

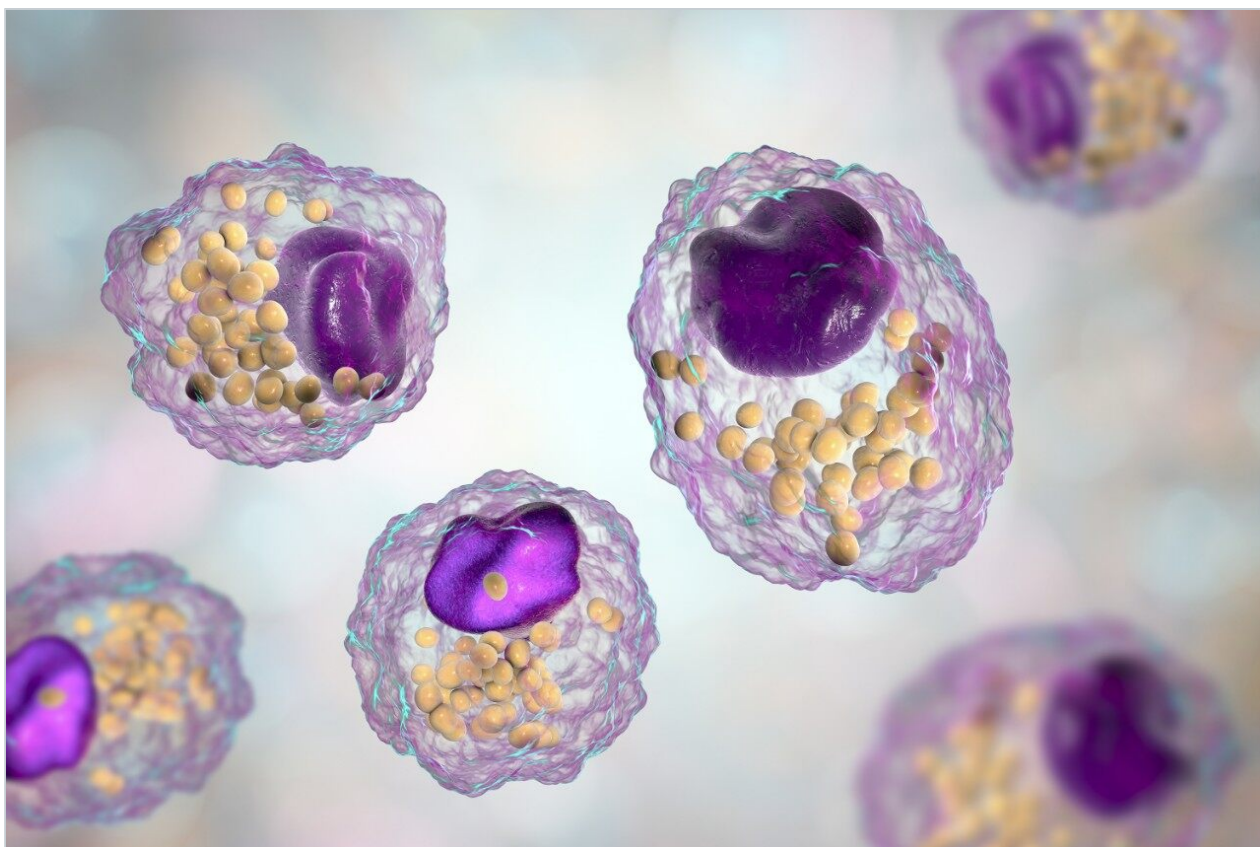
Application Note

## LipidQuan for Comprehensive and High-Throughput HILIC-based LC-MS/MS Targeted Lipid Quantitation

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## Abstract

This application note describes a high-throughput quantitative and comprehensive HILIC method for the analysis of polar and non-polar lipid classes in plasma.

