Validated LipidQuan Method Guide: Analysis of Lipids in Plasma and Serum Samples by LC-MS/MS

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1. Purpose

The purpose of this analysis document is to provide Waters recommended step-by-step instructions for sample preparation followed by HILIC chromatography of human plasma/serum samples for semi-quantitative large cohort lipid analysis. This document includes: details of sample preparation/extraction using IPA, mobile phase buffer preparation and UPLC-MS/MS analysis of the samples.

2. Scope

This document contains Waters recommendations for:

- Internal standard preparation
- Standard curve preparation
- Quality control sample preparation
- Human plasma/serum sample preparation for large cohort lipidomics studies
- Sample analysis by LC-MS/MS

3. Notes

The following method conditions are suitable for the semi-quantification of (polar lipids) phospholipids and ceramides. Non-polar lipids (MG, DG, TG, Cholesterol, Cholesterol Esters, etc.) elute in the solvent front using these conditions and therefore concentration values obtained are for monitoring only and should not be deemed quantitative with this method. Although Free Fatty Acids (negative mode) elute close to the solvent front it is still possible to semi-quantify this class using these conditions.

The LipidQuan MS methods, LC methods, and TargetLynx processing method packages are available for download from the Waters website at www.waters.com/targetedomics. How-To videos on downloading, importing, running, and data processing are available on the site.

A project batch should not consist of more than a 1500 biological samples. Calibration curves will be run at the beginning and end of each batch. If the cohorts are larger than 1500 samples, then it is

advisable to run calibration curves at points throughout the analysis to ensure bracketed data is available that will allow the adjustment for any drift within the run or to flag any analysis failure.

Prior to sample analysis, blanks and quality controls (QC) injections are performed to demonstrate that the instrument is stable and that results are consistent prior to running valuable biological samples. Ensuring that the instrument is stable prior to sample analysis will also help to maintain consistency between the runs.

In addition to running blanks and QCs prior to the start of analytical runs, QC samples are acquired throughout the analytical run; these intra-run QCs will assist with determining analysis stability and data suitability. These frequent injections allow for any instrument changes to be monitored. They can also be used to indicate injection suitability prior to, or post, any instrument issues that result in an analysis stoppage [1].

4. Abbreviations

ACN	Acetonitrile	PC	Phosphatidylcholine
Cer	Ceramide	PE	Phosphatidylethanolamine
CE	Cholesteryl Ester	PG	Phosphatidygylycerols
CL	Cardiolipin	PI	Phosphatidylinositols
DG	Diacylglycerol	PLs	Phospholipids
DAG	Diacylglycerol	PS	Phosphatidylserine
FA	Fatty acyls	QC	Quality Control
FFA	Free Fatty Acid	RSD	Relative standard deviation
GLs	Glycerolipids	RT	Retention time
GPs	Glycerophospholipids	SIL	Stable isotope labelled
IPA	Isopropanol (2-Propanol)	SP	Sphingolipids
LPC	Lysophosphatidylcholine	SM	Sphigomyelin
MG	Monoacylglycerols	TG	Triacylglycerol
MTBE	Methyl-*tert*-butyl ether	TAG	Triacylglycerol
PA	Phosphatidic acid		

5. Materials & Equipment

5.1. Equipment

- Pipettes & Tips (1000 µL, 200 µL and 10 µL minimum requirement)
- Fume Hood
- Centrifuge
- Waters TQ-S, TQ-XS or TQ-S micro mass spectrometer (Xevo TQ-XS recommended)
- Waters UPLC system (ACQUITY I-Class Flow Through Needle (recommended) or Fixed Loop)
- 1.5 mL microcentrifuge (Eppendorf) tubes or 1mL 96 well plate for extractions (Waters p/n: 186002481)
- Total recovery vials (Waters p/n: 186002805) or 1 mL 96 well analytical plates (Waters p/n: 186002481)
- Pre-slit PTFE / Silicone Cap Mat for 96-well (Waters p/n: 186006332)
- Temperature controlled mixer

5.2. Materials for Sample Preparation

- Avanti Deuterated Ceramide LIPIDOMIX™ lipids standards (p/n: 330713)
- Avanti SPLASH LIPIDOMIX™ lipid standards (p/n: 330707)
- Avanti Odd-Chained LIPIDOMIX™ lipid standards (p/n 330711)
- Isopropanol (LCMS grade)
- Acetonitrile (LCMS grade)

5.3. Materials for UPLC-MS/MS analysis

- Water (LCMS grade or 18.2MΩ Milli-Q)
- Acetonitrile (LCMS grade)
- Formic Acid (LCMS grade)
- Isopropanol (LCMS grade)
- Ammonium Acetate (LC eluent grade)
- UPLC BEH Amide, 1.7µm, 2.1 x 100 mm column (Waters p/n: 186004801)

6. Standards and Sample Preparation

6.1. Standard Spiking Mix Preparation

1. Remove a vial of Avanti Odd-Chained LIPIDOMIX™ from the freezer and allow it to equilibrate at room temperature (a minimum of 20 minutes).
2. Prepare spiking solutions 1, 2, and 3 (10x, 20x, and 50x dilution of Avanti Odd-Chained LIPIDOMIX™ standards, respectively). Please see Table 1 below for resulting concentrations.
3. Spiking solution 1: dilute 100 µL of Odd-Chained LIPIDOMIX™ into 900 µL of IPA.
4. Spiking solution 2: dilute 50 µL of Odd-Chained LIPIDOMIX™ into 950 µL of IPA.
5. Spiking solution 3: dilute 20 µL of Odd-Chained LIPIDOMIX™ into 980 µL of IPA.

