Final Report:

Standard Lipidomic Analysis of muscle- and WAT-derived samples.

Client	Laura Formentini CBMSO, Calle Nicolás Cabrera, 1. Campus de Cantoblanco, Universidad Autónoma de Madrid 28049 Madrid Spain
Test site	Lipotype GmbH
Principal Investigator	Christian Klose klose@lipotype.com
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2 Results

2.1 Samples

Samples were shipped on dry ice and arrived frozen on 20.02.2019 Receipt was confirmed by email.

Samples were stored at -80˚C. Prior to analysis they were thawed at 4˚C.

Based on the exploratory experiment samples were diluted and their quantity adjusted for lipid extraction (Appendix A2). All sample material was consumed for the analysis.

2.2 Lipidomics

Only lipids that passed the following criteria were considered for final data analysis (Table 1):

Parameter	Value
noise	5
occupation	0
background	5

Table 1. Parameters for lipids to be reported as true identifications. Lipids with an intensity 5 fold above the noise in mass spectrum and 5 fold above the intensity in blank samples were included in the analysis. Lipids present in any of the replicates were considered.

Extraction and sample processing went without any incidents, the ionization quality was satisfactory and no issues were encountered. The internal standard signal-to-noise ratio was in range of hundreds and more, indicating high quality of spectra. The technical reproducibility, as assessed by quality control reference samples (mammalian full blood) included in the same analytical run was very good, with the median coefficient of variation across all classes being 8.6% (for classes present in all replicates; Figure 1; Appendix A2).

Blank samples included in this analytical batch (internal background control) did not contain any unusual background.

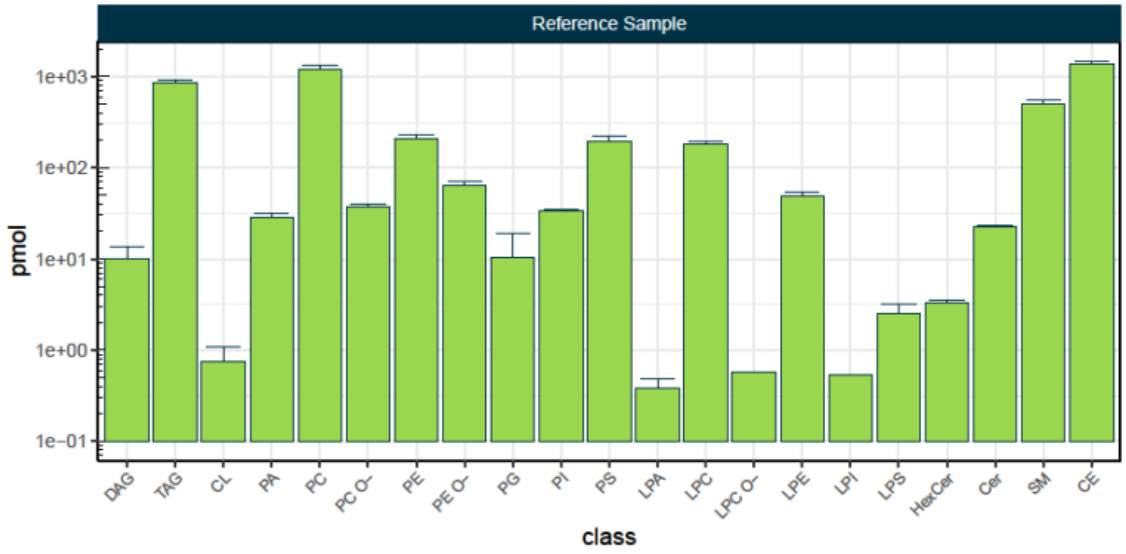

Figure 1. Variation in amounts of lipids measured in quality control reference samples. n=4 ± SD. Lipids present in any of the replicates are shown.

Samples yielded ca. 6600-34150 pmol of lipids. These values were in the optimal range for lipidomic measurements (Figure 2 and Figure 3).

