SUPPLEMENTARY INFORMATION

LipidQuant 1.0: automated data processing in lipid class separation - mass spectrometry quantitative workflows

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1. Materials and methods

1.1 Chemicals and lipid standards

Chemicals and solvents (LC/MS grade, Chromasolv-Honeywell, Riedel-de Haën, Germany) were purchased from Sigma Aldrich (St. Louis, MI, USA) or Merck (Darmstadt, Germany). The following nonendogenous lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) or Nu-Chek Prep (Elysian, MN, USA) and used as internal standards (IS) for the quantitative analysis: MG 19:1/0:0/0:0, DG 12:1/0:0/12:1, TG 19:1/19:1/19:1, D7-CE 16:0, Cer d18:1/12:0, D7-cholesterol, LPC 17:0/0:0, LPE 14:0/0:0, PC 14:0/14:0, PE 14:0/14:0, SM d18:1/12:0, PS 14:0/14:0, PA 14:0/14:0, PG 14:0/14:0, LPG 14:0/0:0, HexCer d18:1/12:0, Hex2Cer d18:1/12:0, and SHexCer d18:1/12:0. Carbon dioxide (scCO$_2$) with 99.995% purity was purchased from Messer (Bad Soden, Germany).

1.2 Samples

Human serum samples were isolated from the whole blood, drawn into tubes without anticoagulant (Sarstedt S-Monovette, Germany), incubated at room temperature for 60 min, centrifuged at 1500 × g for 15 min, the supernatant was transferred to Eppendorf tubes, and immediately frozen at -80°C until the extraction. The study was approved by the institutional ethical committee. All donors signed the informed consent. In total, 43 samples from female donors with an average age of 47 years and 22 samples of male donors with the average age of 44 years were investigated. The QC sample was a pooled sample from all serum samples.

1.3 Internal standard mixture

Stock solutions of all IS in the range of 0.25 to 2.1 µg/µL were prepared and mixed to obtain an IS mixture for spiking. The final concentrations of IS were reported in Table 1 in nmol/mL serum.

Table 1. Concentrations of IS for individual lipid classes

Lipid class	Internal standard	Concentration [nmol/ml]
Monoacylglycerols	MG 19:1/0:0/0:0	113.4
Diacylglycerols	DG 12:1/0:0/12:1	46.4
Triacylglycerols	TG 19:1/19:1/19:1	113.3
Cholesterol esters	D7-CE 16:0:	443.3
Ceramides	Cer d18:1/12:0	11.6
Cholesterol esters	D7-cholesterol	1066.8
Lysophosphatidylcholines	LPC 17:0/0:0	57.7
Lysophosphatidylethanolamines	LPE 14:0/0:0	13.2
Phosphatidylcholines	PC 14:0/14:0	124
Phosphatidylcholines	PE 14:0/14:0	8.8
Sphingomyelins	SM d18:1/12:0	43.3
Phosphatidylserines	PS 14:0/14:0	8.0
Phosphatidic acids	PA 14:0/14:0	9.1
Phosphatidylglycerols	PG 14:0/14:0	4.1
Lysophosphatidylglycerols	LPG 14:0/0:0	43
Hexosylceramide	HexCer d18:1/12:0	6.9
Dihexosylceramide	Hex2Cer d18:1/12:0	7.0
Sulfatides	SHexCer d18:1/12:0	0.1

1.4 Extraction

A modified Folch procedure was used for lipid extraction. Human serum (25 µL), and the mixture of IS (17.5 µL) were homogenized in 3 mL of chloroform - methanol (2:1, v/v) for 10 min in an ultrasonic bath (40°C). When the samples reached ambient temperature, 600 µL of water were added, and the mixture was vortexed for 1 min. After 3 min of centrifugation (3000 rpm), the aqueous layer was removed, and the organic layer was evaporated under a gentle stream of nitrogen. The residue was dissolved in a mixture of 500 µL of chloroform - 2-propanol (1:1, v/v), carefully vortexed, and filtered (0.2 µm syringe filter). The extract was diluted 1:20 with the mixture of hexane - 2-propanol - chloroform (7:1.5:1.5, v/v/v) for ultrahigh-performance supercritical fluid chromatography – mass spectrometry (UHPSFC/MS) analysis.

