

Application Note

## A Robust and Reproducible Reversed-Phase Lipid Profiling Method for Large Sample Sets

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## Abstract

Lipidomic analysis of mammalian plasma from large sample sets such as animal safety assessment studies and epidemiological studies requires a reliable and robust methodology. Rapid and robust methods facilitate the support of longitudinal studies allowing the transfer of methodology between laboratories. To address this need, a high throughput reversed-phase LC-MS methodology was developed using UltraPerformance Liquid Chromatography (UPLC) with Charged Surface Hybrid (CSH) C<sub>18</sub> Technology and accurate mass detection using SYNAPT XS. The methodology showed excellent specificity, robustness, and reproducibility for over 100 injections of human plasma.

### Benefits

- Highly reproducible and robust lipid profiling method
- A high throughput and comprehensive approach to large cohort sample sets
- Excellent chromatographic resolution for complex mixtures
- Optimum lipidomics coverage for positive and negative mode analyses
- Applicable to a broad range of biological samples such as body fluids, tissue samples, and cell cultures

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## Introduction

Lipids play an important role in the energy storage, cellular signaling, and pathophysiology of diseases such as cancer, neurodegenerative diseases, infections, and diabetes. Advances in LC-MS have allowed lipids to be studied with greater sensitivity and specificity, alleviating the effects of co-eluting compounds and isobaric interference and allowing low abundance lipids to be more readily detected. The increased interest in employing lipidomics analysis on large scale drug discovery and epidemiological studies means that there is a need for high throughput, robust, and highly reproducible methodologies, which can be used for longitudinal studies and can be transferred between laboratories.<sup>1,2</sup> For comprehensive lipid profiling, lipids need to be screened in both positive and negative ion modes.

Conventional mass spectrometric analysis of lipids is often performed by direct infusion or reversed-phase (RP) and normal-phase (NP) chromatography. However, each of these methods faces its own challenges. Direct infusion gives rise to ion suppression, limiting lipid detection, and lowering reproducibility. It also does

not allow for separation of isobaric lipids, which can complicate the resultant analysis, necessitating deconvolution. NP chromatography allows for the separation of lipids by class but often suffers from long elution times and the inability to separate isomeric lipids. Traditional RP methods are widely used but similarly suffer from extensive elution times, low peak capacity, and poor run-to-run reproducibility.

In this work we present a high throughput, robust, and reproducible LC-MS methodology RP-UPLC lipid separation using a Waters ACQUITY UPLC I-Class PLUS System with Charged Surface Hybrid (CSH) C<sub>18</sub> chemistry and QToF detection. The combination of sub-2- $\mu$ m particle size with a liquid chromatography system optimized to minimize band spreading novel chemistry allows for a significantly improved RP method. This method maximizes the performance of these particles and is optimized for the analysis of large cohort sample studies.

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## Experimental

### Sample Description:

The Differential Ion Mobility System Suitability Lipidomix (Avanti Polar Lipids, INC.) was used as system suitability to evaluate instrument performance. The mixture contains 1 mg/mL of each lipid and (0.25 mg/mL PI), 0.5 mL per vial in chloroform:methanol (1:1). A working 500 ng/mL solution (125 ng/mL PI) was prepared in (IPA/ACN, 50/50).

A simple plasma sample preparation procedure was adopted using protein precipitation with pre-cooled isopropanol (IPA). 20  $\mu$ L human plasma NIST Standard Reference Material 1950 was mixed with 80  $\mu$ L pre-cooled IPA. The samples were vortex mixed for one minute and placed at -20 °C for 10 minutes. Samples were vortex mixed again for one minute and placed at 4 °C for two hours to ensure complete protein precipitation. The samples were centrifuged at a maximum of 10,300 g for 10 minutes at 4 °C. The supernatant was transferred and dilute with (IPA/ACN, 50/50) in 1:5 ratio before transferring the supernatant to Waters Total Recovery UPLC Vials (p/n: [186005669CV < https://www.waters.com/nextgen/in/en/shop/vials-containers--collection-plates/186005669cv-truview-lcms-certified-clear-glass-12-x-32-mm-screw-neck-total-r.html>](https://www.waters.com/nextgen/in/en/shop/vials-containers--collection-plates/186005669cv-truview-lcms-certified-clear-glass-12-x-32-mm-screw-neck-total-r.html) ) for LC-MS analysis.

