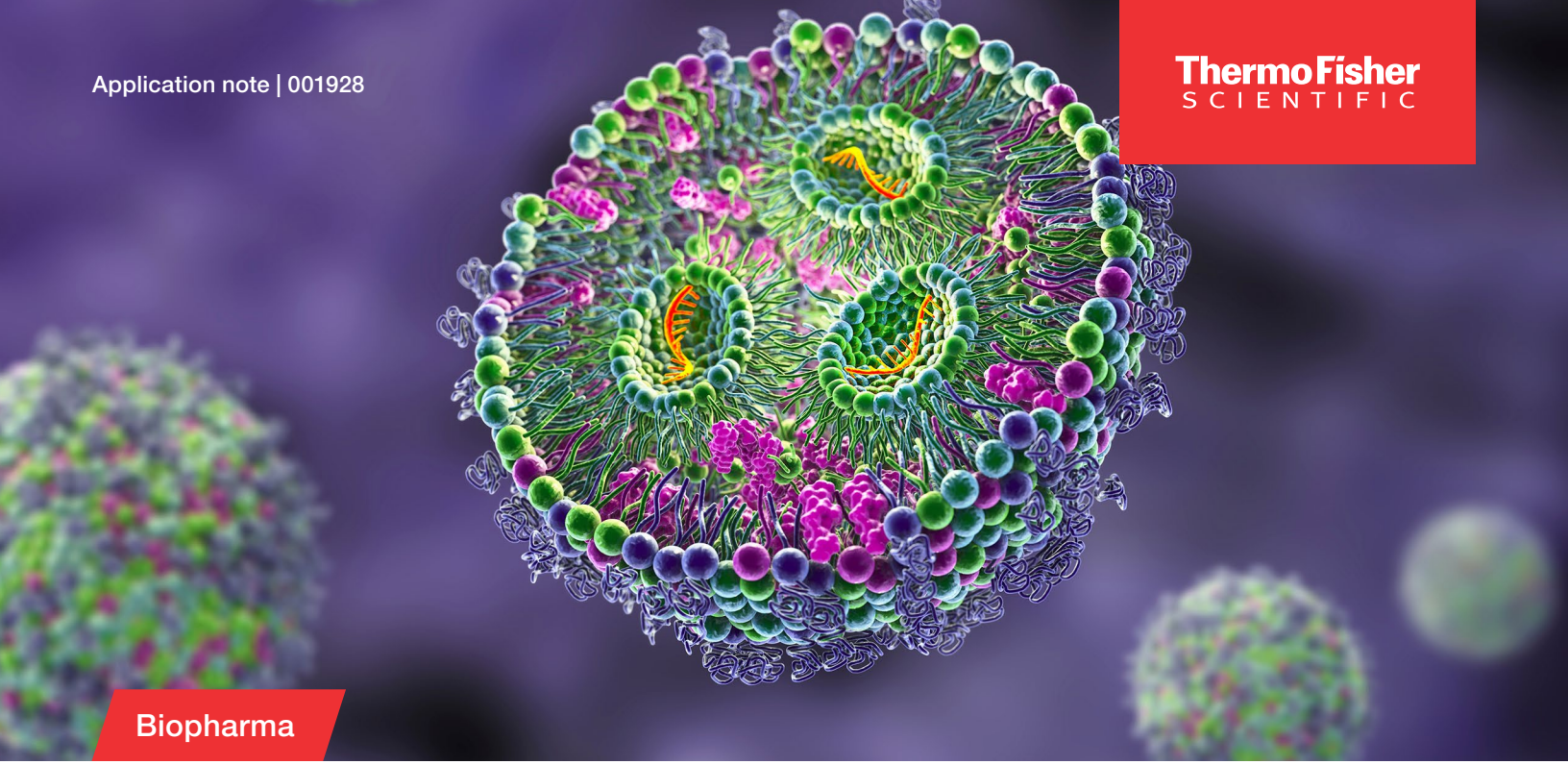
Application note | 001928



Biopharma

Characterization of lipid components in lipid nanoparticle (LNP) formulations

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Keywords

Lipid nanoparticle (LNP) formulation, lipid analysis, UHPLC, charged aerosol detector (CAD), cationic lipid, ionizable lipid, Accucore C30 HPLC column

Application benefits

- A simple UHPLC-CAD method was developed under enterprise compliance-ready Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), providing fast and accurate ID confirmation and molar ratio assessment of lipid components for LNP formulation development and quality control.
- High sensitivity, uniform response, and wide dynamic range provided by CAD greatly simplifies the sample preparation and facilitates method development.

