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LipidQuan: HILIC-Based LC-MS/MS High-Throughput Targeted Phospholipids Screen (PC, LPC, SM)

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APPLICATION BENEFITS

- Rapid quantification of 106 choline containing phospholipids (61 PCs, 24 SMs, and 21 LPCs) in plasma
- Improve identification and specificity of phosphatidylcholines (PC) using MRM transitions from the two fatty acyl chain fragments
- A robust and easy to deploy platform reducing method development and training costs, using Quanpedia™ and SOPs
- Fast data processing and visualization using TargetLynx™ Software and third party informatics (i.e. Skyline) for maximum flexibility
- Faster and more cost effective than comparable workflows

WATERS SOLUTIONS

LipidQuan™

Quanpedia

Xevo TQ-S micro™

Xevo TQ-XS

Xevo TQ-S

ACQUITY™ UPLC™ I-Class System

BEH Amide Column

TargetLynx Software

KEYWORDS

Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), sphingomyelin (SM), lipidomics, targeted, high-throughput

INTRODUCTION

Choline containing lipids, such as phosphatidylcholine (PC) and sphingomyelin (SM), are a major component of cell membranes. PCs accounts for more than 50% of the phospholipids in most eukaryotic membranes.[1] SMs are synthesized by the transfer of a phosphorylcholine group from PC to ceramide forming the only nonglycerol phospholipid in cell membranes. Research interest in measuring these lipids has increased in recent years due to their implication in numerous diseases including multiple sclerosis[2] and Niemann-Pick disease.[3]

Although advances in mass spectrometry (MS) have allowed for more in-depth lipidomic analysis, unambiguous identification and quantification has proven difficult as lipids exhibit a high number of isomeric and isobaric species. Furthermore, MS spectra often contain peaks and fragments from multiple compounds making confident identification and relative quantification of specific molecular species difficult and time consuming. As a result, the transfer of lipidomic data between laboratories is severely hindered, making multi-site study interpretation problematic.

A hydrophilic interaction chromatography (HILIC) based approach for the separation of lipids by class prior to MS analysis is a proven method of reducing identification ambiguity.[4] An additional benefit of separating lipid species by class is that fewer stable isotope labelled (SIL) standards are required for quantification, conferring a cost saving. This application note describes the use of the LipidQuan platform (Figure 1) that utilizes a HILIC-based approach to perform a targeted screen for LPCs, PCs, and SMs.

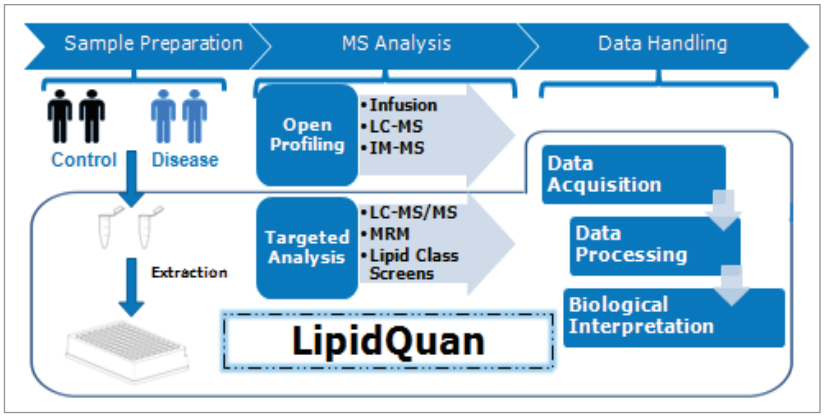

Figure 1. Generic lipidomics workflow used in most research laboratories, with the LipidQuan workflow highlighted.

EXPERIMENTAL

Samples

Pooled healthy human plasma was spiked with stable isotope labeled (SIL) standards, (SPLASH LIPIDOMIX,™ Avanti Lipids, Alabaster, AL) at nine concentration levels to generate calibration curves for quantification of PC (15:0-18:1) (d7) = 16–8000 ng/mL, SM (18:1) (d9) =3–1500 ng/mL, and LPC (18:1) (d7) =2.5–1250 ng/mL.

Six replicates of the NIST Standard Reference Material® 1950 plasma (Sigma Aldrich, Poole, UK) were also spiked with 5% SIL standards, prior to extraction.

Sample preparation

A simple sample preparation procedure was adopted using protein precipitation with pre-cooled isopropanol (IPA) (1:5, plasma:IPA). 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/MS analysis.

