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## Abstract

The recent success of mRNA vaccines for COVID-19 is in part due to the development of lipid nanoparticle (LNP) delivery systems. The LNP contains four lipid components (cholesterol, DSPC, ionizable, and PEGylated lipids) and it is important to control the quality and purity of these lipids. A simple, rapid, and routine LC-MS method was developed for the characterization and analysis of LNP components using an ACQUITY Premier CSH C<sub>18</sub> Column and the BioAccord System. The LNP components, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dilinoeyloxy-3-dimethylaminopropane (MC3), and PEGylated lipid were detected at a concentration level of 5 pg/μL and cholesterol at 250 pg/μL. The developed method was applied for the analysis of complex bovine liver lipid extracts and all the major lipid classes were detected. The method is useful for the simultaneous analysis of the four lipid components in the LNP and other impurities that could be resulted from either synthesis process, degradation, or raw material contamination.

## Benefits

- A simple, rapid, and routine method for the analysis of lipid composition in LNPs
  - Simultaneous analysis of LNP components and impurities in process development and quality control environments
  - The BioAccord System coupled with waters\_connect informatics platform provides a streamlined analytical workflow for data acquisition and processing
  - The BioAccord System provides the detection limit of 5 pg/μL (25 pg on column) for DSPC, ionizable, and
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PEGylated lipids, and of 250 pg/ $\mu$ L (1.25 ng on column) for cholesterol

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## Introduction

Lipid nanoparticles (LNP) are the preferred delivery vessel for mRNA therapies or vaccines. The recent success of mRNA vaccines in SARS-CoV-2 clinical trials is in part due to the development of lipid nanoparticle delivery systems.<sup>1</sup> LNPs can achieve high encapsulation efficiency and the size of the mRNA is almost unlimited, making it unique when compared to other transgene delivery vehicles.<sup>1,2</sup> Specifically, for their role in vaccines, they have the added benefit to stimulate the innate immune system without inducing immunogenicity.<sup>2</sup> Moreover, incorporating the mRNA into LNP protects the mRNA from enzymatic attack and enhances cell uptake and expression.<sup>1</sup> A LNP is typically composed of four lipids (Figure 1), each with their own specific role. The majority of a LNP is comprised of an ionizable/cationic lipid that drives potency, and significant development and intellectual property currently lies with this component that typically consists of half the LNP composition. Early LNPs used in clinical trials relied on 1,2-dilinoeyloxy-3-dimethylaminopropane (Dlin-MC3-DMA (MC3)).<sup>2</sup> Cholesterol is the next most prevalent species; it enhances LNP stability and promotes membrane fusion.<sup>3,4</sup> The two other lipids include a polyethylene glycol-lipid (PEGylated lipid) and a phospholipid like 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), also referred to as the helper lipid. The polyethylene glycol (PEG) reduces aggregation tendencies and shields the LNP from non-specific endocytosis by the immune cells. DSPC and cholesterol are natural lipids, whereas the ionizable and PEGylated lipids are synthesized lipids. A small alternation in the chemical structure of the ionizable lipid, stability of the PEGylated lipid, and the ratio of the four lipids can alter the property and LNP delivery efficiency.<sup>1</sup>



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## Experimental

### Sample Description

Individual lipid standards of cholesterol, DSPC, and MC3 were purchased from Sigma and DMG-PEG-2000 was obtained from Avanti Polar Lipids. A 1 mg/mL stock concentration of cholesterol and DMG-PEG-2000 was prepared in methanol. A 1 mg/mL stock of DSPC and MC3 was prepared in methanol/chloroform (1/1). From the stock solutions various working concentrations of each lipid standard or mixtures thereof were prepared in methanol.

The bovine liver total lipid extract was purchased from Avanti Polar Lipids. The extract was prepared by making a 5 mg/mL stock solution in chloroform/methanol (2/1, v/v). A working 0.1 mg/mL solution was then prepared by diluting the stock solution with methanol.