### Benefits

- A high-throughput (8 minutes), quantitative and comprehensive LC-MS/MS method for the analysis of polar and non-polar lipid classes in plasma
- Fast separation of 16 polar and non-polar lipid classes
- Quantitation of 508 lipid species from plasma sample (261 positive mode injection and 247 negative mode injection)
- A robust and easy to deploy platform reducing method development and training costs, using Quanpedia and SOPs
- Fast data processing and visualization using TargetLynx and third party Informatics (Skyline) for maximum flexibility
- Time and cost savings when compared to competitive products, enabling increased customer productivity

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## Introduction

Traditionally, lipids are associated with cellular roles involving energy storage and used as structural building blocks. Recent developments in biomedical lipid research using mass spectrometric approaches have identified the important role of lipids in a variety of different disease states including cancer, inflammation, and cardiovascular diseases which has generated a resurgence in lipid research.<sup>1</sup>

The extreme structural diversity of lipids in real biological samples is challenging for analytical techniques due to large differences in physicochemical properties (such as acidity, basicity, neutral, polar, and non-polar) of individual lipid species. Currently, the two main analytical strategies used for lipidomics are direct infusion Mass Spectrometry (MS) and high performance reversed phase (RP) LC-MS/MS. Direct infusion MS provides high-throughput analysis but suffers from the inability to separate and resolve isobaric species and to identify low abundant species due to ion suppression. RP-LC is widely used for the separation of individual

lipid molecular species based on their lipophilicity; a characteristic governed by the carbon chain length and number of double bonds. Separation by lipophilicity, however, often results in the co-elution of lipid species from different classes.<sup>2</sup> Normal-phase methods, using solvents such as hexane and chloroform, are also used for the separation of lipid classes but their lengthy elution, limited analyte solubility, and lack of solvent compatibility with mass spectrometer detection makes the technique less attractive.

Hydrophilic interaction chromatography (HILIC) is a type of normal-phase chromatography that can be used for the separation of lipid classes with a reversed phase solvent system to overcome these potential challenges.<sup>3</sup> The lipid class separation using HILIC is more convenient for LC-MS quantitation because lipid class stable isotope labelled (SIL) standards coelute with lipid species inside particular lipid classes under identical mobile phase and matrix composition. This provides identical ionization conditions for the SIL standards and determined lipid class.<sup>(2,4)</sup> An additional benefit of separating lipid species by class is that fewer SIL standards are required for quantification and leads to a significant cost savings over including SIL standards for each lipid of interest. This application note describes a comprehensive and high-throughput HILIC-based analytical method for the separation and quantitation of both polar and non-polar lipid classes (Figure 1).