1.5 Analysis

UHPSFC/MS measurements were carried out on an Acquity Ultra Performance Convergence Chromatography (UPC^2) system hyphenated to the hybrid quadrupole - traveling

wave ion mobility time-of-flight mass spectrometer Synapt G2 Si from Waters by using the commercial interface kit (Waters, Milford, MA, USA). The instrumental setting was the same as in the previous works (Lísa *et al.*, 2015; Lísa *et al.*, 2017). The lipid class separation was achieved by employing a Viridis BEH column (Waters, 100 x 3 mm, 1.7 µm) and the gradient elution. The mobile phase A was $scCO_2$, and the mobile phase B and make-up solvent were MeOH with 1% water and 30 mM NH_4OAc. The linear gradient was employed: 0 min - 1% B, 5 min - 51 % B, 6.5 min - 51% B, 6.8 min - 1% B. The total run time was 7.5 min. The column temperature was 60°C, the automatic back-pressure regulator was set to 1800 psi, the flow rate to 1.9 mL/min, the injection volume to 1 µL, and the make-up flow rate to 0.25 mL/min. Electrospray ionization in the positive-ion mode was used, and the mass range was set to *m/z* 50-1200 in the sensitivity mode. The continuum mode with a scan rate of 0.15 s was used for the analysis. The peptide leucine enkephalin was used as the lock mass with the scan time of 0.1 s and the interval of 30 s. The lock mass was scanned but not automatically applied for mass calibration correction. All samples were measured in duplicate.

1.6 Data processing

The noise reduction was performed on the raw files after measurements using the Waters compression tool. Afterwards, the files were lock mass corrected and converted into centroid data using the exact mass measure tool from Waters. Retention time ranges or mass scan ranges of individual lipid classes were determined by comparing the first and last measured samples to verify that the lipid class peak was still within the determined range even in case of possible retention time shifts. For each lipid class, the combined mass scan range of each lipid class was prepared by MarkerLynx XS (Waters). The peak separation window was 0.05 Da, and the intensity threshold was 3000 counts. Each method was applied for all quantified lipid classes in all samples within the sequence to obtain a summary table containing all features within the defined *m/z* range together with intensities for all samples in MarkerLynx XS. These tables obtained for each lipid class were exported as txt file and further processed by LipidQuant 1.0. The similar protocol may be used for data measured by mass spectrometers from other manufacturers to obtain the final txt file suitable for LipidQuant 1.0 processing.

1.7 Statistical analysis and visualization

SIMCA software, version 13.0 (Umetrics, Umeå, Sweden) was used to perform unsupervised principal component analysis (PCA) and supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA). The scatter plots of the first and second components are shown for PCA. OPLS-DA separates samples into predefined classes, *i.e.,* gender. The results table from LipidQuant 1.0 was copied into the SIMCA software, the studied lipids were defined as variables, and samples were defined as different observations. The data were pretreated by logarithmic transformation, centering, Pareto scaling, and evaluation of outliers. The logarithmic transformation aims to convert each lipid species into a Gaussian distribution. The centering relates the relative changes of lipid species to the average, where the Pareto scaling compensates the concentration differences of lipid species. The scaling allows that low abundant species contribute to the model to the same extent by dividing the centered species by the root of the standard deviation (Pareto scaling). To evaluate lipids of statistical relevance, a two-sided two sample T-test assuming unequal variances (Welch test) was performed for female and male samples in Microsoft Excel. P-values <0.05 were considered to indicate statistical significance. For better visualization of differences in lipid concentrations between males and females, the S-plot was generated from the OPLS-DA plot (in SIMCA), box plots were constructed in R free software environment (https://www.r-project.org) using readxl and ggplot2 packages and diagram types from Microsoft Excel.