### LC Conditions

LC-MS system:	BioAccord LC-MS System
Vials:	Waters Total Recovery UPLC Vials (p/n: 186005669CV)
Column(s):	ACQUITY Premier CSH C <sub>18</sub> 2.1 x 100 mm, 1.7 μm (p/n: 186009461)
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	5 μL
Flow rate:	400 μL/min
Mobile phase A:	600/390/10 (ACN/Water/1 M aqueous ammonium

formate) in 0.1% formic acid

Mobile phase B:

900/90/10 (IPA/ACN/1 M aqueous ammonium formate) in 0.1% formic acid

## Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.4	50	50	Initial
0.5	0.4	47	53	6
4.0	0.4	45	55	6
7.0	0.4	35	65	6
7.5	0.4	20	80	1
10.0	0.4	1	99	6
11.0	0.4	1	99	1
12.0	0.4	50	50	1

## MS Conditions

MS system:

ACQUITY RDa Mass Detector

Mode:

Full scan MS with fragmentation (Simultaneous dual-function acquisition at low (fixed) and high (ramped) cone voltage)

Mass range:	50–2000 <i>m/z</i>
Polarity:	ESI positive (1.5 KV)
Scan rate:	10 Hz
Cone voltage:	30 V
Fragmentation cone voltage:	120–200 V

## Data Management

UNIFI Scientific Information System under waters\_connect with accurate mass screening workflow was used for data acquisition and processing.

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## Results and Discussion

LC-MS methods are widely used to separate and analyze lipids of various origins into lipid classes or molecular species. Normal-phase (NP) chromatography and hydrophilic interaction chromatography (HILIC) are two common techniques used to separate lipid species into classes while reversed-phase (RP) chromatography is mostly useful for the separation of lipid molecular species within a class. The lipid ingredients for LNPs were separated using a RP ACQUITY Premier CSH C<sub>18</sub> Column to provide improved coverage and resolution over other RP columns.<sup>5,6</sup> These lipids possess low or no UV absorbance, and an alternative detector such as the ACQUITY RDa Mass Detector should be used to improve the sensitivity of analysis. The standard mixture which was prepared by diluting the stock solution with methanol to yield the target concentration was analyzed by the BioAccord System. A typical extracted ion chromatogram is shown in Figure 2A. The lipids were eluted in the order of DMG-PEG-2000, cholesterol, ionizable lipid MC3, and DSPC. Figure 2B shows corresponding spectra for the four representative lipids of DMG-PEG-2000, cholesterol (*m/z* 369.352), ionizable lipid MC3 (*m/z* 642.627), and DSPC (*m/z* 790.618). The combined spectra for DMG-PEG-2000 is shown in Figure 2B (top panel). DMG-PEG-2000 forms an ammonium adduct under the LC-MS conditions with multiple charge states (+2, +3, and +4)

observed in the spectrum.

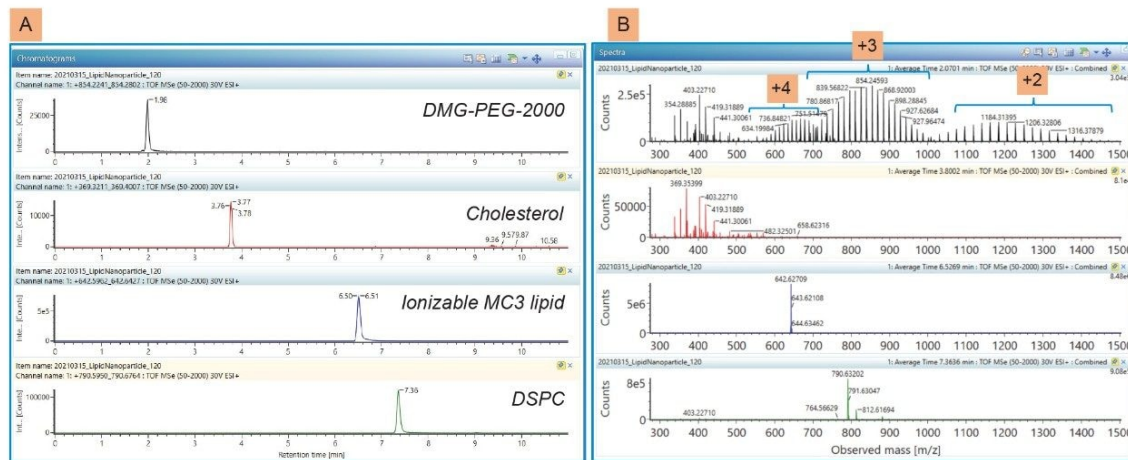


Figure 2. (A) Positive ion mode extracted ion chromatograms and (B) Corresponding spectra of DMG-PEG-2000, cholesterol ( $m/z$  369.352), ionizable MC3 lipid ( $m/z$  642.627), and DSPC ( $m/z$  790.618). The PEGylated lipid exits as multiply charged series of +2, +3, and +4 with 38-50 polyethylene glycol repeat units. The spectra are color coded to their corresponding chromatogram.