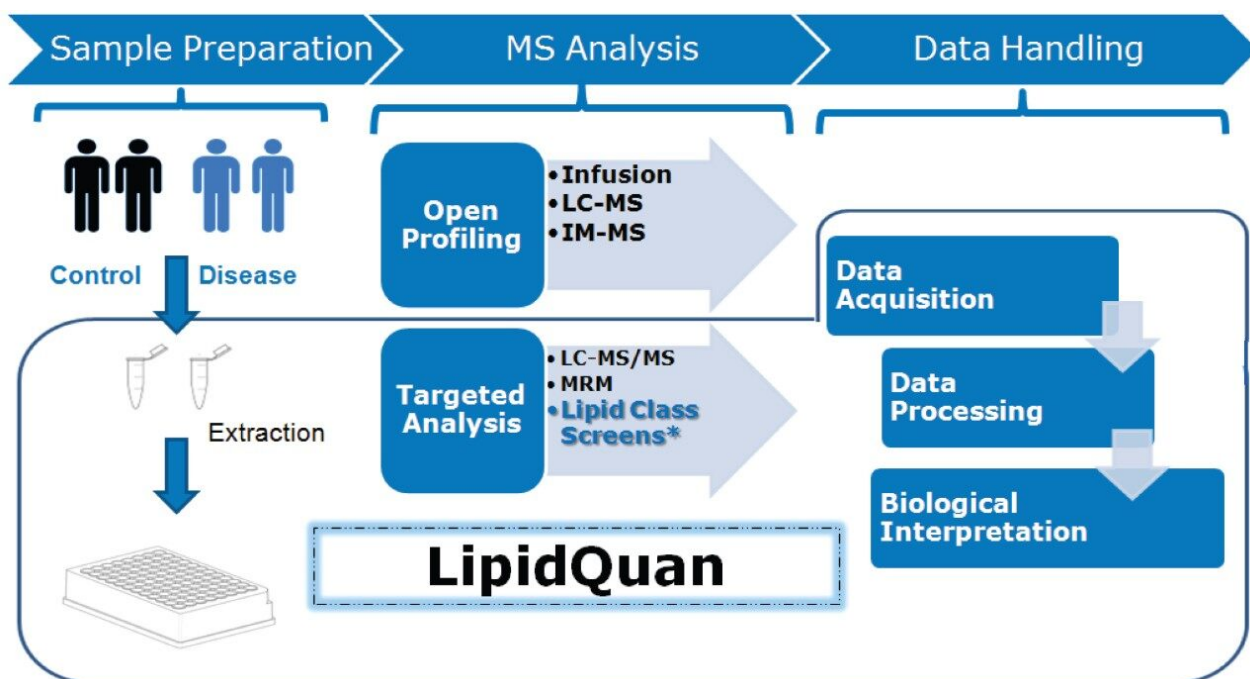


Figure 1. General lipidomic workflow using LipidQuan.

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## Experimental

### Samples

Pooled healthy control plasma was spiked with SIL standards, (SPLASH LIPIDOMIX, Avanti Lipids, Alabaster, AL) at nine concentration levels to generate calibration curves for quantification. Table 1 shows the list of SIL standards spiked into the NIST Standard Reference Material 1950 plasma (Sigma Aldrich, Poole, UK) extract and other calibration parameters in positive and negative ion mode.

Six replicates of the NIST Standard Reference Material 1950 plasma were also spiked with 5% SIL prior to extraction.

### Sample preparation

A simple sample preparation procedure was adopted using protein precipitation with a pre-cooled isopropanol (IPA) at 4 °C (1:5, plasma:IPA).<sup>5</sup> Samples were vortex mixed for 1 minute and placed at -20 °C for 10 minutes. Samples were vortex mixed again for 1 minute and placed at 4 °C for 2 hours to ensure complete protein precipitation. The extracted samples were centrifuged at a maximum of 10,300 g for 10 minutes at 4 °C before transferring the supernatant to glass vials for LC-MS analysis.

### LC conditions

LC system:	ACQUITY UPLC H-Class PLUS
Detector:	ACQUITY UPLC PDA and ACQUITY QDa
LC system:	ACQUITY I-Class UPLC, Fixed Loop or Flow through Needle (FTN)
Column(s):	ACQUITY UPLC BEH Amide 1.7 µm, 2.1 × 100 mm
Column temp.:	45 °C
Flow rate:	0.6 mL/min

Mobile phase A:	95:5 Acetonitrile/water + 10 mM ammonium acetate
Mobile phase B:	50:50 Acetonitrile/water + 10 mM ammonium acetate
Gradient:	0.1% to 20.0% B for 2 minutes, then 20% to 80% B for 3 minutes followed by 3 minutes re-equilibration
Run time:	8 minutes
Injection volume:	1 $\mu$ L

## MS conditions

LC system:	ACQUITY UPLC H-Class PLUS
Detector:	ACQUITY UPLC PDA and ACQUITY QDa
MS systems:	Xevo TQ-XS or Xevo TQ-S
Ionization mode:	ESI (+/-)
Capillary voltage:	2.8 kV (+)/1.9 kV (-)
Acquisition mode:	MRM
Source temp.:	120 °C
Desolvation temp.:	500 °C
Cone gas flow:	150 L/hr
Desolvation flow:	1000 L/hr

Nebulizer gas:	7 bar
Ion guide offset 1:	3 V
Ion Guide offset 2:	0.3 V

## Informatics

A LipidQuan Quanpedia method file (version 1.4) that contains the LC conditions, MS method, and associated TargetLynx processing method (including retention times) was generated. The resulting data were processed with either TargetLynx or Skyline (MacCoss Lab Software, University of Washington).