### Method Conditions

## LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS
Detection:	SYNAPT XS
Vials:	Total Recovery UPLC Vials
Column(s):	ACQUITY UPLC CSH C <sub>18</sub> (2.1 x 100 mm, 1.7 μm)
Column temp.:	55 °C
Sample temp.:	10 °C
Injection volume:	2 μL (pos mode) and 4 μL (neg mode)
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

Time (min)	Flow (mL/min)	%A	%B	Curve
7.5	0.4	20	80	1
10	0.4	1	99	6
11	0.4	1	99	1
12	0.4	50	50	1

## MS Conditions

MS system:	SYNAPT XS
Ionization mode:	ESI positive and negative
Acquisition range:	100–1200
Capillary voltage:	3.0 kV (pos mode) and 2.5 kV (neg mode)
Collision energy:	Linear ramp (transfer CE) 25–45 eV
Cone voltage:	30 V

## Data Management

Chromatography software:	MassLynx V4.2
MS software:	MassLynx V4.2

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## Results and Discussion

Previously we developed a 20 min CSH C<sub>18</sub> method for the deep profiling of lipids in plasma and tissue.<sup>3,4</sup> To address the requirement for faster analysis, a 12 min method was developed as an alternative approach for studies with larger sample numbers where higher-throughput is required. The resulting methodology showed clear resolution of the lipid standards from the different lipid classes in the Avanti Polar Lipid IMS mix. The average chromatographic peak width was determined to be 6.5 seconds giving an overall peak capacity of 90 for the 10 minute separation. The data displayed in Figure 1 shows the separation of the Avanti Polar Lipid IMS mix standards in both positive and negative ion modes.