For the identification of individual lipid components, a custom database (library) containing the various lipid compounds was first created within the UNIFI scientific library. Detailed step-by-step explanation on how to create a database using the UNIFI scientific library is provided elsewhere.<sup>7</sup> An example of the sample analysis results, including component summary, chromatogram, and spectrum views utilizing the UNIFI component analysis workflow are shown in Figure 3. Figure 3A shows the component summary table that lists all components that are identified from the custom lipids-LNP database. For each identified lipid, the extracted ion chromatogram (Figure 3B), low energy parent ion spectrum (Figure 3C), and corresponding fragment ion spectrum (Figure 3D) are displayed. UNIFI compares and matches predicted *in silico* fragments with experimentally derived HRMS fragments for structural elucidation. If needed, each fragment ion spectrum can be examined by clicking on the blue icon and send to the UNIFI Scientific library to create a custom lipid-LNP database that contains fragment ion information.

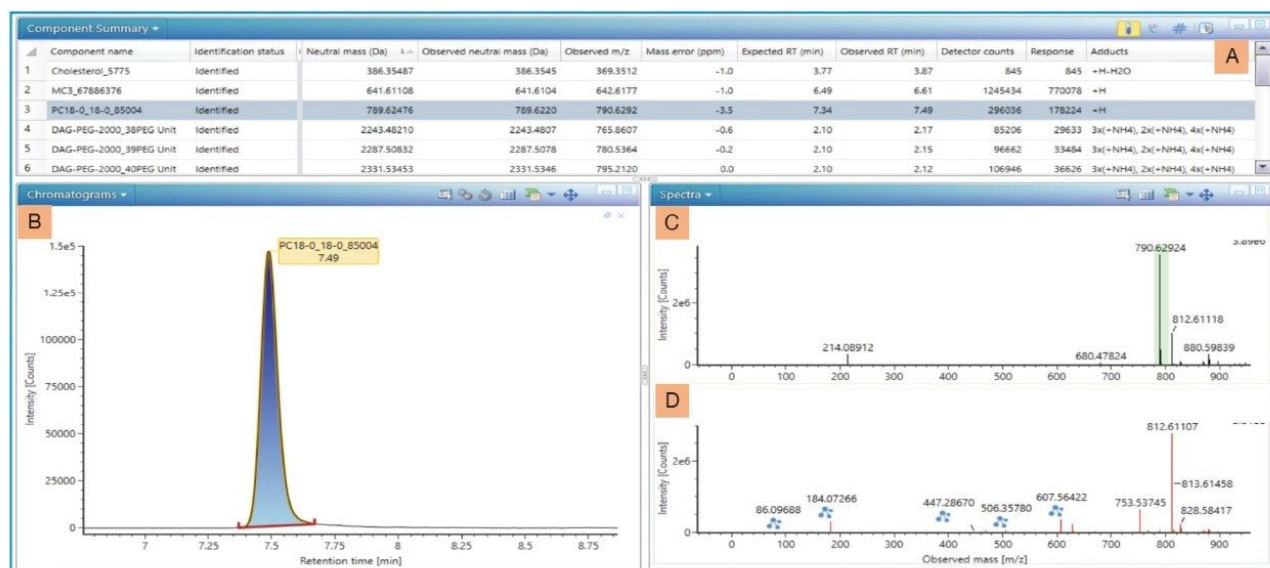


Figure 3. Component summary plot showing (A) the identified lipid nanoparticles of cholesterol, cationic lipid MC3, DSPC, and 13 different DMG-PEG-2000 (B) example extracted ion chromatogram of DSPC (C) Low energy exact mass of DSPC and (D) Corresponding fragment ion spectrum of DSPC. The blue icon indicates matched predicted *in silico* and experimental fragment ions.

