Electronic Supplementary Material (ESI) for Analyst.

Supporting information

LILY-Lipidome Isotope Labeling of Yeast: *In vivo* synthesis of ^{13}C labeled reference lipids for quantification by mass spectrometry

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Supporting information

This section contains the extended methods section and additional information on RP-LC-MS elution profile of four isotopically labeled lipids and their natural analogues (S1), comparison of the theoretical ^{13}C isotopic enrichment and the observed isotopic pattern of PE 34:1 (S2), fragmentation pattern of labeled and unlabeled PA 34:1 and PS 34:1 (S3), trueness of observed fold changes for 20 lipids analyzed by a RP-LC-MS method based on Full-MS quantification (S4) as well as calibration curves of PA 34:1 with and without internal standardization (S5). Additionally, a comprehensive list of ^{13}C isotopically enriched lipids identified in *Pichia pastoris* (S6) is provided.

Materials

Acid washed glass beads (425-600 µm particle size), chloroform, ammonium bicarbonate (ABC), ammonium acetate (NH_4Ac) and ammonium hydroxide (NH_4OH) were acquired from Sigma Aldrich (Steinheim, Germany). Water with 0.1% ammonium acetate (LC-MS grade) was purchased from Fluka (Buchs, Switzerland). Acetonitrile, 2-propanol and methanol, UPLC-grade were obtained from Biosolve (Valkenswaard, The Netherlands). Tris(hydroxymethyl)-aminomethane (Tris) was purchased from Applichem (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Roth (Karlsruhe, Germany) and sodium chloride (NaCl) from Merck (Darmstadt, Germany). Bicinchoninic acid (BCA) assay was purchased from Thermo Scientific (Schwerte, Germany).

Lipid extraction

For extraction, 1 mL of non-labeled or ^{13}C yeast cell suspension corresponding to $4x10^8$ cells was thawed on ice and centrifuged at 10000 rcf (4°C, 10 min). The supernatant was collected and 500 µL of 150 mM ABC buffer was added in each vial. The cells were transferred in a 2 mL polypropylene tube containing glass beads corresponding to a liquid equivalent of 200 µL. Solvent blanks (N=5) as well as extraction blanks (N=3) for each of the identification and quantification steps were prepared using the same equipment and solvent as for the actual samples. Cell opening was performed by mechanical disruption facilitating the TissueLyser II (Qiagen, Hilden, Germany) with 3x 7 min and 30s/min frequency. The cells lysates were pooled and stored at -80°C until further processing. Aliquots of 100 µL yeast lysates were transferred to new polypropylene tubes. For the absolute quantification of lipids, a calibration curve was performed by adding the indicated amounts (2 fmol to 20 pmol) of 5 glycerophospholipid standard mixture to the ^{13}C yeast extract matrix. Further, non-labeled yeast extract aliquots were spiked with ^{13}C yeast extract (N=4) for internal standardization prior to (N=4) or after Folch extraction (N=4). Folch extraction was performed by adding 990 µL of chloroform/methanol 2:1 and incubation at 4 °C and 950 rpm for 1h. After 1 h 198 µL 0.1% ammonium acetate buffer was added to all samples to induce phase separation. The samples were mixed and centrifuged 10 min at 10000 rcf at 4 °C. The upper phase was removed and the lower lipid containing phase was further dried under nitrogen flow and stored at -80 °C until analysis. Protein pellets of the interphase and the methanol phase were subjected to protein precipitation, by adding MeOH in a final ratio of 1:4 (H_2O:MeOH), for 1h at -20 °C. After protein precipitation, the samples were centrifuged at 4 °C for 30 min at 13 500 rcf. The supernatant was removed and the pellet resuspended in 1% SDS lysis buffer.

Protein concentration was determined with the BCA assay. For the relative quantification experiment, 500 µL of pooled (prepared by the some sample preparation step as for the absolute quantification experiment) ^{13}C yeast extracts (corresponding to 10^7 cells and phospholipid concentrations in the low µM range) were mixed with natural *Pichia pastoris* yeast extract (N=4) in defined ratios ($^{12}C/^{13}C$: 0.02, 0.05, 0.10, 0.14, 0.20, 0.33, 0.5, 1, 2, 3, 5, 7; reference points were derived from 500 µL endogenous yeast extract corresponding to 10^7 cells and phospholipid concentrations in the low µM range).

Lipid analysis, direct infusion MS and MS/MS analysis

For the first lipid identification in yeast, 50 µL of 2-Prop/MeOH/$CHCl_3$ (4:2:1, v/v/v) with 7.5 mM ammonium acetate were added to the nitrogen dried lipid extracts in a 96 well plate (Eppendorf, Hamburg, Germany) and then infused *via* robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca NY, USA) into a Q Exactive Plus instrument (Thermo Fisher Scientific, Bremen, Germany) using chips with spraying nozzles of 4.1 µm. Shotgun parameters were applied as previously described[1,2].