LC conditions

LC system:	ACQUITY UPLC I-Class System Fixed Loop (FL) or Flow Through Needle (FTN)
Column(s):	ACQUITY UPLC BEH Amide 2.1 × 100 mm, 1.7 µm
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 µL

MS conditions

MS systems:	TQ-S micro, TQ-XS, or 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 Software processing method (including retention times) was generated. The resulting data were processed with either TargetLynx Software or Skyline (MacCoss Lab Software, University of Washington).

RESULTS AND DISCUSSION

A rapid, specific LC-MS/MS method was developed for the analysis of PC, LPC, and SM lipids in human plasma employing a HILIC-based chromatographic separation and MRM MS detection in both positive and negative ion mode. The HILIC methodology facilitated the elution of lipids in discrete classes with the PCs eluting first (~1.52 mins) followed by SMs (~1.92 mins) and LPCs (~2.15 mins) (Figure 2). The resulting method was capable of measuring 61 PCs, 21 LPCs, and 24 SMs in eight minutes with a linear dynamic range over four orders of magnitude. The method sensitivity easily facilitated the detection of these lipids in human plasma at normal circulating levels from 50 µL of plasma.

Product ion scans of these three lipid classes showed the characteristic 184 *m/z* choline head group fragment in positive ion mode (Figure 3), that results in unambiguous lipid assignment if employed in an MRM transition. To improve identification and specificity, highly specific MRM transitions containing fatty acyl chain fragments for 106 PCs (positive ion) and 278 PCs (negative ion) were developed. Although the MRM transitions for LPC and SM species employ the choline head group fragment, chromatographic separation on the basis of class reduces isomeric and isobaric interferences (Figure 4). Additionally, isobaric effects are further minimized since LPC and SM precursors have mass ranges of 440–608 Da and 648–776 Da , respectively, and, therefore, do not overlap.

The development of a LipidQuan Quanpedia method file allows for the simple download of MRM transitions and chromatographic conditions representing PCs, LPCs, and SMs and thereby, eliminates manual input of LC-MS/MS methods and reduces possible transcription errors.

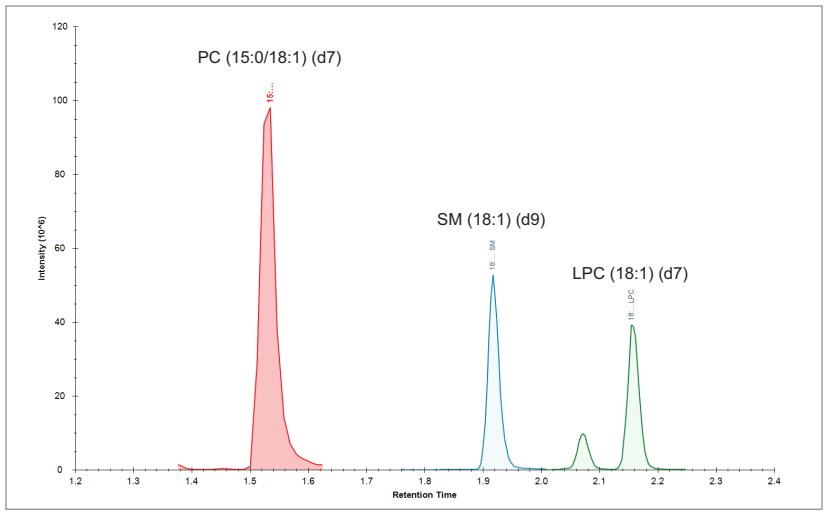

Figure 2. Chromatogram representing HILIC separation of PC, SM, and LPC from the SPLASH LIPIDOMIX™ standard mixture in positive ion mode.

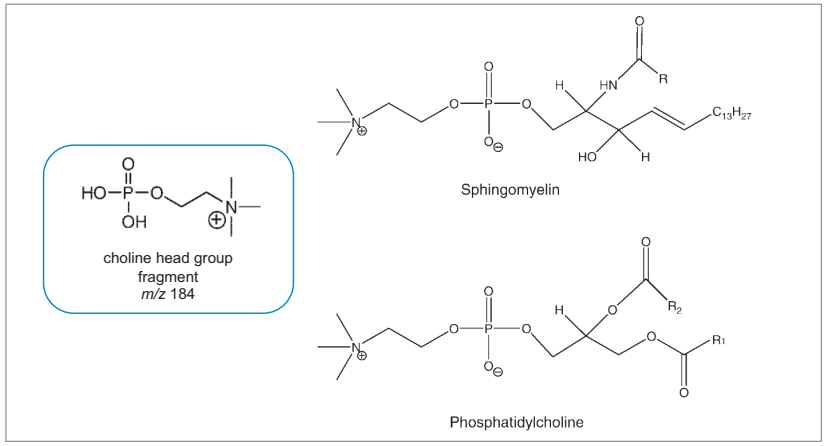

Figure 3. Structural representation of SM and PC. Both species are choline-containing phospholipids that generate the characteristic phosphocholine head group fragment (m/z 184).

