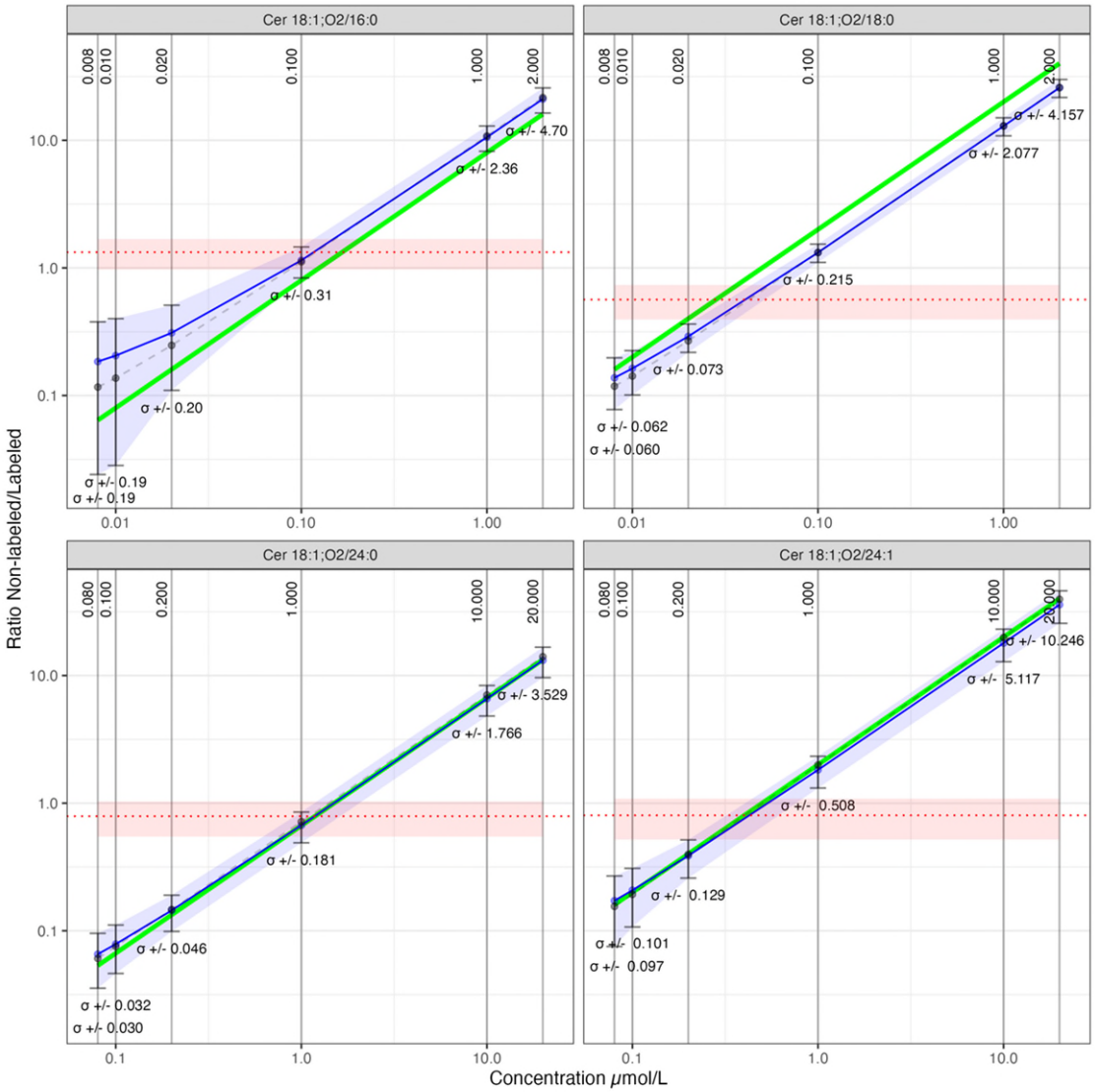
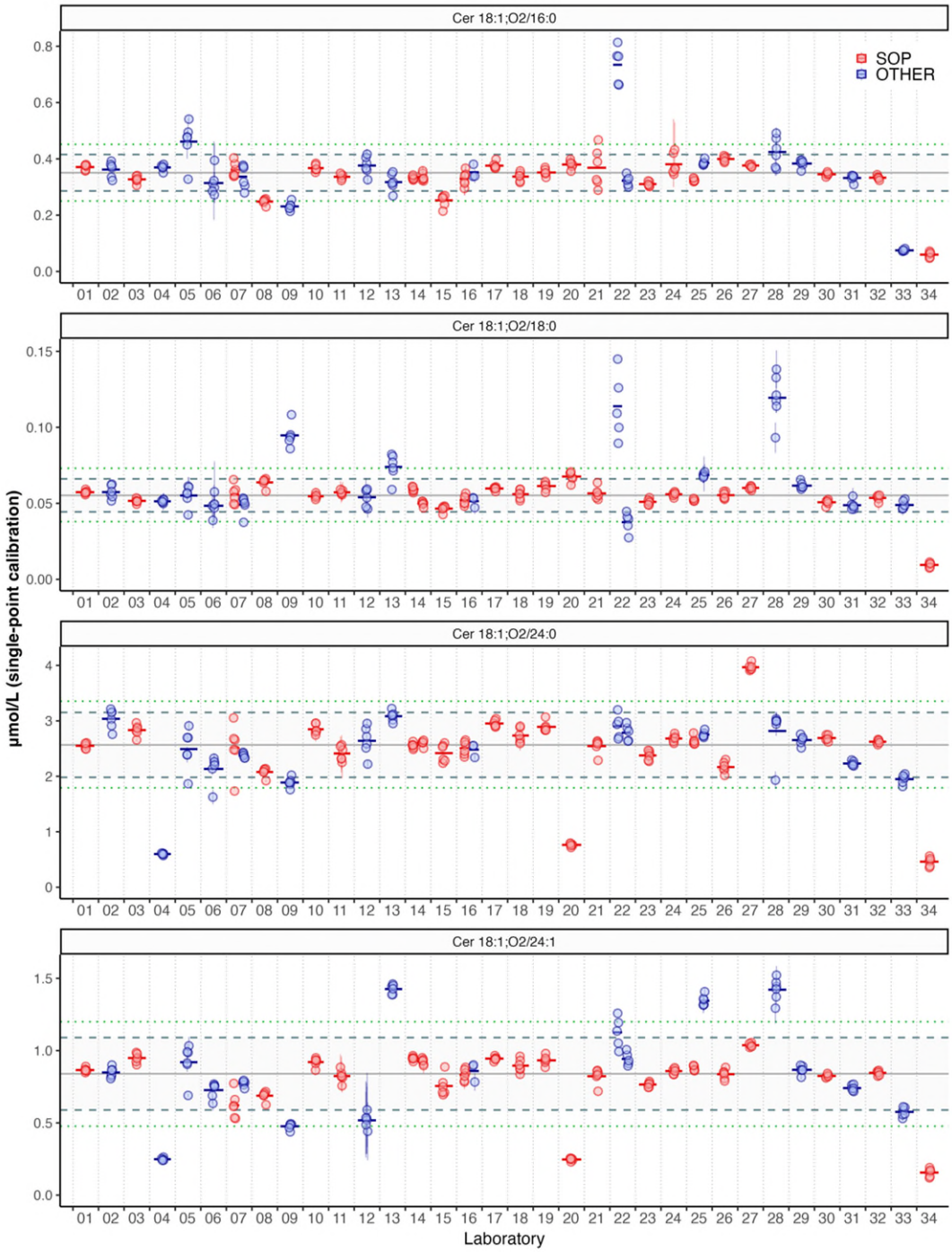
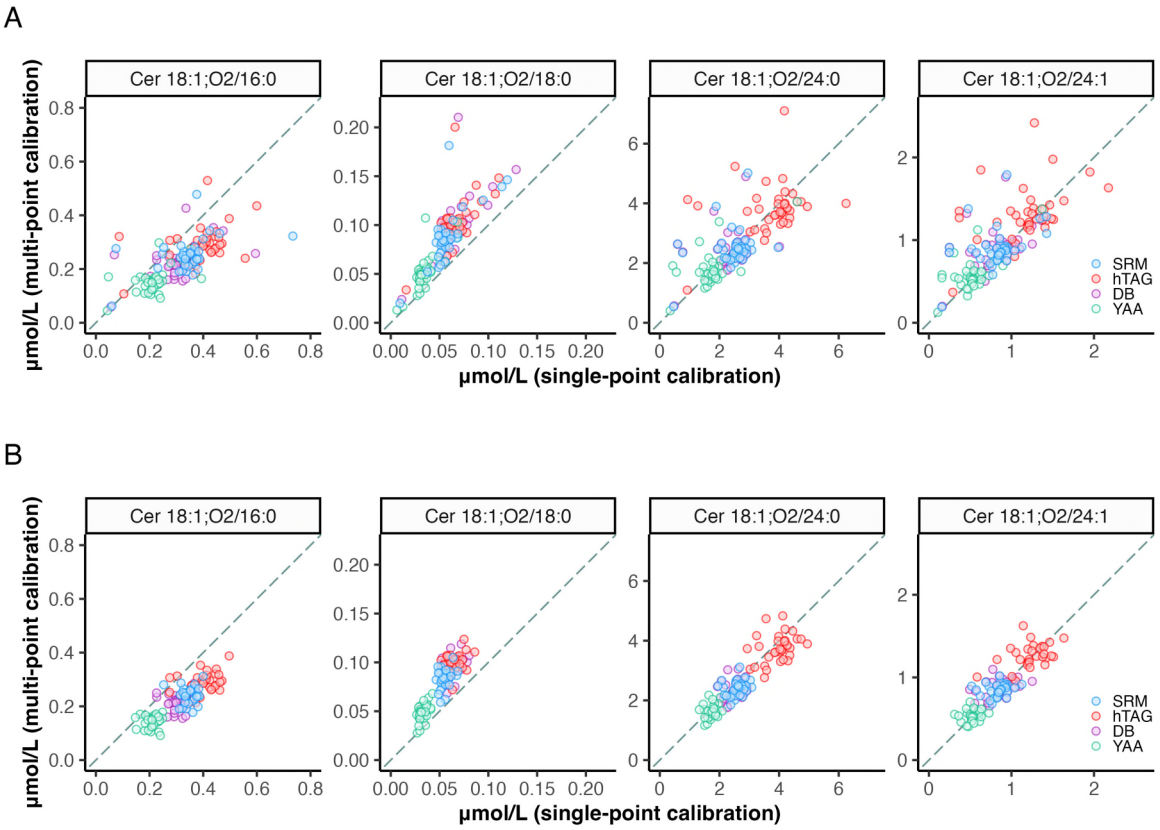
SUPPLEMENTARY FIGURES