   These volumes can be scaled up or down. However, ensure that whenever preparing the calibration curve and QCs that the total volume v/v of Avanti Odd-Chained LIPIDOMIX™ *is no more than 5% at any time.*

	17:1 LPG (Na Salt)	17:1 LPA (NH4 Salt)	17:1 LPI (NH4 Salt)	17:1 LPS (Na Salt)	17:1 LPC	17:1 LPE	17:0-17:0 DG	17:0-17:0-17:0 TAG	12:0 SM (d18:1/12:0)	17:0-14:1 PC	17:0-14:1 PS (NH4 Salt)	17:0-14:1 PG (NH4 Salt)	17:0-14:1 PA (NH4 Salt)	17:0-14:1 PE	17:0-14:1 PI (NH4 Salt)	17:0 Chol Ester
Solution	Concentrations (µg/mL)*															
Neat	13	15	13	13	575	12	300	1500	650	3775	180	90	15	120	200	8475
Actual Conc	13	15	13	13	575	12	300	1500	650	3775	180	90	15	120	200	8475
Spiking solution 1	1.3	1.5	1.3	1.3	57.5	1.2	30	150	65	377.5	18	9	1.5	12	20	847.5
Spiking solution 2	0.65	0.75	0.65	0.7	28.75	0.6	15	75	32.5	188.75	9	4.5	0.75	6	10	423.75
Spiking solution 3	0.26	0.3	0.26	0.3	11.5	0.2	6	30	13	75.5	3.6	1.8	0.3	2.4	4	169.5

Table 1: Spiking Solution Preparation using Avanti Odd-Chained LIPIDOMIX™

**please note these concentrations may vary depending upon the original concentration of the* Avanti Odd-Chained LIPIDOMIX™*, please check the provided certificate of analysis for true values.*

6. Vortex mix each solution for 15 seconds to ensure homogeneity.
7. The spiking solutions may be stored at -20°C (±5°C) for up to 6 months.

6.2. System Blank

1. System blank should be a vial of IPA/ACN 1:2 v/v (chilled to 4-8°C).
2. This solution must be prepared using the processes described in section 6.5.

6.3. Internal Standards Solution Preparation for Plasma Lipid Extraction

A 500x dilution of the SPLASH (p/n: 330707) and Deuterated Ceramide (p/n: 330713) LIPIDOMIX™ is prepared in 1:2 v/v IPA/ACN (**Table 2**). This IPA/ACN solution is used to perform the extraction procedure.

Table 2: Internal Standard Preparation

Solutions	PC (d7)	PE(d7)	PS(d7)	PG(d7)	PI (d7)	PA (d7)	LPC (d7)	LPE(d7)	Chol Ester (d7)	MG (d7)	DG (d7)	TG (d7)	SM (d7)	Cholesterol (d7)	C16 Ceramide-d7	C18 Ceramide-d7	C24 Ceramide-d7	C24:1 Ceramide-d7
Neat (µg/mL)	160	5	5	30	10	7	25	5	350	2	10	55	30	100	21.8	11.5	26.3	13.1
1:500 dilution (µg/mL)	0.32	0.01	0.01	0.06	0.02	0.014	0.05	0.01	0.7	0.004	0.02	0.11	0.06	0.2	0.0436	0.023	0.0526	0.0262
1:500 dilution (ng/mL)	**320**	**10**	**10**	**60**	**20**	**14**	**50**	**10**	**700**	**4**	**20**	**110**	**60**	**200**	**43.6**	**23**	**52.6**	**26.2**

6.4. System Suitability Test Mix

1. Transfer 10 µL of spiking solution 1 (Table 1) into suitable glass HPLC vials.
2. Dilute with 990 µL of Internal Standard Solution (6.3) (chilled to 4-8°C).
3. Vortex mix for 15 seconds to ensure the solution is homogenous.

6.5. Calibrant, QC, and Matrix Blank sample preparation

A pool composed of an aliquot from every sample to be analysed should be prepared. To calculate the amount to remove from each sample for the pool, divide the number of samples by the total volume of pool required (#samples/pool = µL/sample to be removed). The pooled samples will be representative of all the samples in the study and will be combined with the standards mix to form the QCs and the calibration curve standards. Pooled plasma can be aliquoted to microcentrifuge tubes (6.5.2) and stored at -80ºC a day prior to the analysis. At

least one aliquot of blank matrix should be prepared from the pooled samples; the blank matrix includes the QCs but not the calibrants (Table 3).