Goal

Development of a simple UHPLC-CAD method to determine the molar ratio of lipid components in LNP formulations

Introduction

A variety of materials have been developed for *in vitro* transcribed mRNA delivery, including lipid nanoparticles (LNP), which have been thoroughly studied and successfully used in clinical products. Notably, two FDA fully approved Covid-19 vaccines, mRNA-1273 and BNT 162b, use LNP to deliver antigen coding mRNA. Many other lipid nanoparticle-oligonucleotide formulations have been developed and are under clinical evaluation for the prevention of virus infections and treatment of cancer and genetic diseases.[1]

Oligonucleotide loaded LNP systems are complex structures of approximately 100 nm. They are commonly composed of amino lipids (ionizable or cationic amino lipids) as the key component alongside phospholipids, cholesterol, and a PEGylated lipid.[2]

According to the published information of mRNA-LNP drug products in clinical phase III trials,[3] two LNP formulations encapsulated with poly-A as mock payload instead of mRNA were selected and formulated. An efficient method has been developed to quantify the concentration and molar ratio of each lipid in the LNP formulations. The column used is a Thermo Scientific™ Accucore™ C30 column to achieve fast, high-resolution separations of hydrophobic, long-chain compounds. A Thermo Scientific™ Vanquish™ Charged Aerosol Detector is used to quantify the lipids, taking advantage of the near-universal detection capabilities. The system is a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system with integrated biocompatibility to achieve increased separation speed and resolution for valuable biomolecules.

Experimental

Chemicals and consumables

- Water, Optima™ LC/MS grade, Fisher Chemical™ (P/N W64)
- Isopropanol, Optima™ LC/MS grade, Fisher Chemical™ (P/N A461-4)
- Acetonitrile (ACN), Optima™ LC/MS grade, Fisher Chemical™ (P/N A955-4)
- Methanol (MeoH), Optima™ LC/MS grade, Fisher Chemical™ (P/N A456-4)
- Ethyl alcohol (EtOH), 200 proof, 99.5+%, Thermo Scientific Chemicals (P/N 61519-0010)
- Formic acid (FA), Optima™ LC/MS grade, Fisher Chemical™ (P/N A11710X1AMP)
- Fisherbrand™ Disposable Controlled-Drop Pasteur Pipets (P/N 13-678-30)
- Fisherbrand™ Borosilicate Glass Disposable Serological Pipets (P/N 13-678-25A)
- Thermo Scientific™ Chromacol™ GOLD-Grade Inert Vials and Inserts (P/N 13-622-351)

Instrumentation

Vanquish Flex System consisting of:

- Vanquish System Base Horizon/Flex (P/N VF-S01-A)
- Vanquish Binary Pump F (P/N VF-P10-A-01)
- Vanquish Split Sampler FT (P/N VF-A10-A)
- Vanquish Column Compartment H (P/N VH-C10-A-02)
- Vanquish Charged Aerosol Detector H (P/N VH-D20-A)

Lipid nanoparticle formulation

The lipids used in this study are highlighted in Table 1. Working stock concentrations were generated by diluting or resuspending the lipid components in 99% ethanol. The payload used in this formulation was polyadenylic acid potassium salt (poly-A). 1 mg/mL of poly-A in water was first generated. From this stock solution a 0.13 mg/mL poly-A working solution was made up using 100 mM citric acid. Lipid nanoparticles were formulated using an Ignite™ NanoAssemblr™. The parameters used for the Ignite NanoAssemblr are outlined in Table 2. The aqueous payload (C) and the organic lipids (R) were added to individual 10 mL BD syringes. These syringes were attached to the Ignite™ NxGen™ Cartridge in the Ignite NanoAssemblr. 15 mL falcon tubes were added to the waste and sample collection holders in the NanoAssemblr and the instrument method was run. Post formulation purification and concentration was carried out using a 3kDa molecular weight cut off filter (MWCO) to remove residual ethanol, bringing the final volume to 1 mL. Encapsulation efficiency was calculated using Invitrogen™ Quant-It™ RiboGreen™ Assay (P/N R11490). LNP size was measured using dynamic light scattering (DLS). These formulations were then frozen at -20 °C. After removal from the freezer, the formulations were gently inverted a few times then placed in the UHPLC autosampler.