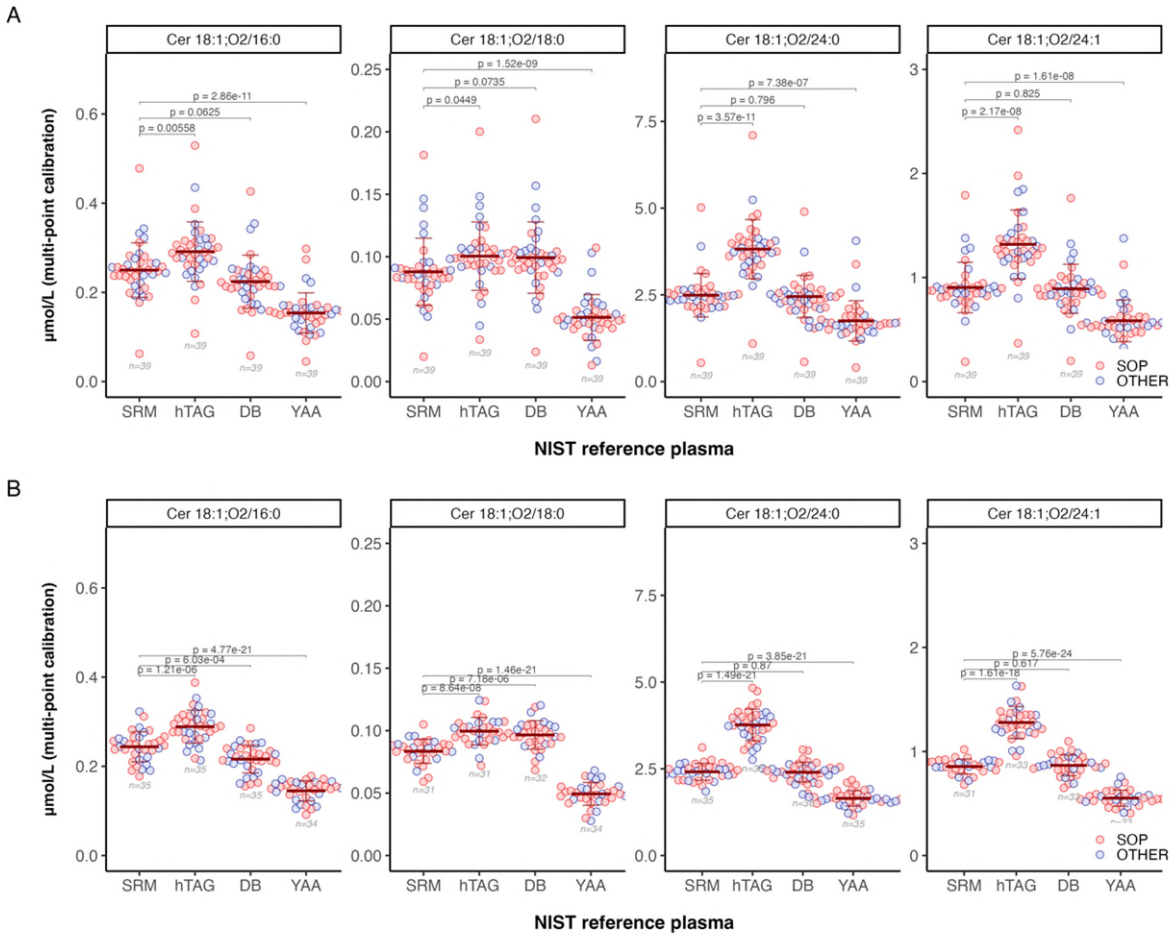
Supplementary Figure 1. Log-log plot of mean calibration lines over all participating labs and protocols. Each concentration step of the calibration line samples is depicted as a blue dot, steps are connected by a blue solid line. The blue ribbons highlight the respective mean ±1SD. Median values are indicated by black dots and connected by a grey dashed line. The green line corresponds to the theoretical equimolar response of spiked-in non-labelled and labelled standards. The red line and ribbon correspond to the actual mean area ratios in the measured NIST SRM 1950 RM samples and the mean ±1SD, respectively, illustrating the suitability of the calibration lines to capture the expected concentrations of the four ceramides. Source data are provided as Source Data file.



