Application Note

Biopharmaceuticals

LC/MS Analysis of Lipid Composition in an mRNA LNP Formulation: A Stability Study

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Abstract

Lipid-based nanocarriers, known as lipid nanoparticles (LNPs), can deliver a wide range of bioactive molecules, including nucleic acids. As a crucial quality aspect of LNPs, lipid identification and ratio composition must be precisely monitored. In this study, the lipid components in an LNP formulation were analyzed with a liquid chromatography/mass spectrometry (LC/MS) technique using the Agilent 1290 Infinity II LC system and Agilent 6545XT AdvanceBio LC/Q-TOF. The LC/MS method was applied to study the influence of storage conditions on mRNA LNPs.

Introduction

mRNA-based biopharmaceuticals have emerged as a new class of therapeutics. mRNA molecules must cross several extracellular and intracellular obstacles to function in vivo.[1] With recent advances in the nanomedicine field, LNPs are becoming well-known in vivo mRNA delivery systems. Encapsulating mRNA into the LNP system protects nucleic acids from degradation and aids in cellular absorption and expression.[2,3]

The LNP formulation usually consists of an ionizable amino lipid, phospholipid, cholesterol, and polyethylene glycol (PEG)-lipid conjugate. The role of lipids includes ionizable lipids to facilitate nucleic acid encapsulation, PEG-lipids to prevent aggregation, cholesterol, and helper lipids to enhance nanoparticle stability during circulation.[4,5] When aqueous (RNA) and organic (lipid) solutions are mixed, the positively charged amine groups on the lipid and the negative charge on the RNA phosphate backbone electrostatically interact to produce mRNA LNPs. This technique yields robust nanoparticles (with fixed lipid molar ratio) in shorter periods; however, it is uncertain how stable the nanoparticles will be under prolonged storage conditions. LNP stability is one of the key quality attributes since aggregation and degradation processes during storage could result in imbalance of the lipid ratio. The changes in the lipid content may affect the efficiency and safety of mRNA vaccines. Therefore, lipid identification and quantification are critical to the stability and functionality of LNPs and call for robust stability-indicating analytical methods.

Previously, LC and GC techniques coupled with aerosol, flame ionization, and MS detection have been used to analyze lipid components.[6-9] Among these, LC/MS offers high-resolution and simultaneous analysis of lipid composition in LNPs.[10] Although LC/MS was used for lipid identification, the technique was not applied in the investigation of LNP stability studies.

In this application note, the lipid composition of an mRNA LNP sample was analyzed with the previously developed LC/MS method.[11] The LC/MS method was applied to examine LNP stability under various formulation and storage conditions.

Experimental

Materials

Heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl)amino) octanoate (SM-102), 6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium (ALC-0315), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2K), dioleoylphosphatidylethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and cholesterol were supplied from MedChemExpress. Methanol (MeOH) was obtained from Agilent Technologies. Formic acid and acetonitrile (ACN) were obtained from Fisher Chemical. Sodium acetate and Tris were obtained from Sigma. Monarch RNA cleanup kit spin columns were obtained from New England Biolabs.

Instrumentation

The 1290 Infinity II LC system, which was coupled to the 6545XT AdvanceBio LC/Q-TOF, comprised the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Column

The column used in this study was the Agilent InfinityLab Poroshell 120 Phenyl-Hexyl column (2.1 × 50 mm, 1.9 µm; part number 699675-912).

Software

The following software packages were used:

- Agilent MassHunter Workstation Data Acquisition software (version 11)
- Agilent MassHunter Qualitative Analysis software (version 10)

Standard and calibration curve

An 8 mM lipid standard was prepared in MeOH. Different working concentrations of each lipid standard or mixture of lipids were prepared in MeOH using the stock solutions. To generate calibration curves, a stock solution containing 2 mM SM-102, 2 mM DMG-PEG 2K, 2 mM DSPC, and 20 mM cholesterol was freshly prepared in MeOH. The calibration solution was then serially diluted in methanol to the minimum concentration of 0.1 fmol SM-102, 0.1 fmol DMG-PEG 2K, 0.1 fmol DSPC, and 10 pmol cholesterol.