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## Results and Discussion

Lipidomics involves the analysis of large scale sample sets and a high-throughput method is required. A rapid and comprehensive LC-MS/MS method was developed for the analysis of polar and non-polar lipid classes in human plasma employing a HILIC-based lipid class separation and MRM MS quantitation in both positive and negative ion mode. Table 1 shows the list of SIL standards spiked into the NIST 1950 plasma extract and the calibration parameters in positive and negative ion mode. Table 2 shows the different lipid classes measured with their corresponding MRM retention time window, acquisition mode used, and the total Quanpedia MRM transitions. The LipidQuan Quanpedia database for this method contains 2041 MRM transition lipid species from 16 polar and non-polar lipid classes. A mixture of SIL lipid standards representing different lipid classes from Avanti (SPLASH LIPIDOMIX) were used to demonstrate the separation of the lipid classes. As shown in Figure 2, lipids are mainly separated into lipid classes according to their polarity within 8 minutes yielding a quantitative method suitable for the lipidomic analysis of large sample sets. Retention time increases with the increase in lipid polarity.

SIL standard	Retention time (min)	Acquisition mode	Calibration range (ng/mL)	Correlation coefficient
15:0/18:1(d7)/15:0 TG	0.40	+ve	5.50-2750	0.994
18:1(d7) LPC	2.08	+ve	2.50-1250	0.9936
18:1(d7) LPE	2.27	+ve	0.50-250	0.9877
15:0/18:1(d7) PC	1.50	+ve	16.00-8000	0.9934
18:1/18:1(d9) SM	1.88	+ve	3.00-1500	0.9933
<b>Average pos mode</b>				<b>0.9924</b>
15:0/18:1(d7) PC	1.50	-ve	16.00-8000	0.9882
15:0/18:1(d7) PE	1.60	-ve	0.50-250	0.9644
15:0/18:1(d7) PG	1.20	-ve	3.0-1500	0.9840
15:0/18:1(d7) PI	2.40	-ve	1.00-500	0.9845
<b>Average neg mode</b>				<b>0.9803</b>

Table 1. Calibration parameters of some stable isotope labeled (SIL) standards spiked in plasma.

Lipid class	Retention time (min)	Acquisition mode	Number of MRM transition	Number of lipid species
SIL standards		+ve	9	9
Cholesterol ester	0.42	+ve	10	10
MG	0.50	+ve	7	7
DG	0.51	+ve	73	39
TG	0.41	+ve	38	20
Ceramide	0.90	+ve	24	24
Hexosyl Ceramide	0.90	+ve	23	23
LPC	2.08	+ve	22	22
LPE	2.25	+ve	24	24
PC	1.50	+ve	175	59
SM	1.85	+ve	24	24
<b>Total pos mode</b>			<b>429</b>	<b>261</b>
SIL		-ve	6	6
FFA	0.51	-ve	27	27
LPA	2.80	-ve	5	5
LPI	3.00	-ve	6	6
PA	2.20	-ve	36	20
PC	1.50	-ve	123	64
PE	1.60	-ve	92	49
PG	1.20	-ve	35	20
PI	2.40	-ve	59	32
PS	2.15	-ve	30	18
<b>Total neg mode</b>			<b>419</b>	<b>247</b>

*Table 2. Total Quanpedia MRM transitions and number of lipid species used for the different lipid classes.*

The development of a LipidQuan Quanpedia method file allows for the simple download of all the MRM transitions and chromatographic conditions representing the different lipid classes with a total of 2041 MRM transitions, therefore eliminating manual input of LC-MS methods and reducing method development training costs and possible transcription errors.