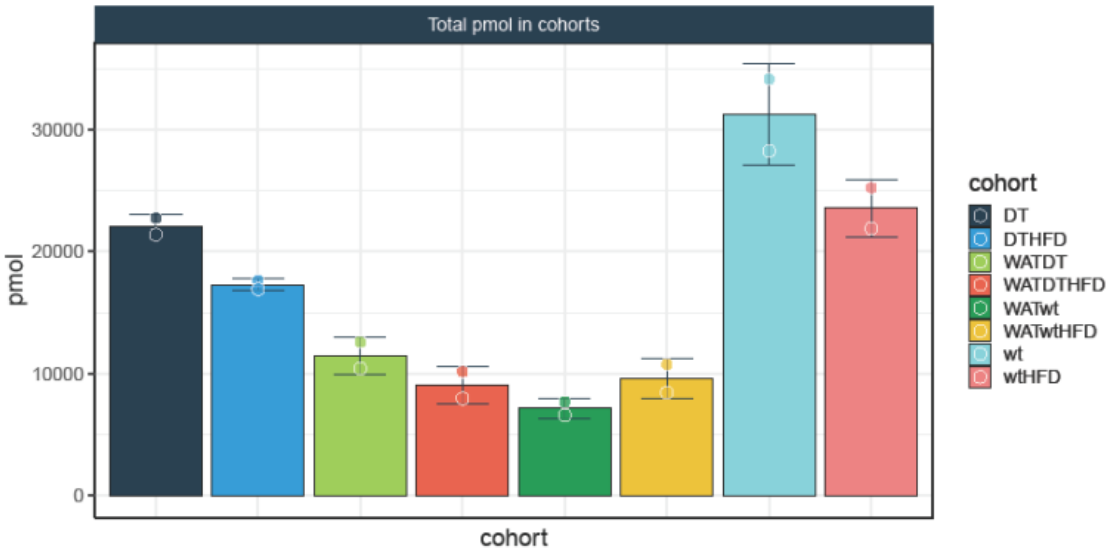

Figure 2. Total lipid measured in samples. Bars represent averages ± SD, with the individual samples shown as points.

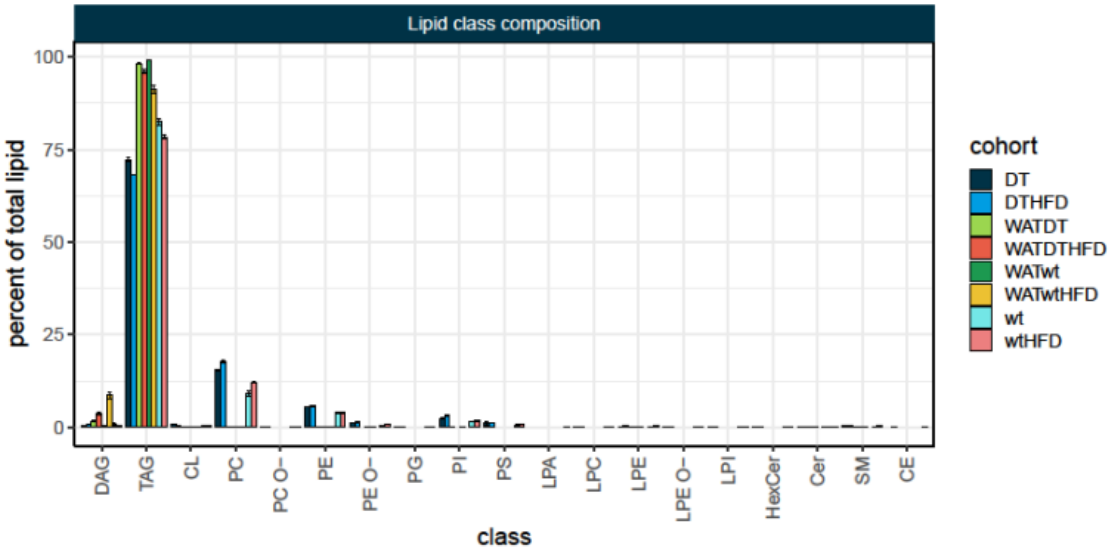

Figure 3. Lipid composition of analyzed samples.

A principal component analysis (PCA) with the mol% data for the lipid species shows that replicates are close to each other (Figure 4).