Liquid chromatography/ mass spectrometry

LC/MS lipid separation was performed on an InfinityLab Poroshell 120 Phenyl-Hexyl column (2.1 × 50 mm, 1.9 µm) using a seven-minute gradient. LC/MS conditions are detailed in Table 1. mRNA-LNP samples were dissolved in water, and MeOH dilution aliquots were injected into the LC/MS system.

Preparation of mRNA LNPs

The LNPs produced and used in this application note were similar in composition to the Moderna COVID-19 vaccine LNP. mRNA was in vitro transcribed from a PCR-amplified dsDNA template, purified using spin columns, then dissolved in 1 mM sodium acetate buffer (pH 4.7) to form the aqueous phase.

Table 1. LC/MS parameters

Parameter	Value
Agilent 1290 Infinity II LC System	
Column	InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 50 mm, 1.9 µm
Sample Thermostat	25 °C
Mobile Phase A	90% MeOH in 10 mM ammonium acetate
Mobile Phase B	90% ACN in 10 mM ammonium acetate
Gradient	Time %A %B 0.00 100 0 2.00 100 0 7.00 0 100
Stop Time	7 min
Column Temperature	55 °C
Flow Rate	0.4 mL/min
Agilent 6545XT AdvanceBio LC/Q-TOF	
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	250 °C
Drying Gas Flow	10 L/min
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Fragmentor Voltage	150 V
Skimmer Voltage	65 V
Octupole Ion Guide Voltage	750 V
Reference Mass	922.009798
Acquisition Mode	Data were acquired in Extended Dynamic Range (2 GHz)
MS Mass Range	*m/z* 110 to 1,700
Acquisition Rate	8 spectra/s
MS Range	*m/z* 350 to 3,200
MS Acquisition Rate	2 spectra/s

For the Spikevax LNP formulation[4], SM-102, DMG-PEG 2K, DSPC, and cholesterol were dissolved in ethanol at the molar ratio of 50:1.5:10:38.5 to form the organic phase. The mRNA was dispersed in 25 mM sodium acetate to form the aqueous phase. These two phases were mixed using the benchtop microfluidic device at the volume ratio 3:1, and the total flow rate was 12 mL/min. The N:P ratio was 5.67:1. Then, the formed mRNA LNPs were buffer exchanged with 20 mM Tris (pH 7.4) and concentrated by ultracentrifuge tubes with a molecular weight cutoff of 30 kDa at 4 °C × 2,500 g × 60 minutes to a total lipid concentration of approximately 4 mg/mL. The formed mRNA LNPs were subjected to lyophilization. The LNPs were mixed and diluted to reach a final concentration of 200 ng/µL mRNA and an 8% w/v lyoprotectant (trehalose, sucrose, and mannitol). Then, the lyophilization process was carried out using a chamber benchtop freeze dryer (FreeZone Triad, Labconco). The aliquoted samples were frozen at –40 °C for 24 hours. The primary drying cycle was conducted under vacuum at –55 °C for 24 hours. Then, the temperature was increased gradually, and the secondary drying cycle was conducted at 10 °C for 4 hours. After drying, the lyophilized samples were sealed immediately and stored at different conditions for the stability test.

Results and discussion

Previously, an LC/MS method to separate and identify different lipid classes employed in LNPs preparation[11] was developed using an accurate mass LC/Q-TOF system. The method demonstrated high-resolution separation of lipids with high precision.

In this study, the LC/MS method was used to examine the different mRNA-LNP formation production samples.

To quantify the lipids, first the linearity of the LC/MS method was assessed at a 0.1 to 1,000 pmol concentration range for four lipids: cholesterol, DMG-PEG 2K, SM-102, and DSPC. Calibration curves (Figure 1) were established on a logarithmic scale by plotting log peak area versus log lipid concentration and fitted with a linear curve. The coefficients of determination $R^2 \geq 0.99$ demonstrated a strong linearity on the concentration ranges under study. The extracted ion chromatogram (EIC) overlay of the lipids employed for the quantitative determination is displayed in Figure 2.