Table 1. Sample information for method development

Formulation #1 (Total working stock concentration: 20 mM)				
Lipid	Molecular weight (g/mol)	Working stock (mM)	Molecular %	Supplier
ALC-0315	766.29	9.26	46.3	SINOPEG (P/N 2036272-55-4)
DSPC	790.145	1.88	9.4	Avanti (P/N 850365)
Cholesterol	386.654	8.54	42.7	Sigma-Aldrich (P/N C1231)
ALC-0159	2330	0.32	1.6	SINOPEG (P/N 1849616-42-7)
Formulation #2 (Total working stock concentration: 40 mM)				
Lipid	Molecular weight (g/mol)	Working stock (mm)	Molecular %	Supplier
SM-102	710.2	20	50	Cayman Chemical (P/N 33474)
DSPC	790.145	4	10	Avanti (P/N 850365)
Cholesterol	386.654	15.4	38.5	Sigma-Aldrich (P/N C1231)
PEG2000-DMG	2509.2	0.6	1.5	Avanti (P/N 880151)

Table 2. Ignite NanoAssemblr instrument method parameters

Parameter	Value
Flow rate ratio (C:R)	3.00 : 1.00
Total volume	2 mL
Total flow rate	20.00 mL/min
Starting waste	0.25 mL
End waste	0.05 mL

Preparation of calibration mixed standard for formulation #1

1. Stock solution preparation: Each lipid standard (Table 3) was weighed directly in a dry volumetric flask (10 mL) and ethanol was added. All flasks were placed in an ultrasonic water bath and sonicated for 10 minutes. The flask was brought up to volume with additional ethanol if needed.

Table 3. Concentration of each lipid stock solution of formulation #1

Lipid	Concentration (mg/mL)
ALC-0135	8.92
ALC-0159	1.01
Cholesterol	3.05
DSPC	1.54

2. Calibration mixed standards: Using glass pipets, 500 µL of each prepared stock solution was transferred to a glass vial and vortexed to mix well. This solution was Mix #1.
3. A series of dilutions using methanol was performed as shown in Table 4.

Table 4. Calibration mixed standard dilution table

	Volume, µL	Methanol, µL
Mix #1	500 x 4	0
Mix #2	300, Mix #1	300
Mix #3	200, Mix #1	400
Mix #4	200, Mix #1	600
Mix #5	200, Mix #1	1,400

Preparation of calibration standards for formulation #2

1. Stock solution preparation: Each lipid standard was weighed directly in a dry volumetric flask (10 mL) and brought to volume with ethanol.

Table 5. Concentration of each lipid stock solution of formulation #2

Lipid	Concentration (mg/mL)
SM-102	3.33
DMG-PEG 2000	0.99
Cholesterol	5.39
DSPC	2.02

2. Calibration standards: A series of dilutions using methanol was performed as shown in Table 6.

Table 6. Calibration standards dilution table

	Volume, µL	Methanol, µL
Standard #1	2,000	0
Standard #2	300, Standard #1	300
Standard #3	200, Standard #1	400
Standard #4	200, Standard #1	600
Standard #5	200, Standard #1	1,400

Chromatographic conditions

Parameter	Value		
Column	Accucore C30, 3.0 × 100 mm, 2.6 µm (P/N 27826-103030)		
Mobile phase	A: 0.1% FA in 100% water		
Mobile phase	B: 0.1% FA in 60% IPA, 30% ACN, 10% water		
Gradient	Time (min)	%A	%B
	0	100	0
	2	100	0
	6	0	100
	13.0	0	100
	13.1	100	0
	15.0	100	0
Flow rate	0.9 mL/min		
Column temperature	50 °C		
Autosampler temperature	10 °C		
Injection volume	1 µL		
Injection wash solvent	Mobile phase B		

Detector settings

CAD setting	Value
Power function	1.0
Evaporator temperature	35 °C
Data rate	2Hz
Filter	3.6

Chromatography Data System

Chromeleon CDS 7.2.10 MUd was used for data acquisition and analysis.

Results and discussion

Initial attempts with higher organic (75%) solvent in the starting point gave a not retained peak, which was off the scale for the CAD detector (Figure 1). This could be due to intact LNP breaking through the column. Also, the ALC-0315 had poor resolution and ALC-0159 was hard to integrate. An optimized method with higher aqueous (100%) solvent was developed that gave improved resolution and good integration of ALC-0315 and ALC-0159 (Figure 2). The large breakthrough of intact LNP in the void volume was prevented and the signals of the individual lipids were increased by starting the gradient with a higher aqueous condition. The poly-A encapsulated material can now be observed as an early eluting peak. The second peak needs to be confirmed for future work. Formulation #2 had very similar separation with the optimized method.