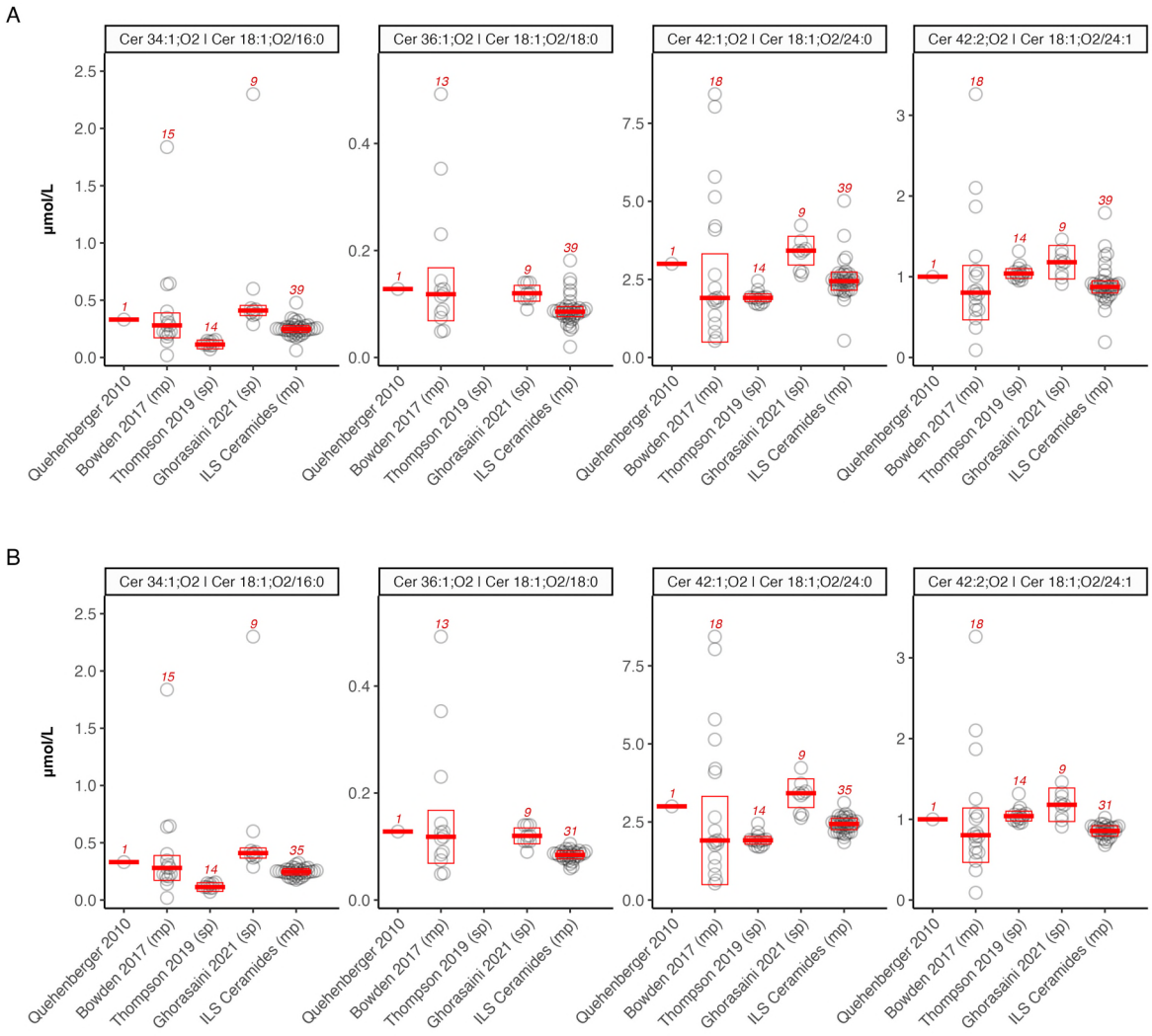
Supplementary Figure 2. Consensus concentrations of ceramides in NIST SRM 1950 across all study participants. Concentration of the 4 reported ceramides in NIST SRM 1950 based on direct quantification using the internal standard (single-point calibration). Each circle corresponds to the mean of 1 to 6 replicate measurements from a vial of NIST SRM 1950 shipped to each of the participating laboratories. The error bars depict ±1×SD, which in some cases is smaller than the diameter of the plotted circle or absent if only 1 measurement was made. Horizontal bars represent the mean values of the mean measured concentration per NIST vial. The outer green dotted lines indicate Tukey's Q1–1.5×interquartile range (IQR) and Q3+1.5×IQR used for as cut-off values for outlier removal. The mean ± 2×SD (solid grey and dashed grey lines) is based on the data after outlier removal. SOP (red) and OTHER (blue) refer to the Standard vs OTHER methods, respectively. Source data are provided as Source Data file.



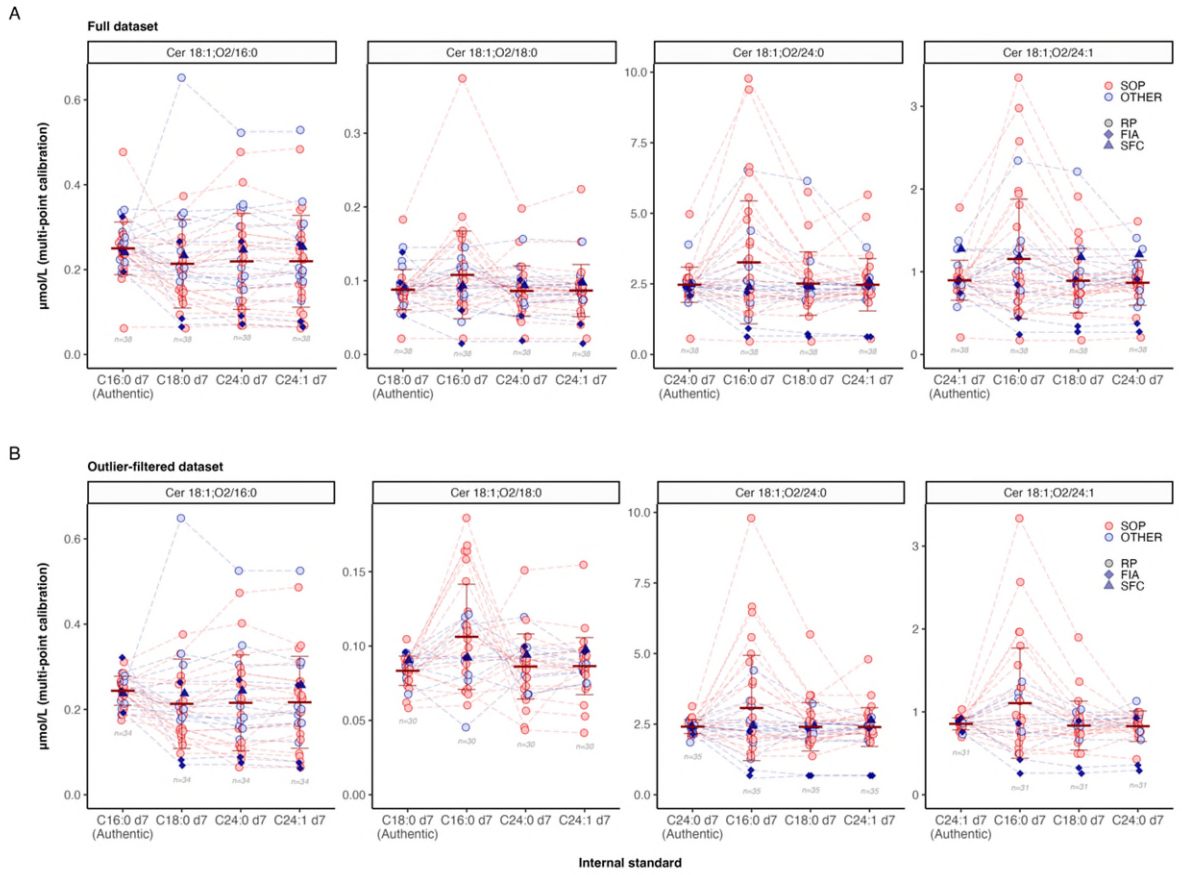
<u>Supplementary Figure 3</u> – Comparison of single-point and multi-point calibrations of the full (A) and outlier-filtered (B) dataset. Molar concentrations derived from single-point vs. multi-point calibration based quantifications. The diagonal line indicates equal results between single-point (horizontal axis) and multi-point (vertical axis) calibration-based quantification. Outlier measurements were removed for each ceramide species in each reference plasma (see Fig. 1 and text). Each circle corresponds to the mean value reported by one laboratory (n=6). NIST SRM 1950 (SRM, blue); hypertriglyceridemic (hTAG, red), diabetic (DB, purple), and Young African American (YAA, green), which are part of NIST RM 8231. Source data are provided as Source Data file.