The results of all identified lipids can be displayed as a component-confirmed table (Figure 4A) or as a component plot (Figure 4B). Figure 4A shows the complete component summary list of identified lipids for DMG-PEG-2000, cholesterol, ionizable lipid MC3, and DSPC. The PEGylated lipid displays as multiple charge state of +2, +3, and +4 in Figure 4A under ESI positive ion mode. In addition, DMG-PEG-2000 exists as a non-uniform polydisperse polymer and contains uneven PEG chain lengths ranging from 38 to 50 (CH<sub>2</sub>CH<sub>2</sub>O) units. Monodisperse polymers are uniform polymers in which all molecules have the same degree of PEG polymerization and it provides the same single *m/z* value. A total of 13 different PEGylated lipids were identified based on the polydispersity of PEG unit.



Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Detector counts	Response	Adducts
1 Cholesterol_5775	Identified	386.35487	386.3545	369.3512	-1.0	3.77	3.87	845	845	+H-H2O
2 MC3_67886376	Identified	641.61108	641.6104	642.6177	-1.0	6.49	6.61	1245434	770078	+H
3 PC18-0_18-0_85004	Identified	789.62476	789.6220	790.6292	-3.5	7.34	7.49	296036	178224	+H
4 DAG-PEG-2000_38PEG Unit	Identified	2243.48210	2243.4807	765.8607	-0.6	2.10	2.17	85206	29633	3x(+NH4), 2x(+NH4), 4x(+NH4)
5 DAG-PEG-2000_39PEG Unit	Identified	2287.50832	2287.5078	780.5364	-0.2	2.10	2.15	96662	33484	3x(+NH4), 2x(+NH4), 4x(+NH4)
6 DAG-PEG-2000_40PEG Unit	Identified	2331.53453	2331.5346	795.2120	0.0	2.10	2.12	106946	36626	3x(+NH4), 2x(+NH4), 4x(+NH4)
7 DAG-PEG-2000_41PEG Unit	Identified	2375.56074	2375.5646	809.8887	1.6	2.10	2.10	112716	38467	3x(+NH4), 2x(+NH4), 4x(+NH4)
8 DAG-PEG-2000_42PEG Unit	Identified	2419.58696	2419.5936	824.5650	2.7	2.10	2.07	116200	40352	3x(+NH4), 2x(+NH4), 4x(+NH4)
9 DAG-PEG-2000_43PEG Unit	Identified	2463.61317	2463.6206	839.2407	2.9	2.10	2.05	116558	40105	3x(+NH4), 2x(+NH4), 4x(+NH4)
10 DAG-PEG-2000_44PEG Unit	Identified	2507.63939	2507.6484	853.9166	3.5	2.10	2.02	115456	39762	3x(+NH4), 2x(+NH4), 4x(+NH4)
11 DAG-PEG-2000_45PEG Unit	Identified	2551.66560	2551.6727	868.5914	2.7	2.10	2.00	110101	37981	3x(+NH4), 2x(+NH4), 4x(+NH4)
12 DAG-PEG-2000_46PEG Unit	Identified	2595.69182	2595.7000	883.2671	3.1	2.10	1.98	102827	35470	3x(+NH4), 2x(+NH4), 4x(+NH4)
13 DAG-PEG-2000_47PEG Unit	Identified	2639.71803	2639.7264	897.9426	3.1	2.10	1.96	92260	31092	3x(+NH4), 2x(+NH4), 4x(+NH4)
14 DAG-PEG-2000_48PEG Unit	Identified	2683.74425	2683.7498	912.6171	2.0	2.10	1.94	81179	27228	3x(+NH4), 2x(+NH4), 4x(+NH4)
15 DAG-PEG-2000_49PEG Unit	Identified	2727.77046	2727.7738	927.2918	1.2	2.10	1.92	69132	22560	3x(+NH4), 2x(+NH4), 4x(+NH4)
16 DAG-PEG-2000_50PEG Unit	Identified	2771.79668	2771.8041	941.9685	2.6	2.10	1.90	56491	17903	3x(+NH4), 2x(+NH4), 4x(+NH4)

