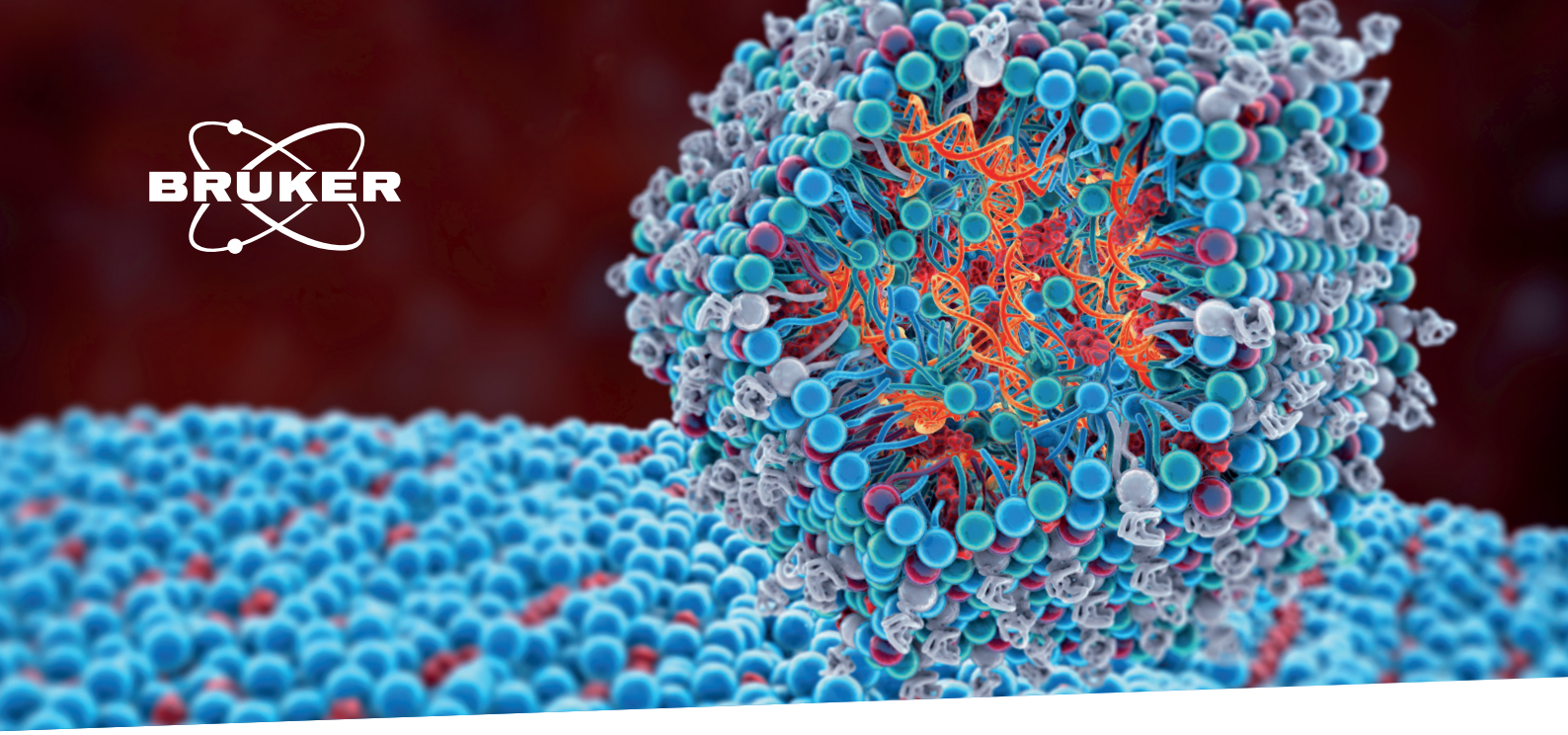

# 4D analysis of lipid nanoparticles using Elute-timsTOF Pro 2 with VIP-HESI

A rapid method utilizing ultra-high performance liquid chromatography, trapped ion mobility spectrometry, and high-resolution mass spectrometry (UHPLC-TIMS-HRMS) is demonstrated for the sensitive and specific analysis of lipid nanoparticle components.