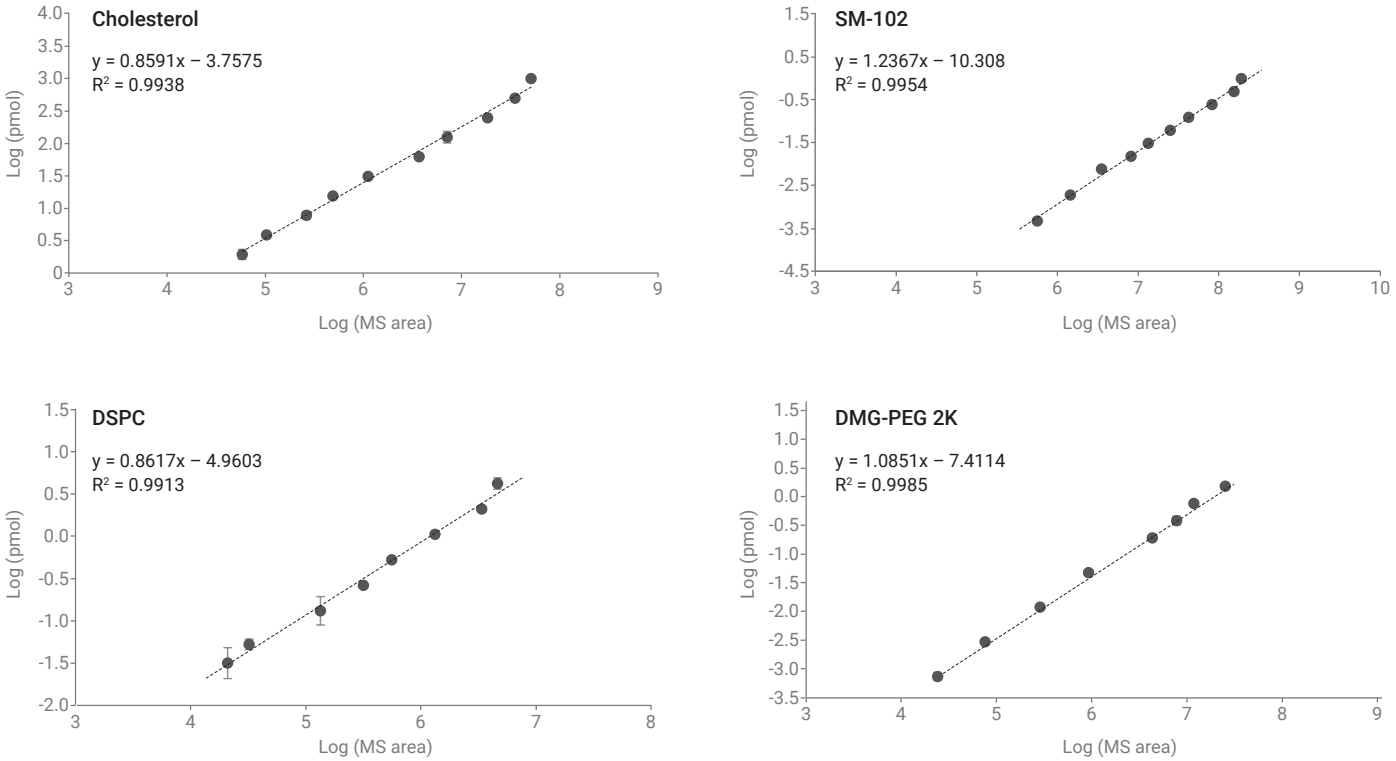

Figure 1. Calibration curve of lipid standards (n = 3).