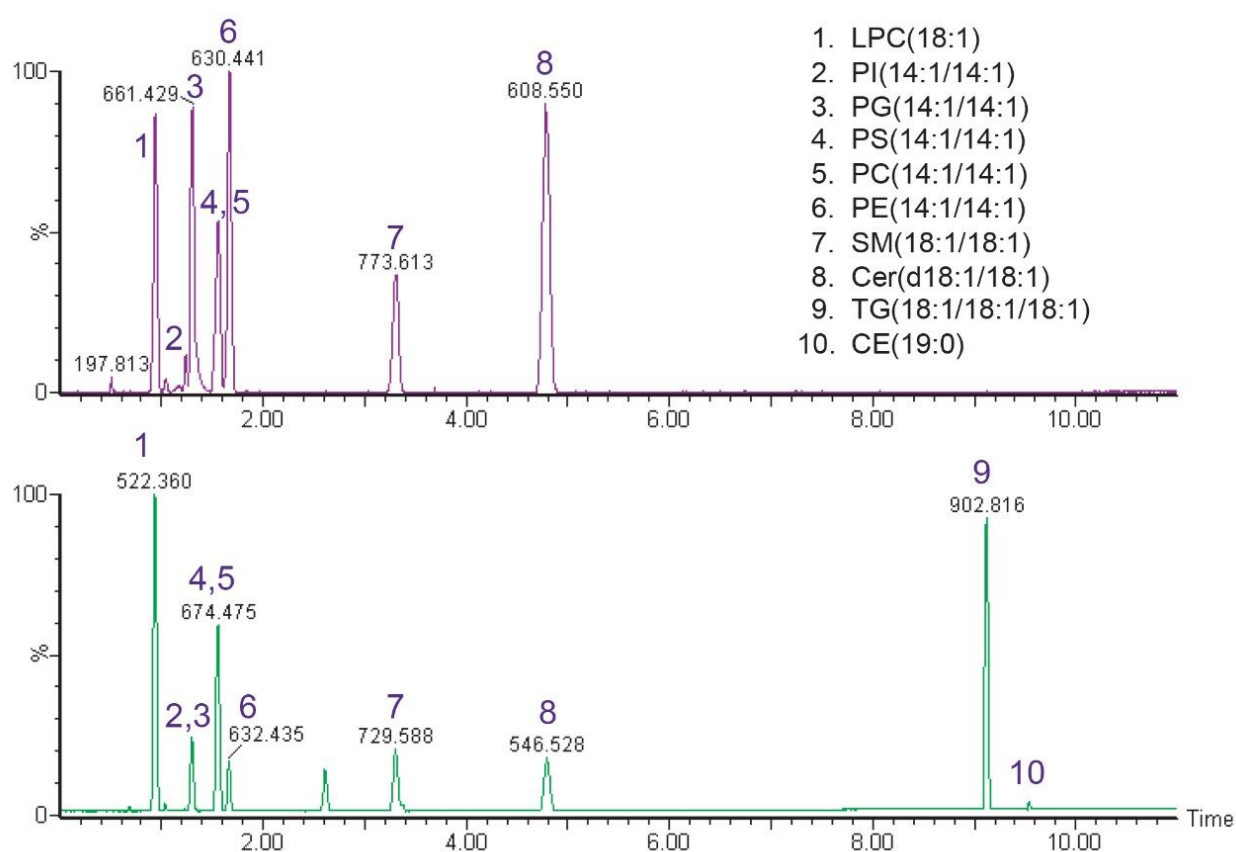


Figure 1. The Avanti Differential Ion Mobility System Suitability standard Lipidomix Kit was used as system suitability solution. Negative mode base peak intensity chromatogram with 4  $\mu$ L injection volume (top) and positive mode base peak intensity chromatogram with 2  $\mu$ L injection volume (bottom). A concentration of 500 ng/mL in IPA/ACN (50/50) was prepared from the Avanti stock solution.

For comprehensive screening, lipidomics experiments are performed in both positive and negative ionization modes. To assess the lipid coverage and applicability of this methodology in real biological samples, total lipid extracts from human plasma NIST SRM 1950 were analyzed using the ACQUITY UPLC I-Class PLUS

System with CSH C<sub>18</sub> column in MS<sup>E</sup> continuum, resolution mode. The results demonstrated that the methodology was capable of clearly resolving the plasma lipids in the sample and was able to separate the major lipid classes with high resolution, sensitivity, and lipid coverage, as shown in Figures 2. As can be seen from this data, the lysophospholipids and free fatty acids (FFA) elute first in the first 2 minutes of the separation (these lipids elute early due to their great polarity). The less polar lipids (diglycerides, triglycerides, and cholesterol esters) elute at the end of the separation between 8 and 10 minutes. The various phospholipids and sphingolipids with intermediate polarity (PI, PG, PS, PC, PE, SM, and Cer) elute between 2 and 8 minutes depending on the fatty acyl chain and number of double bonds.