## Abstract

Here we combine the Elute UHPLC system with the timsTOF Pro 2 to enable powerful multidimensional separation of lipid nanoparticle (LNP) components including synthetic PEGylated lipids from the complex lipidome of human plasma. A heated electrospray ionization source with a vacuum insulated probe (VIP-HESI) provides exceptional sensitivity enhancement in comparison to conventional ESI sources, completing the hyphenated system as a powerful solution for studying the composition of LNPs and their interaction in complex biological systems.



Keywords:
timsTOF, CCS, VIP-HESI, lipid nanoparticles, lipidomics, gene therapy


## Introduction

Lipid nanoparticles have emerged from the recent COVID-19 pandemic as effective delivery vehicles for mRNA vaccines (1, 2), and pharmaceutical formulations in nucleic acid-based drugs. LNPs are typically composed of four critical lipid species components:

1. Cholesterol, a natural structural lipid that can enhance particle stability (3).
2. Phospholipids such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), which acts as a natural cell membrane component that stabilizes the structure of lipid nanoparticles (4).
3. Synthetic ionizable lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) with a positive charge for binding and encapsulating negatively-charged mRNA (5).
4. PEGylated lipids such as 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG-2000) for reducing particle aggregation and increase stability (6).


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As LNPs become more commonly considered for use by drug and vaccine manufacturers, the research interest in their composition, stability, and biological interactions is driving a need for bioanalytical solutions capable of in-depth sample characterization. Bruker's 4D-Lipidomics™ solution leverages powerful multidimensional separation by combining ultra-high performance liquid chromatography (UHPLC) with trapped ion mobility spectrometry (TIMS) and high-resolution mass spectrometry (HRMS) in a single hyphenated system capable of separating and describing sample components by their retention time, collision cross section (CCS), and mass-to-charge (*m/z*) measurements, respectively. The "4D" workflow is completed using quadrupole-time-of-flight HRMS and parallel acquisition serial fragmentation (PASEF) technology for the rapid (>100 Hz) acquisition of TIMS-separated tandem mass spectrometry (MS/MS) fragmentation spectra. The UHPLC-TIMS-HRMS system is further enhanced by the use of a vacuum insulated probe – heated ESI (VIP-HESI) source which considerably reduces heat transfer to the analytes in the LC-eluate and dramatically enhances their signal for increased analytical sensitivity. Its active exhaust system ensures the ion source is kept clean for high robustness and low background across prolonged periods of analysis.

In this application note, four components of LNPs are analyzed using the Elute–timsTOF Pro 2 system with CCS-capability via a VIP-HESI source. Analysis of LNP lipid species in a complex biological matrix is also investigated.

## Experimental

### Sample and Materials

Chemical standards of DSPC, DOTAP, and DMG-PEG-2000 were purchased from Avanti Polar Lipids and cholesterol from Sigma-Aldrich. Stock solutions of 1 mg/mL cholesterol and DMG-PEG-2000 were prepared in methanol/chloroform (50/50 v/v), DOTAP and DSPC were in methanol. Working solutions with concentrations ranging from 1 ppb - 100 ppm of the resulting LNP component mixture were diluted in methanol. The structures of the four lipid species are listed in Figure 1.

NIST SRM 1950 plasma was obtained from Sigma-Aldrich. Lipids were extracted from plasma by an MTBE protocol (7). Thirty µL of NIST SRM 1950 plasma was extracted by MTBE and reconstituted in 300 µL 9:1 MeOH:DCM. The reconstituted plasma extract was split into two aliquots, one aliquot was analyzed as is and the other aliquot was spiked with LNP component mixture (5 ppm cholesterol, 1 ppm DMG-PEG 2000, and 50 ppb DOTAP/DSPC).