2. Discussion

2.1 General remarks

Lipid class separation hyphenated with high-resolution MS allows the robust analysis of lipids with a high coverage of the lipidome (Holčapek, M. *et al.*, 2018). The identification of lipids is based on the accurate *m/z* values and retention times. The separation is based on the interaction of the lipid head group with the stationary phase, therefore isomers differing in the fatty acyl chain structure are not chromatographically resolved or only partially separated. Hence, only the sum composition of lipid species can be analyzed resulting in less lipid identifications in comparison to chromatographic methods resolving isomers, *i.e.,* reversed-phase UHPLC/MS. However, lipid class separation approaches have the advantage that the analysis times can be kept relatively short with usually less than 10 min, which allows high-throughput lipidomic analysis. The main issue is that the coelution of lipid class IS and analytes from the same class guarantees the coionization, which is a key requirement for robust MS-based quantitation due to the identical matrix effects for IS and analytes. LipidQuant 1.0 was developed to deal with large data sets from clinical cohorts generated with high-throughput lipid class separation techniques hyphenated with QTOF or other high-resolution mass spectrometers, which allows the automated lipid identification and quantitation of 2500 samples in one batch or unlimited amount of samples in multiple processing batches. The successful application of LipidQuant 1.0 has been proven in our recent lipidomic studies (Chocholoušková M. *et al.*, 2021; Peterka, O. *et al.*, 2020; Wolrab, D. *et al.*, 2020a; Wolrab, D. *et al.*, 2020b; Wolrab D. *et al.*, 2021).
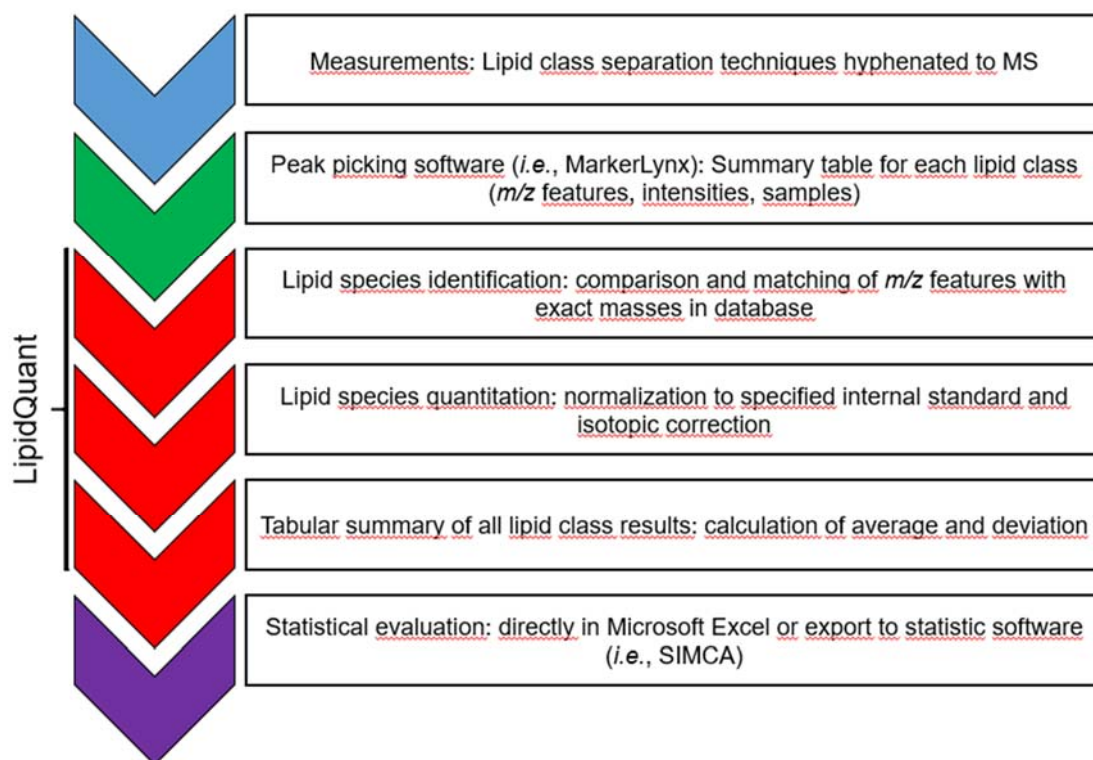
2.2 LipidQuant 1.0

2.2.1 Input

MarkerLynx (Waters) generates summary tables of all *m/z* features with the corresponding intensities in the measured samples, which can be exported as txt files. This txt file can be imported into LipidQuant 1.0 for the identification using the embedded database and the quantitation of lipid species. The LipidQuant 1.0 is applicable for MS data from any vendor, but it only requires to arrange the data in a summary table for each lipid class containing *m/z* features with the corresponding intensities for all samples. The output of peak picking software

has to contain *m/z* features in the first column with the heading of *m/z*, followed by the individual samples containing the intensity or other quantitative measures for each *m/z* feature.

