Robust and Sensitive LC-MS/MS Based Plasma Lipid Profiling on a Thermo Scientific Q Exactive HF-X Mass Spectrometer

Tabiwang N. Arrey[1], Elena Sokol[2], Angela Criscuolo[1], Claire Dauly[3], Alexander Harder[1], Thermo Fisher Scientific, [1]Bremen, Germany, [2]Hemel Hempstead, UK, [3]Villebon-sur-Yvette, France

ABSTRACT

Purpose: Develop a reproducible workflow for deep plasma profiling of lipids including sample extraction and LC-MS parameters on a Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer (MS) with acquisition in positive and negative ionization modes in a single injection.

Methods: Analysis of lipid extracts from human plasma. An isotopically labeled lipid standard mixture (SPLASH™ Lipidomix™ Mass Spec Standard, Avanti Polar Lipids) was spiked into each sample prior to lipid extraction. A full workflow optimization was performed including the determination of optimal plasma volume, MS source conditions and detection parameters. Thermo Scientific™ LipidSearch™ software was used for lipid identification and relative quantification. Limits of quantification were evaluated for all detected SPLASH standards using the optimized workflow.

Results: A robust and sensitive MS based workflow for plasma lipid profiling was developed, resulting in the confident identification and relative quantification of more than 500 lipids across 13 lipid classes. Low limits of quantification were demonstrated with LOQs down to 3 ng/mL.

INTRODUCTION

Lipids play an important role in biology. Understanding changes in their metabolism and associated signaling pathways requires robust and sensitive analytical workflow that enables identification and quantification of multiple lipid classes. Accurate detection and quantification of lipids can be compromised by a number of factors including their extraction from biological samples, chromatographic separation, ionization conditions and detection by mass spectrometry. The Q Exactive HF-X is a new Orbitrap based mass spectrometer with increased scan speed and sensitivity, both of which should benefit large scale lipid profiling experiments. In this study, we optimized a complete workflow including lipid extraction from plasma and mass spectrometry parameters to achieve high quantitative performances, which could be applicable to large cohort studies.

MATERIALS AND METHODS

Sample Preparation

Human plasma was purchased from Sigma (P9623-1ml). Plasma aliquots of different concentrations were prepared by mixing 0 to 80 µL of plasma with varying volumes of 155 mM Amonium Acetate to a total of 200 uL. 10 µL of SPLASH standard (Avanti) was added to each sample before lipid extraction. Lipids were extracted using chloroform/methanol solution (2:1), dried down and reconstituted into 100 µL of 7.5 mM Acid Formic, Ch/MeOH/PrOH (1:2:4) prior to LC-MS analysis. A SPLASH standard dilution series was prepared by serial dilutions of the SPLASH standard spiked into the optimized plasma volume (80 µL) before lipid extraction.

Chromatography Conditions

Column: Thermo Scientific™ Accucore™ C30 column (2.1×150mm, 2.6µm)
Column temperature: 50 °C
Mobile phase:
A = 5 mM Acid Formic in acetonitrile : water (50:50 v/v)
B = 5 mM Acid Formic in 2-propanol : acetonitrile : water (85:10:5 v/v/v)
Flow rate: 325 mL/min

Table 1. HPLC Gradient

Time (min)	B%
0	25
20	86
22	90
24	95
26	95
26.1	25
32	25

Mass Spectrometry Conditions

A Q Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer was used in positive/negative switching mode. Table 2 shows the MS instrument setup.

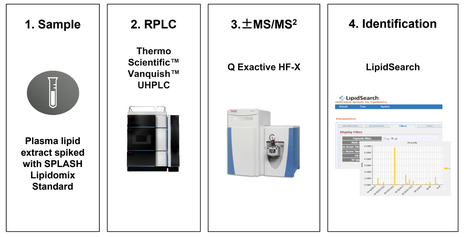
Table 2. Mass Spectrometry Conditions.

HESI Source	Q Exactive HF-X
Sheath gas: 35 Aux gas: 10	MS, R = 120K FWHM at 200 *m/z*, positive/negative switching, mass range; Pos: 250–1200 *m/z*: Neg: 500–1000 m/z
Spray voltage; Pos: 3900 V; Neg: 3500 V	Data Dependent MS/MS, Top10, R = 15K FWHM at 200 *m/z*, max injection time: 60 ms, Dynamic exclusion: 8 s
RF Lens level: 35	MS/MS Isolation Width: 1.0 *m/z*
Capillary Temp.: 250 °C	Stepped NCE; Pos: 25, 30; Neg: 25, 30
Heater Temp: 350 °C	MS AGC Target: 1E+6, MS/MS AGC Target: 1E+5

Data Processing

LipidSearch 4.2 software was used for lipid identification and quantitation.