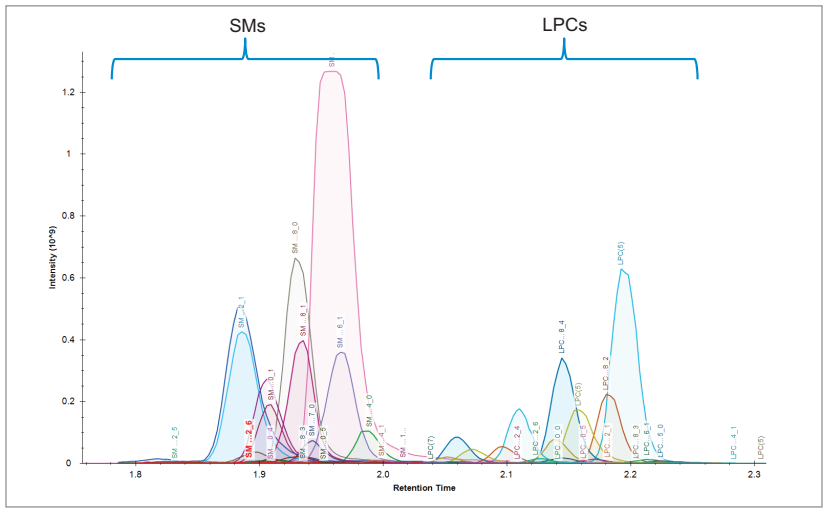

Figure 4. Implementation of HILIC chromatography shows the class separation of endogenous SMs and LPCs in plasma, and thereby minimizes possible isobaric effects.

Quantification was achieved using calibration curves of plasma spiked with known concentrations of SIL standards prior to extraction. By using surrogate standards prepared and analyzed under identical conditions as those of endogenous lipids, the quantification of endogenous lipids within the same class was achieved. The use of a single SIL standard per lipid class rather than a SIL standard for each measured lipid significantly reduces the overall cost of large cohort studies. Example calibration curves for SIL standards SM (18:1) (d9) and LPC (18:1) (d7) are shown in Figure 5A/5B, and can be used for quantification of endogenous LPCs and SMs. Additional example curves representing PCs in positive and negative ion are also provided (Figure 5C/5D).

The data shown here was acquired using the curated LipidQuan plasma screens (LipidQuan Quanpedia file v1.4). Typical R^2 values of 0.95 and deviations from the line of best fit (CVs <30%) are routinely achieved (Tables 1–4).