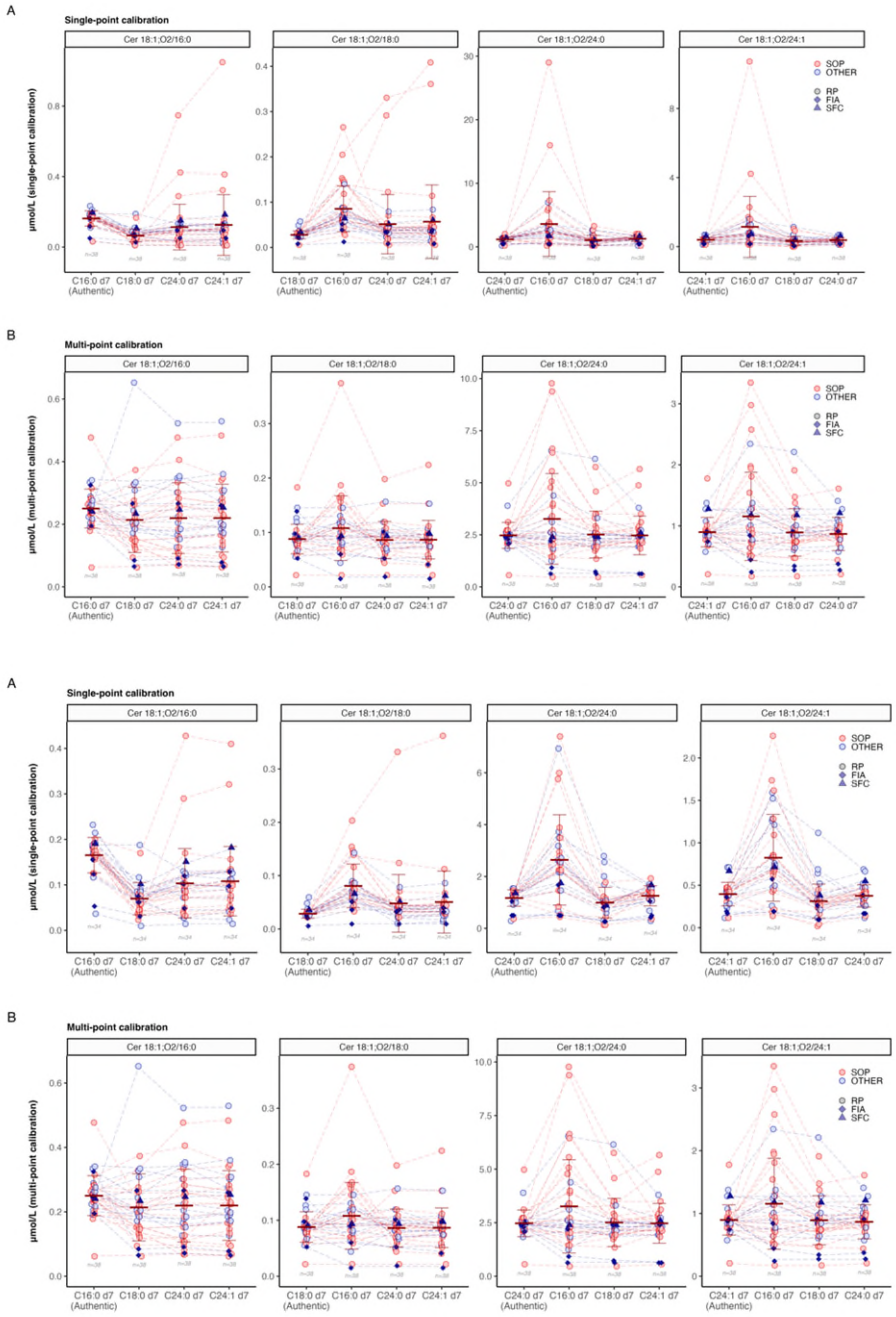
<u>Supplementary Figure 4</u> – Concentration values of ceramides in NIST SRM 1950 and the suite of human plasma RM contained in RM 8231 of the full (A) and outlier-filtered (B) dataset. Concentrations of the 4 ceramides in different NIST reference plasma samples. Each circle corresponds to the values reported by one laboratory. P values are based on paired t-tests of complete datasets. The horizontal bars correspond to the mean ±1×SD of all points per group. Outlier measurements were removed in (B) for ceramide species in each reference plasma (see Fig.1 and text). SOP (red) and OTHER (blue) refer to the Standard vs Other methods, respectively. Source data are provided as Source Data file.