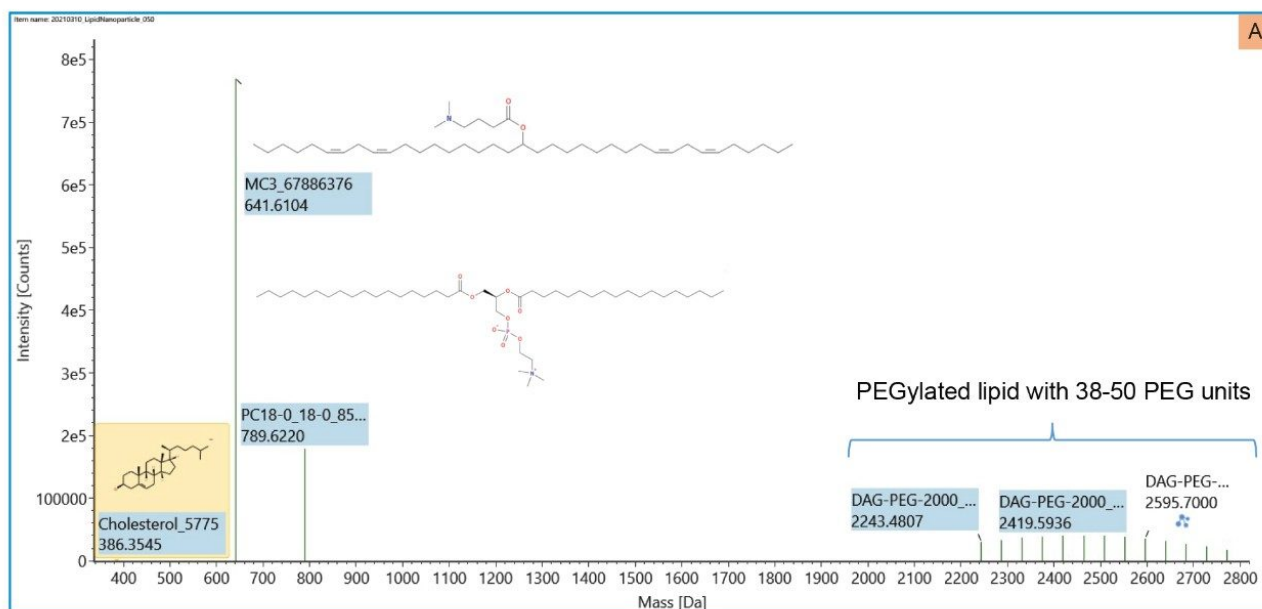


Figure 4. (A) Component summary list of identified lipid nanoparticle components of cholesterol, ionizable MC3 lipid, DSPC, and DMG-PEG-2000. (B) Component plot of the identified four lipids with corresponding compound names and structures. Clicking on the blue icon allows to visualize the lipid structure.

A serial dilution of each lipid stock solution in a concentration range of 0.1-500 pg/ $\mu$ L was prepared and analyzed at six concentration levels to evaluate the detection limit of the BioAccord System. Figure 5 shows a representative summary plot for ionizable lipid MC3 at a concentration level of 1, 5, 50, 100, 250, and 500 pg/ $\mu$ L. The lower limit of detection was determined to be 5 pg/ $\mu$ L (25 pg on column) for ionizable lipid MC3, DSPC, and DMG-PEG-2000, and 250 pg/ $\mu$ L (1.25 ng on column) for cholesterol.