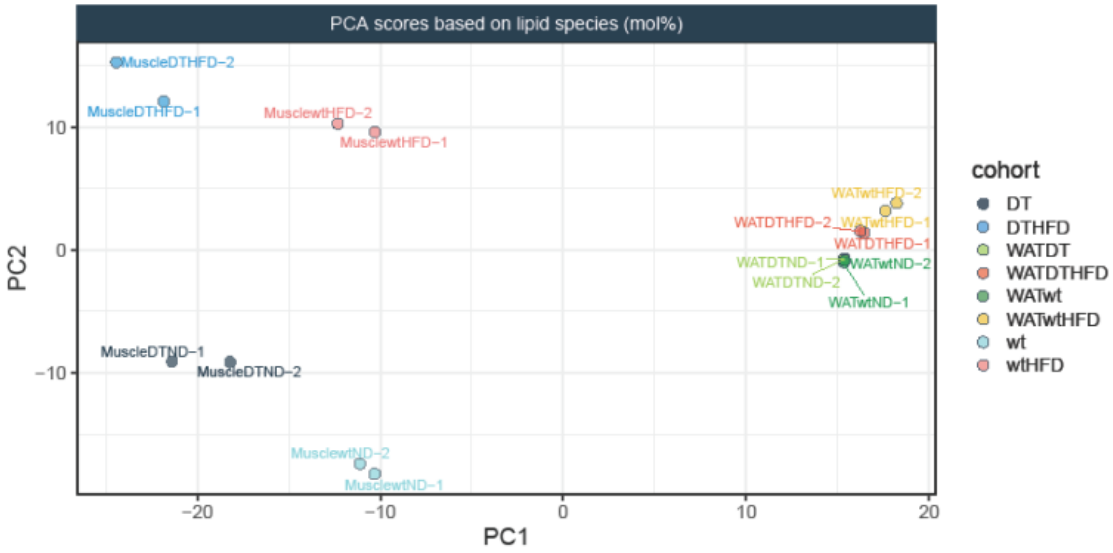

Figure 4. PCA analysis of individual samples. Lipid species mol% values per sample were used as input data.

The complete results are presented in the appendix A2 to A4.

2.3 Intended use of results

The results are intended for research, non-clinical, non-diagnostic purposes only.

3 Material and Methods

3.1 The Lipotype Shotgun Lipidomics technology

The Lipotype Shotgun Lipidomics platform consists of the automated extraction of samples, an automated direct sample infusion and high-resolution Orbitrap mass spectrometry including lipid class-specific internal standards to assure absolute quantification of lipids. An in-house developed software – LipotypeXplorer – is used for identification of lipids in the mass spectra. Further data processing and analyses is performed using the Lipotype laboratory information and management system and LipotypeZoom – web browser-based data visualization tool (Figure 5).

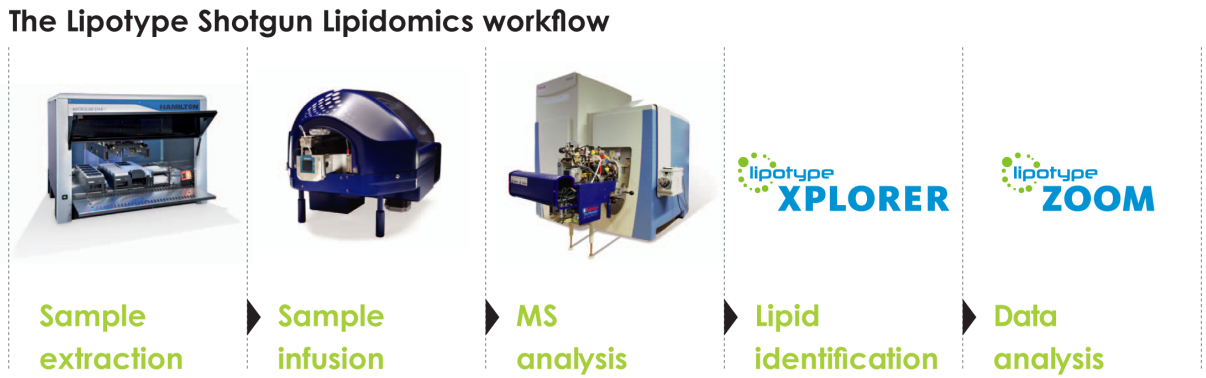

Figure 5. The Lipotype ShotgunLipidomics workflow.

3.2 Range of analysis

Lipotype is delivering quantitative lipidomics analyses of the membrane and storage lipids present in the sample (Table 2).

Lipid Class	MS mode	Structural detail level
CER	MS	species
CL	MS	species
HEXCER	MS	species
LPA	MS	species
LPC/LPC O-	MS	species
LPE/LPE O-	MS	species
LPG	MS	species
LPI	MS	species
LPS	MS	species
PA	MSMS	subspecies
PC/PC O-	MSMS	subspecies
PE/PE O-	MSMS	subspecies
PG	MSMS	subspecies
PI	MSMS	subspecies
PS	MSMS	subspecies
SM	MS	species
CE	MSMS	subspecies
DAG	MSMS	subspecies
TAG	MSMS	species

Table 2: List of analyzed lipid classes. MSMS mode (i.e. the fragmentation of the lipid molecules) delivers additional confidence of lipid identification and additional structural information.