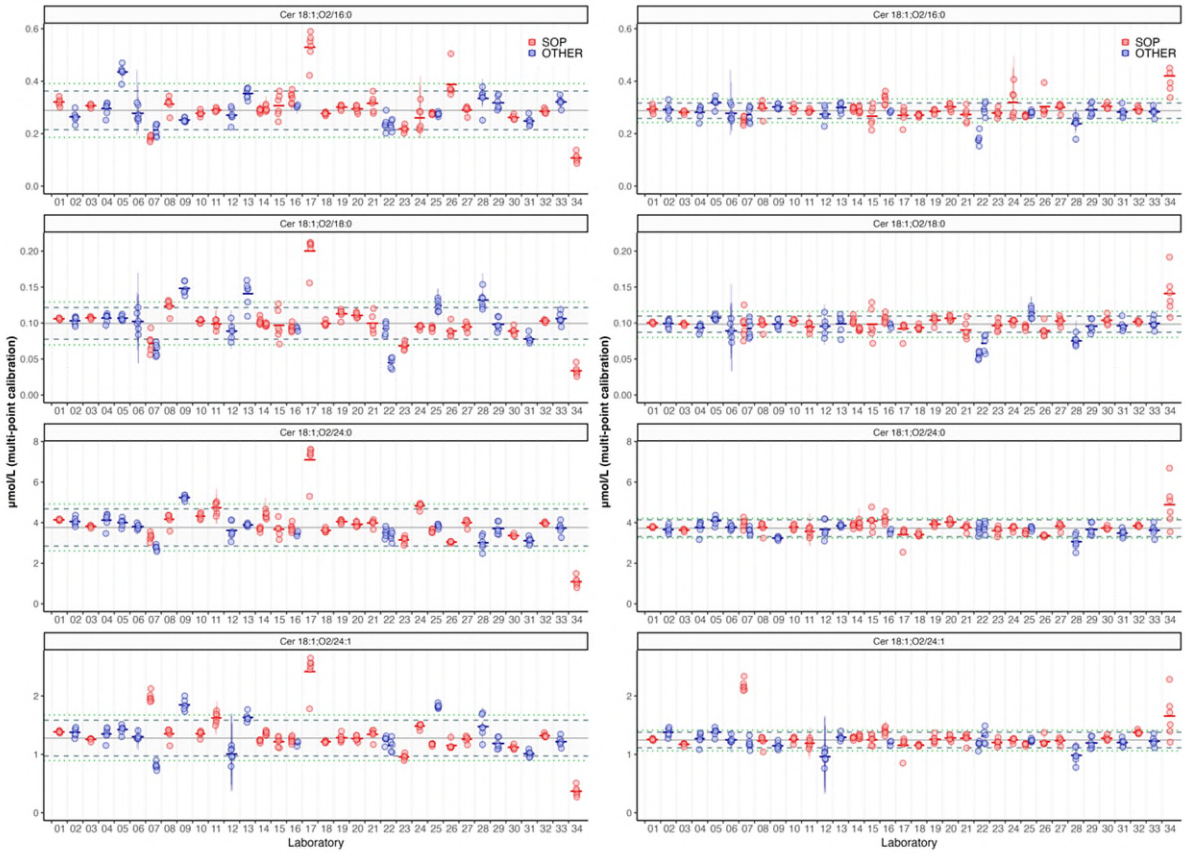
<u>Supplementary Figure 5</u> – Comparison of concentration values obtained in this and previous studies covering ceramides by MS-platforms in NIST SRM 1950 with the full (A) and outlier-filtered (B) ILS Ceramides dataset. Comparison of inter-laboratory and/or cross-platform trials reporting ceramide concentrations in NIST SRM 1950. Each open circle corresponds to the reported value from one laboratory in each of the respective studies referenced by first author and year of publication. (sp) refers to a single, standardized protocol and platform employed by all laboratories while (mp) denotes studies in which multiple protocols and platforms were used. The red boxes and numbers indicate median ± MAD (mean absolute deviation) and the number of participating laboratories, respectively. Depending on the precise nature of the study and/or reporting laboratory, concentrations were either reported with sphingoid-base and fatty acyl chain details (e.g. Cer 18:1;O2/16:0, left panel) or as sum compositions (i.e. Cer 34:1;O2, left panel). In the latter case, isobaric ceramide species other than the 4 ceramides targeted in this study may therefore have contributed to the reported concentrations. Details of the individual studies are presented in Table S3. No outlier removal has been applied for any of the studies. Source data are provided as Source Data file.



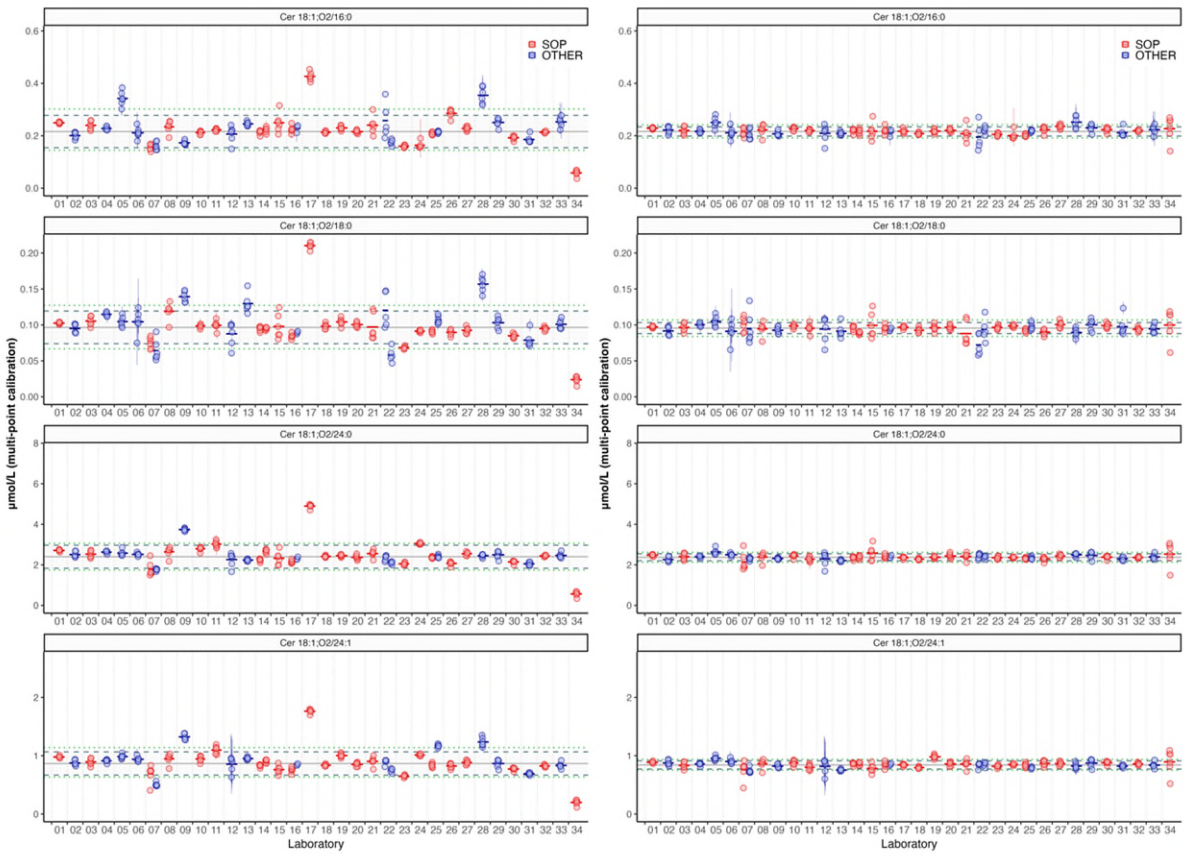
<u>Supplementary Figure 6</u> – Authentic vs non-authentic internal standard normalization and its effects on quantification bias and variability. Concentration of ceramides in SRM 1950 RM for the full (A) and outlier-filtered (B) dataset are based on multi-point calibration. The horizontal bars correspond to the mean and ±1×SD of all points per group. Data originating from the same laboratory are connected by dashed lines. SOP (red) and OTHER (blue) refer to the Standard vs other methods, respectively. Outliers in the authentic internal standard groups have been removed (see Fig. 1 and text). In panel B, outliers (found in the authentic internal standard datasets) have been removed in all groups for each ceramide species (see Fig.1 and text). Dataset 11 was excluded for Cer16:0 in panels A and B, as it showed 15x higher concentration than the second highest data point for C24:0 D7 normalization. The number of data points is indicated for each dataset. Source data are provided as Source Data file.