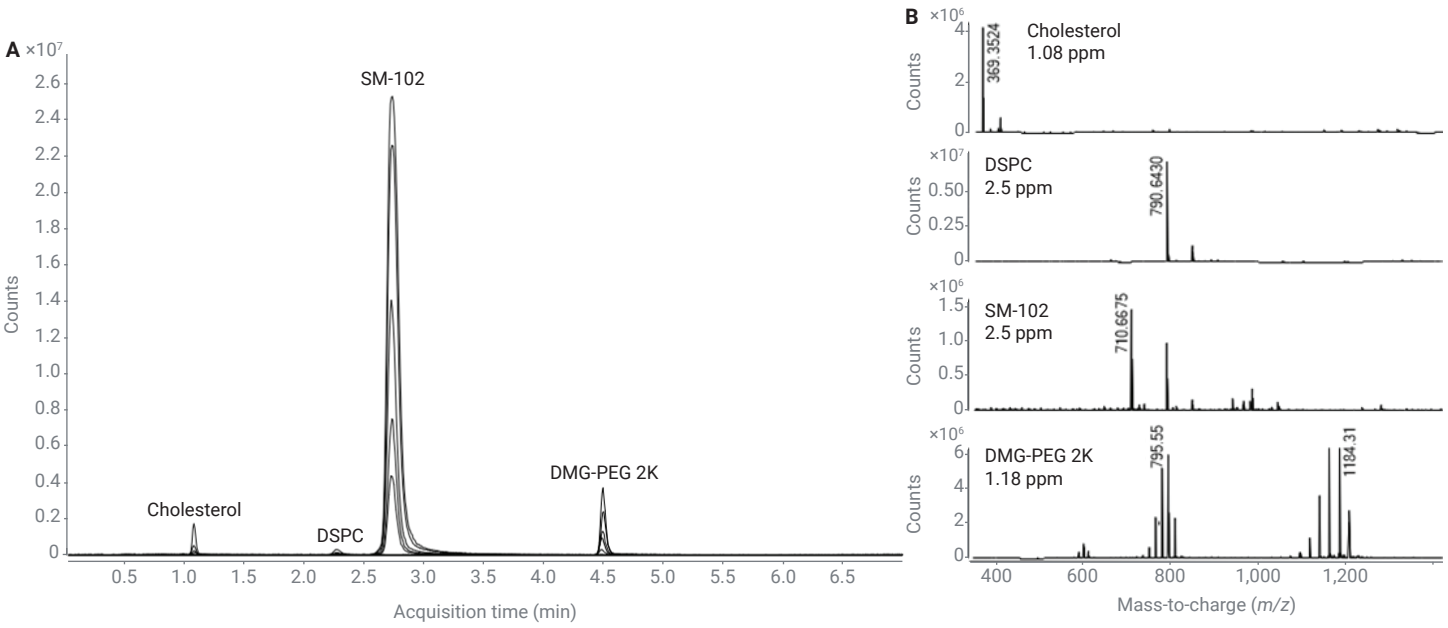

Figure 2. (A) Selected EIC overlay of lipid standards used to prepare LNPs. Cholesterol (20 to 625 pmol); DSPC, SM-102, DMG-PEG 2K (0.06 to 1 pmol). (B) Mass spectra of the lipid components.

The applicability of the LC/MS method was evaluated as a quality control tool by examining the lipid content in different mRNA LNP formulation conditions. The percent molar ratios of lipids determined by LC/MS under cryoprotectant conditions are displayed in Figure 3. The lipid content in all the samples was consistent with the targeted mRNA LNP formulation (SM-102:DMG-PEG 2K:DSPC:cholesterol at 50:1.5:10:38.5), demonstrating the reliability of the LC/MS method.

mRNA LNPs pose a significant challenge for transportation and long-term storage. To gain a deeper understanding of mRNA-loaded LNP stability and the impact of storage conditions, the stability of LNPs was evaluated with and without cryoprotectants (sucrose, trehalose, or mannitol) under the conditions of freezing and lyophilization processes at multiple time points over the course of four weeks.

mRNA LNP % Molar Ratios (Expected)	SM-102	DSPC	DMG-PEG 2K	Cholesterol
	50	10	1.5	38.5