The developed method was used for the quantitative analysis of lipid species in NIST Standard Reference Material 1950 plasma samples using SIL standards for each lipid class. Known concentration SIL standards were added to the NIST plasma sample before extraction. Figures 3 and 4 show representative chromatogram HILIC lipid class separation in positive and negative ion modes, respectively. A total of 508 lipid species are identified and quantified from the NIST plasma (261 positive mode injection and 247 negative mode injection). The platform can also be used for more in-depth targeting of a specific class of



lipids of interest. Table 1 shows calibration parameters of SIL standards spiked into the NIST plasma extract using positive and negative ion mode. Calibration curves were constructed for each SIL standards and are linear within tested calibration ranges with average correlation coefficients of greater than 0.992 (positive mode) and 0.980 (negative mode). Representative calibration curves for SIL standards 15:0/18:1(d7) PC in positive ion mode with 0.993 correlation coefficient and 15:0/18:1(d7) PG in negative ion mode with 0.984 correlation coefficient are shown in Figures 5A and 5B respectively.

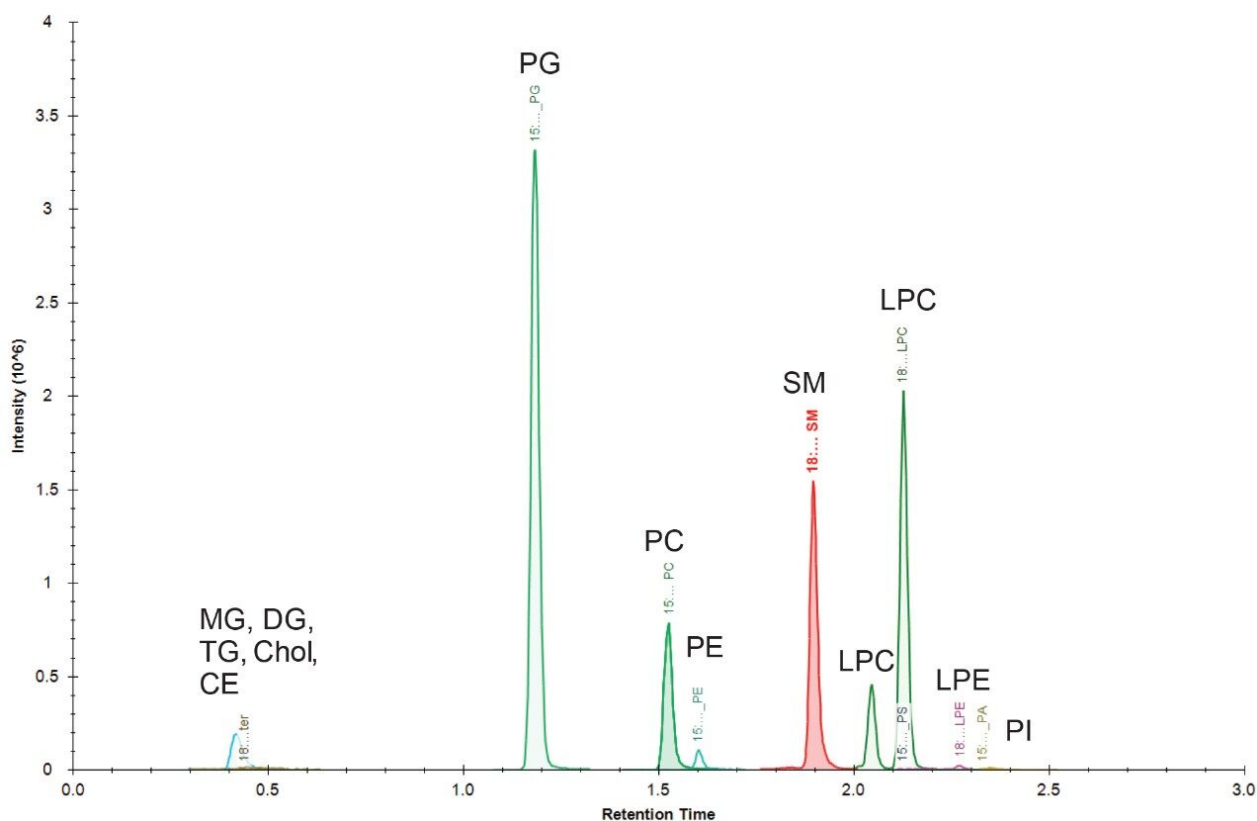


Figure 2. Positive ion mode chromatogram representing HILIC separation of the SPLASH LIPIDOMIX standard mixture.