1. Remove the pooled plasma from the freezer and allow it to equilibrate at room temperature for 1 hour (± 10 minutes) prior to use.
2. Prepare the calibration curve using the solutions prepared in 6.1 and the pooled samples. Use the volumes as described below in Table 3.

Table 3: Calibration Curve Preparation

Curve	Solution used	Solution	Pool**	Final
		*Volumes in µL**		
Cal 1	Odd-Chained LIPIDOMIX™	10	190	200
Cal 2	Odd-Chained LIPIDOMIX™	10	240	250
Cal 3	Odd-Chained LIPIDOMIX™	10	340	350
Cal 4	Odd-Chained LIPIDOMIX™	5	245	250
Cal 5	Odd-Chained LIPIDOMIX™	5	495	500
Cal 6	Spiking 1	10	190	200
Cal 7	Spiking 1	10	240	250
Cal 8	Spiking 2	10	240	250
Cal 9	Spiking 3	10	240	250
Cal 10	Spiking 3	5	245	250
Matrix Blank	NA	0	200	200

**these volumes can be scaled up if more aliquots are required, it is NOT advised to pipette volumes smaller than 5 µL due to the higher potential for pipetting inaccuracies.*
***It is preferred to use pool test plasma samples. If there is not enough test plasma pool available from the test samples to be analysed any available plasma sample can be used to mimic the matrix effect for the calibration curve.*

3. The resulting concentrations for the curve are listed below in Table 4.

4. Prepare the QC samples using the standard solution mix prepared in 6.1 and the pooled sample volumes as described below in Table 5. Use High (HQC), Middle (MQC), and Low

(LQC) QC samples prepared at 70%, 40%, and 6.4% of the highest concentration of the calibration curve for a typical routine analysis.

	17:1 LPG (Na Salt)	17:1 LPA (NH4 Salt)	17:1 LPI (NH4 Salt)	17:1 LPS (Na Salt)	17:1 LPC	17:1 LPE	17:0-17:0 DG	17:0-17:0-17:0 TAG	12:0 SM (d18:1/12:0)	17:0-14:1 PC	17:0-14:1 PS (NH4 Salt)	17:0-14:1 PG (NH4 Salt)	17:0-14:1 PA (NH4 Salt)	17:0-14:1 PE	17:0-14:1 PI (NH4 Salt)	17:0 Chol Ester
	Concentration in ng/mL															
Cal 1	650	750	650	650	28750	600	15000	75000	32500	188750	9000	4500	750	6000	10000	423750
Cal 2	520	600	520	520	23000	480	12000	60000	26000	151000	7200	3600	600	4800	8000	339000
Cal 3	371	429	371	371	16429	343	8571	42857	18571	107857	5143	2571	429	3429	5714	242143
Cal 4	260	300	260	260	11500	240	6000	30000	13000	75500	3600	1800	300	2400	4000	169500
Cal 5	130	150	130	130	5750	120	3000	15000	6500	37750	1800	900	150	1200	2000	84750
Cal 6	65	75	65	65	2875	60	1500	7500	3250	18875	900	450	75	600	1000	42375
Cal 7	52	60	52	52	2300	48	1200	6000	2600	15100	720	360	60	480	800	33900
Cal 8	26	30	26	26	1150	24	600	3000	1300	7550	360	180	30	240	400	16950
Cal 9	10	12	10	10	460	10	240	1200	520	3020	144	72	12	96	160	6780
Cal 10	5	6	5	5	230	5	120	600	260	1510	72	36	6	48	80	3390

Table 4: Resulting Individual Avanti Odd-Chained LIPIDOMIX™ Calibration Curve Concentrations

Table 5: Suggested QC Concentration Preparation

QCs	Solution used	Solution	Pool**	Final
		Volumes in µL		
HQC	Odd-Chained LIPIDOMIX™	7	193	200
MQC	Odd-Chained LIPIDOMIX™	5	245	250
LQC	Spiking 1	8	242	250

***It is preferred to use pool test plasma samples. If there is not enough test plasma pool available from the test samples to be analysed any available plasma sample can be used to mimic the matrix effect for the QC.*

5. The resulting QC concentrations for the curve are listed below in Table 6.

Table 6: Individual Avanti Odd-Chained LIPIDOMIX™ QC Concentrations

	17:1 LPG (Na Salt)	17:1 LPA (NH4 Salt)	17:1 LPI (NH4 Salt)	17:1 LPS (Na Salt)	17:1 LPC	17:1 LPE	17:0-17:0 DG	17:0-17:0-17:0 TAG	12:0 SM (d18:1/12:0)	17:0-14:1 PC	17:0-14:1 PS (NH4 Salt)	17:0-14:1 PG (NH4 Salt)	17:0-14:1 PA (NH4 Salt)	17:0-14:1 PE	17:0-14:1 PI (NH4 Salt)	17:0 Chol Ester
	Concentration in ng/mL															
HQC	455	525	455	455	20125	420	10500	52500	22750	132125	6300	3150	525	4200	7000	296625
MQC	260	300	260	260	11500	240	6000	30000	13000	75500	3600	1800	300	2400	4000	169500
LQC	41.6	48	42	41.6	1840	38.4	960	4800	2080	12080	576	288	48	384	640	27120

6.6. Lipid Extraction and Protein Precipitation

A simple protein precipitation sample preparation procedure should be used with pre-cooled (4-8°C) 1:2 v/v IPA/ACN (**Section 63** [2].