2.2.2 Structure of LipidQuant 1.0

LipidQuant 1.0 (**Fig. 1**) was designed for lipid data processing obtained with lipid class separation techniques hyphenated to QTOF, *e.g.*, UHPSFC/MS (Lísa *et al.*, 2015; Chocholoušková M. *et al.*, 2021), hydrophilic interaction liquid chromatography (HILIC)/MS (Chocholoušková M. *et al.*, 2021; Peterka, O. *et al.*, 2020; Wolrab, D. *et al.*, 2020a; Wolrab, D. *et al.*, 2020b), or normal-phase ultrahigh-performance liquid chromatography (UHPLC)/MS (Holčapek *et al.*, 2015). LipidQuant 1.0 is based on a Microsoft Excel script, therefore it can be used on every PC without any special requirements of software licenses, and it is flexible for modifications according to the needs of the acquired data and the employed method. The following requirements have to be fulfilled: decimal numbers separated by points instead of commas, all macros allowed, and workbook calculations set to automatic calculations. LipidQuant 1.0 is structured in various sheets, where individual sheets represent a lipid class database, together with the IS information and the isotopic correction percentages. In the support sheet, it is necessary to define the number of lipid species included for each lipid class and the name according to the individual sheet names. The database of individual lipid species for each lipid class was created by collecting the exact masses from the elemental composition and the isotopic abundances of M+2 isotopes using the Isotope Pattern tool from Bruker Daltonics. The isotopic correction is applied for lipid species within the lipid class differing by DB, as the intensity of lipid species mass peak can be the sum of M+2 and the lipid species with one DB less, when applying lipid class separation methods. The lipid species in the database were annotated in line with the shorthand notation for lipids derived from MS data (Liebisch *et al.*, 2013), *i.e.*, the lipid class abbreviation followed by the total carbon number in fatty acyl chains and DB number, *e.g.*, PC 38:2.

Fig. 1. Overview of the LipidQuant 1.0 workflow for automated processing of lipidomic data obtained using any lipid class separation technique hyphenated with high-resolution MS.

The general LipidQuant 1.0 structure is based on different table sheets (**Fig. 2**), whereby the first sheet named "Start" is mainly used for data processing. The following sheets represent the databases for individual lipid classes, containing the exact mass of the lipid species, the annotation of the lipid species, the percentage of isotopic correction, the tolerance range for lipid identification, and information about the IS. In general, lipid classes not required for data processing can be removed by deleting the table sheet, *i.e.,* when lipid classes are not detected in the sample, *i.e.,* due to the lack of sensitivity of the method. However, special care has to be taken that the "Support" sheet has to be updated regarding the included lipid class databases and their list position. Furthermore, the database of lipid species can be modified by adding or removing lipid species and also the lipid classes on additional sheets. However, the general alignment has to be followed together with all information, like exact mass, lipid species annotation, isotopic correction, and the information of the position of the IS, which should be applied for the quantitation. In the "Support" sheet, the number of lipid species included in the database has to be defined for all lipid classes. Furthermore, it has to be defined in the "Support" sheet, if the samples were measured as single or multiple injections. For multiple injections, the
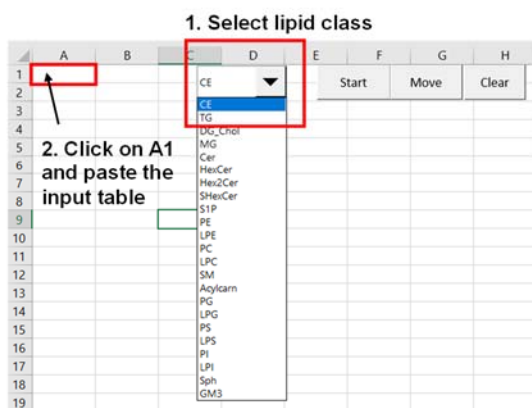
repetitive samples have to be subsequently ordered in the summary table generated by MarkerLynx or other peak picking software. The final sheets are for summarizing the quantitative results in the "Results" sheet, all concentrations in all samples for all lipid classes can be inserted by pressing the button "Insert data". For multiple injections, the summary table for the average of lipid concentrations and the standard deviation for the repetitive samples is generated in the sheets "Average" and "Deviation".
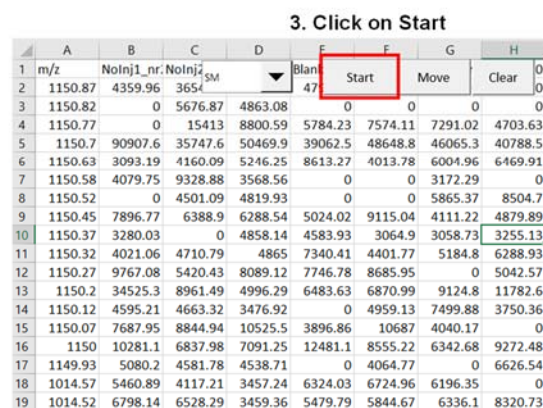

Fig. 2. Overview of LipidQuant 1.0 workflow (description of individual steps is explained in the discussion).

