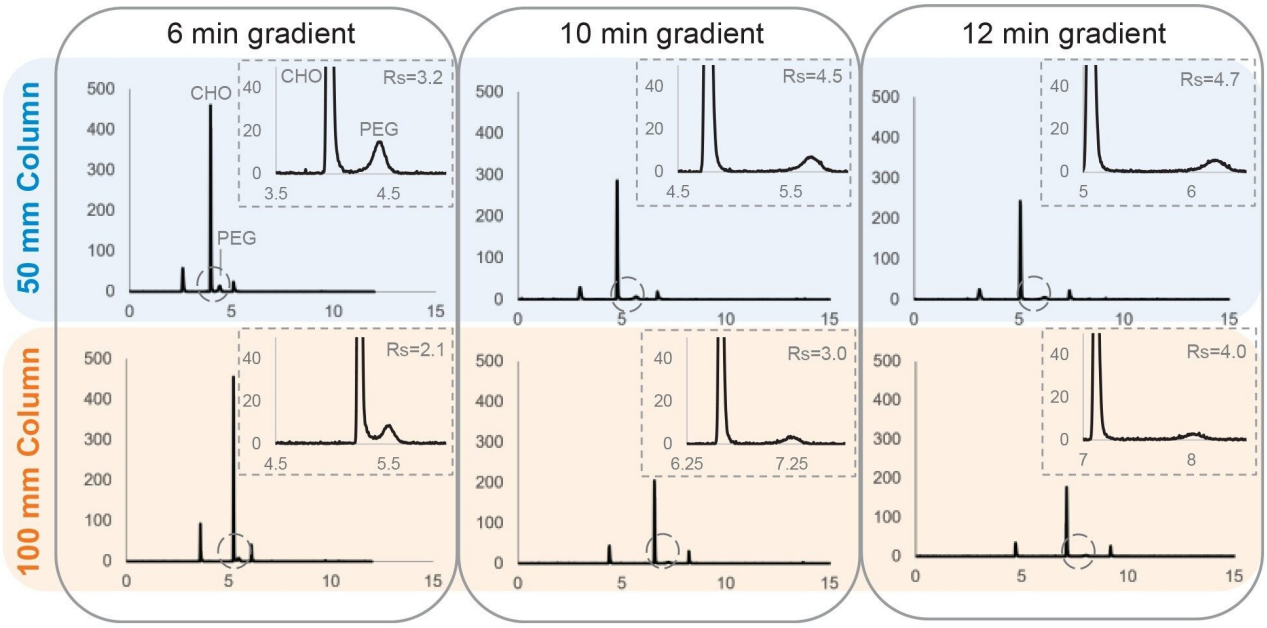


Figure 3. The effect of separation length on chromatographic resolution, including three different gradient lengths (left to right, 6 min, 10 min, and 12 min) with two different column lengths (top 50 mm, bottom 100 mm). The insets show the broadening effect on the PEGylated lipid peak due to increasing retention time.

To improve peak resolution without sacrificing detection of PEG, the mobile phase composition was evaluated in terms of elution strength to improve mass transfer and overall peak shape. Previous studies have shown isopropyl alcohol (IPA) increases elution strength of the mobile phase, which can improve the separation of lipids in reversed-phase techniques.[5] In this study, the addition of IPA to mobile phase B (0.1% formic acid in acetonitrile) up to 50% v/v was evaluated. As shown in Figure 4, increasing the amount of IPA in mobile phase B up to 25% (v/v) improves PEG's peak shape, leading to an increase in resolution between CHO and PEG. At 50% IPA, the increased elution strength causes CHO and PEG to coelute again when using the same gradient. In this respect, using an IPA concentration between 10% and 25% in mobile phase B leads to the greatest resolution between peaks with the six-minute gradient shown.

