Robust Lipidomic Profiling to Routinely Quantitate Ceramides and Confidently Identify Unknown Lipids

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ABSTRACT

Purpose: Develop targeted lipidomic profiling to routinely quantitate selected serum ceramides.

Methods: LC-MS quantitation of four ceramides in fetal bovine serum (FBS) samples, with different treatment conditions, was performed on Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer using C18 HPLC separation. Quantitation data were acquired in Selective Ion Monitoring (SIM) mode with simultaneous acquisition of Full-MS data for untargeted lipidomics analysis. Calibration and concentration calculations were done using isotopically labeled internal standards. Data-dependent MS/MS (DD-MS/MS) acquisition was performed on pooled FBS sample for unknown lipids annotation.

Results: Linear calibration curves of the investigated ceramides were reconstructed for concentration range of 0.25 (or 0.1) – 10,000 ng/mL. Data of internal standards showed very good reproducibility of both retention times (CV < 0.1 %, n = 94) and integrated peaks areas (in FBS samples; CV ≤ 5.2 %, n = 63). Concentration levels of the tested ceramides in FBS samples were calculated in range of 10 – 150 ng/mL. Variations in ceramide levels in FBS samples vary up to tens of percent. Finally, no significant variation was observed in ceramide concentrations dependence on FBS treatment or origin. Full-MS data of several LPC and PC lipids were retrospectively analyzed. Observed decrease of two LPC lipids suggests that dialysis might affect abundance of some small lipids.

INTRODUCTION

Fetal bovine serum (FBS) is commonly added to cell culture media as a growth supplement in *in vitro* cell culture applications. FBS contains several nutritional and macromolecular components including a variety of small molecules like amino acids, sugars, lipids, and hormones that are essential for cell growth and proliferation. Concentration of such compounds (e.g. ceramides, sphingomyelins, and triacylglycerides) may differ within the variety of commercially available FBS material and may further depend on processing protocols and geographical regions. A targeted lipidomic profiling experiment was applied to assess potential phenotypic variation among different FBS samples, which can be important for successful and reproducible cell culture production.

MATERIALS AND METHODS

Sample Preparation

FBS samples were obtained from commercially available sources. Ceramides Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1), and Cer(d18:1/24:0) were purchased at Cayman Chemical Company. The corresponding isotopically D7-labeled ceramides were purchased at Avanti Polar Lipids and used as internal standards (IS). Stock solution (1 mg/mL) of ceramides standards was prepared in chloroform. This stock was then used to prepare calibration solutions in isopropanol. IS-stock solution (10 ng/mL) was prepared by mixing and diluting of the D7-labeled ceramides in isopropanol. Final calibration solutions were prepared by mixing of stock STD solutions (50 µL) with IS-stock solution (450 µL). FBS samples (50 µL) were mixed with IS-stock solution (450 µL), vortexed, shaken (30 min), and centrifuged (9000 rpm for 10 min). Supernatants were transferred into glass HPLC vials. All FBS samples were processed at three technical replicates.

Liquid Chromatography

LC system: Thermo Scientific™ Vanquish™ Flex binary UHPLC system. Autosampler temp.: 15 ° C
HPLC Column: Thermo Scientific Accucore™ C18 (2.1 x 150 mm, 2.6 µm) at 45 ° C.
Injection Volume: 10 µL; Sample loop (50 µL) was placed between injector and column as a mixer.
Mobile Phase: (A) 10 mM $HCOONH_4$ in 60% ACN (v) + 0.1% HCOOH (v)
(B) 10 mM $HCOONH_4$ in IPA / ACN 90:10 (v/v) + 0.1% HCOOH (v)

HPLC Gradient:

Time	A%	B%	Time	A%	B%
0.00	85	15	13.00	1	99
0.10	85	15	15.00	1	99
0.10	70	30	15.10	85	15
3.00	60	40	19.00	85	15

Flow Rate: 400 µL/min

Divert Valve: To MS = 0 – 0.52 min
To Waste = 0.52 – 1.30 min
To MS = 1.30 – 19.00 min

Mass Spectrometry

Mass spectrometer: Orbitrap Exploris™ 120 mass spectrometer equipped with heated ESI probe. Ion source settings: Spray Voltage = 3.25 kV (pos. polarity) or 3.0 kV (neg. polarity), Vaporizer = 325 ° C, Transfer Tube = 275 ° C, RF Lens = 75 %, Sheath Gas = 60, Aux. gas = 17, Sweep Gas = 1.