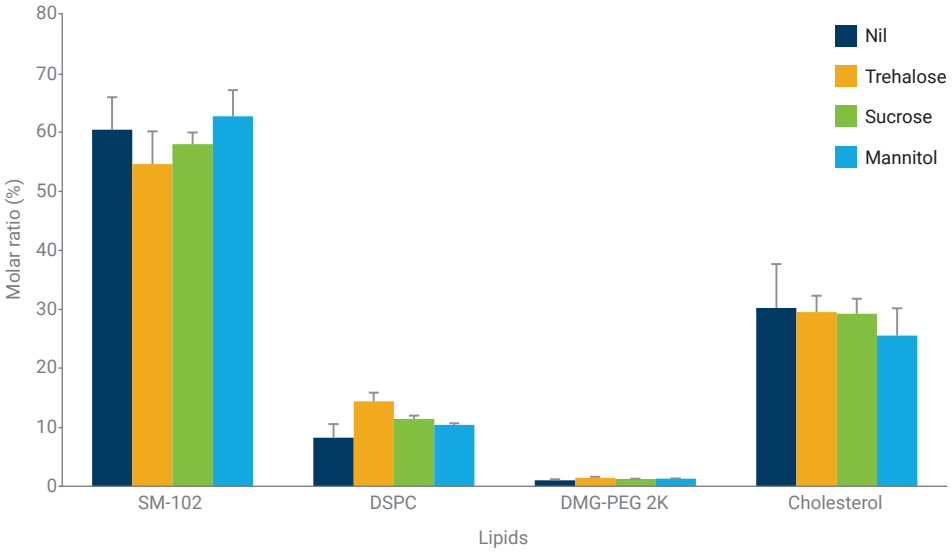

Figure 3. LC/MS analysis of lipid composition of mRNA-LNP lyophilized samples (stored at –70 °C). LNP encapsulating firefly luciferase mRNA (n = 3 measurements).

Both lyophilized and nonlyophilized mRNA LNPs with cryoprotectants were stored at 4, –20, and –70 °C. The samples were reconstituted with double distilled water and further diluted in MeOH for LC/MS analysis. At different time intervals—0 (control), 1, and 4 weeks—the stability of LNPs was evaluated by measuring the molar ratio of lipids.

Figure 4 depicts the percent molar ratio changes of the lipid composition of nonlyophilized mRNA LNPs under different formulation and storage conditions. At –70 °C, freshly prepared (control) LNPs maintained their lipid percent molar ratio, and there were no changes in lipid composition under the conditions of different cryoprotectants.

Regardless of cryoprotectants, however, significant lipid composition alterations occurred during different storage conditions (both 1 and 4 weeks at –20 and –70 °C). These results demonstrated that it is not advisable to store mRNA LNPs for extended periods of time in nonlyophilized conditions.