3.3 Equipment and Software

The equipment and software used in the study are listed in Table 3.

Analysis step	Equipment or software
Extraction	Hamilton Robotics STARlet
Sample infusion	Advion Triversa Nanomate
Mass spectrometry	Thermo Scientific Q-Exactive
Lipid Identification	LipotypeXplorer
Data processing	Lipotype LIMS and LipotypeZoom

Table 3: List of equipment and software.

3.4 Reagents

Chemicals and solvents of HPLC/LC-MS analytical grade were used.

3.5 Lipid extraction

Lipids were extracted using chloroform and methanol (1). Samples were spiked with lipid class-specific internal standards prior to extraction. After drying and re-suspending in MS acquisition mixture, lipid extracts were subjected to mass spectrometric analysis.

3.6 Spectra acquisition

Mass spectra were acquired on a hybrid quadrupole/Orbitrap mass spectrometer equipped with an automated nano-flow electrospray ion source in both positive and negative ion mode.

3.7 Data processing and normalization

Lipid identification using LipotypeXplorer (2) was performed on unprocessed (*.raw format) mass spectra. For MS-only mode, lipid identification was based on the molecular masses of the intact molecules. MSMS mode included the collision-induced fragmentation of lipid molecules and lipid identification was based on both the intact masses and the masses of the fragments.

Prior to normalization and further statistical analysis lipid identifications were filtered according to mass accuracy, occupation threshold, noise and background.

Lists of identified lipids and their intensities were stored in a database optimized for the particular structure inherent to lipidomic datasets. Intensity of lipid class-specific internal standards was used for lipid quantification (Figure 6).

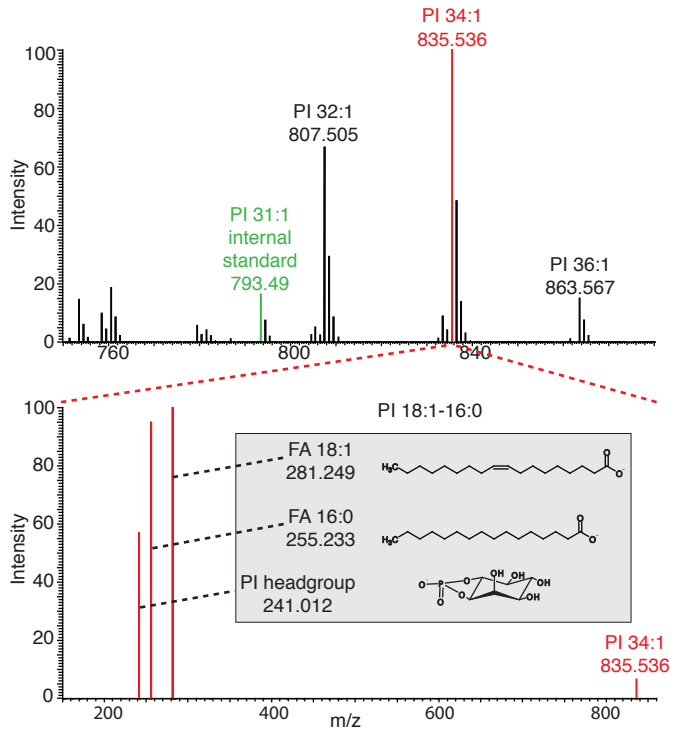

Figure 6. Quantitative and structural analysis of lipids by mass spectrometry. Without fragmentation (upper panel), high-resolution MS allows for assigning the sum composition of a lipid molecule to a peak in the spectrum. For example, PI 34:1 denotes a PI molecule with 2 fatty acids that in sum contain 34 carbon atoms and 1 double bond. Fragmentation of the molecule reveals the exact fatty acid composition. For example, in addition to the PI headgroup, fragmentation, PI 34:1 releases the fatty acids C18:1 (oleic acid) and C16:0 (palmitic acid). Thus, normalization to the added standard lipid allows for absolute quantification, and fragmentation specifies the molecular identity of the lipid in question (3).

3.8 Study Documentation

Sample information, handling and processing details; reagents; equipment; procedures; raw data (mass spectra) as well as processed data are stored and documented in the Lipotype LIMS.