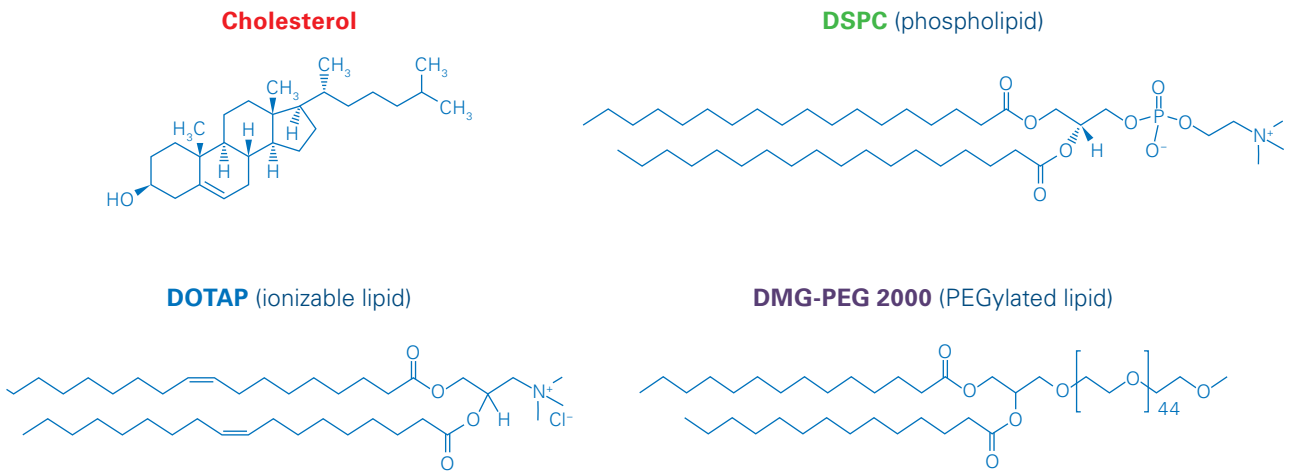


**Figure 1**
**Chemical structures of four LNP components analyzed in this study.**

| LC conditions | | | |
|---|---|---|---|
| **LC system** | **Elute** | | |
| **Column** | YMC-Triart C18, 100 x 21 mm, 1.9 µm, P/N TA12SP9-10Q1PT | | |
| **Mobile phase A** | 600/390/10 ACN/Water/10 mM ammonium formate in 0.1% formic acid | | |
| **Mobile phase B** | 900/90/10 IPA/ACN/10 mM ammonium formate in 0.1% formic acid | | |
| **Gradient** | **Time [min]** | **% A** | **% B** |
| | 0.0 | 50 | 50 |
| | 0.5 | 47 | 53 |
| | 4.0 | 45 | 55 |
| | 7.0 | 35 | 65 |
| | 7.5 | 20 | 80 |
| | 10.0 | 1 | 99 |
| | 13.0 | 1 | 99 |
| | 13.1 | 50 | 50 |
| **Flow rate** | 0.4 mL/min | | |
| **Column temp.** | 55°C | | |
| **Sample temp.** | 4°C | | |
| **Injection volume** | 2 µL | | |

| MS parameters | | |
|---|---|---|
| **MS system** | **timsTOF Pro 2** | |
| **Method** | default 4D-Lipidomics_pos | |
| **VIP-HESI Source Parameter** | **Parameter** | **Value** |
| | End Plate Offset | 500 V |
| | Capillary Voltage | 4500 V |
| | Nebulizer | 2.0 Bar |
| | Dry Gas | 8.0 L/min |
| | Dry Temp | 230°C |
| | Prob Gas Temp | 400°C |
| | Probe Gas | 4.0 L/min |
| **VIP-HESI Source Parameter** | **Parameter** | **Value** |
| | End Plate Offset | 500 V |
| | Capillary Voltage | 4000 V |
| | Nebulizer | 2.2 Bar |
| | Dry Gas | 10.0 L/min |
| | Dry Temp | 220°C |