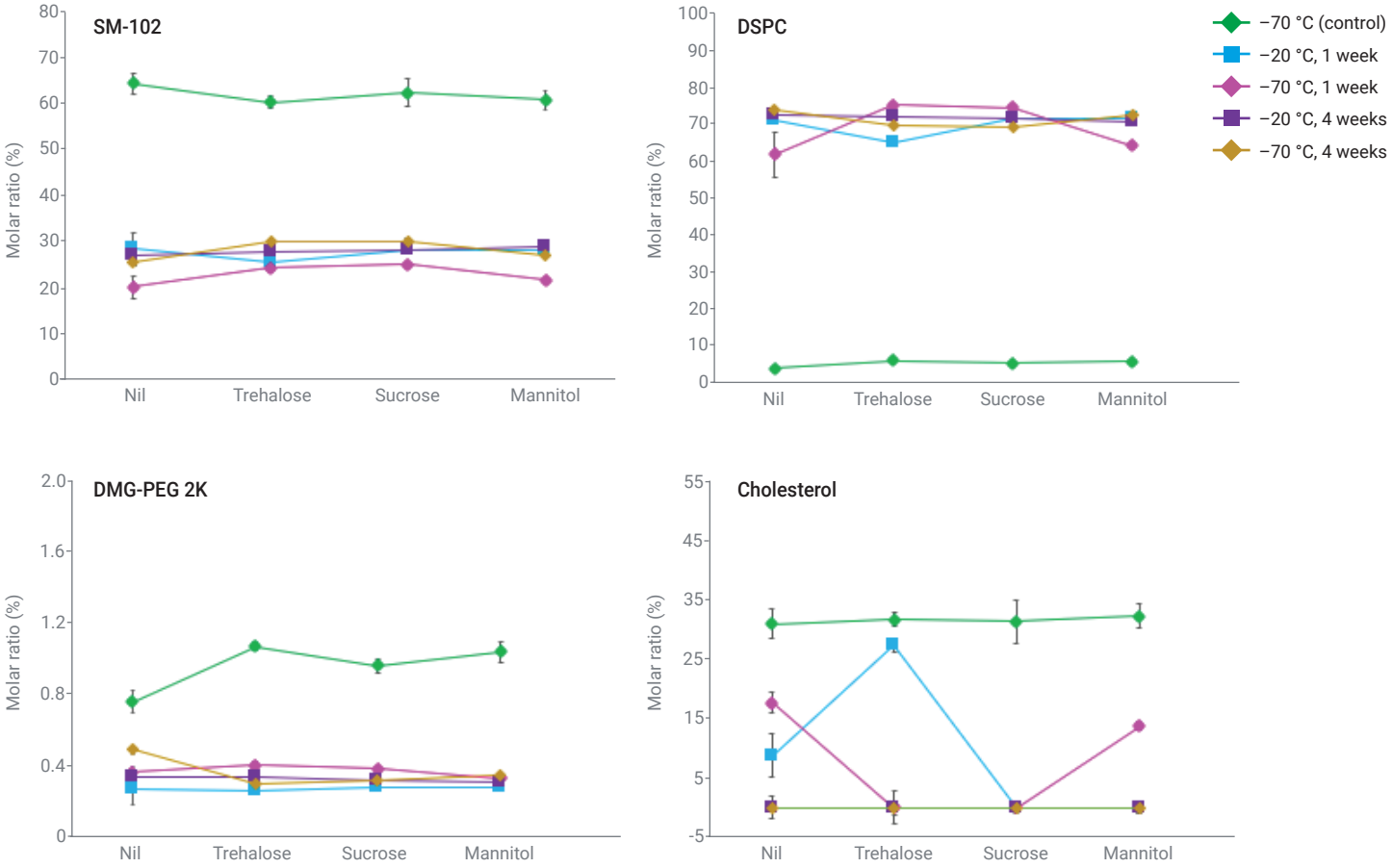

Figure 4. LC/MS analysis of nonlyophilized mRNA LNPs stored at –70 and –20 °C, for 0 (control), 1, and 4 weeks.

Since mRNA LNPs cannot be kept in aqueous conditions for an extended period, their stability under lyophilized conditions was examined. Figure 5 depicts the percent molar ratio changes of the lipid composition of postlyophilized mRNA LNPs under different formulation and storage conditions.

Again, the percent molar ratios of the lipid components of lyophilized mRNA LNPs were consistent with the target molar ratio used for the preparation of LNPs at −70 °C. Lipid composition was maintained in sucrose and mannitol conditions at all the storage conditions, except for DMG-PEG 2K at 4 weeks at 4 °C, which showed decreased levels.

Storage of lyophilized LNPs for the 12 weeks tested did not result in any changes in the lipid composition, demonstrating the stability and integrity of lyophilized LNPs. In general, lyophilized conditions provided improved stability over nonlyophilized samples.