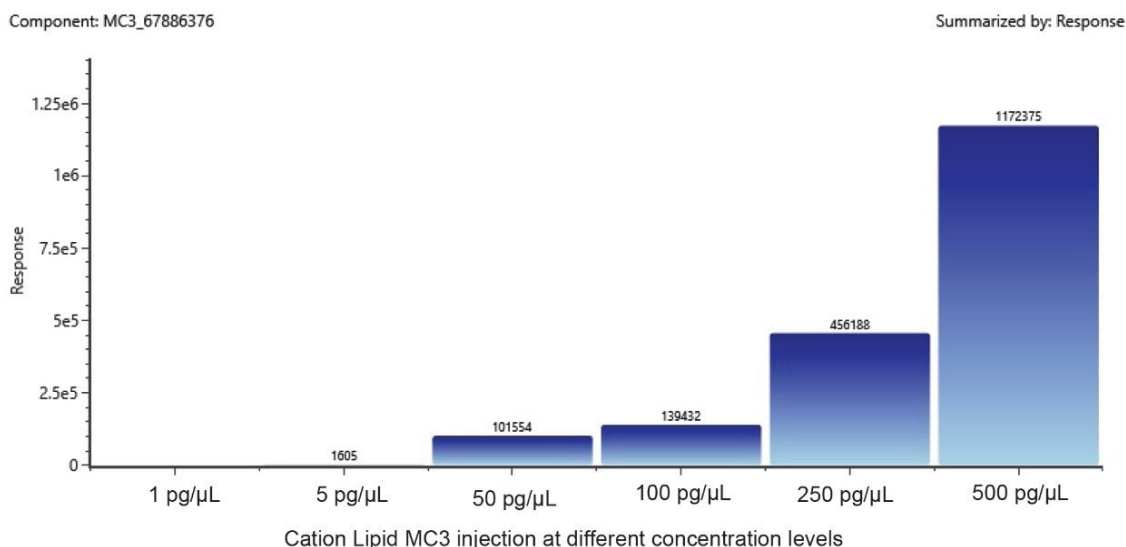


Figure 5. Example summary plot providing a visual response for ionizable MC3 lipid at different concentration levels.

The binary comparison functionality in UNIFI can be used to directly compare the analysis results of an unknown lipid sample with those of a reference sample. This is a useful feature, with the LC-MS profile of the raw lipid material or product of interest being generated, to allow differentiation from similar products, to identify uncommon impurities, or to compare the quality of lipids from multiple production batches. One can also use the function to compare the abundance of individual lipid species in unknown samples based on the extracted ion chromatograms (Figure 6A) or specified spectra (Figures 6C and 6E) with those of the reference sample and displayed as a mirror image plot. In this binary sample comparison mode, users can set specific matching criteria (with respect to RT, and mass accuracy) to determine how closely peaks in the unknown and reference samples need to be before the two peaks are considered as the same lipid component. Figure 6A shows a mirror chromatogram comparison from an injection of 0.1 mg/mL Avanti bovine liver lipid extract (reference) and an injection of 0.1 mg/mL Avanti bovine liver lipid extract spiked with DMG-PEG 2000 and ionizable lipid MC3 (labelled as 'unknown') sample. Two unique peaks at a retention time of 2.02 and 6.62 min were observed only in the unknown (i.e. spiked-in) sample indicating the presence of the spiked DMG-PEG 2000 and MC3 lipids respectively. The chromatogram difference plot (Figure 6B) shows those two peaks were only identified in the unknown sample. For the best comparison outcome under the binary comparison functionality, the LC-MS analysis would need reproducible RT measurement, and consistent MS response across all injections in a sample

set.

The binary comparison of the combined spectra (at retention time 2.02 min) for the unknown sample of DMG-PEG 2000 and the reference sample is shown in Figure 6C. The spectrum difference plot between the reference and unknown samples is shown in Figure 6D. The unique PEG spectrum pattern with charge states +2, +3, and +4 is clearly only observed in the unknown spectrum (Figures 6C and 6D). Similarly, the binary comparison of the unknown sample of ionizable lipid MC3 (at a retention time 6.62 min) with the reference sample is shown in Figure 6E. The difference plot between the reference and unknown samples is shown in Figure 6F. The presence of the MC3 ( $m/z$  642.601) only in the unknown sample as well as the spectrum difference plot which shows that the MC3 is unique in the unknown sample (Figure 6E and 6F).