## Results and discussion

### UHPLC-TIMS-HRMS analysis of LNP components

UHPLC-TIMS-HRMS analysis of a LNP mixture (2 µL) containing 100 ppb DOTAP, 100 ppb DSPC, 20 ppm DMG-PEG-2000, and 10 ppm cholesterol is displayed in Figure 2. Panel I shows the extracted ion chromatograms of the PEGylated lipid (represented by most abundant peak for +3 charge state), cholesterol, an ionizable lipid, and a phospholipid. The four lipid species were readily separated and detected by the Elute-timsTOF Pro 2 system. Corresponding mass spectra for the four components of DMG-PEG-2000 (+2, +3, and +4 charge states), DOTAP (*m/z* 662.6087), cholesterol (*m/z* 369.3516 with water loss), and DSPC (*m/z* 790.6320) are displayed in Panel II.

In addition to LC-MS separation, these four LNP components are separated by TIMS, providing an additional dimension of separation. Panel III shows the extracted ion mobilogram of the four lipid species. Mobility $1/K_0$ is measured by TIMS and converted to CCS. CCS provides an additional physical property of the molecules and can be used for increased confidence in compound annotation.

### VIP-HESI source for improved LNP component detection

LNP components at various concentrations were analyzed using the VIP-HESI source and compared to a conventional ESI source. Figure 3 shows the peak areas of each compound at varying concentrations with the VIP-HESI source providing 5-25 fold increased signal.