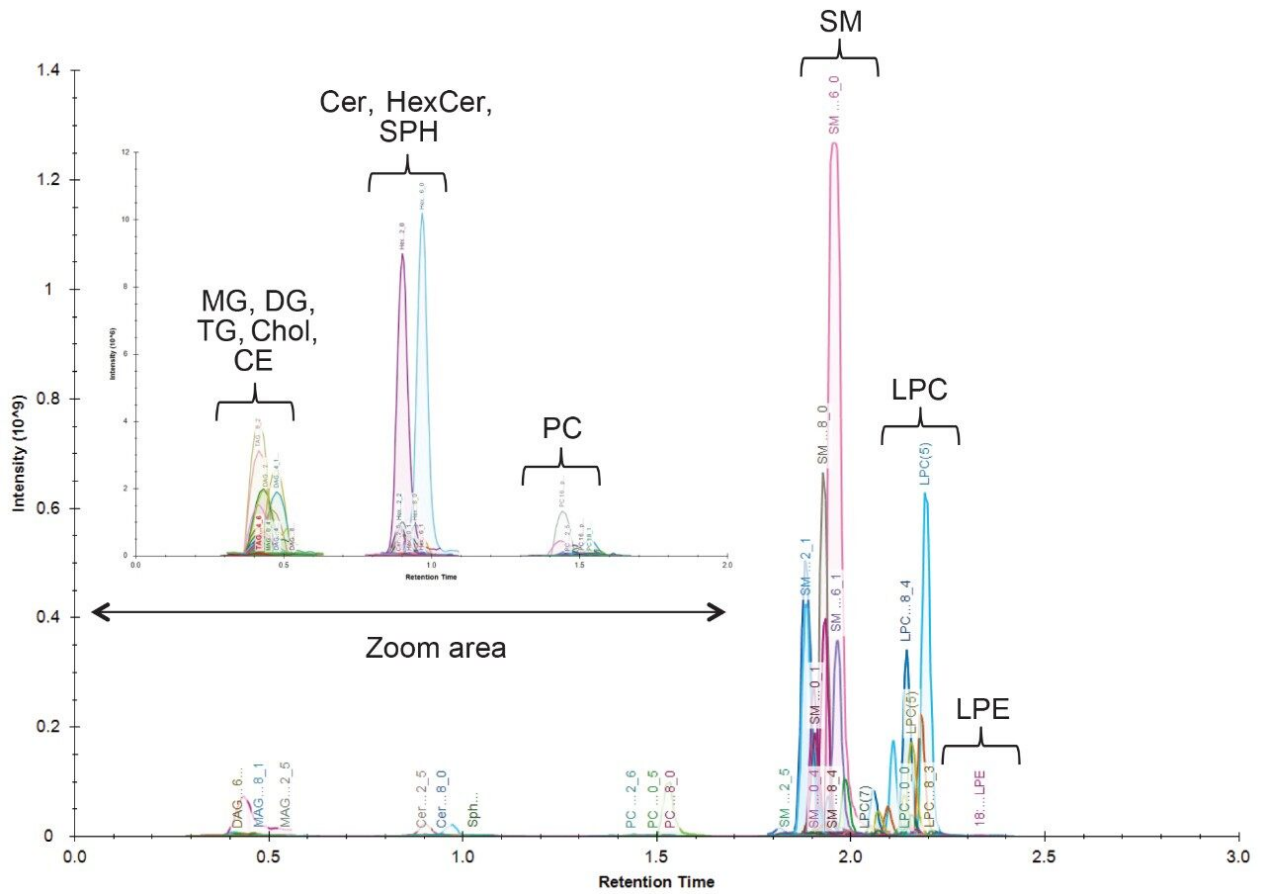


Figure 3. Positive ion mode chromatogram representing HILIC separation of polar and non-polar lipid classes in plasma. The inset chromatogram shows a zoom of the different lipid classes.