A) Quantitation data acquisition settings: Alternating scan Event 1 (MS) and Event 2 (SIM).

Scan Event 1) MS	Scan Event 2) SIM			
RT Time = 0 - 19 min	Compound	m/z	RT (min)	RT Window (min)
Mass Range = 250-1600 Da	Cer(16:0)	538.5194	6.46	0.7
	D7-Cer(16:0)	545.5633	6.46	0.7
	Cer(18:0)	566.5507	7.19	0.7
	D7-Cer(18:0)	573.5946	7.19	0.7
	Cer(24:1)	648.6289	8.37	0.7
	D7-Cer(24:1)	655.6729	8.37	0.7
	Cer(24:0)	650.6446	9.11	0.7
	D7-Cer(24:0)	657.6885	9.11	0.7

Positive ion mode, Trapping = Mild, AGC = Standard, Resolution = 120,000, Max Injection Time = 150 ms for MS and 200 ms for SIM.

B) Qualitative dd-MS/MS data acquisition settings:

Top-4 experiment, AGC = Standard, Trapping = Mild, Max Injection Time = 80 ms for MS^2, Resolution = 60,000 for MS and 15,000 for MS^2, SIM Isolation = 2.0 Da, Dynamic Exclusion = 4 s. Collision Energy (NCE) = 25 - 30 for positive and NCE = 20 - 30 - 40 for negative ion mode. Exclusion mass lists for both polarity modes were created from solvent injection runs by averaging mass spectra (~1.4 – 15.5 min) and exporting m/z values of the 150 most intense ions.

Data Analysis

All data were acquired using Thermo Scientific™ Xcalibur™ Software. Quantitation data were processed in Thermo Scientific™ TraceFinder™ Software 5.1 using a 5 ppm mass tolerance filter. Qualitative dd-MS/MS data were automatically processed using Thermo Scientific™ LipidSearch™ Software 4.2.

RESULTS

Data Acquisition

Ceramides ESI(+) LC-MS quantitation data were acquired via Selective Ion Monitoring (SIM) mode analysis and IS utilization, simultaneously, Full-MS mode data was acquired for lipids untargeted profiling (Figure 1). Compared to Full-MS, SIM provided significantly better sensitivity due to enhanced S/N (spectrum signal/electronic-noise ratio) as displayed on Cer(18:0) in Figure 2. Non-selective nature of Full-MS data can be used for later retrospective analysis and quantitation of other lipids. Data acquisition rate of the instrument provided sufficient number of scans (≥ 10) across a typical chromatographic peak (including time ranges of alternating MS/SIM scans as seen in Figure 2).

Figure 1. ESI(+) Total ion chromatogram (MS + SIM) of pooled FBS sample.

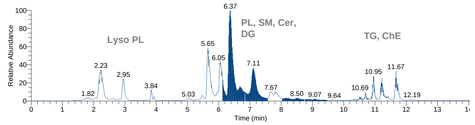
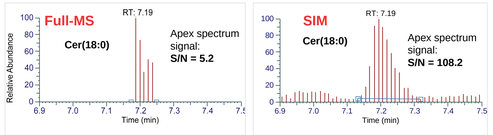


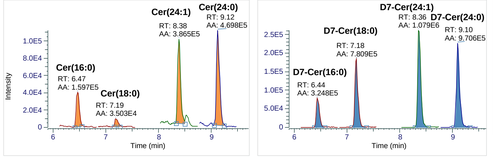
Figure 2. Cer(18:0) chromatographic peaks (XIC m/z 566.5507) of pooled FBS sample acquired (simultaneously) in Full-MS and SIM data



Data Acquisition

Samples were acquired in the following order: Blanks – Calibration samples (consequent triplicate injections) – Blank – Pooled FBS sample – FBS samples (three sets, randomized order) – Pooled FBS sample. An example of quantified ceramides and related ISTDs is shown in Figure 3.

Figure 3. Ceramides' chromatographic peaks (XIC) of pooled FBS sample acquired in SIM mode.