2.2.3 Identification via embedded database

The embedded database of lipids is based on lipid species typically identified in body fluid samples (plasma, serum, or urine), cell lines, and tissues mainly from human biological samples based on previous works and experiences in our group (Holčapek *et al.*, 2018; Wolrab *et al.*, 2019). The LipidQuant 1.0 has separate versions for both polarity modes, where the positive-ion mode database contains 23 lipid classes with 1470 lipid species, and the negative-ion mode database contains 24 lipid classes with 1999 lipid species (versions for formate and acetate adducts). The lipid species in the database are in line with Lipid MAPS (Fahy, E. *et al.*, 2009) and shorthand nomenclature for lipid species derived from MS (Liebisch, G. *et al.*, 2013). The first step is to define the mass tolerance window in the individual database sheets according to the instrumental characteristics and measurement conditions. For HILIC-UHPLC and UHPSFC hyphenated to QTOF, a tolerance window of $\pm$ 0.01 Da or $\pm$ 0.005 Da are commonly employed. The lipid class of interest is selected in the dropdown list, and the summary table from the peak picking software for the defined lipid class is copied in cell A1 of the "Start" sheet. The *m/z* feature filtering is applied when the button "Start" is pressed. The exact masses of the lipid species defined in the database are compared with experimentally obtained *m/z* features, and annotated with a color tag, when the *m/z* feature is within the defined mass tolerance window or within two times the specified mass tolerance range. Lipid species marked as green are within the specified mass range, and only a single *m/z* feature fulfilled this criterion. Lipid species marked as red are within the specified mass range, but more than one *m/z* feature fulfills this criterion. Lipid species marked as yellow are within two times the mass tolerance range.

2.2.4 Quantitation and isotopic correction

After the identification of all lipid species for a given sample, the isotopic correction has to be performed in the lipid class mass spectrum. The deisotoping calculation algorithm starts from the left side of the mass spectrum (*i.e.*, the lowest detected *m/z* value), and then the theoretical M+2 isotopic contribution is subtracted from *m/z* feature with *m/z* value two units higher. The same step is repeated for all observed *m/z* values in the lipid class mass spectrum until the last *m/z* value on the right side of the mass spectrum (*i.e.*, the highest detected *m/z* value) is reached.

For the quantitation of identified lipid species, it is necessary to define the IS used for quantitation together with the known concentration in the upper orange colored panel of each lipid class database. In total, 3 IS can be defined, where the IS written in B3-E3 is defined as the first IS, the second IS is in B4-E4, and the third IS is in B5-E5 (**Fig. 3**).

The position of the IS used for the quantitation has to be defined for each lipid species by typing the position of IS in the database to the right column with IS. In general, only one IS can be used per lipid species, but different lipid species can be quantified with different IS. This may be advantageous for lipid species with different response factors, *i.e.,* lipid species with short fatty acyl chains can be quantified with an exogenous IS with short fatty acyl chains, and the lipid species with higher carbon number with IS with long fatty acyl chains. The similarity criterion may be applied for DB number as well. This may compensate the quantitation errors caused by differences in the ionization efficiency for short and long fatty acyl chain lengths. The quantitation of lipid species is initiated in the "Start" sheet. The filtered and colored tagged *m/z* features obtained during the identification have to be sorted before the deisotoping step. No action is needed for green colored lipid species. The yellow tag of lipid species means that *m/z* values are within two times the specified mass tolerance range. The order in the database has to be inserted in the yellow colored column (E), or the line is deleted and therefore not considered for quantitation. The red tag means that multiple *m/z* features match the specified mass tolerance range, therefore the mass tolerance window should be adjusted, or the correct *m/z* feature is selected, and other incorrect options are removed by deleting the line.