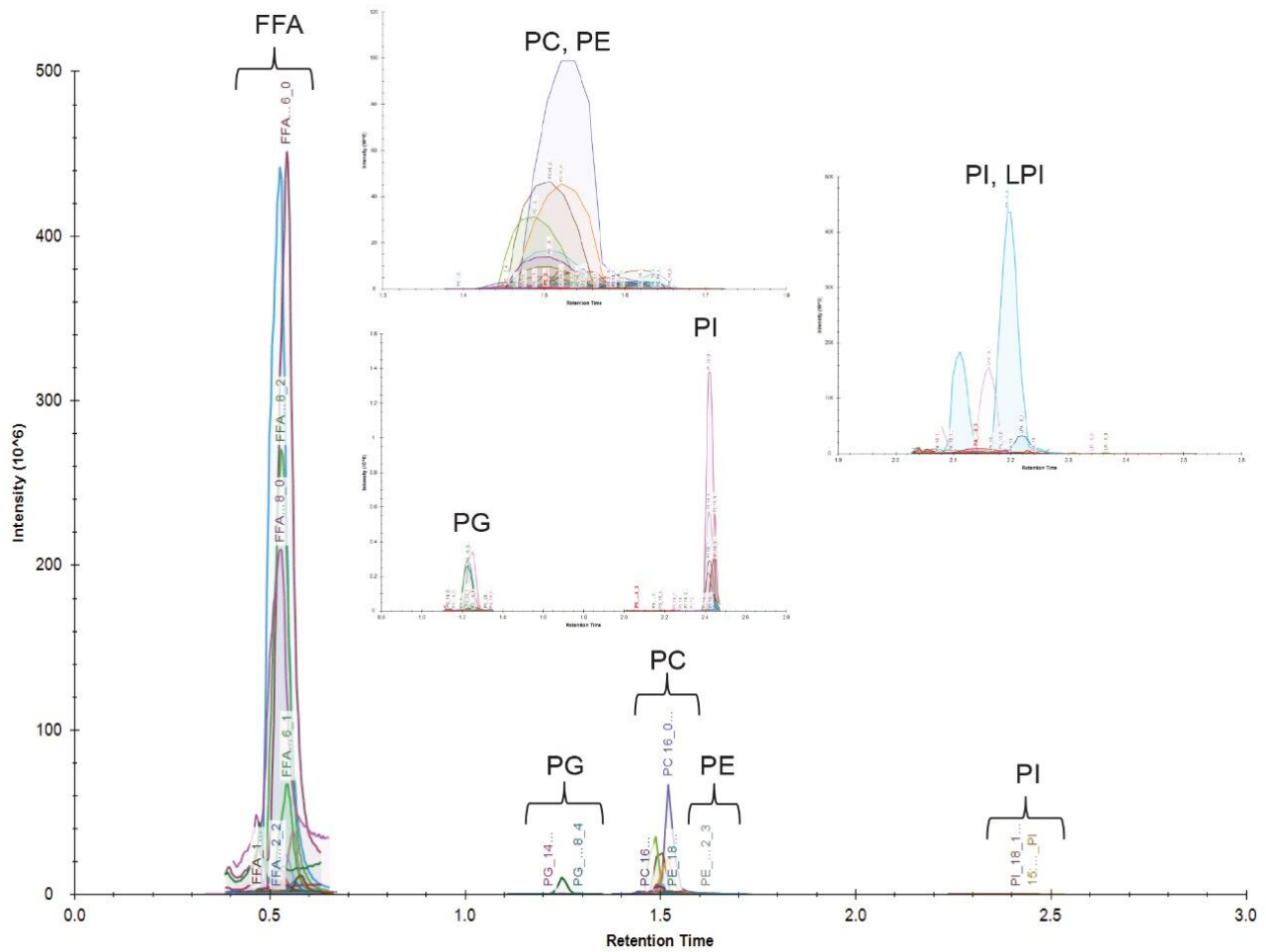


Figure 4. Negative ion mode chromatogram representing HILIC separation of different lipid classes in plasma. The inset chromatogram shows a zoom of the different lipid classes.