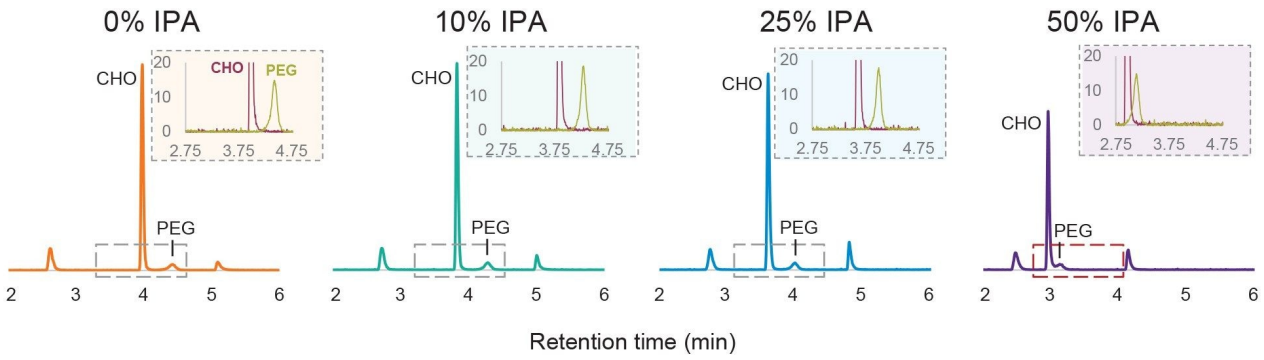


Figure 4. The effect of increasing volume percent of isopropyl alcohol (IPA) in mobile phase B on the chromatographic separation of four lipid nanoparticle components. Insets show the effect on resolution of cholesterol and PEG.

ELSD Parameters

The three main ELSD parameters that affect aerosol and particle formation are the drift tube temperature, nebulizer power, and rate of gas flow throughout the instrument. It is generally recommended to optimize these parameters for each application, as they can vary with mobile phase, flow rate, and sample stability.

The drift tube is where particles are produced from the droplets formed in the nebulizer. Shown in Figure 5 is the effect of drift tube temperature on the peak response of cholesterol. Each peak showed the highest intensity at a drift tube temperature of 48 °C. To ensure that the increased signal did not also lead to an increase in baseline noise, the signal-to-noise ratio (S/N) was also calculated for each peak. The trend was similar to that of the peak intensity, where the lower drift tube temperature (48 °C) improved S/N for each peak. However, lowering the temperature to below 48° C may result in a loss of signal, indicating that the mobile phase is not able to evaporate from the analyte compounds.