Extraction of calibrants, QCs, blank IPA, pool, and test samples should follow the same conditions to ensure uniformity.

1. Allow calibrants (Table 3), QCs (Table 5), matrix blank (Table 3), extraction blank and test samples to equilibrate at 4-8°C for 1 hour prior to preparation.
2. Pipette 50 µL aliquots of the calibrants, QCs, matrix blank, pooled plasma and test samples to 1.5 mL microcentrifuge tubes (or 1 mL 96 well plates).
3. Add 250 µL of IPA/can with IS (1:2) containing internal standards (solution from 6.3) (chilled to 4-8°C).
4. If your sample volume is limited, then these volumes can be reduced as long as the ratio of 1:5 (sample:IPA/ACN with IS) is maintained. However, do not let the plasma sample fall below 10 µL as it may be challenging to detect and quantify the low abundant lipid species.
5. Vortex mix for 30 seconds.
6. Incubate the mixture at 2-8°C for 2 hours with agitation using a temperature-controlled shaker (preferred) to ensure complete protein precipitation. (If shaker has no temperature

control, remove sample from fridge and shake for 5 minutes every 30 minutes before returning to 2-8°C).

7. Centrifuge at 10,300 g for 10 minutes at 4°C.
8. Carefully transfer 250 µL of each supernant to vials (110 µL to each vial if separate vials or 96 well plates are required for positive and negative mode). The recommend acquisition list is shown in section 9.

7. Mobile phase Preparation

Please purge lines for a minimum of 2 minutes when new mobile phases or solvents are added.

7.1. Mobile phase A

95:5 Acetonitrile:Water, 10mM Ammonium acetate

1. Accurately measure out 50 mL of LCMS grade water into a 1 L Duran bottle.
2. Add 0.77 g (+/-0.01g) of ammonium acetate to the water and mix until it visually appears to have dissolved; about 2 minutes.
3. Accurately measure 950 mL of LCMS grade ACN and slowly add to the ammonium acetate solution whilst swirling to ensure good mixing.
4. The solution may become 'cloudy'; sonicate for 10 minutes to ensure proper dissolution and mixing.

7.2. Mobile phase B

50:50 Acetonitrile:Water, 10mM Ammonium acetate,

1. Accurately measure out 500 mL of LCMS grade water into a 1L Duran bottle.
2. Add 0.77 g (+/-0.01g) of ammonium acetate to the water and mix until it visually appears to have dissolved; about 2 minutes.
3. Accurately measure out 500 mL of LCMS grade ACN and slowly add to the ammonium acetate solution whilst swirling to ensure good mixing.
4. Sonicate for 10 minutes to ensure complete dissolution and mixing.

Scale up as necessary. It is recommended to prepare ALL required analysis mobile phases in one large batch and aliquot into suitable quantities. It is best practice to use a single batch of all solvents and additives during the entire study. This will minimise batch differences seen through the analysis.

7.3. Wash Solutions

Seal Wash: *10% acetonitrile in water*

1. Measure 900 mL of water
2. Top up the bottle with 100 mL of acetonitrile

Weak wash (Fixed Loop): *95:5 (v/v) Acetonitrile:Water*

3. Measure 50 mL of water
4. Top up bottle with 950 mL of acetonitrile

Strong wash (Fixed Loop), Needle Wash & Purge (Flow Through Needle): 100% isopropanol

8. Instrument Set-up

8.1. UPLC system set-up

Table 7: UPLC system set-up

Variable	Description
Mobile Phase A	95:5 Acetonitrile:Water + 10 mM Ammonium Acetate
Mobile Phase B	50:50 Acetonitrile:Water + 10 mM Ammonium Acetate
Seal Wash	10:90 Acetonitrile:Water, set to run every 2 minutes
Weak Wash	95:5 Acetonitrile:Water
Strong Wash	Isopropanol
Column	ACQUITY UPLC BEH Amide column (2.1 x100mm, 1.7 µm)
Column Temp.	45°C
Injection Volume	1 µL positive, 2 µL negative
Run Time	8.5 mins (including injection time)
Sample Cooler	4-8°C

8.2. UPLC Gradient

Table 8: UPLC gradient

#	Time (Mins)	Flow (ml/min)	% A	% B	Curve
1	Initial	0.6	99.9	0.1	Initial

2	2	0.6	80	20	6
3	5	0.6	20	80	6
4	5.1	0.6	99.9	0.1	6
5	8	0.6	99.9	0.1	6

8.3. MS Source Settings

Table 9: MS Source Settings

Variable	Description	
Polarity	Negative	Positive
Capillary voltage	1.9 kV	2.8 kV
Desolvation temperature	500 °C	
Source temperature	120 °C	
Desolvation gas flow	1000 L/hr	
Cone gas flow	150 L/hr	
Nebuliser gas	7.0 bar	
Collision gas flow	0.13 L/hr	
Ion Guide Offset 1	3.0 V	
Ion Guide Offset 2	0.3 V	