Figure 1. Experimental workflow.

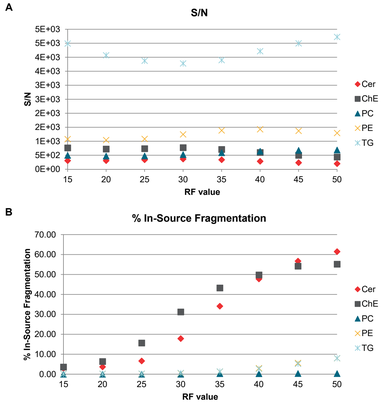


RESULTS

Signal Optimization

An optimization of the ion transfer conditions was conducted in order to minimize the in-source fragmentation of labile lipids and avoid excessive losses of lipid ions. Different ion source and ion transfer parameters were tested by looking at labile lipids such as Cholesterol esters without compromising the detection of other lipid classes. A set of 5 standard lipids (Cer (d34:2), ChE (18:2), PC (34:1), PE (36:4), and TAG (50:2)) were mixed and analyzed using different capillary temperatures and RF values. The capillary temperature was shown to have little influence on the lipid detection, and 250 °C was set as an optimum value (data not shown). Eight ion funnel RF levels were tested. Signal over noise (S/N) values and percentage of in-source fragmentation were calculated for all 5 standards. An RF value of 35 was identified as the best compromise the signal and in-source fragmentation of Ceramides and Cholesterol Esters (Figure 2).

Figure 2. Ion funnel RF optimization. S/N (A) and % of fragmentation (B).



Plasma Extraction

When doing lipid profiling, the main objective is to obtain a broad coverage of lipids identified and quantified with good precision, which can be compromised by their extraction from biological samples. When working with an increasing amount of total lipids, high abundant species tend to get extracted with a higher yield while low abundant lipids could be missed. Different plasma extraction volumes were tested in order to maximize the number of identified lipid species (replicate extractions). This evaluation was conducted by varying the amount of plasma from 0 µL to 80 µL and extracting the ion signals for 4 known plasma lipids ranging in abundance from low (Cer (34:1;2) and Cer (38:1;2)) to high (PC (34:2)). 80 µL was identified as the best extraction volume, yielding a higher detection level for all measured lipids (Figure 3). Two replicate extractions were performed for each plasma volume confirming the reproducibility of the method with coefficient of variations below 25% for all detected SPLASH standards (Table 3).

Figure 3. Plasma extraction optimization.

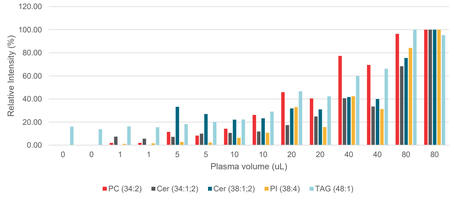


Table 3. Detected SPLASH standards in replicate plasma lipid extractions

Compound	Concentration (µg/mL)	Main Ion	*m/z*	RT	80_1	80_2	CV (%)
PC 15:0-18:1(d7)	160.7	H+	753.6134	14.69	3.22E+08	4.24E+08	19
PE 15:0-18:1(d7)	5.7	H-	709.5519	15.12	3.52E+06	4.07E+06	10
PS 15:0-18:1(d7)	4.2	H+	755.5563	13.63	1.55E+06	1.55E+06	0
PG 15:0-18:1(d7)	29.1	H-	740.5465	13.78	1.76E+07	2.22E+07	16
PI 15:0-18:1(d7)	9.1	NH4+	847.6036	13.34	1.75E+06	2.15E+06	15
PA 15:0-18:1(d7)	7.4	H-	666.5097	14.71	3.19E+05	2.60E+05	14
LPC 18:1(d7)	25.5	H+	529.3994	4.48	8.52E+07	9.87E+07	10
LPE 18:1(d7)	5.3	H+	487.3524	4.68	7.24E+06	8.52E+06	11
ChoE 18:1(d7)	356.1	NH4+	675.6779	25.42	1.41E+07	1.01E+07	23
MG 18:1(d7)	2	NH4+	381.3704	nd	nd	nd	nd
DG 15:0-18:1(d7)	9.4	NH4+	605.5844	17.65	8.38E+06	8.67E+06	2
TG 15:0-18:1(d7)-15:0	57.3	NH4+	829.7985	23.97	2.10E+08	1.85E+08	9
SM d18:1-18:1(d9)	30.9	H+	738.647	13.84	3.02E+07	3.90E+07	18
Cholesterol	98.4	NH4+	411.4326	nd	nd	nd	nd