Shotgun Data analysis

All spectra were imported by LipidXplorer 1.2.6 into a MasterScan database under the following settings: mass tolerance 5 ppm; range of m/z 350-1200; min occupation of 1; intensity threshold 1 x 10^4 and lipid identification was carried on as previously described[1,3]. Isotopic correction was performed and intensities were exported to carry out an intensity based quantification.

Lipid analysis, LC-MS and MS/MS analysis

Reversed-phase chromatography

The chromatographic reversed-phase separation was performed on an Ascentis Express C18 column (150 mm × 2.1 mm, 2.7 µm, Supelco) fitted with a guard cartridge (50 mm × 2.1 mm, 2.7 µm, Supelco)[4]. The temperatures of the autosampler and the column oven were set to 10 and 60 °C, respectively. Solvent A was ACN/H_2O (3:2, v/v), and solvent B was IPA/ACN (9:1, v/v). Both solvents contained 0.1% formic acid, 0.1% phosphoric acid and 10 mM ammonium formate. The separation was carried out at a flow rate of 500 µL min^{-1} with the following gradient: 0.0−3.0 min 30% B, 3.0−15.0 min ramp to 75% B, 15.0−17.0 min ramp to 100% B, 17.0-30.0 to 5% B, 30.0-30.1 to 30% B, 30.0-35.0 30% B. The injector needle was

washed with 80% chloroform, 20% H_2O, 0.1% formic acid prior to each injection. Dried samples were reconditioned in 50 µL of the chromatography starting eluent composition (30% B). Samples and standards were injected with volumes of 2-5 µL (N=4).

MS parameters

For the lipid identification, a Q Exactive Plus instrument (Thermo Fisher Scientific, Bremen, Germany) was used and MS1 resolution was set to 70000, MS2 to 17500 (data dependent top 5 method) applying a relative collision energy of 24 (negative mode) or 21 (positive mode). The following ESI source parameters were applied: Spray voltage 4 and 3 kV for positive and negative mode, respectively, 285 °C capillary temperature, S-lens RF level of 60, 370 °C auxillary gas heater temperature, auxillary gas flow rate of 20, sheath gas flow rate of 50. For the absolute quantification a PRM list of exact monoisotopic masses for ^{12}C and ^{13}C analogs (PA 34:1- ^{12}C= 673.4814, ^{13}C=710.6055; PC 34:1- ^{12}C=804.5760, 13C=846.7169; PE 34:2- ^{12}C=714.5079, ^{13}C=753.6388; PI 34:1- ^{12}C= 835.5342, 878.6785; PS 34:1- ^{12}C=760.5134, ^{13}C=800.6476) of 5 glycerophospholipids was used and measured in negative mode. For the relative quantification (N=4 analytical replicates) experiment a PRM (consecutive cycles of 8 PRMs with MS2 resolution of 15000 followed by one Full-MS scan with 30000 resolution) was performed using a Q Exactive HF instrument (Thermo Fisher Scientific, Bremen, Germany). The PRM list included 20 exemplary yeast lipids (CL 70:6, CL 72:6, IPC 42:0,4, LPE 18:0, PA 34:1, PA 36:2, PC 34:2, PC 36:2, PC 38:2, PE 34:1, PE 34:2, PG 34:1, PI 34:1, PI 34:2, PS 34:1, PS 34:2, MIPC 42:0,4, HexCer 35:2, DAG 34:3, DAG 36:2) with their exact monoisotopic masses for C12 and C13 analogs and corresponding retention times windows of +/- 0.5 min. To compare targeted PRM and Full-MS quantification, a Full-MS experiment (MS1 resolution: 120000) was performed and combined with ddMS2 in a separate run (MS1 resolution: 60000, MS2: 30000) followed after all replicates (N=3 analytical replicates) of one sample.