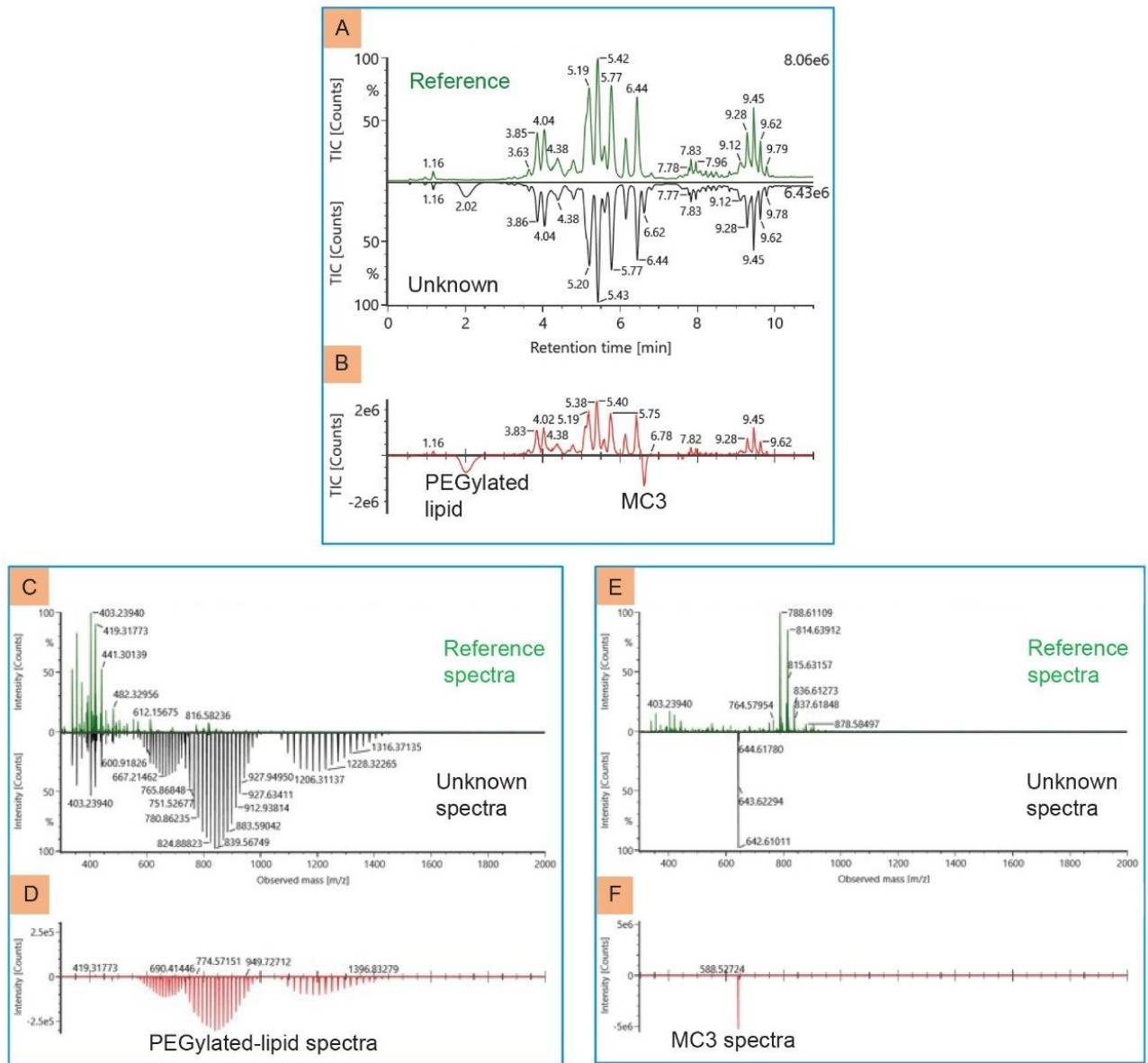


Figure 6. (A) Chromatogram binary comparison of 0.1 mg/mL Avanti bovine liver lipid extract (top panel: component amount in reference chromatogram) and 0.1 mg/mL Avanti bovine liver lipid extract spiked with ionizable MC3 lipid and DMG-PEG 2000 (lower panel: component amount in unknown chromatogram). (B) Chromatogram difference plot between the reference and unknown samples (by point-to-point subtraction). (C) Combined spectra binary comparison of DMG-PEG 2000 (retention time 2.02 min). (D) Spectra difference plot between the reference and unknown samples from figure 6C. (E) Combined spectra binary comparison of ionizable MC3 lipid (retention time 6.62 min).

(F) Spectra difference plot between the reference and unknown samples from figure 6E.