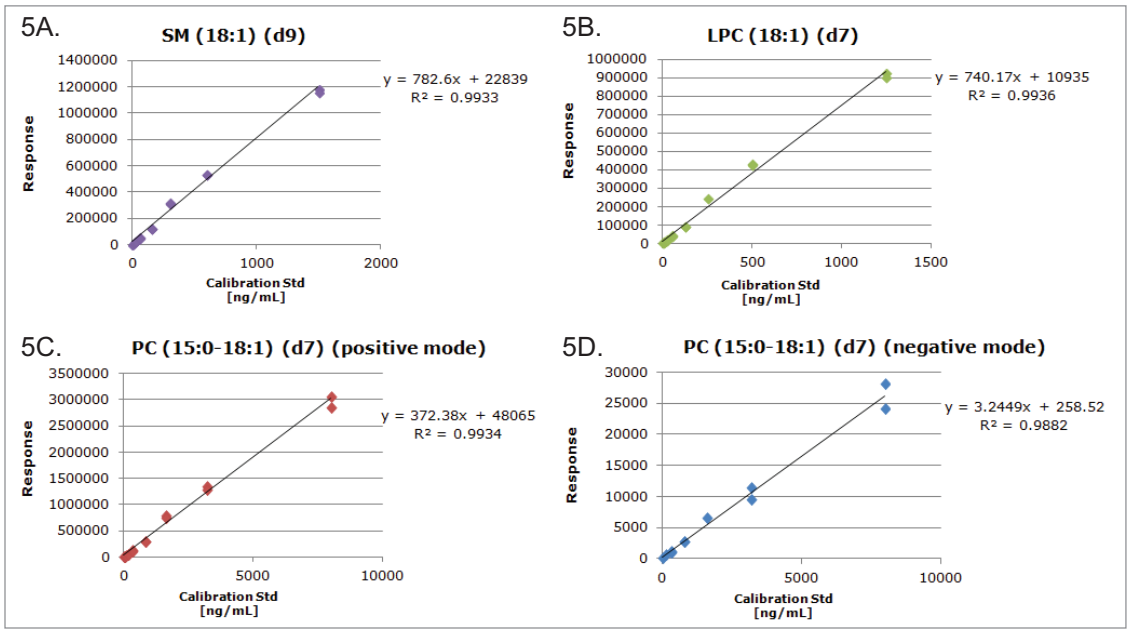

Figure 5. Calibration curves for SM (18:1) (d9) (3–1500 ng/mL) (A), LPC (18:1) (d7) (2.5–1250 ng/mL) (B) and PC (15:0/18:1) (d7) (16–8000 ng/mL) (C) in positive ion mode from a typical plasma screen. The calibration curve for PC (15:0/18:1) (d7) (16–8000 ng/mL) (D) in negative mode from a typical plasma screen. Typically, R^2 values of >0.95 and deviations from the line of best fit (CV <30%) are specified as acceptance criteria.

Table 1. The back calculated concentrations of spiked SIL standards are included below as well as the actual concentration of SIL standards spiked into the NIST plasma in (n=12).

SPLASH LIPIDOMIX™	RT (mins)	MRM transition	Actual conc (ng/mL)	Calculated Conc (n=12)	Std dev	CV (%)
LPC (18:1) (d7)	2.15	529.4>184.1	1250.0	1201.2	87.8	7.31
SM (18:1) (d9)	1.92	738.6>184.1	1500.0	1629.2	138.4	8.50
PC (15:0-18:1) (d7)	1.52	811.6>288.3 (-ve)	8000.0	7918.8	782.5	9.88

Table 2. MRM transitions of endogenous LPC lipids in NIST Standard Reference Material® 1950 plasma with CVs <30%. The calibration range of LPC (18:1) (d7) was 2.5–1250 ng/mL.

Lysophosphatidylcholine (LPC)	RT (mins)	MRM transition
LPC 15_0	2.22	482.3>184.1
LPC 16_0	2.20	496.3>184.1
LPC 16_1	2.21	494.3>184.1
LPC 18_0	2.15	524.4>184.1
LPC 18_1	2.16	522.4>184.1
LPC 18_2	2.18	520.3>184.1
LPC 18_3	2.20	518.3>184.1
LPC 20_0	2.10	552.4>184.1
LPC 20_3	2.14	546.4>184.1
LPC 20_4	2.14	544.3>184.1
LPC 20_5	2.16	542.3>184.1
LPC 22_1	2.18	578.4>184.1
LPC 22_4	2.11	572.3>184.1
LPC 22_6	2.13	568.3>184.1
LPC 24_0	2.03	608.5>184.1
LPC O- 16_0	2.22	482.4>184.1
LPC O- 18_0	2.17	510.4>184.1
LPC P-18_1	2.04	506.4>184.1
LPC P-20_0	2.14	536.4>184.1

Table 3. MRM transitions of endogenous PC lipids in NIST Standard Reference Material® 1950 plasma with CVs <30%. The calibration range of PC (15:0/18:1) (d7) was 16–8000 ng/mL.