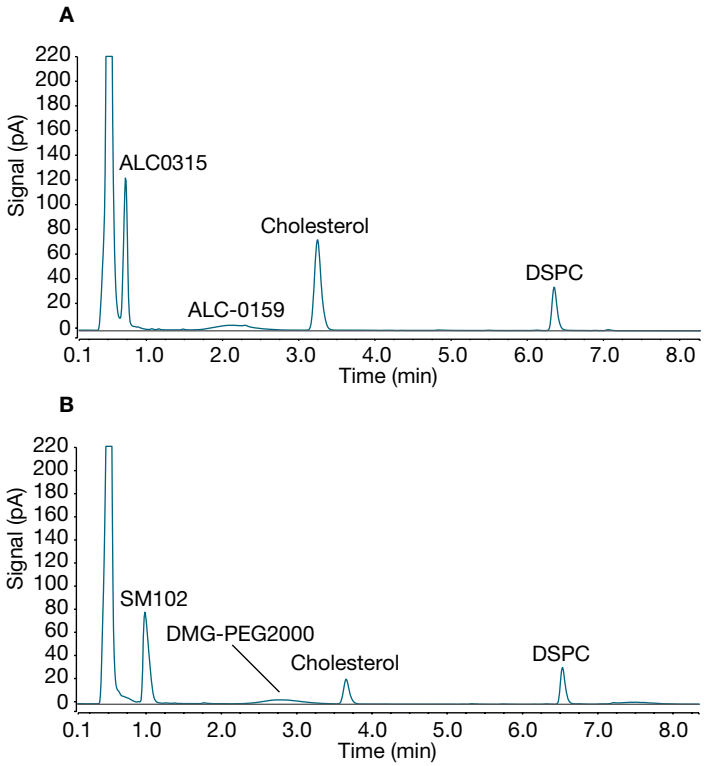


Figure 1. Initial attempt to separate lipid components in formulations. (A) Formulation #1, (B) Formulation #2

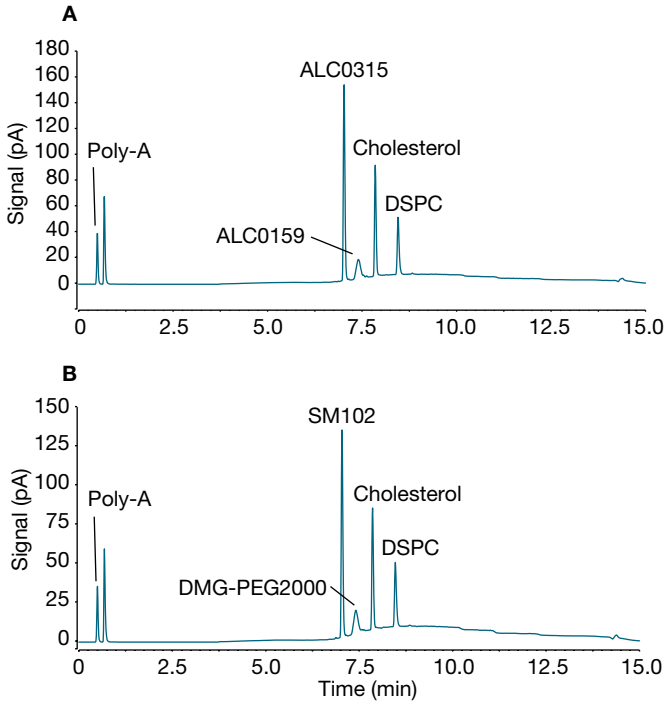


Figure 2. Separation of formulations with optimized method. (A) Formulation #1, (B) Formulation #2

After good separation was achieved, the calibration curves for all lipid components were conducted in a series of single runs for formulation #1. A test injection of the formulation was made to decide the highest concentration of each standard. Since the mixed four standards were used for formulation #1, the stock concentrations (4x Mix1) needed to be four times the highest concentration (Mix1). A series of single runs were performed using the calibration standards mix. The calibration curves for all lipids showed a calibration coefficient larger than 0.998 (quadratic fit).

For formulation #2, an individual calibration curve was performed for quantification of each lipid where the stock concentration was the highest concentration of each lipid standard. A series of single runs were performed for each lipid individually. Figure 4 shows SM-102 with calibration details as an example. Calibration standard mixtures were used when a sufficient amount of lipid standard material was available, otherwise individual standards should be used.