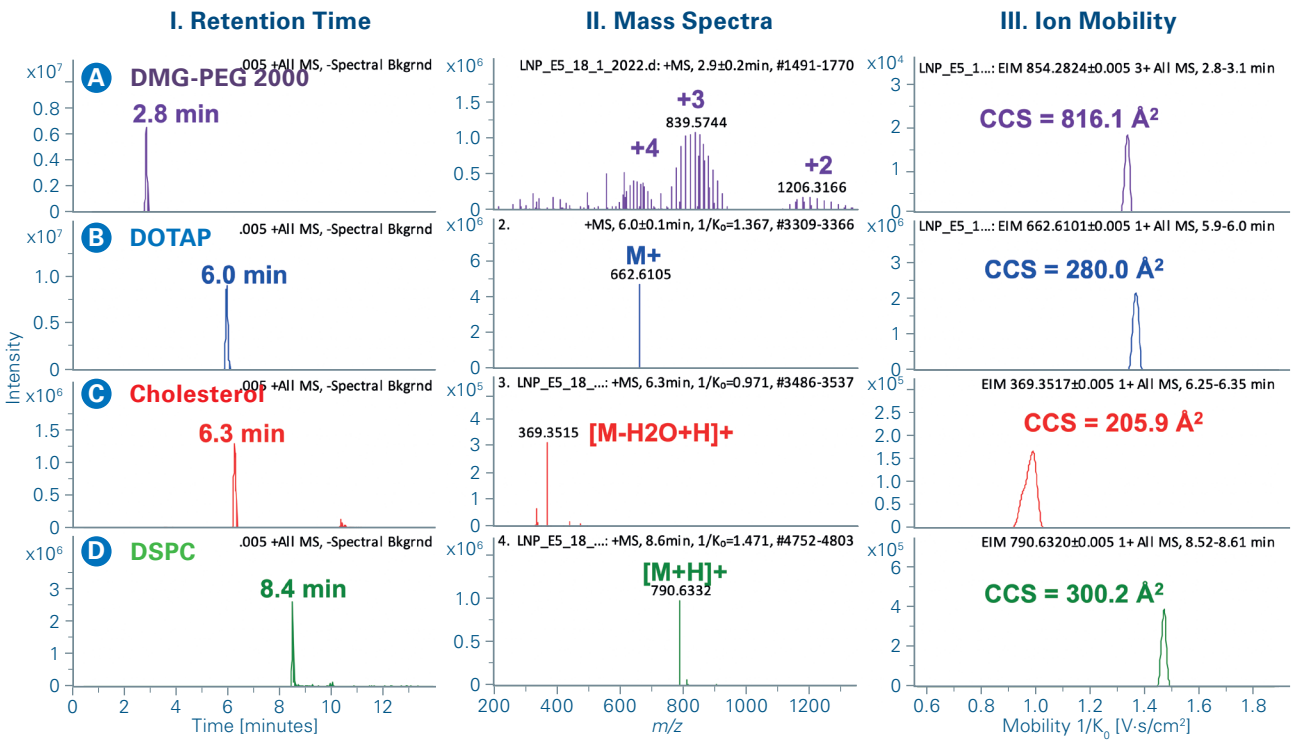


**Figure 2**

**Results of (I) extracted ion chromatogram, (II) extracted mass spectra, and (III) calculated CCS for the analysis of a mixture of four LNP components:** Ⓐ DMG-PEG-2000 (*m/z* 854.2496 for +3 of 44 repeating PEG unit), Ⓑ DOTAP for the cation (*m/z* 662.6087), Ⓒ Cholesterol with water loss (*m/z* 369.3516), and Ⓓ DSPC (*m/z* 790.6320).