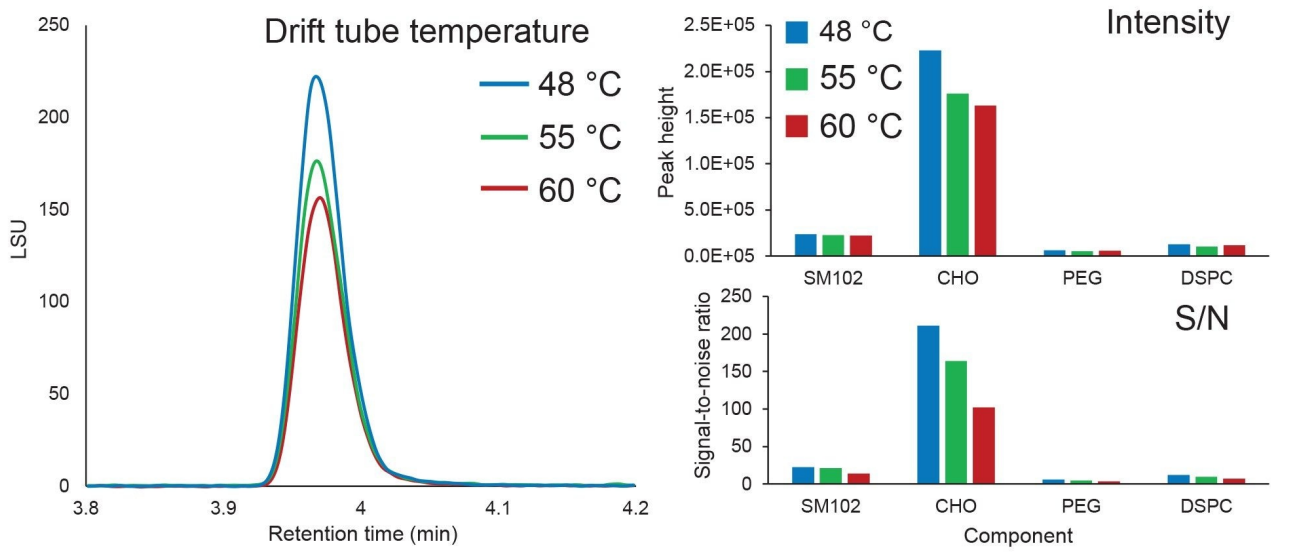


Figure 5. Effect of drift tube temperature on analyte response, including (left) an overlay of chromatograms for cholesterol, and numerical values for (top) peak intensity and (bottom) signal to noise ratio (S/N) for each component, including an ionizable lipid (SM-102), cholesterol (CHO), PEGylated lipid DMG-PEG 2000 (PEG), and distearoylphospatidylcholine (DSPC).

The nebulizer power is set as a percentage relative to the drift tube temperature. For example, if the drift tube temperature is set to 48 °C, a nebulizer power of 100% indicates 48 °C, while 80% indicates 42 °C, etc. During nebulization, heating or cooling can be applied to affect the creation of aerosol as the mobile phase is mixed with the carrier gas. Figure 6 shows the effect of the nebulizer power on the resulting peak signal, including cooling mode and five different power levels in heating mode. As the nebulizer power increases, both the intensity and S/N increase for each component. This trend can also be visualized in the chromatogram in Figure 6. For this study, a nebulizer power of 80% was found to have the highest S/N where power settings greater than 80% had negligible impact on S/N.

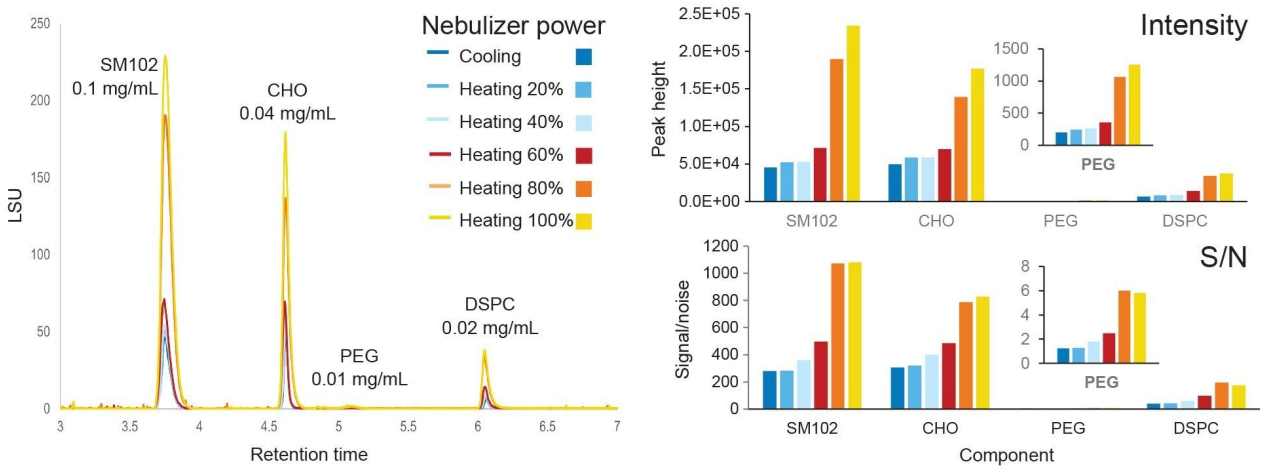


Figure 6. Effect of nebulizer power on analyte response, including (left) an overlay of chromatograms, and numerical values for (top) peak intensity and (bottom) signal to noise ratio (S/N) for each component, including an ionizable lipid (SM-102), cholesterol (CHO), PEGylated lipid DMG-PEG 2000 (PEG), and distearoylphospatidylcholine (DSPC).