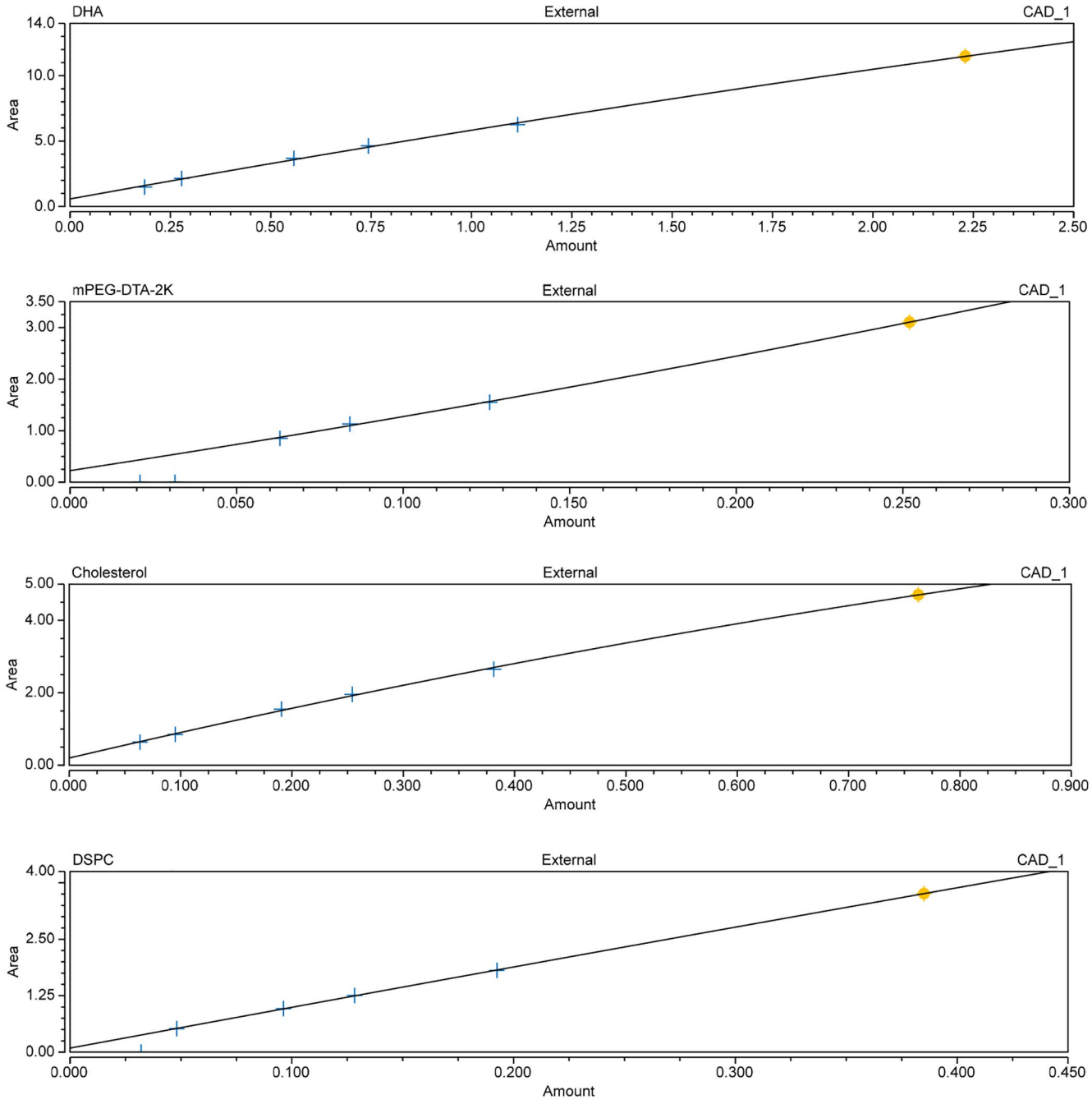
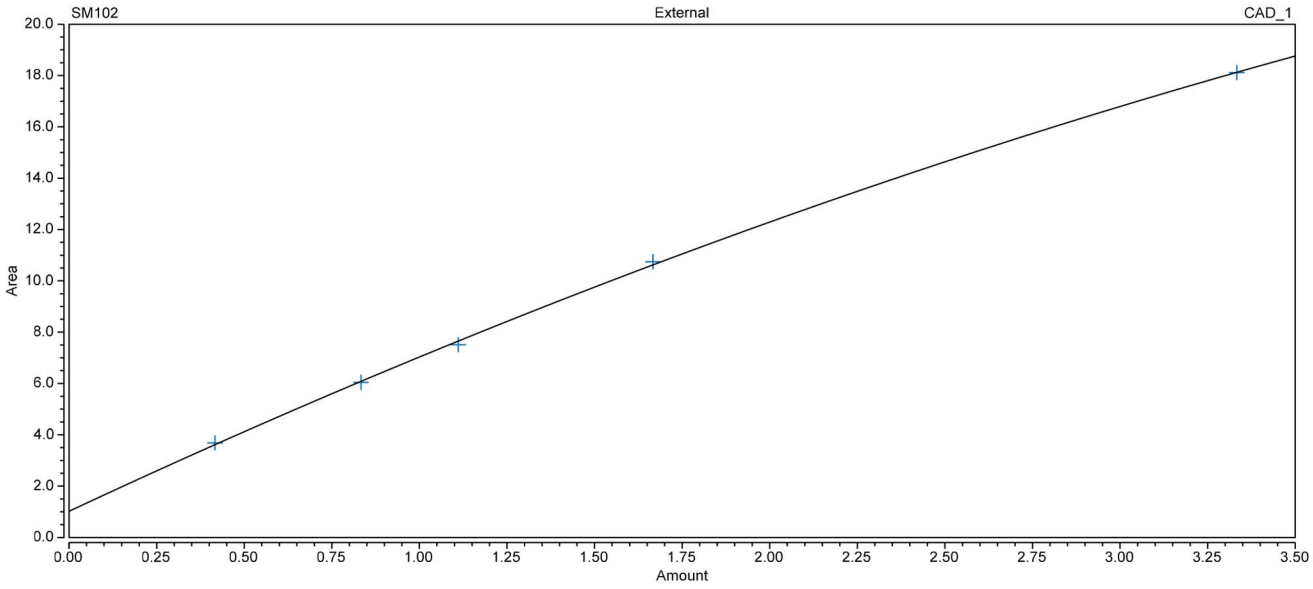