Fig. 3. Exemplary screenshot of the database structure

For quantitation, the intensities of a lipid species are divided by the intensity of the IS and multiplied by the known concentration of the IS. However, as the intensity of a lipid species can be the sum of the intensity of the target lipid species and the M+2 of lipid species with one DB more, an isotopic correction has to be performed. Therefore, the intensity of the target lipid species is subtracted from the M+2 abundance of the lipid species with an additional DB (**Equation 1**). The automated quantitation is performed by pressing the button "Move" in the "Start" sheet, and lipid species concentrations are summarized in the corresponding lipid class sheet.

$$c\,(\text{species x})$$
$$= \frac{\text{Intensity (species x)} - \left(\text{Intensity (species x + 1DB)} \times \dfrac{\%\,(M + 2, \text{species x} + 1DB)}{100}\right)}{\text{Intensity (IS)}}$$
$$\times\, c\,(\text{IS})$$

Equation 1. Calculation of isotopic correction for lipid species differing by one DB.

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The lipid species identification and quantitation have to be performed for each lipid class separately. Therefore, the identified lipid species in the "Start" sheet have to be removed by pressing the button "Clear", and the next lipid class has to be chosen in the dropdown list and processed in the same way. If it is intended to remove all concentrations for all lipid classes, "Clear all concentrations" a button has to be pressed in the "Start" sheet. If it is aimed to remove only concentrations for one lipid class, "Clear concentrations" button in the corresponding database has to be triggered.

2.2.5 Output

Once all lipid classes are processed, a summary table can be generated by triggering the "Insert data" button in the "Results" sheet (**Fig. 4**). All lipid species concentrations for all samples will be inserted in the results table, whereby also lipid classes, which were not investigated, will be included with empty entries. For multiple injections (defined in the Support sheet), the average and deviation will be automatically calculated and summarized in the corresponding sheets. For a better view, empty entries of lipid species, *i.e.,* lipid classes not investigated or lipid species, which have no values for over 20% of investigated samples, are usually deleted before further data processing. For statistical analysis using SIMCA, the quantitative data have to be further processed. Therefore, all zero values for lipid species are replaced by 80% of the minimum concentration determined for all samples.

Database					Concentrations after isotopic correction					
PC		Isotopic correction			Sample ID					
M+H	Species	M+2	M+1	IS	QC_1	QC_2	43_1	43_2	48_1	48_2
678.5068	PC 28:0	0.00%	0.00%	1	60.00	60.00	60.00	60.00	60.00	60.00
690.5432	PC O-30:1/P-30:0	0.00%	0.00%	1	Not quantified					
692.5589	PC O-30:0	10.30%	0.00%	1						
704.5225	PC 30:1	0.00%	0.00%	1						
706.5382	PC 30:0	10.51%	0.00%	1	0.16	0.18	0.27	0.34	0.52	0.52
716.5589	PC O-32:2/P-32:1	0.00%	0.00%	1						
718.5745	PC O-32:1/P-32:0	11.24%	0.00%	1	0.13		0.25	0.16	0.30	0.28
720.5902	PC O-32:0	11.25%	0.00%	1						
730.5382	PC 32:2	0.00%	0.00%	1	0.20	0.15	0.31	0.34	0.62	0.63
732.5538	PC 32:1	11.46%	0.00%	1	1.82	1.72	2.41	2.34	4.08	4.14
734.5695	PC 32:0	11.47%	0.00%	1	1.00	1.09	1.84	1.78	2.38	2.44
742.5745	PC O-34:3/P-34:2	0.00%	0.00%	1	0.38	0.33	0.77	0.74	1.30	1.31
744.5902	PC O-34:2/P-34:1	12.23%	0.00%	1	0.85	0.90	1.76	1.83	2.60	2.60
					Concentration [nmol/mL]					