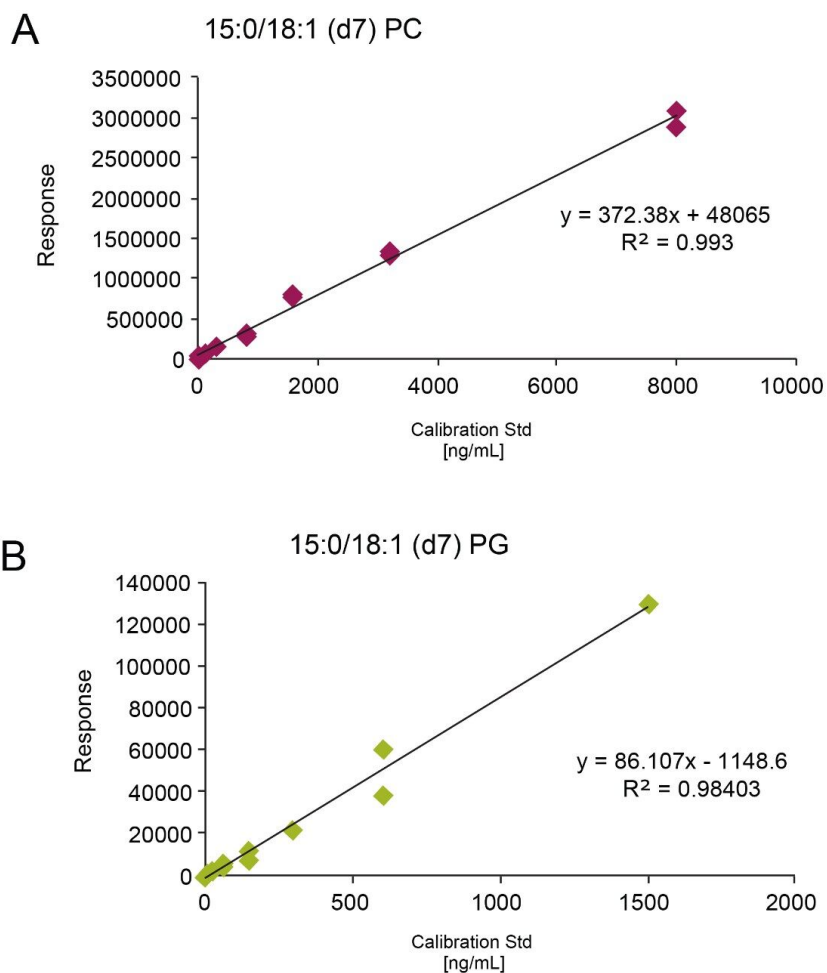


Figure 5. (A) Representative calibration curves for 15:0/18:1(d7) PC in positive ion mode and (B) 15:0/18:1(d7) PG in negative ion mode spiked in a NIST plasma sample.

## Conclusion

- A high-throughput quantitative and comprehensive HILIC method was developed for the analysis of polar and non-polar lipid classes in plasma.
- This research based method enables the separation of 16 polar and non-polar lipid classes in 8 minutes making it suitable for the lipidomics analysis of large sample sets.
- A total of 508 lipid species are identified and quantified from the NIST plasma (261 positive mode

injection and 247 negative mode injection).

- The method was shown to be linear over 4-orders of magnitude and had sufficient sensitivity to allow for the analysis of lipids at systemic levels in human plasma.
- Employing HILIC-based chromatography allowed lipids to elute according to class, thereby reducing potential isomeric/isobaric interferences and the number of stable label isotopes required for quantification, leading to cost reductions.
- The simple download of the LipidQuan Quanpedia method file allows for a robust and easy to deploy platform reducing method development and training cost.

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