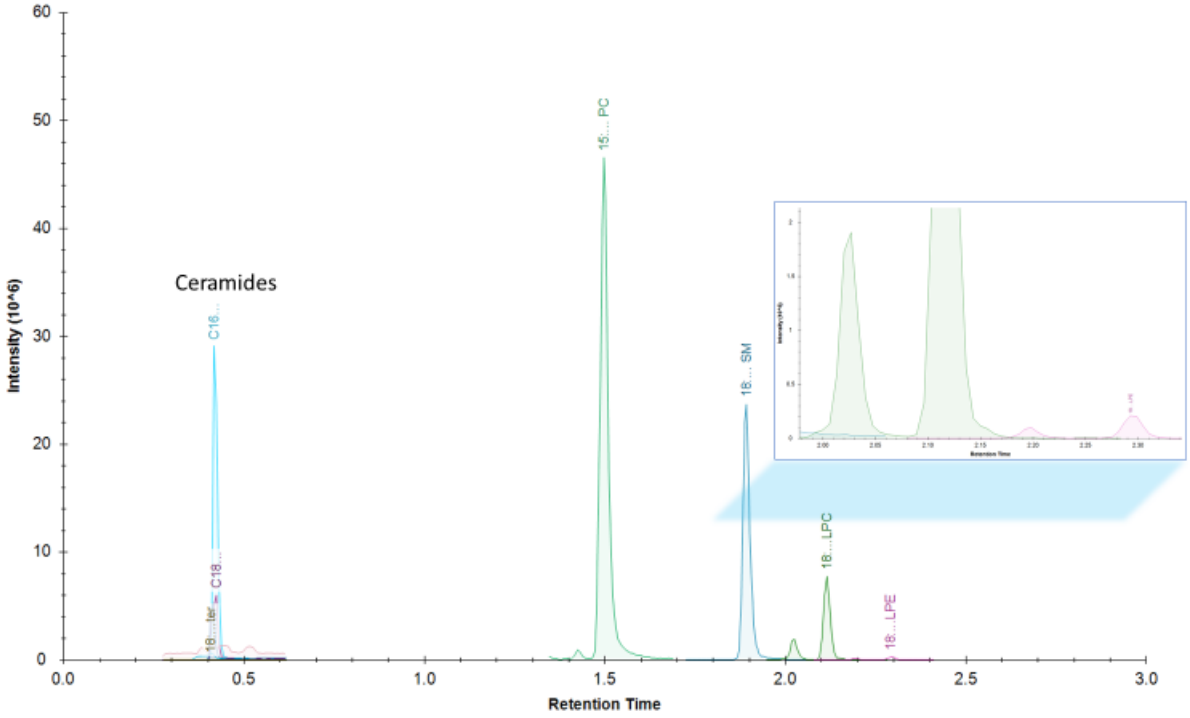
8.4. Inlet & MRM Method

The LipidQuan MS and LC (contained in the LipidQuan Quanpedia file) settings as well as the TargetLynx™ processing files are available for download from the Waters website at www.waters.com/targetedOmics.

1. Please use the Quanpedia files or MS methods provided for transitions.
2. Please ensure that the retention windows are 0.5 minutes.
3. Please ensure that the dwell time is not below 0.0008 seconds for any transition or the signal will be compromised and quantitation may be less accurate.

8.5. Pre-analysis Checks

1. Ensure the cone is clean (See Waters video for guide on cleaning the cone) [3].
2. Using the Console: Perform leak tests for all pumps.
3. Using the system suitability mix, check that all peaks can be seen, check for any retention time shifts, and update the MS method if required (Figures 1-4).