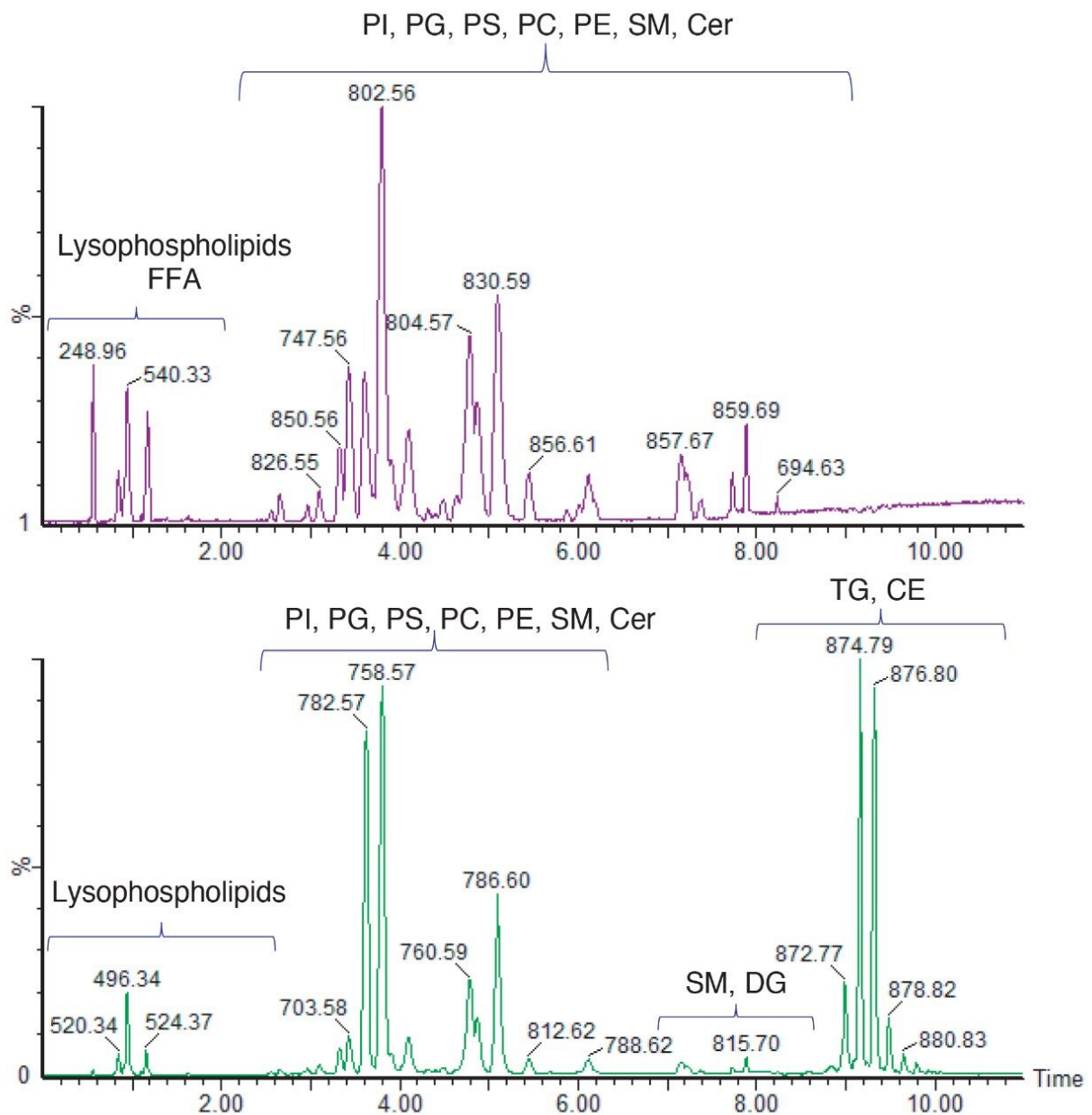


Figure 2. Negative (top) and positive mode (bottom) base peak intensity chromatogram of human plasma Standard Reference Material (SRM 1950).

Assay reproducibility is a key factor to support a large sample number with lipidomics information. This is especially important for large cohort studies, batch-to-batch comparison, or inter laboratory data. The ACQUITY UPLC I-Class PLUS System with CSH C<sub>18</sub> Column demonstrated excellent retention time reproducibility from multiple injections (%RSD <0.19; n=100), as shown in Figure 3. This is critical for lipidomic analysis, which requires the alignment and comparison of a large number of LC-MS



chromatograms derived from multiple sample sets.