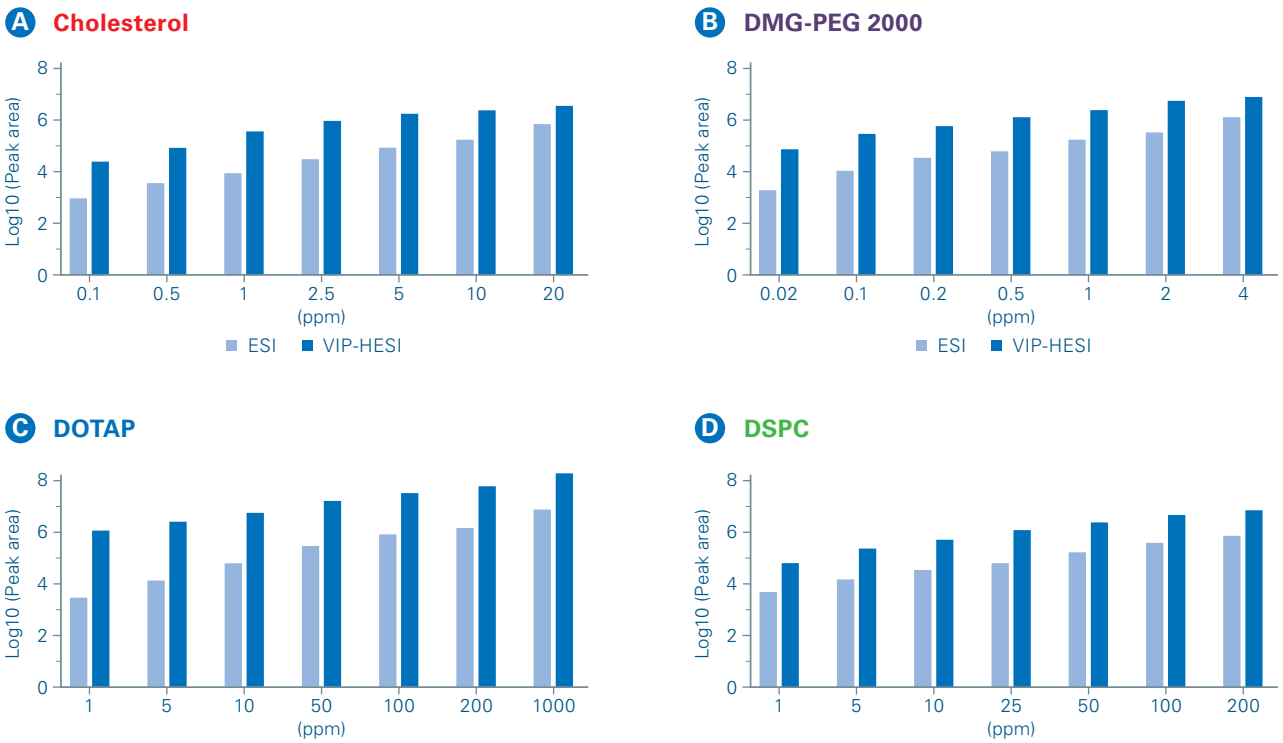


**Figure 3**

**Log10 of peak area of representative EIC peaks from the four lipid species comparing ESI vs. VIP HESI sources for** Ⓐ Cholesterol at *m/z* 369.3516, Ⓑ DMG-PEG-2000 at *m/z* 854.2496, Ⓒ DOTAP at *m/z* 662.6087, and Ⓓ DSPC at *m/z* 790.6320.

### UHPLC-TIMS-HRMS VIP analysis of LNP components in plasma

To evaluate the analysis of LNP components in a complex biological matrix, NIST SRM 1950 plasma extract was spiked with the LNP component mixture and analyzed. Base peak chromatograms (BPC) of plasma (A) with and (B) without the LNP spike are displayed in Figure 4. The retention time range where DMG-PEG-2000 elutes (2.4-3.7 min) is highlighted in blue and shown explicitly in Figure 4C.

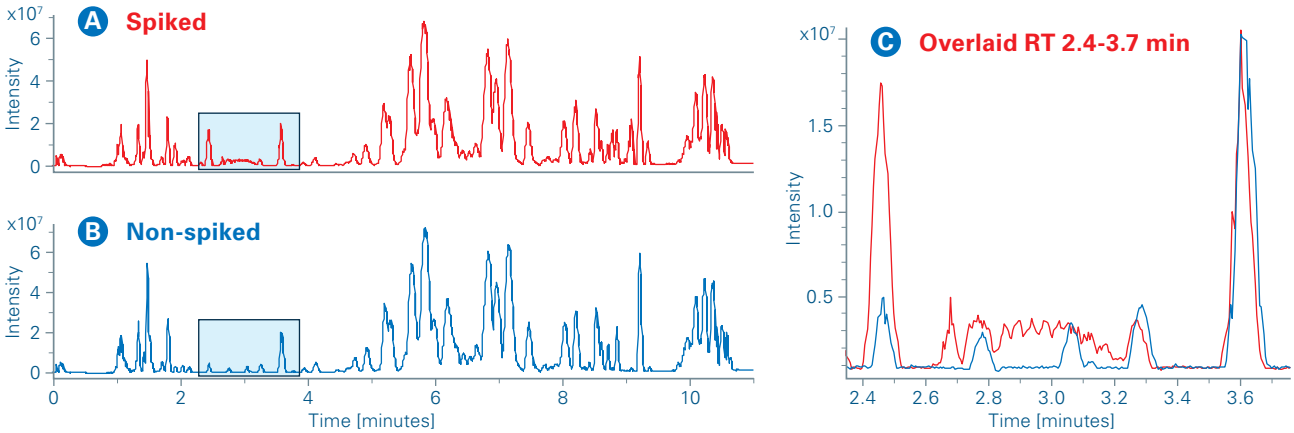


**Figure 4**

**Base peak chromatogram of SRM 1950 lipid extract (A) with and (B) without LNP component mixture spike, (C) overlaid chromatogram of RT 2.4-3.7 region.**