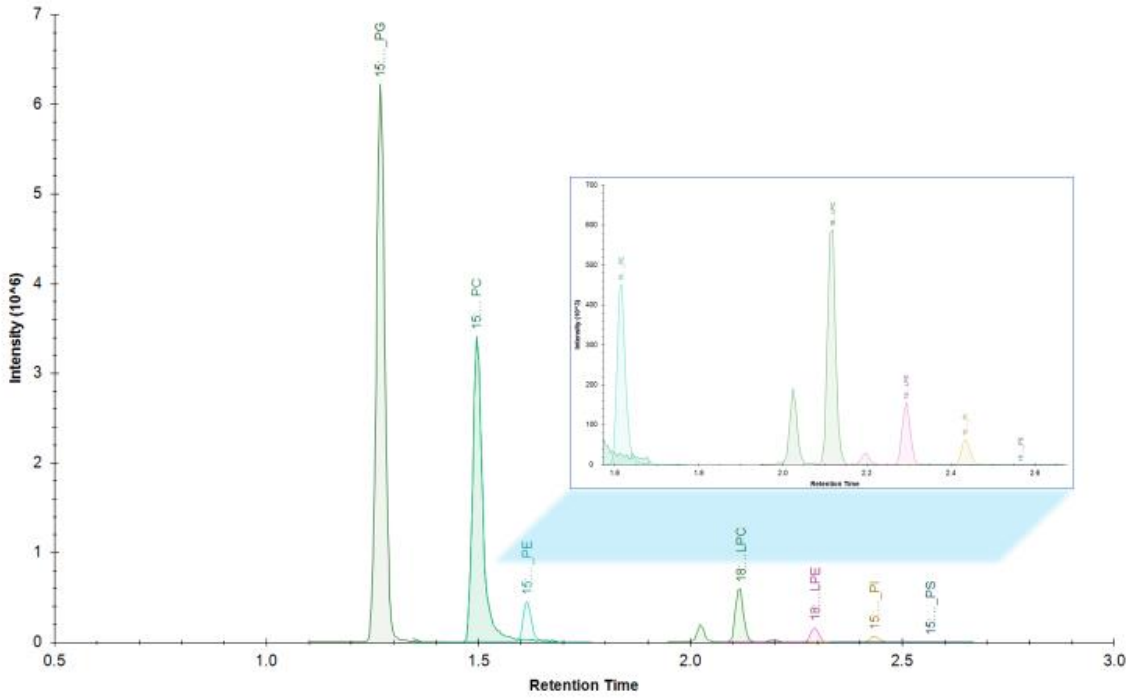


Standard Type	Lipid	Average RT (min) +/-0.08
Splash Mix Int Stds Pos	15:0-18:1 (d7) DG	0.41
Splash Mix Int Stds Pos	15:0-18:1 (d7)-15:0 TG	0.41
Splash Mix Int Stds Pos	18:1 (d7) Chol Ester*	0.41
Splash Mix Int Stds Pos	18:1 (d7) MG*	0.41
Ceramide Lipidomix Int Stds	C24:1 Ceramide-d7 (d18:1/24:1(15Z))*	0.41
Ceramide Lipidomix Int Stds	C16 Ceramide-d7 (d18:1/16:0)	0.42
Ceramide Lipidomix Int Stds	C18 Ceramide -d7(d18:1/18:0)	0.42
Ceramide Lipidomix Int Stds	C24 Ceramide-d7 (d18:1/24:0)	0.42
Splash Mix Int Stds Pos	15:0-18:1 (d7) PC	1.50
Splash Mix Int Stds Pos	18:1 (d9) SM	1.88

Splash Mix Int Stds Pos	18:1 (d7) LPC	2.12
Splash Mix Int Stds Pos	18:1 (d7) LPE	2.30
Splash Mix Int Stds Pos	Cholesterol (d7)*	N/A

Figure 1: Screen shot of internal standard responses by positive/negative mode switching method