Fig. 4. Extract of summary table of lipid class species quantitation

2.2.6 Application lipidomic analysis of serum samples

The LipidQuant 1.0 has been already used in recent lipidomic studies (Chocholoušková M. *et al.*, 2021; Peterka, O. *et al.*, 2020; Wolrab, D. *et al.*, 2020a; Wolrab, D. *et al.*, 2020b; Wolrab D. *et al.*, 2021), which proved a significant reduction of working effort for data processing of large clinical cohorts. Here we present an illustrative example of the lipidomic quantitation of serum samples from healthy volunteers using UHPSFC/MS data set. QC samples were analyzed in duplicate after every twenty samples. In total, 152 lipid species from 8 lipid classes (CE, TG, DG, MG, Cer, PC, LPC, SM) were quantified. The PCA shows the clustering of the QC sample, indicating no measurement errors (**Fig. 5a**). Furthermore, a partial separation according to gender can be observed in the PCA, indicating gender-related differences in the lipidome. The calculation of p-values reveals that 89% of CE, 20 % of TG, 31 % of DG, 0 % of MG, 100 % of Cer, 79 % of PC, 0% of LPC, and 100% of SM lipid species are significantly different for males and females. The sum of average lipid species concentrations per lipid class for each gender shows higher concentrations of CE, Cer, PC, LPC, and SM for females, and higher concentrations of glycerolipids (TG, DG, and MG) for males. OPLS-DA analysis shows a clear differentiation of samples obtained from males or females (**Fig. 5b**). The S-plot illustrates the lipid species regulation, whereby lipid species in the right upper corner are upregulated and downregulated in the left lower corner for females and vice versa for males (**Fig. 5c**). The general trend is that glycerophospholipids are upregulated in females, and glycerolipids are upregulated for males. The boxplots of the most regulated lipid species PC 36:1 and TG 47:0 are shown in **Fig. 5d**.
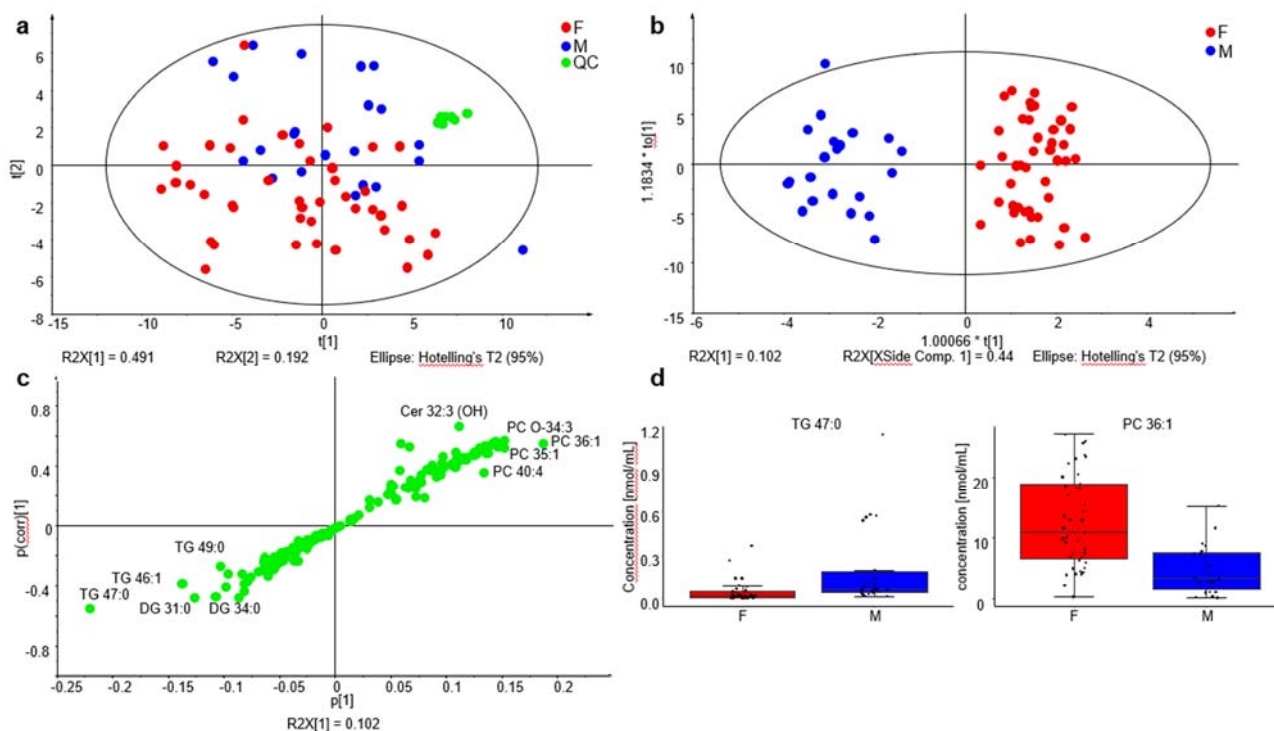
Fig. 5. Multivariate data analysis of the lipidomic data for human serum plasma of female and male healthy volunteers: a/ PCA, b/ OPLS-DA, c/ S-plot, and d/ selected boxplot for TG 47:0 and PC 36:1.

3. Acknowledgements

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