3.9 Data Quality

The dynamic range for cell culture samples was determined prior to analysis. Based on these data, limits of quantification and coefficients of variation for the different lipid classes were determined. Limits of quantification are in the lower µM to sub-µM range, depending on the lipid class. The average coefficient of variation for a complete set of quantified lipid classes is around 10-15%.

Each analysis is accompanied by a set of blank samples to control for a background and a set of quality control reference samples to control for intra-run reproducibility and sample specific issues.

4 Data structure

4.1 Lipid nomenclature

Depending on the nature of the analysis, lipid molecules may be identified as species or subspecies (Table 2). Fragmentation of the lipid molecules in MSMS mode delivers subspecies information, i.e. the exact acyl chain (e.g. fatty acid) composition of the lipid molecule. MS only mode, acquiring data without fragmentation, cannot deliver this information and provides species information only. In that case the sum of the carbon atoms and double bonds in the hydrocarbon moieties is provided.

Lipid species are annotated according to their molecular composition as NAME <sum of the carbon atoms in the hydrocarbon moiety>:<sum of the double bonds in the hydrocarbon moiety>;<sum of hydroxyl groups>. For example PI 34:1;0 denotes phosphatidylinositol with a total length of its fatty acids equal to 34 carbon atoms, total number of double bonds in its fatty acids equal to 1 and 0 hydroxylations. In case of sphingolipids, SM 34:1;2 denotes a sphingomyelin species with a total of 34 carbon atoms, 1 double bond, and 2 hydroxyl groups in the ceramide backbone.

Lipid subspecies annotation contains additional information on the exact identity of their acyl moieties and their *sn*-position (if available). For example PI 18:1;0_16:0;0 denotes phosphatidylinositol with octadecenoic (18:1;0) and hexadecanoic (16:0;0) fatty acids, for which the exact position (*sn*-1 or *sn*-2) in relation to the glycerol backbone cannot be discriminated (underline "_" separating the acyl chains). On contrary, PC O-18:1;0/16:0;0 denotes an ether-phosphatidylcholine, where an alkyl chain with 18 carbon atoms and 1 double bond (O-18:1;0) is ether-bound to sn-1 position of the glycerol and a hexadecanoic acid

(16:0;0) is connect via an ester bond to the sn-2 position of the glycerol (slash "/" separating the chains signifies that the *sn*-position on the glycerol can be resolved).

4.2 Quantification and normalization

The identified lipid molecules were quantified by normalization to a lipid class-specific internal standard. The amounts in pmoles of individual lipid molecules (species of subspecies) of a given lipid class were summed to yield the total amount of the lipid class. The amounts of the lipid classes may be normalized to the total lipid amount yielding mol% per total lipids.

4.3 Other parameters

Total Double Bond Index – The quantities of the lipid species containing the same number of double bonds are summed and these values are normalized to the total amount of the given lipid class. The values are thus given as mol% of the lipid class.

Total Carbon Length Index - The quantities of the lipid species containing the same number of carbon atoms in the hydrocarbon moiety are summed and these values are normalized to the total amount of the given lipid class. The values are thus given as mol% of the lipid class.

Total Hydroxylation Index - The quantities of the lipid species containing the same number of hydroxyl groups are summed and these values are normalized to the total amount of the given lipid class. The values are thus given as mol% of the lipid class.

Taken together, these indices provide a qualitative description of the lipid classes.

5 Scientific publications – materials and methods section

If required, please use the following text for the relevant "Materials and Methods" section regarding Lipotype GmbH analysis of samples.

Lipid extraction for mass spectrometry lipidomics

Mass spectrometry-based lipid analysis was performed by Lipotype GmbH (Dresden, Germany) as described (Sampaio et al. 2011). Lipids were extracted using a two-step chloroform/methanol procedure (Ejsing et al. 2009). Samples were spiked with internal lipid standard mixture containing: cardiolipin 16:1/15:0/15:0/15:0 (CL), ceramide 18:1;2/17:0 (Cer), diacylglycerol 17:0/17:0 (DAG), hexosylceramide 18:1;2/12:0 (HexCer), lyso-phosphatidate 17:0 (LPA), lyso-phosphatidylcholine 12:0 (LPC), lyso-phosphatidylethanolamine 17:1 (LPE), lyso-phosphatidylglycerol 17:1 (LPG), lyso-phosphatidylinositol 17:1 (LPI), lyso-phosphatidylserine 17:1 (LPS), phosphatidate 17:0/17:0 (PA), phosphatidylcholine 17:0/17:0 (PC), phosphatidylethanolamine 17:0/17:0 (PE), phosphatidylglycerol 17:0/17:0 (PG), phosphatidylinositol 16:0/16:0 (PI), phosphatidylserine 17:0/17:0 (PS), cholesterol ester 20:0 (CE), sphingomyelin 18:1;2/12:0;0 (SM), triacylglycerol 17:0/17:0/17:0 (TAG). After extraction, the organic phase was transferred to an infusion plate and dried in a speed vacuum concentrator. 1st step dry extract was re-suspended in 7.5 mM ammonium acetate in chloroform/methanol/propanol (1:2:4, V:V:V) and 2nd step dry extract in 33% ethanol solution of methylamine in chloroform/methanol (0.003:5:1; V:V:V). All liquid handling steps were performed using Hamilton Robotics STARlet robotic platform with the Anti Droplet Control feature for organic solvents pipetting.