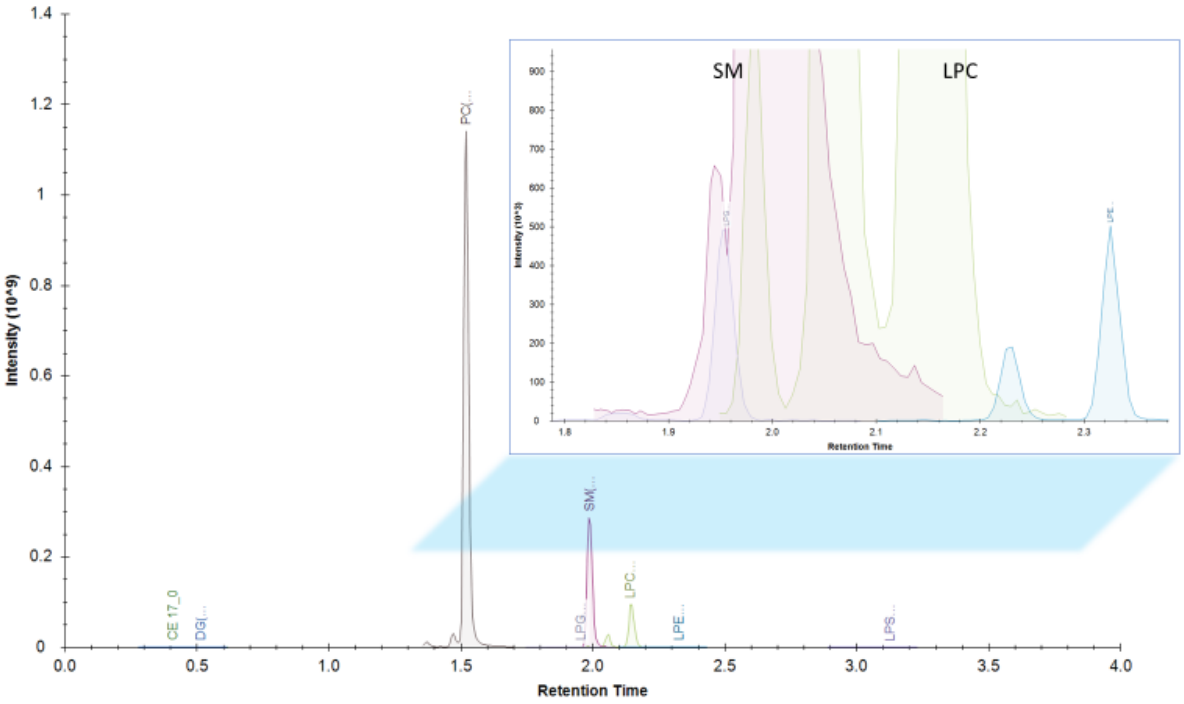
* Poor peak shape



Standard Type	Lipid	Average RT (min) +/-0.08
Splash Mix Int Stds Neg	15:0-18:1_(d7)_PG	1.25
Splash Mix Int Stds Neg	15:0-18:1 (d7) PC	1.50
Splash Mix Int Stds Neg	15:0-18:1_(d7)_PE	1.62
Splash Mix Int Stds Neg	15:0-18:1_(d7)_PA*	2.07
Splash Mix Int Stds Neg	18:1 (d7) LPC	2.12
Splash Mix Int Stds Neg	18:1 (d7) LPE	2.30
Splash Mix Int Stds Neg	15:0-18:1_(d7)_PI	2.44
Splash Mix Int Stds Neg	15:0-18:1_(d7)_PS*	N/A

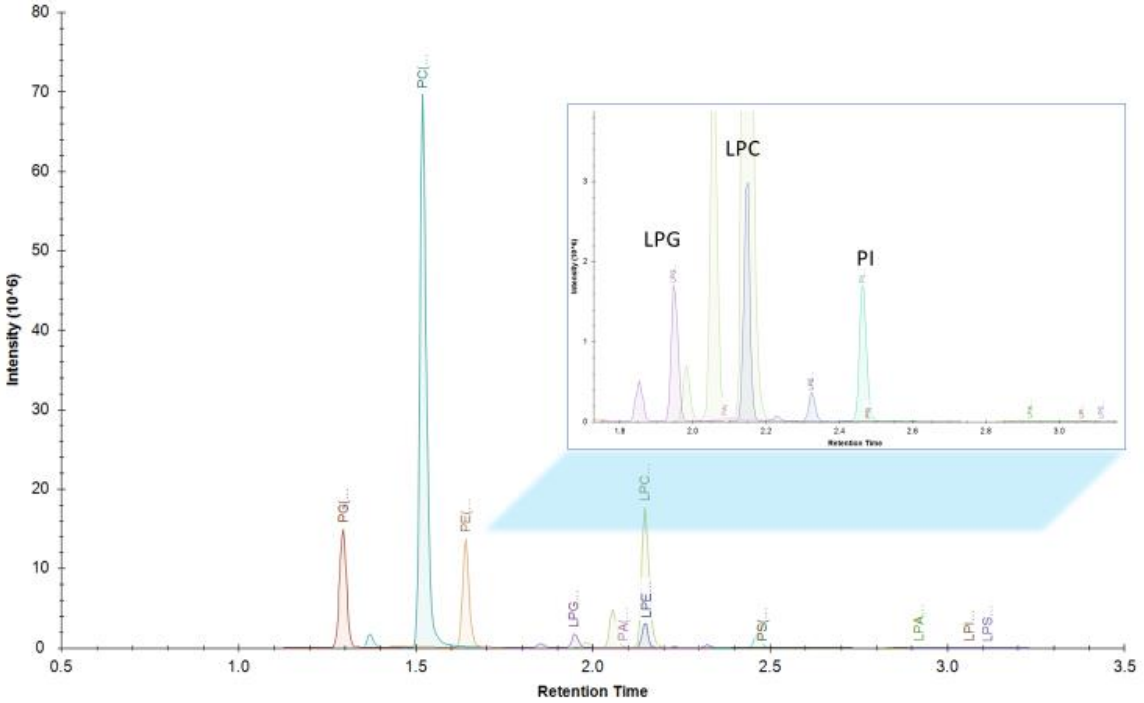
Figure 2: Screen shot of internal standard responses by positive/negative mode switching method

* Poor peak shape



Standard Type	Lipid	Average RT (min) +/-0.08
Odd Chain Lipidomix Cal Stds Pos	DG(17:0/17:0)	0.41
Odd Chain Lipidomix Cal Stds Pos	CE 17_0	0.41
Odd Chain Lipidomix Cal Stds Pos	TG(17:0/17:0/17:0)	0.41
Odd Chain Lipidomix Cal Stds Pos	PC(17:0/14:1)	1.52
Odd Chain Lipidomix Cal Stds Pos	LPG(17:1)	1.92
Odd Chain Lipidomix Cal Stds Pos	SM(d18:1/12:0)	1.99
Odd Chain Lipidomix Cal Stds Pos	LPC(17:1)	2.15
Odd Chain Lipidomix Cal Stds Pos	LPE(17:1)	2.33
Odd Chain Lipidomix Cal Stds Pos	LPS(17:1)*	3.11