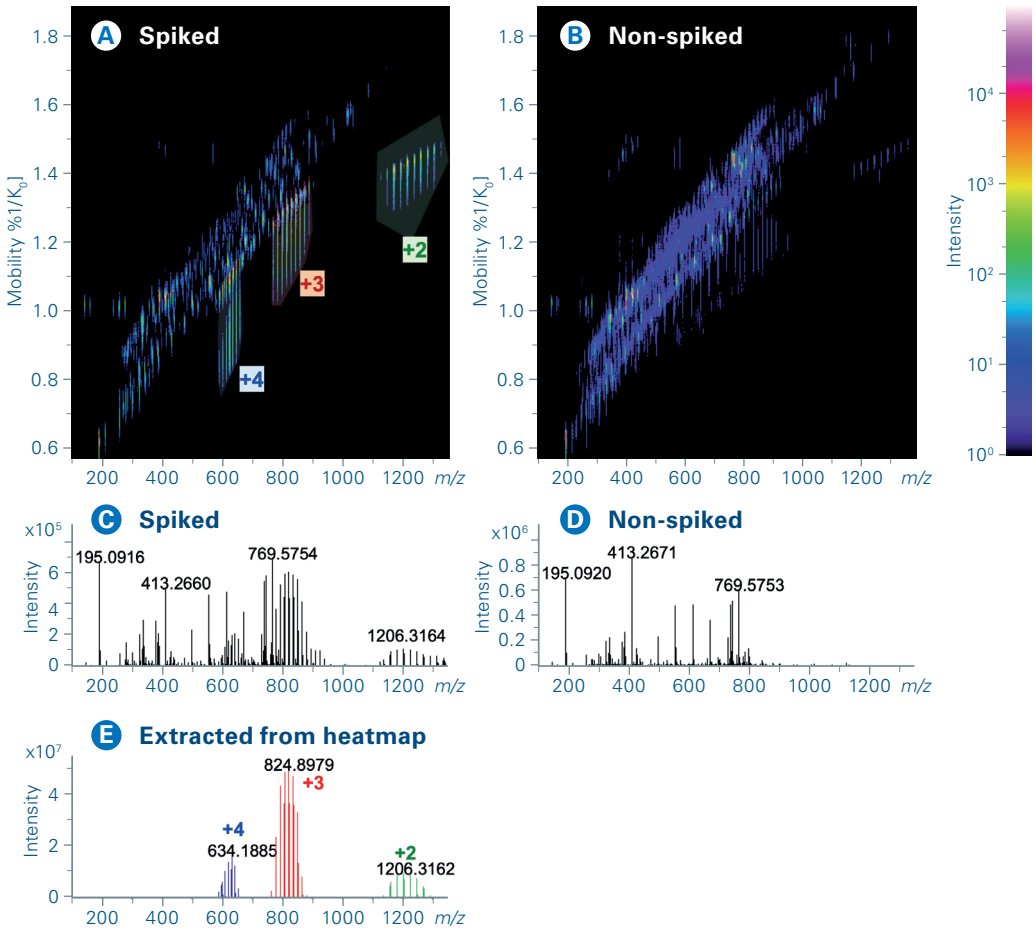


**Figure 5**

**Heatmap of plasma extract from 2.3-3.2 min for (A) with and (B) without LNP spike. ((C)-(D)) Mass spectra extracted from 2.8-3.2 min from LC chromatogram. (E) Mass spectrum extracted from heatmap.**

Heatmaps of these regions where the PEGylated lipid is eluted are shown in Figure 5 for SRM plasma extract (A) with and (B) without the LNP component mixture spike. Mass spectra extracted from the corresponding RT range are displayed from (C) with and (D) without LNP spiked plasma samples. DMG-PEG-2000 signals are observed but co-eluted with other compounds or background in the plasma (BPC in Figure 4C and mass spectrum in 5C). They are not observed in plasma without spiking (Figure 5D). Figure 5E displays overlaid mass spectra extracted directly from regions of the heatmap using the polygon tool, the clean mass spectrum of +2 to +4 charge states of DMG-PEG-2000 displays clear separation between the PEGylated lipid and other compounds extracted from plasma in the ion mobility dimension. These results demonstrate the efficient ion mobility separation of DMG-PEG-2000 from the complex matrix.

## Conclusion

Rapid analysis of LNP components is provided by UHPLC-TIMS-HRMS. The VIP-HESI source demonstrates significant sensitivity enhancement for ionizing LNP materials. The additional dimension of ion mobility spectrometry provides CCS information for LNP compound identification and can separate PEGylated lipids from complex plasma samples.

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