Figure 3. Calibration curves of lipid components for formulation #1. ALC-0135 was 0.186–2.230 mg/mL, ALC-0159 was 0.021–0.252 mg/mL, cholesterol was 0.0635–0.763 mg/mL, and DSPC was 0.032–0.385 mg/mL.



Calibration Details	SM102		
Calibration Type	Quad, WithOffset	*Offset (C0)*	1.0206
Evaluation Type	Area	*Slope (C1)*	6.3896
Number of Calibration Points	5	*Curve (C2)*	-0.3771
Number of disabled Calibration Points	0	*R-Square*	0.9997

Figure 4. Calibration curve of SM-102 for formulation #2. SM-102 was 0.417–3.333 mg/mL, DMG-PEG2000 was 0.124–0.99 mg/mL, cholesterol was 0.337–5.39 mg/mL, and DSPC was 0.253–2.02 mg/mL (figures not shown).

Five repetitive injections were performed with formulations #1 and #2 separately. Using the reported amount of each injection from Chromeleon CDS, the average amount (concentration) was calculated for five injections of each lipid (Tables 7 and 8), then the molar ratio was calculated by dividing by the formula weight. Refer to the data of molar ratio% as shown in Table 1, the measured molar ratio corresponded very well, especially for formulation #1.

Table 7. Test result of formulation #1

Formulation #1		Measured result	Reference (Table 1)
	Average amount (mg/mL)	Molar ratio%	Molar ratio%
ALC0315	1.3497	45.71	46.3
ALC0159	0.1619	1.75	1.6
Cholesterol	0.6468	43.42	42.7
DSPC	0.2777	9.12	9.4

Table 8. Test result of formulation #2

Formulation #2		Measured result	Reference (Table 1)
	Average amount (mg/mL)	Molar ratio%	Molar ratio%
SM-102	0.9069	47.46	50
DMG-PEG2000	0.1557	2.31	1.5
Cholesterol	0.3894	37.43	38.5
DSPC	0.2722	12.8	10

Conclusion

- The same method can be used to measure individual components of both LNP formulations. The measured molar ratios agreed well with the original molar ratios of the sample.
- The Accucore C30 column provides good separation of lipid components along with other excipients of LNP formulations.
- Using Chromeleon software, the calibration curve of each lipid compound can be conducted with a mixed standard in a series of single runs or an individual standard in a series of single runs.
- The calibration curve from the CAD provides good accuracy and uniform equation across the entire dynamic range.

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