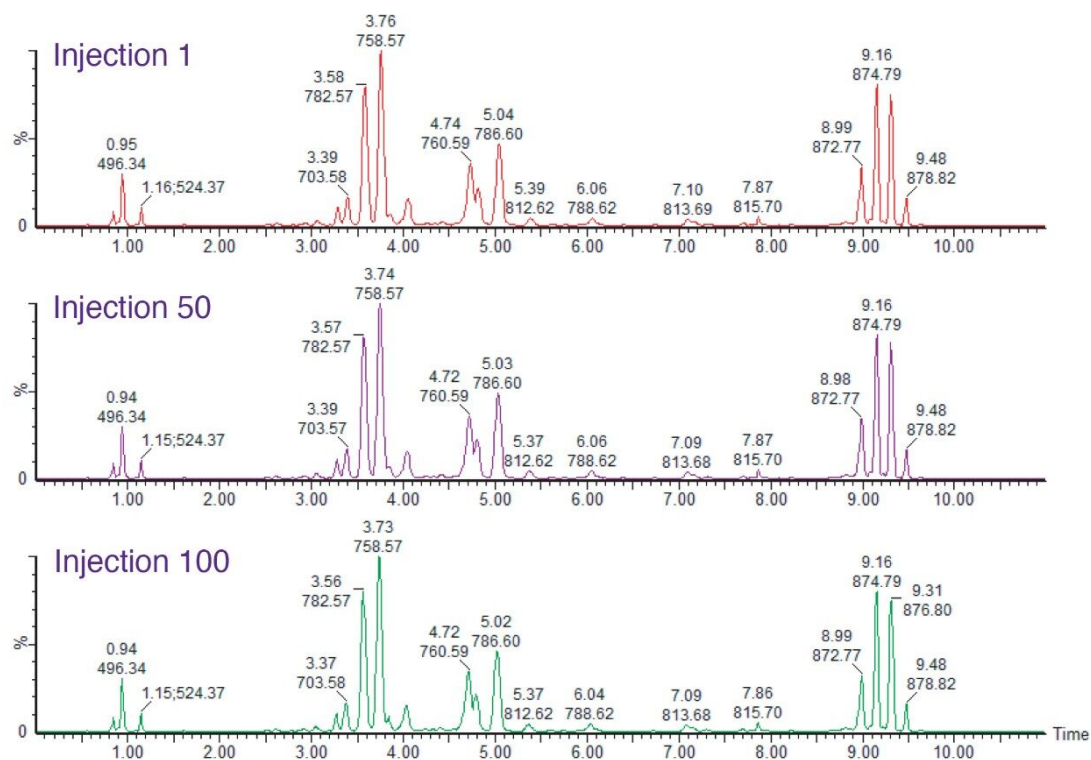


Figure 3. Retention time reproducibility for selected injection numbers 1, 50, and 100 based on LPC(16:0)  $m/z$  496.34, PC(16:0\_18:1)  $m/z$  758.57, SM(d18:1\_24:1)  $m/z$  813.69, and TAG(52:3)  $m/z$  874.79 with average retention time RSD value  $<0.19\%$  ( $n=100$ ).

## Conclusion

A robust and reproducible high resolution UPLC-MS method has been developed for the detection and identification of lipid species in plasma, with average retention time percent relative standard deviation of less than 0.19 across the analysis ( $n=100$ ). Despite the short analysis time, 10 minutes, the method produced a peak capacity of 90, allowing for the excellent separation of the various lipid classes in the NIST human plasma sample. The developed method is readily applicable to a broad range of biological samples such as body fluids, tissue samples, and cell cultures.

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## References

1. Stephenson DJ, Hoeflerlin LA, Chalfant CE. Lipidomics in Translational Research and the Clinical Significance of Lipid-Based Biomarkers. *Transl Res*. 2017 Nov;189:13–29.
2. Audano M, Maldini M, Fabiani ED, Mitro N, Caruso D. Gender-related Metabolomics and Lipidomics: From Experimental Animal Models to Clinical Evidence, *J Proteomics* 2018 Apr 30;178:82–9.
3. Isaac G, McDonald S, Astarita G. Lipid Separation using UPLC with Charged Surface Hybrid Technology. Waters Application Note [720004107en](#) <<https://www.waters.com/webassets/cms/library/docs/720004107en.pdf>> . 2011 Sep.
4. Damen CWN, Isaac G, Langridge J, Hankemeier T, Vreeken RJ. Enhanced Lipid Isomer Separation In Human Plasma Using Reversed-Phase UPLC with Ion-Mobility/High-Resolution MS Detection. *J Lipid Res*. 2014 Aug;55(8):1772–83.

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720006959, July 2020