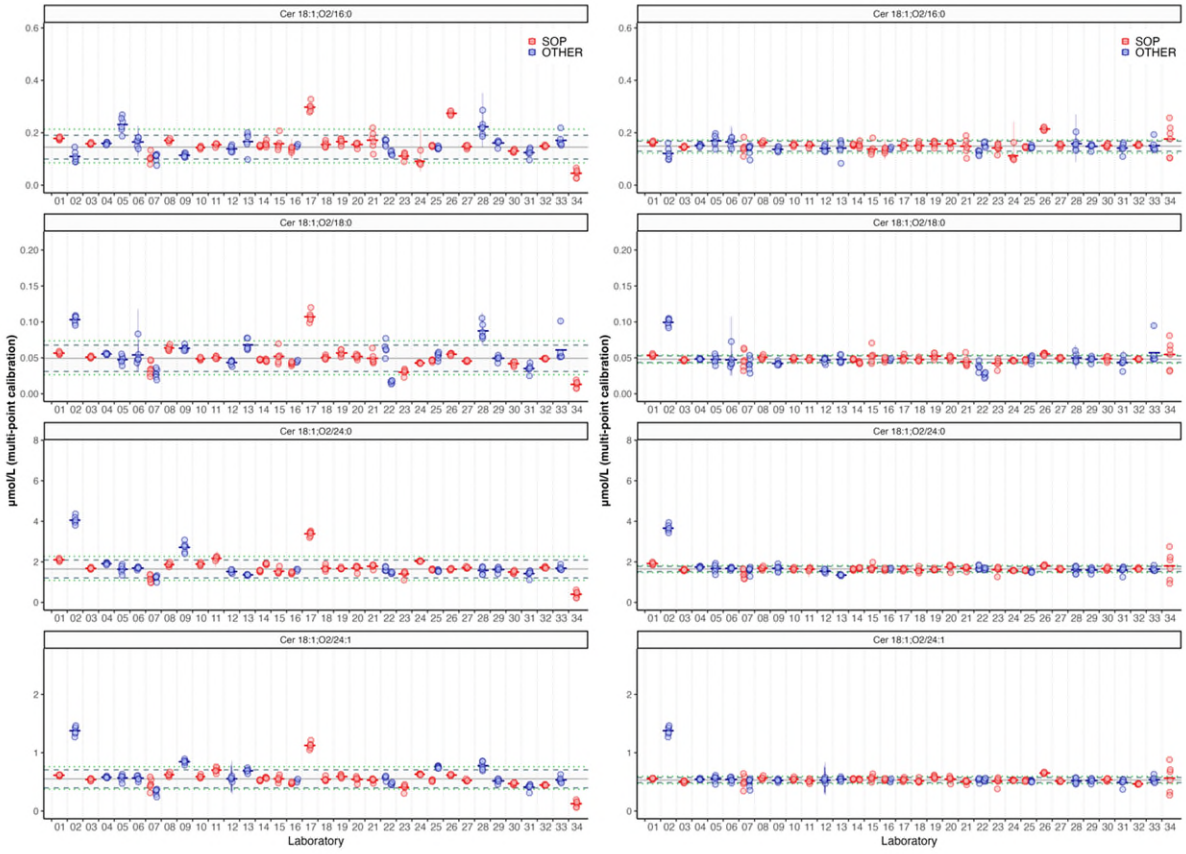
<u>Supplementary Figure 7</u> – Authentic vs non-authentic internal standard normalization and its effect on quantitation bias and variability for SRM 1950 RM when using single-point (A) and multi-point (B) calibrated concentrations. The horizontal bars correspond to the mean and ±1×SD of all points per group. Data originating from the same laboratory are connected by dashed lines. SOP (red) and OTHER (blue) refer to the Standard vs other methods, respectively. The full dataset is shown here, except of dataset 11 in Cer16:0, excluded from panels A and B as it showed 15x higher concentrations than the second highest data point for C24:0 D7 normalization. The number of data points is indicated for each dataset. Source data are provided as Source Data file.



Supplementary Figure 8. Re-calibration of hTAG using SRM 1950 after multi-point calibration. All datasets are shown, while the indicated %CVs were calculated after exclusion of outliers (see Fig. 1 and text). Source data are provided as Source Data file.



Supplementary Figure 9. Re-calibration of DB using SRM 1950 after multi-point calibration. All datasets are shown here, while the indicated %CVs were calculated after exclusion of outliers (see Fig. 1 and text). Source data are provided as Source Data file.



Supplementary Figure 10. Re-calibration of YAA using SRM 1950 after multi-point calibration. All datasets are shown here, while the indicated %CVs were calculated after exclusion of outliers (see Fig. 1 and text).

SUPPLEMENTARY TABLES

Supplementary Table 1. Concentrations and inter-lab %CVs in all reference materials. ALL and Filt correspond to values calculated from the full or the outlier-filtered dataset, respectively. All concentrations were calculated using multi-point calibration. *n* corresponds to the number of datasets. Source data provided as a Source Data file.

Matrix	Ceramide	µmol/L (ALL)					µmol/L (Filt)				
		n	mean	SD	median	%CV	*n*	mean	SD	median	%CV
SRM	Cer 18:1;O2/16:0	39	0.2500	0.0612	0.2481	24.5	35	0.2438	0.0336	0.2468	13.8
SRM	Cer 18:1;O2/18:0	39	0.0879	0.0269	0.0857	30.6	31	0.0835	0.0097	0.0844	11.6
SRM	Cer 18:1;O2/24:0	39	2.4888	0.6266	2.4439	25.2	35	2.4116	0.2441	2.4368	10.1
SRM	Cer 18:1;O2/24:1	39	0.9039	0.2422	0.8739	26.8	31	0.8554	0.0724	0.8563	8.5
hTAG	Cer 18:1;O2/16:0	39	0.2914	0.0667	0.2902	22.9	35	0.2888	0.0369	0.2902	12.8
hTAG	Cer 18:1;O2/18:0	39	0.1004	0.0274	0.0994	27.3	31	0.0995	0.0110	0.0994	11.0
hTAG	Cer 18:1;O2/24:0	39	3.8190	0.8533	3.8073	22.3	36	3.7641	0.4587	3.7790	12.2
hTAG	Cer 18:1;O2/24:1	39	1.3186	0.3307	1.2701	25.1	33	1.2784	0.1534	1.2606	12.0
DB	Cer 18:1;O2/16:0	39	0.2242	0.0594	0.2219	26.5	35	0.2161	0.0309	0.2155	14.3
DB	Cer 18:1;O2/18:0	39	0.0993	0.0286	0.0979	28.8	32	0.0966	0.0114	0.0975	11.8
DB	Cer 18:1;O2/24:0	39	2.4525	0.6096	2.4428	24.9	36	2.4013	0.2847	2.4289	11.9
DB	Cer 18:1;O2/24:1	39	0.8919	0.2358	0.8757	26.4	33	0.8663	0.0996	0.8663	11.5
YAA	Cer 18:1;O2/16:0	39	0.1539	0.0451	0.1541	29.3	34	0.1450	0.0227	0.1504	15.6
YAA	Cer 18:1;O2/18:0	39	0.0514	0.0183	0.0494	35.6	34	0.0494	0.0092	0.0494	18.6
YAA	Cer 18:1;O2/24:0	39	1.7503	0.5800	1.6475	33.1	35	1.6485	0.2217	1.6364	13.4
YAA	Cer 18:1;O2/24:1	39	0.5842	0.2006	0.5520	34.3	33	0.5518	0.0775	0.5432	14.0