Calibration Data

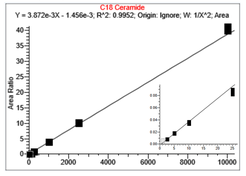
Calibration curves were reconstructed for the quantified ceramides using internal calibration (Cer(18:0) is displayed in Figure 4 as an example). For all compounds, linear fit (R^2 > 0.99) and $1/x^2$ weighing were used. All calibration levels showed a CV ≤ 20% and an average calculated difference CV ≤ 20%. Summary of concentration levels with related %CV and Lower Limit of Quantitation (LOQ) are tabulated in Table 1. Concertation of 2.5 ng/mL was determined as LOQ for Cer(16:0), Cer(18:0), and Cer(24:0), and 1.0 ng/mL for Cer(24:1).

Presented concentration level values are related to concentration in stock STDs samples (and formally FBS sample). In fact, the physical concentration levels in each injected STD sample is 10 x lower due to sample dilution.

Table 1. Summary table of calibration standards.

Level [ng/mL]	Cer(16:0) %CV	Cer(18:0) %CV	Cer(24:0) %CV	Cer(24:1) %CV
1.0	(16.5)	(24.4)	(6.7)	***(LOQ)*** 9.6
2.5	***(LOQ)*** 4.8	***(LOQ)*** 2.7	***(LOQ)*** 5.4	5.0
5.0	11.6	3.5	7.0	0.8
10	4.7	5.4	4.0	3.2
25	1.9	4.9	0.9	1.8
100	0.7	1.1	1.4	1.1
250	0.5	1.1	1.2	0.3
1000	1.2	1.3	1.0	0.5
2500	1.5	1.5	1.2	1.3
10000	1.6	1.4	0.8	2.6

Figure 4. Reconstructed calibration curve of Cer(18:0)



Data Reproducibility

Retention times reproducibility of the deuterated ceramides was evaluated (Figure 5). All compounds showed very low RT variation with CV < 0.1% for 94 injections.

Reproducibility of integrated peak areas of the deuterated ceramides was evaluated on FBS samples (n = 63 injections). As displayed in Figure 6, all compounds exhibited stable peak areas with calculated CVs in a range of 1.18 – 5.21 %.

Figure 5. Reproducibility of retention times of deuterated IS (n = 94 injections)

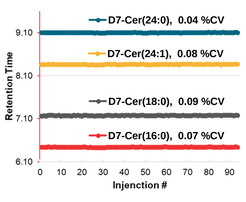
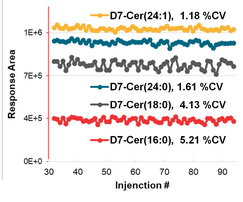


Figure 6. Reproducibility of integrated peak areas of deuterated ISTDs in FBS samples (n = 63 injections)



Measured Ceramides Levels in FBS samples.

Calculated concentration levels of the investigated ceramides are summarized in Figure 7. Each sample is labeled with a vendor code, country of origin, and type of treatment. Relatively for individual FBS samples, highest concentration level was measured for Cer(24:0) followed by Cer(16:0) and Cer(24:1). The lowest concertation was measured for Cer(18:0). All FBS samples were processed with three technical replicates and data recorded with randomized acquisition list. Measured concentrations of ceramides in individual samples show low variations of avg. CV <2 % for Cer(24:0), Cer(16:0), Cer(24:1), and avg. CV < 5% for Cer(18:0).

Comparing individual FBS samples, concentration of each of the quantified ceramides varies up to tens of percents. The biggest overall difference was observed between FBS-15 (Panama, untreated) and FBS-11 (Canada, heated) samples. Compared to FBS-15, concentration level in FBS-11 is higher by 63% for Cer(16:0), 68% for Cer(18:0), 118% for Cer(24:0), and 74% for Cer(24:1). On the other hand, low or no statistical significance was observed for dependence of the ceramides concentration on origin or treatment. For example, concentration levels of the investigated compounds show only insignificant differences on treatment type (Figure 8).

Figure 7. Ceramides' concentration in individual FBS samples (n = 3).