The developed method was applied for the analysis of a complex bovine liver extract to demonstrate its generally applicability to analyze other types of lipid compounds. All the major lysophospholipids, phospholipids, and neutral lipids were detected (Figure 7) by the method. Therefore, the method is useful for the simultaneous determination of the composition of LNPs and any other impurities produced due to potential degradation, oxidation, hydrolysis, hydrogenation, aggregation, and dimer formation during LNP formulations.

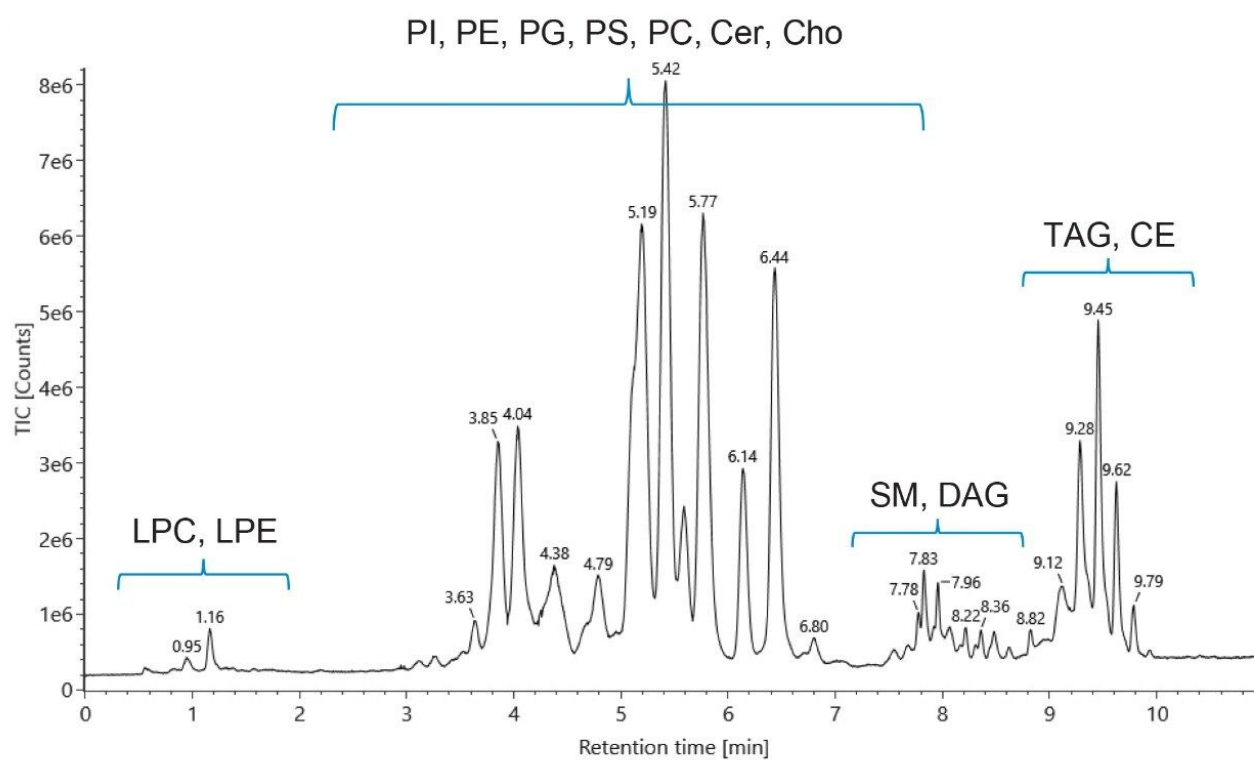


Figure 7. Representative positive mode total ion chromatogram of Avanti Polar Lipids bovine liver total lipid extract. Abbreviations: DAG, diacylglycerols; TAG, triacylglycerols; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PS, phosphatidylserines; PI, phosphatidylinositol; Cer, ceramides; SM, sphingomyelins; Cho, cholesterol; CE, cholesterol esters.

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## Conclusion

A simple, rapid, and routine reversed-phase LC-MS method was developed for the analysis of LNP composition. The performance of the method was evaluated on the BioAccord System, demonstrating it can be used for the simultaneous analysis of LNP components and impurities in process development and quality control.

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