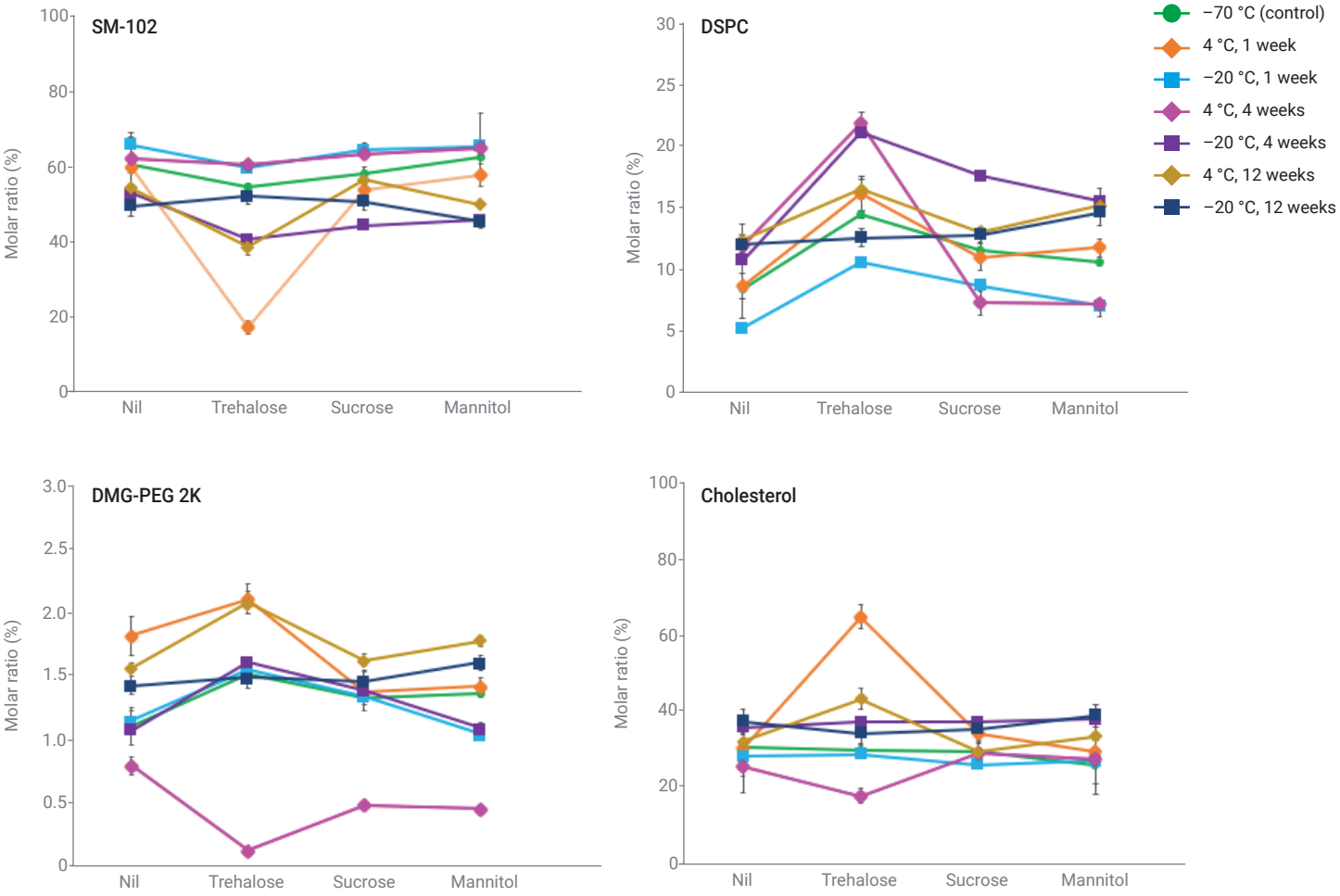

Figure 5. LC/MS analysis of postreconstitution of lyophilized mRNA LNPs stored at −70, −20, and 4 °C, for 0 (control), 1, 4, and 12 weeks.

Conclusion

In this application note, an LC/MS method using the Agilent 1290 Infinity II LC system and 6545XT AdvanceBio LC/Q-TOF was employed to analyze the lipid composition of LNPs. Further, the mRNA LNP stability was investigated under various formulation and storage conditions. The results demonstrate that lyophilized conditions exhibit greater stability compared to nonlyophilized samples. The described LC/MS method is ideal for determining the lipid content and identity, and monitoring the stability of LNP formulations, and can be applied to LNP development and the quality control process.

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