Figure 3: Screen shot of odd chained calibration standard responses by positive/negative mode switching method. * Poor peak shape



Standard Type	Lipid	Average RT (min) +/-0.08
Odd Chain Lipidomix Cal Stds Neg	PG(17:0/14:1)	1.30
Odd Chain Lipidomix Cal Stds Neg	PC(17:0/14:1) -ve	1.52
Odd Chain Lipidomix Cal Stds Neg	PE(17:0/14:1)	1.63
Odd Chain Lipidomix Cal Stds Neg	LPG(17:1) -ve	1.95
Odd Chain Lipidomix Cal Stds Neg	PA(17:0/14:1)*	2.10
Odd Chain Lipidomix Cal Stds Neg	LPC(17:1)	2.15
Odd Chain Lipidomix Cal Stds Neg	LPE(17:1) ES-	2.33
Odd Chain Lipidomix Cal Stds Neg	PI(17:0/14:1)	2.47
Odd Chain Lipidomix Cal Stds Neg	PS(17:0/14:1)*	2.49
Odd Chain Lipidomix Cal Stds Neg	LPA(17:1)	2.95
Odd Chain Lipidomix Cal Stds Neg	LPI(17:1)	3.07
Odd Chain Lipidomix Cal Stds Neg	LPS(17:1)*	3.10

Figure 4: Screen shot of odd chained calibration standard responses by positive/negative mode switching method. * Poor peak shape

8.6. Sample Acquisition

1. The run is now ready to begin.
2. During the run, it is advised that you process the calibration curve and check QCs periodically to ensure the run is progressing correctly.

9. Acquisition

1. Suggested analysis running order*:

Table 10: Suggested analysis running order

Analysis Section	Number of injections	Description
Pre-Sample runs	3	System Blanks
	3	System Suitability Solution
	30	Pooled Blank conditioning
	10	Concentration Curve
	3	QC Injections
Sample Analysis	X	Analytical plates
End	3	QC Injections
	10	Concentration Curve
	3	System Blanks

**it is recommended that no more than 1500 samples are injected continuously. The instrument should be cleaned and checked between each set of 1500 samples.*
Every injection from row 12 on your plate will be a QC.

2. System blanks and suitability test mix should be checked prior to continued acquisition. To ensure the system is functioning as expected.

3. Should the analysis halt for any reason and the analysis session requires a restart, the analyst should assess the previous analysis. The subsequent course of action will be down to operator discretion.
    - Should the issue have impacted multiple injections it may be necessary to re-prepare the affected plate and re-analyse.

- If the issue has not impacted previously injected samples then it is possible to re-start the analysis continuing sample analysis from the last injection. Once the system is operational, the operator must inject a new concentration curve to bracket the new analysis set. The initial set may need to be processed with only a single calibration curve.

10. Quality Control

10.1. Curve Linearity

1. The curve of each lipid within the Avanti Odd-Chained LIPIDOMIX™ should be assessed and is deemed acceptable if the R^2 value is >0.95 with no more than 20% of the points being removed as outliers.
2. The following table shows the lipids from the Avanti Odd-Chained LIPIDOMIX™ indicating whether they pass the curve acceptance criteria using this method, and in which mode they are analysed.

Table 11: List of standards that meet the quantification criteria and those suitable for monitoring. Weighting can be adjusted as appropriate

Lipid	Typical Weighting	Curve achieved >0.95
17:1 LPG *	1/x	Yes
17:1 LPA	None	Yes
17:1 LPI *	None	Yes
17:1 LPS *	None	Yes
17:1 LPC +ve	1/x	Yes
17:1 LPC -ve	1/x	Yes
17:1 LPE +ve	1/x	Yes
17:1 LPE -ve*	1/x	Yes
17:0-17:0 DG *	1/x	Yes
17:0-17:0-17:0 TAG *	None	No
12:0 SM (d18:1/12:0)	1/x^2	Yes
17:0-14:1 PC +ve	1/x	Yes
17:0-14:1 PC -ve	1/x	Yes
17:0-14:1 PS **	1/x	Yes
17:0-14:1 PG	1/x	Yes
17:0-14:1 PA *	None	No
17:0-14:1 PE	1/x	Yes
17:0-14:1 PI	1/x	Yes
17:0 Chol Ester *	None	No

** no Internal Standards*

*** LPC (d7) Internal Standards*

10.2. Quality Control Injections

1. The quality control (QC) injections are deemed acceptable if they fall within ±30% from the absolute concentration. The absolute concentration is the value of calibration point 1 (the highest level in the curve).
2. The assay is deemed acceptable if >67% of the quality control injections pass the above criteria.

References

1. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB. Metabolomics. 2018;14(6):72.
2. Magali H. Sarafian, Mathieu Gaudin, Matthew R. Lewis, Francois-Pierre Martin, Elaine Holmes, Jeremy K. Nicholson, and Marc-Emmanuel Dumas, Anal. Chem., 2014, 86 (12):5766–5774
3. http://videos.waters.com/detail/video/5235769453001/cleaning-the-sample-cone-and-gas-cone

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