MS data acquisition

Samples were analyzed by direct infusion on a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). Samples were analyzed in both positive and negative ion modes with a resolution of $R_{m/z=200}$=280000 for MS and $R_{m/z=200}$=17500 for MSMS experiments, in a single acquisition. MSMS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1 Da increments (Surma et al. 2015). Both MS and MSMS data were combined to monitor CE, DAG and TAG ions as ammonium adducts; PC, PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as deprotonated anions. MS only was used to monitor LPA, LPE, LPE O-, LPI and LPS as deprotonated anions; Cer, HexCer, SM, LPC and LPC O- as acetate adducts.

Data analysis and post-processing

Data were analyzed with in-house developed lipid identification software based on LipidXplorer (Herzog et al. 2011; Herzog et al. 2012). Data post-processing and normalization were performed using an in-house developed data management system. Only lipid identifications with a signal-to-noise ratio >5, and a signal intensity 5-fold higher than in corresponding blank samples were considered for further data analysis.

References

- Ejsing, C.S. et al., 2009. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proceedings of the National Academy of Sciences of the United States of America, 106(7), pp.2136–41.
- Herzog, R. et al., 2011. A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome biology, 12(1), p.R8.

- Herzog, R. et al., 2012. LipidXplorer: a software for consensual cross-platform lipidomics. PloS ONE, 7(1), p.e29851.
- Sampaio, J.L. et al., 2011. Membrane lipidome of an epithelial cell line. Proceedings of the National Academy of Sciences of the United States of America, 108(5), pp.1903–7.
- Surma, M.A. et al., 2015. An automated shotgun lipidomics platform for high throughput, comprehensive, and quantitative analysis of blood plasma intact lipids. European journal of lipid science and technology, 117(10), pp.1540–1549.

6 Abbreviations

CE	Cholesterol esters
CER	Ceramide
CHOL	Cholesterol
CL	Cardiolipin
DAG	Diacylglycerol
FA	fatty acid
GL	Glycerolipid
GPL	Glycerophospholipid
HEXCER	Hexosylceramide
LPA	lyso-Phosphatidate
LPC (O-)	lyso-Phosphatidylcholine (-ether)
LPE (O-)	lyso-Phosphatidylethanolamine (-ether)
LPG	lyso-phosphatidylglycerol
LPI	lyso-Phosphatidylinositol
LPS	lyso-Phosphatidylserine
MEM	Membrane lipid
MS	mass spectrometry
MS	mass spectrometry
MSMS	tandem mass spectrometry
PA	Phosphatidate
PC (O-)	Phosphatidylcholine (-ether)
PE (O-)	Phosphatidylethanolamine (-ether)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SM	Sphingomyelin
ST	Sterol
STE	Sterol ester
STO	Storage lipid
TAG	Triacylglycerol

7 References

1. Sampaio, J. L. *et al.* Membrane lipidome of an epithelial cell line. *Proceedings of the National Academy of Sciences* **108**, 1903–1907 (2011).
2. Herzog, R. *et al.* A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. *Genome Biol.* **12**, R8 (2011).
3. Klose, C., Surma, M. A. & Simons, K. Organellar lipidomics--background and perspectives. *Curr Opin Cell Biol* **25**, 406–413 (2013).

8 Appendices

Appendix A2:

Data on sample amounts used for extraction and coefficient of variations for lipid classes measured in reference samples (*.xlsx).

Appendix A3:

List of lipid species and their abundances in pmol per sample (*.csv).

Appendix A4:

List of lipid species and their abundances in mol%/sample (*.csv).