Phosphatidylcholine (PC)	RT (mins)	MRM transition
PC 14_0–16_0	1.56	764.5>227.3
		764.5>255.3
PC 16_0–16_0	1.54	792.6>255.3
PC 16_0–16_1	1.54	790.6>253.3
		790.6>255.3
PC 16_0–18_0	1.50	820.6>255.3
		820.6>283.3
PC (34:1) PC 16_0–18_1	1.50	818.6>255.3
		818.6>281.3
PC (34:1) PC 16_1–18_0	1.50	818.6>253.3
		818.6>283.3
PC (34:2) PC 16_0–18_2	1.51	816.6>255.3
		816.6>279.3
PC (34:2) PC 16_1–18_1	1.50	816.6>253.3
		816.6>281.3
PC (34:3) PC 16_0–18_3	1.51	814.6>255.3
		814.6>277.3
PC (34:3) PC 16_1–18_2	1.51	814.6>253.3
		814.6>279.3
PC (36:1) PC 16_0–20_1	1.49	846.6>255.3
		846.6>309.2
PC (36:1) PC 18_0–18_1	1.48	846.6>281.3
		846.6>283.3
PC (36:2) PC 16_0–20_2	1.49	844.6>255.3
		844.6>307.2
PC (36:2) PC 18_0–18_2	1.49	844.6>279.3
		844.6>283.3
PC (36:2) PC 18_1–18_1	1.48	844.6>281.3
PC (36:3) PC 16_0–20_3	1.48	842.6>255.3
		842.6>305.2
PC (36:3) PC 18_0–18_3	1.48	842.6>277.3
		842.6>283.3
PC (36:3) PC 18_1–18_2	1.49	842.6>279.3
		842.6>281.3
PC (36:4) PC 16_0–20_4	1.47	840.6>255.3
		840.6>303.2
PC (36:4) PC 16_1–20_3	1.48	840.6>253.3
		840.6>305.3
PC (36:4) PC 18_1–18_3	1.49	840.6>277.3
		840.6>281.3
PC (36:4) PC 18_2–18_2	1.49	840.6>279.3
PC (36:5) PC 16_0–20_5	1.47	838.6>255.3
		838.6>301.2
PC (36:5) PC 16_1–20_4	1.47	838.6>253.3
		838.6>303.2
PC 18_1–20_0	1.45	874.7>281.3
		874.7>311.2
PC (38:2) PC 18_0–20_2	1.47	872.6>283.3
		872.6>307.2
PC (38:2) PC 18_1–20_1	1.47	872.6>281.3
		872.6>309.2
PC (38:2) PC 18_2–20_0	1.46	872.6>279.3
		872.6>311.2
PC (38:3) PC 16_0–22_3	1.46	870.6>255.3
		870.6>333.2
PC (38:3) PC 18_1–20_2	1.47	870.6>281.3
		870.6>307.2
PC (38:3) PC 18_0–20_3	1.46	870.6>283.3
		870.6>305.2
PC (38:4) PC 16_0–22_4	1.47	868.6>255.3
		868.6>331.2
PC (38:4) PC 18_2–20_2	1.48	868.6>279.3
		868.6>307.2
PC (38:5) PC 16_0–22_5	1.46	866.6>255.3
		866.6>329.2
PC (38:5) PC 18_0–20_5	1.45	866.6>283.3
		866.6>301.2
PC 16_0-22_6	1.46	864.6>255.3
		864.6>327.2
PC 18_0-22_4	1.44	896.6>283.3
		896.6>331.2
PC 18_0-22_5	1.44	894.6>283.3
		894.6>329.2

Table 4. MRM transitions of endogenous SM lipids in NIST Standard Reference Material® 1950 plasma with CVs <30%. The calibration range of SM (18:1) (d9) was 3–1500 ng/mL.

Sphingomyelin (SM)	RT (mins)	MRM transition
SM d18_1–12_0	2.02	647.5>184.1
SM d18_1–14_0	1.99	675.5>184.1
SM d18_1–14_1	2.00	673.5>184.1
SM d18_1–16_0	1.96	703.6>184.1
SM d18_1–16_1	1.96	701.6>184.1
SM d18_1–17_0	1.94	717.6>184.1
SM d18_1–18_0	1.93	731.6>184.1
SM d18_1–18_1	1.93	729.6>184.1
SM d18_1–18_2	1.93	727.6>184.1
SM d18_1–18_3	1.93	725.6>184.1
SM d18_1–18_4	1.96	723.5>184.1
SM d18_1–20_0	1.91	759.6>184.1
SM d18_1–20_1	1.91	757.6>184.1
SM d18_1–20_2	1.90	755.6>184.1
SM d18_1–20_3	1.91	753.6>184.1
SM d18_1–20_4	1.90	751.6>184.1
SM d18_1–22_0	1.89	787.7>184.1
SM d18_1–22_1	1.89	785.7>184.1
SM d18_1–22_2	1.90	783.6>184.1
SM d18_1–22_3	1.90	781.6>184.1
SM d18_1–22_4	1.89	779.6>184.1
SM d18_1–22_5	1.89	777.6>184.1
SM d18_1–22_6	1.89	775.6>184.1

CONCLUSIONS

- A rapid quantitative method was developed for the analysis of PC, LPC, and SM lipids in plasma.
- The methodology allowed for the analysis of 61 PCs, 21 LPCs, and 24 SMs within eight minutes.
- The method was shown to be linear over four orders of magnitude and had sufficient sensitivity to allow for the analysis of lipids at normal 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 isotope labeled standards required for quantification (i.e. cost reductions).

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