Supplementary Table 2. Concentrations and inter-lab %CV of ceramide species from the two protocol types. The mean concentrations given in µmol/L are based on multi-point calibration after exclusion of outliers. The *P* values are from the comparison of individual values reported by SOP vs OTHER protocols (Welch's two-sided *t*-test). *n* corresponds to the number of datasets after outlier removal. Source data provided as a Source Data file

Matrix	Ceramide	*n* (SOP)	*n* (OTHER)	µmol/L (SOP)	µmol/L (OTHER)	*P* value	%CV (SOP)	%CV (OTHER)
SRM	Cer 18:1;O2/16:0	21	14	0.2438	0.2439	0.993	12.9	15.5
hTAG	Cer 18:1;O2/16:0	21	10	0.0827	0.0851	0.502	12.4	10.2
DB	Cer 18:1;O2/16:0	20	15	2.4463	2.3652	0.319	11.0	8.7
YAA	Cer 18:1;O2/16:0	20	11	0.8575	0.8517	0.823	9.2	7.4
SRM	Cer 18:1;O2/18:0	20	15	0.2954	0.2801	0.244	11.4	14.4
hTAG	Cer 18:1;O2/18:0	20	11	0.0994	0.0999	0.906	10.7	12.2
DB	Cer 18:1;O2/18:0	21	15	3.8826	3.5983	0.060	12.1	11.2
YAA	Cer 18:1;O2/18:0	20	13	1.2808	1.2745	0.915	10.9	14.1
SRM	Cer 18:1;O2/24:0	21	14	0.2177	0.2135	0.702	14.2	14.9
hTAG	Cer 18:1;O2/24:0	21	11	0.0945	0.1005	0.180	11.3	12.1
DB	Cer 18:1;O2/24:0	21	15	2.4446	2.3406	0.262	12.9	9.9
YAA	Cer 18:1;O2/24:0	21	12	0.8664	0.8662	0.995	12.7	9.6

SRM	Cer 18:1;O2/24:1	20	14	0.1468	0.1424	0.590	15.4	16.4
hTAG	Cer 18:1;O2/24:1	21	13	0.0482	0.0512	0.411	15.3	22.6
DB	Cer 18:1;O2/24:1	21	14	1.7039	1.5653	0.052	14.0	10.7
YAA	Cer 18:1;O2/24:1	21	12	0.5467	0.5609	0.645	13.0	16.1

Supplementary Table 3. Inter-laboratory/platform trials measuring ceramides in NIST SRM 1950. Source data available from the publications reported in the 'Study' column (see main text for reference details).

Study	**Year**	**ISTD**	**Extraction**	**Platform**	**Laboratories (Datasets)**
Quehenberger at al. (LIPID MAPS)	2010	Cer18:1;O2/12:0	$CH_3OH/CHCl_3$ + KOH-Hydrolysis	RP-LC/MRM	1 (1)
Bowden et al.	2017	Multiple (in-house)	Multiple (in-house)	Multiple	18 (18)
Thompson et al	2019	Unknown	CH_3OH	FIA-HRMS (Biocrates p400HR)	14 (14)
Ghorasaini et al.	2021	Cer18:1;O2[d2]/16:0	(1) MTBE (2) Bligh-Dyer	FIA-IMS-MRM (Lipidyzer)	9 (9)
This study (ILS Ceramides)	2023	Cer18:1;O2[D7]/16:0 Cer18:1;O2[D7]/18:0 Cer18:1;O2[D7]/24:0 Cer18:1;O2[D7]/24:1	(1) SOP: ethyl acetate:IPA Multiple (in-house)	SOP: RP-LC/MRM Multiple	34 (39)

SUPPLEMENTARY NOTES

Supplementary Note 1. Standard Operating Protocol Harmonization Ceramides

Summary: This protocol describes the extraction and LC-MRM procedure for absolute quantification of Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/24:0 and Cer d18:1/24:1 in human plasma according to Kauhanen et al, Anal Bioanal Chem (2016) 408:3475–3483.
A calibration curve is built by injecting several dilutions of the non-labelled standards mixed with a fixed concentration of labelled standards. Each calibration point is prepared and analysed in triplicates (n=3).
To establish the endogenous concentration of the four selected ceramides in the study samples (NIST SRM1950, NIST high TAG, NIST T1D, and NIST young AA) each sample is extracted and analyzed in six replicates (n =6).
Prepare the pooled plasma quality control sample (pooled plasma QC) by mixing 100 µL each of NIST SRM1950, NIST high TAG, NIST T1D, and NIST young AA (to obtain in total 400 µL of pooled plasma).
We suggest proceeding first to measure the ceramides concentration in the four study samples and then continue with the intra-assay validation to avoid wasting material.

Extraction

Table 1.

Chemicals, solvents and materials
Ethyl acetate (EtAc)
2-propanol (IPA)
5% Bovine Serum Albumin (BSA)
96-well plates/tubes
Sealing foil
2 mL deep well plates/tubes/LC vials

Table 2.

Sample type			
Pooled Quality Control (QC) samples	10 µL pooled plasma QC	20 µL Labelled IS	570 µL EtAc:IPA 2:8 (v/v)
Matrix Blank (MB)	10 µL Matrix (pooled plasma QC) only	-	590 µL EtAc:IPA 2:8
Study Samples (S)	10 µL Human plasma (NIST SRM1950, high TAG, T1D, young AA)	20 µL Labelled IS	570 µL EtAc:IPA 2:8
Total Blank (TB)	10 µL 5% BSA	-	590 µL EtAc:IPA 2:8
Calibration line standards (STD)	10 µL 5% BSA + 20 µL non-labelled STDx mixture (Table 5)	20 µL Labelled IS	550 µL EtAc:IPA 2:8

Table 3. **Labelled internal standard** mixture solution (provided). Keep at -20° C. Equilibrate at RT and sonicate 10 min before use.

Labelled internal standard mixture in EtAc:IPA 2:8 (v/v)	Concentration, pmol/µL [µM]	Vendor
D7-Cer d18:1/16:0	0.125	Avanti Polar Lipids
D7-Cer d18:1/18:0	0.050	Avanti Polar Lipids
D7-Cer d18:1/24:0	1.500	Avanti Polar Lipids
D7-Cer d18:1/24:1	0.500	Avanti Polar Lipids

Table 4. **STD1** mixture solution (provided)

Non-labelled standards in EtAc:IPA 2:8 (v/v)		Concentration [pmol/µL]	Vendor
Non-labelled standards mixture	Cer d18:1/16:0	2	Avanti Polar Lipids
	Cer d18:1/18:0	2	Avanti Polar Lipids
	Cer d18:1/24:0	20	Avanti Polar Lipids
	Cer d18:1/24:1	20	Avanti Polar Lipids

Table 5. Serial dilutions of STD1 non-labelled standards **for calibration line**.