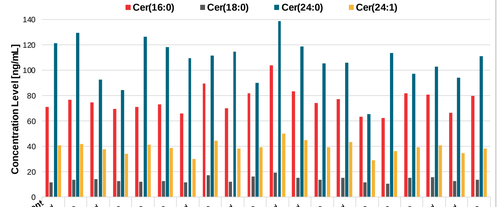
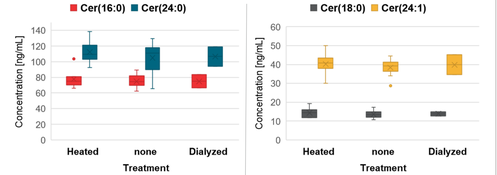


Figure 8. Box and whisker plots of Ceramides concentration dependency on Treatment type



Untargeted Lipidomics Data Acquisition and Processing

Proper annotation of lipid species is essential for possible retrospective data analysis of previously acquired Full-MS data of FBS samples. Therefore, untargeted DD-MS/MS lipidomics profiling data were acquired from pooled FBS sample. Data were acquired in both positive and negative ion modes with duplicate injections and processed in LipidSearch software. Compound detection and annotation in the software is based on both MS and MS/MS data (example in Figure 9). Number of annotated lipids is summarized in Table 2. Filtered Count data (n = 320) show the most relevant lipid species (636 species without data quality filtering) .

Retrospective data analysis of Full-MS data was used on several LPC lipids and related (same FA chain) PC lipids (Figure 10). For LPC(16:0) and LPC(18:0) lipids, dialyzed FBS samples showed statistically significant lower response compared to non-treated and Heated samples. On the other hand, LPC(18:1) and LPC(20:4) did not show such statistically significant change for Dialyzed samples (Figure 10 A and B). Interestingly, structurally related PC lipids (same FA chains) did not show any significant response area change comparing dialyzed and non-treated FBS samples. Only some response increase can be observed for Heated samples when compared to non-treated samples (Figure 10 C and D). This suggests a possibility that Dialysis of FBS samples can reduce amount of some small molecule size lipids, such as certain LPC. On the other hand, bigger lipids (e.g. PC) cannot migrate via dialysis membrane and dialysis does not affect their amount in FBS samples.

Figure 9. LipidSearch MS/MS annotation and chromatographic peak integration of PC(18:0/20:4) in pooled FBS sample.

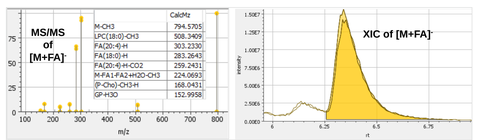
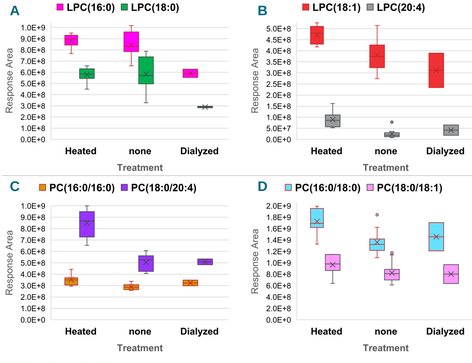


TABLE 2. Number of lipid species annotated in pooled FBS sample.

Class	Filtered Count	Total Count
AcCa	5	14
CL	0	1
Cer	15	21
ChE	17	36
Co	1	2
DG	0	1
LPA	7	9
LPC	50	94
LPE	5	10

Class	Filtered Count	Total Count
LPI	1	1
LPS	0	2
PA	0	1
PC	94	219
PE	6	11
PI	14	21
SM	57	102
SPH	0	3
TG	48	88
Total	**320**	**636**

Figure 10. Box and whisker plots of LPC and PC lipids' response area on Treatment type



CONCLUSIONS

- LC-MS method for sensitive quantitation of Ceramides Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/24:1), and Cer(d18:1/24:0) in FBS samples was developed on Orbitrap Exploris 120. Quantitation data for the analytes and isotopically-labeled internal standards were acquired in SIM mode. Simultaneously acquired Full-MS data can be used for retrospective data analysis.
- LOQs of the investigated ceramides were determined at 2.5 or 1.0 ng/mL. Linear calibration curves were reconstructed from LOQ levels up to 10,000 ng/mL.
- Acquired data of internal standards showed very good reproducibility of retention times (in all samples; CV < 0.1 %) and integrated peaks areas (in FBS samples; CV ≤ 5.2 %).
- Concentration of investigated ceramides vary up to tens of percent within the FBS samples. No significant variation was observed in ceramide concentrations dependence on FBS treatment or origin.
- Processing of untargeted DD-MS/MS data via Lipid Search led to the annotation of 320 lipid species in pooled FBS sample. Full-MS data of several LPC and PC lipids were retrospectively analyzed. Observed decrease of two LPC lipids suggests that dialysis might affect abundance of some small lipids.

TRADEMARKS/LICENSING

PO66120 EN0921S