The carrier gas is responsible for both nebulizing droplets at the entrance of the ELSD and carrying the particles through the drift tube to the detector. The gas pressure can be set between 20 and 60 psi, which corresponds to approximately 1.5 L/min to 3.5 L/min gas flow. Gas pressures were tested across the entire range, and the results are shown in Figure 7. Lower gas pressures led to a higher intensity and S/N for each peak, which can also be visualized in the chromatogram shown. These results indicate that lower gas pressures can improve the detection of lipid nanoparticle components.

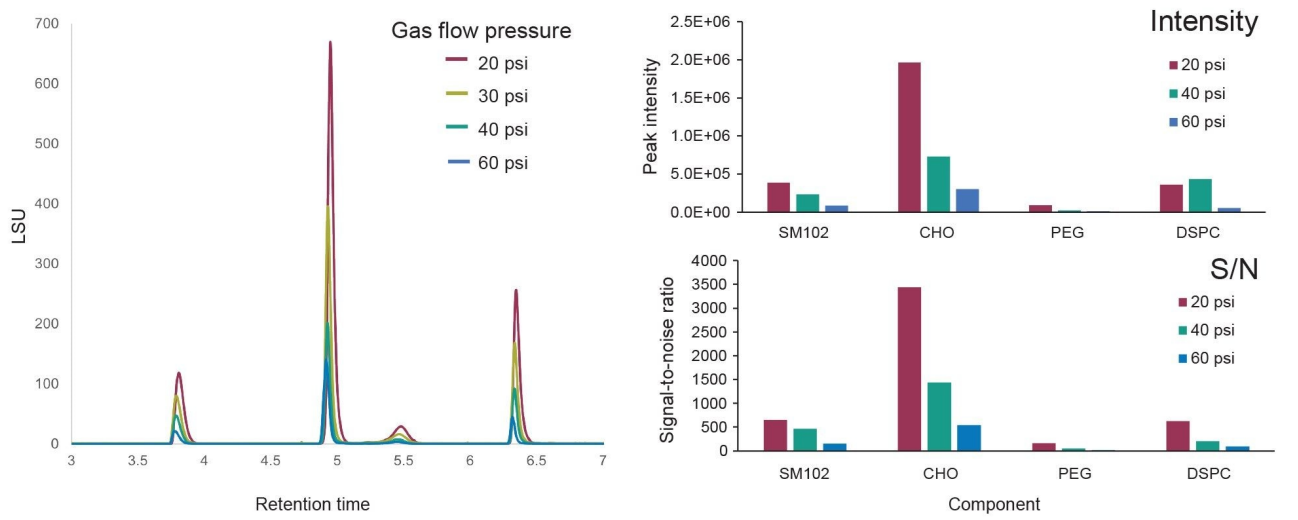


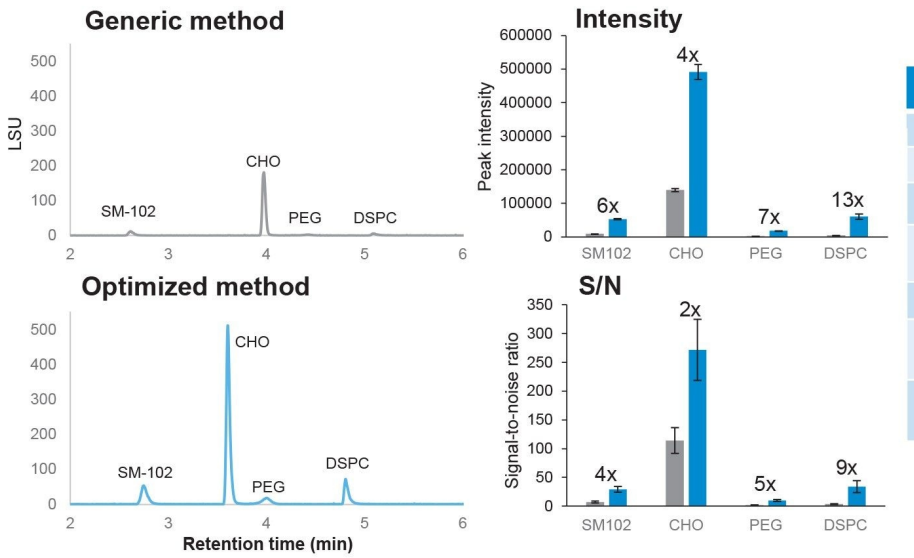
Figure 7. Effect of carrier gas pressure on analyte response, including (left) an overlay of chromatograms, and numerical values for (top) peak intensity and (bottom) signal to noise ratio (S/N) for each component, including an ionizable lipid (SM-102), cholesterol (CHO), PEGylated lipid DMG-PEG 2000 (PEG), and distearoylphospatidylcholine (DSPC).

Several other parameters related to the detector that are often overlooked include the gain, sampling rate, and time constant. Increasing the detector gain increases both peak signal and S/N (data not shown). However, each detector has an upper saturation limit that should be considered. While increasing the detector gain allows for lower limits of detection, it will not improve the dynamic range of individual components and should be adjusted based on the amount of sample being analyzed. Stepped gain settings can also be used to adjust the gain for analytes of varying responses based on their retention times, as has been demonstrated elsewhere.[6] The sampling rate and time constant refer to the number of data points acquired per second and the high-frequency noise filter applied to the data, respectively. Inappropriate settings for these parameters can lead to distorted peaks and a loss of sensitivity. Therefore, it is recommended that these settings also be considered when optimizing a method.

An important consideration when processing ELSD data compared to optical detection is that the detector response generated is dependent on aerosol generation, which results in a nonlinear relationship between amount and area best modeled by a power function ($A=aM^b$), where A=area, M=concentration, and a and b are constants. However, a linear relationship can be achieved when plotting calibration curves by performing a