Add 20 µL of		**STD1**	STD2	STD3	STD4	STD5	STD6	
Non-labelled STD mixture	Cer d18:1/16:0	**2.00**	1.00	0.10	0.02	0.01	0.008	pmol/µL
	Cer d18:1/18:0	**2.00**	1.00	0.10	0.02	0.01	0.008	
	Cer d18:1/24:0	**20.00**	10.00	1	0.20	0.1	0.08	
	Cer d18:1/24:1	**20.00**	10.00	1	0.20	0.1	0.08	

Extraction procedure:

1) Prepare different dilutions of STD1 by diluting the provided mixture (table 4) with EtAc:IPA 2:8 as suggested in Table 5.
2) Thaw plasma samples on ice (+4°C) and bring them to room temperature prior to extraction. Mix by pipetting up and down several times before use.
3) Prepare samples in plates/tubes manually or with a robotic liquid handler, according to specifications in Column 2 of Table 2.
4) Add 20 µL of labelled internal standard mixture to samples (Column 3 Table 2).
5) For the calibration line, add 20 µL of different dilutions of the non-labelled mixture (STD1-6).
6) Add 590 µL of ethyl acetate:isopropanol (2:8, vol/vol) to Total Blank and Matrix Blank; 550 µL to calibration line (STD); 570 µL to study samples and QCs (Column 4 Table 2)
7) Mix samples 10 min by automated pipetting or vortexing
8) Centrifuge samples for 10 min at 3000 x *g* at room temperature
9) Transfer 50 µL of the clear supernatant to MS vials or MS plates, close/seal, store samples at -20°C prior to LC-MRM analysis.

LC-MRM

Table 6.

Chemicals and solvents
Ammonium acetate
Formic acid
Ultra-pure water
Acetonitrile

Table 7.

LC-MS details	Specifications
Mass spectrometry	QQQ
LC	UPLC
Injection volume (indicative)	5 µL
Weak wash	Water
Strong wash	Acetonitrile
Pre-column	Waters Acquity BEH C18, 1.7 µm VanGuard Pre-Column
Column	Waters Acquity BEH C18, 2.1 × 50 mm id. 1.7 µm
Column temperature	60 °C
Mobile phase A	10 mM ammonium acetate in water with 0.1% formic acid
Mobile phase B	10 mM ammonium acetate in acetonitrile:2-propanol (4:3, vol/vol) with 0.1% formic acid

Table 8.

LC gradient			
Time	A%	B%	Flow (µL/min)
0.00	15	85	500
0.50	15	85	500
1.50	0	100	500
4.00	0	100	500
4.10	15	85	500
5.00	15	85	500

Table 9 (example based on the published reference).

MS conditions (this only applies to Sciex QTRAP 6500)	
Polarity mode	Positive
Ion spray	5000 V
Curtain gas	25 psi
Source temperature	300 °C
Gas 1	50 psi
Gas 2	35 psi
DP	30 V
EP	10 V
Collision cell exit potential	20 V
Collision energy CE	40 eV

Table 10.

Mass transitions used for absolute quantification. With different systems RT and CE might be different.					
Analyte	Mass transitions (MRM)	IS	Mass transitions (MRM)	RT (min)	CE (not applicable to different instruments)
Cer d18:1/16:0	538.5 – 264.3 $C_{34}H_{68}NO_3 – C_{18}H_{34}N$	D7- Cer d18:1/16:0	545.6 - 271.3	1.9-2.0	40
Cer d18:1/18:0	566.6 – 264.3 $C_{36}H_{72}NO_3 – C_{18}H_{34}N$	D7- Cer d18:1/18:0	573.6 – 271.3	2.1-2.2	40
Cer d18:1/24:0	650.6 – 264.3 $C_{42}H_{84}NO_3 – C_{18}H_{34}N$	D7- Cer d18:1/24:0	657.7 – 271.3	2.4-2.5	40
Cer d18:1/24:1	648.6 – 264.3 $C_{42}H_{82}NO_3 – C_{18}H_{34}N$	D7- Cer d18:1/24:1	655.7 – 271.3	2.3-2.4	40

Suggested sample sequence. Samples and QC for intra-assay validation can be measured in one single batch

Total Blank 1
Matrix blank
Calibration line 1 from STD6 to 1 (n=3)
[…]
Pooled QC
Pooled QC
SRM 1950-1
SRM 1950-2
SRM 1950-3
SRM 1950-4
SRM 1950-5
SRM 1950-6
Pooled QC
Pooled QC
T1D-1
T1D-2
T1D-3
T1D-4
T1D-5
T1D-6
Total Blank 2
Pooled QC
Pooled QC
Young AA-1
Young AA-2
Young AA-3
Young AA-4
Young AA-5
Young AA-6
Pooled QC
Pooled QC
highTAG-1

highTAG-2
highTAG-3
highTAG-4
highTAG-5
highTAG-6
Pooled QC
Pooled QC
Calibration line 2 from STD1 to 6 (n=3)
LLQC 1 LLQC 2 LLQC 3 LLQC 4 LLQC 5 LLQC 6 LQC 1 LQC 2 LQC 3 LQC 4 LQC 5 LQC 6 MQC 1 MQC 2 MQC 3 MQC 4 MQC 5 MQC 6 HQC 1 HQC 2 HQC 3 HQC 4 HQC 5 HQC 6 HLQC 1 HLQC 2 HLQC 3 HLQC 4 HLQC 5 HLQC 6
Matrix blank
Total Blank 3

Clarifications: In the corresponding Excel table we have 3 cells for each STD concentration (1-6), so we mean that we inject only once from each vial (each vial is a replicate that you prepared). The calibration curves are 2: one at the beginning and one at the end.
Then for the study samples we ask to prepare 6 replicates. Then in the Excel we have 3 cells for each sample, which means this time that each vial is injected 3 times.

Table 11.

Data processing	
Processing software	(Add here name and version of the software used)

Quality controls for intra-assay variation

Prepare the pooled plasma QC sample containing equal volumes of the four NIST reference plasma you received.
The pooled QC representing middle quality control (MQC), low QC (LQC), and lowest level of QC (LLQC) is prepared by diluting MQC (e.g. two-(1:1) and three-fold (1:2)) with water. High QC (HQC) and highest level of QC (HLQC) are prepared by adding to MQC additional 10 µL and 20 µL respectively of STD3 endogenous standards from table 5. Internal standards are added to all QC samples.

Only intra-assay variation will be measured

The precision and accuracy of the assay is determined for each of the four ceramides measured in LLQC, LQC, MQC, HQC, and HLQC.
Intra-assay variation, precision, and accuracy is calculated for each of the ceramide in replicates of six (n = 6) at each QC concentration.

The intra-assay precision (percentage coefficient variance, %CV) and accuracy (percentage accuracy, %Accuracy) are calculated from the nominal concentrations according to the formulas reported in the corresponding Excel tables attached to this document.

Recovery estimation is not necessary for the published method but only for different ones.
Stability estimation is not necessary for the published method but only for different ones.
Calibration line is prepared in 5% BSA in water, no specific purity/type of BSA is required.

Results

Please **report the peak areas** values for Calibration lines, Samples and Intra assay QC in tables 12-15 of the corresponding Excel file.