Plasma Lipid Profiling

Data dependent parameters were optimized in order to maximize the number of high quality MS2 spectra generated in view of the higher scan rate and sensitivity of the instrument. A high resolution survey scan (120K) was generated in both positive and negative modes followed by up to 15 high resolution (15K) MS/MS spectra for both polarities. . Lipid species were identified by product search with mass tolerance of 5 ppm for both precursor and fragment ions. Twenty one lipid classes were searched (LPC, PC, LPE, PE, LPS, PS, LPG, PG, LPI, PI, LPA, PA, SM, phSM, MG, DG, TG, SPH, SPPH, Cer, ChE). For PCs and LPCs only species with main ion "M+H" were considered. For PEs, species detected as sodiated main ion adducts were rejected. For TGs and MGs only species with main ion detected as "M+NH4" were allowed.

This optimization process resulted in a deep plasma lipidome coverage with 577 lipids identified over the 13 classes of compounds selected (Table 3). The majority of the identified lipid species showed reproducible peak areas over three replicate injections, yielding precise quantitation results. Figure 4 shows the identification and relative quantification of a low abundant phosphoserine (PS) with coefficient of variation (CV) below 10% in both replicate extractions. Figure 5 shows the relative abundance of all detected lipid species summed by lipid class in the two samples. In this study, 515 lipid species were quantified with less than 30% CVs in both extraction replicates.

Table 4. Lipids confidentially identified per lipid class.

Class	Count		Class	Count
Cer	29		PE	75
ChE	5		PG	4
DG	41		PI	28
LPC	36		PS	13
LPE	7		SM	67
LPI	4		TG	112
PC	156			

Figure 4. Confident identification and relative quantification of low abundant PS (18:2-18:2) lipid.

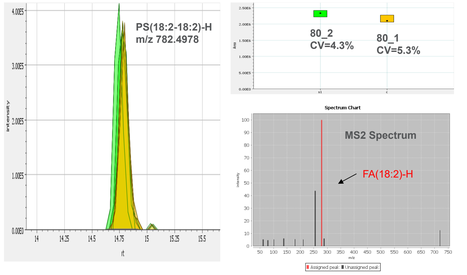
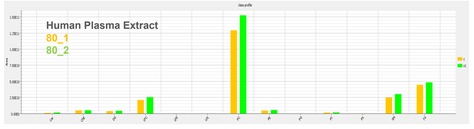


Figure 5. Detected SPLASH standards in replicate plasma extractions



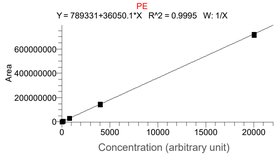
Limits of Quantification

In data dependent LC-MS/MS experiments, lipids are selected for MS/MS and quantified based on the peak areas in MS1 while fragmentation spectra are used to assign their structural composition. The method's sensitivity is therefore directly linked to the detection of lipid species in the MS1 survey scan. A dilution series of the SPLASH standard was generated in plasma and calibration curves were generated to determine the linearity of detection and limits of quantification (LOQ) expressed as the lowest calibration point with % of difference below 20% and a CV below 20% (triplicate injections) (Table 5, Figure 6).

Table 5. LOQ for all detected SPLASH standards in plasma lipid extracts.

Compound	LOQ (ng/mL)
PC 15:0-18:1(d7)	16
PE 15:0-18:1(d7)	3
PS 15:0-18:1(d7)	269
PG 15:0-18:1(d7)	3
PI 15:0-18:1(d7)	116
PA 15:0-18:1(d7)	94
LPC 18:1(d7)	3
LPE 18:1(d7)	14
ChoE 18:1(d7)	182
DG 15:0-18:1(d7)	24
TG 15:0-18:1(d7)-15:0	6
SM d18:1-18:1(d9)	16

Figure 6. PE 15:0-18:1(d7) calibration curve in plasma



CONCLUSIONS

- A robust and sensitive MS based workflow for plasma lipid profiling was developed using the Q Exactive HF-X mass spectrometer.
- Lipid extraction from 80 µL of plasma produced the highest recovery of lipids across the dynamic range of plasma concentration while number of detected volume was determined, resulting in the confident identification and relative quantification of more than 500 lipids across 13 lipid classes from 80 µL of plasma.
- Capillary temperature and ion funnel RF levels were optimized to maximize ion signal while minimizing in-source fragmentation of labile lipids like Ceramides and Cholesterol Esters.
- More than 500 lipids were confidentially identified and relatively quantified across 13 lipid classes in two separate plasma extracts.
- Low limits of quantification were demonstrated with LOQs down to 3 ng/mL.

TRADEMARKS/LICENSING

PO65282-EN 0518S

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