logarithmic transformation on both sides of the equation (log A = b log M + log a).[7]

To demonstrate the value of systematically optimizing a method, Figure 8 shows a side-by-side comparison of a generic ELSD method before optimization and the final optimized method. As shown in the chromatograms, the peaks are more readily detectable when using the optimized ELSD parameters and LC mobile phase. The peak intensity increased by up to 13-fold, and the S/N increased by up to 9-fold, indicating improved detection of LNP components. The table in Figure 8 lists the final optimized parameters used for the LNP analysis.



Optimized conditions	
Column length	50 mm
Gradient	6 min; 60-100% B
Mobile phase A	0.1% FA in water
Mobile phase B	0.1% FA in 25/75 IPA/ACN
Drift tube temp	48° C
Nebulizer power	Heating 80%
Carrier gas pressure	20 psi

Figure 8. Summary of the improvement gained through optimizing workflow parameters, including (left) chromatograms visualizing the improvement in detection and numerical values for (top) peak intensity and (bottom) signal to noise ratio (S/N) for each component, including an ionizable lipid (SM-102), cholesterol (CHO), PEGylated lipid DMG-PEG 2000 (PEG), and distearoylphospatidylcholine (DSPC). The numbers next to each set indicate the fold increase obtained with the optimized method. The final optimized conditions are shown in the right table.

Conclusion

Careful analysis of LNP related species throughout the development and manufacturing process is critical to

ensuring their efficacy as therapeutic delivery vehicles. Taking the time to systematically optimize conditions across the entire analytical workflow can substantially improve analytical performance. This study explored the effect of parameters from the solvent in which the samples are reconstituted to the appropriate detector settings for sensitive ELSD analysis. The improvements in analyte response demonstrate the value of optimizing methods specific for each application. The settings and conditions described in this study can be used to enhance LNP analysis via ELSD across the various analytical workflows.

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