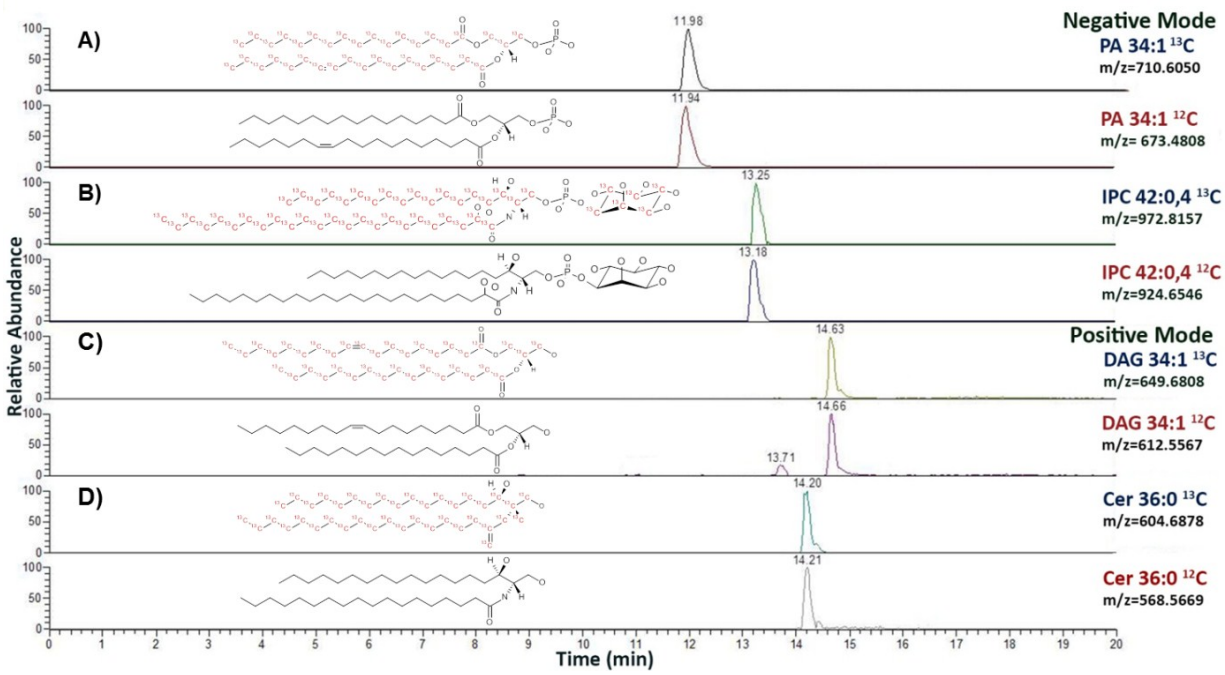
Lipid identification and LC-MS/MS Data analysis

Comprehensive lipid identification was performed using data derived from data dependent RP-LC-MS/MS (top 5). Accurate mass was determined from the MS spectra (mass tolerance of 5 ppm) and structure specific fragments recognized in the MS/MS spectra were used. Therefore, for lipid identification in negative mode we used the acyl anions of the fatty acid moieties and the headgroup specific fragments (e.g. 152.9953 m/z for the GPLs, 241.0113 m/z for PI, 421.0752 for MIPC) or the neutral losses of the headgroups (-87.0320 m/z for PS)

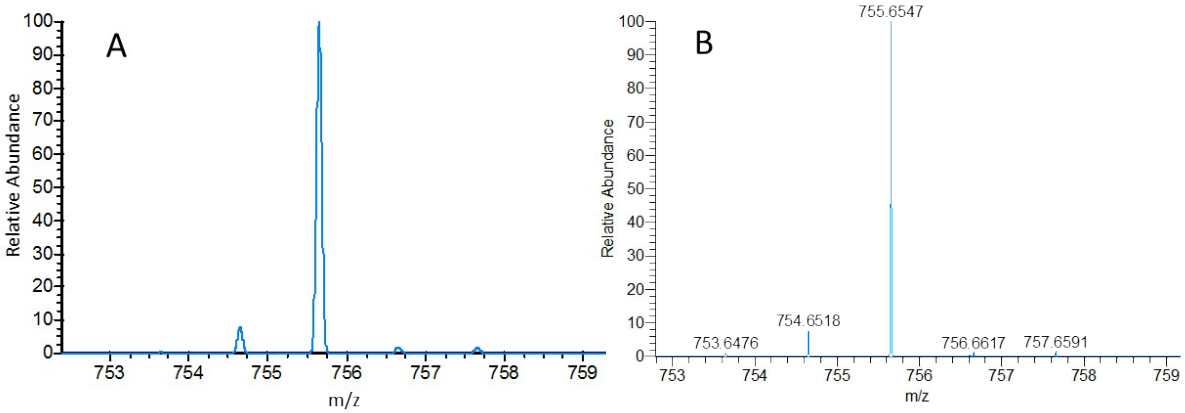
where present. Positive mode was used mainly for the identification of glycerolipids, Cer and HexCer species where we based on the neutral loss of the fatty acids for GLs and on the presence of the LCB fragments for ceramides respectively. No 13C enriched lipids were detected in any extraction blank (N=3) or solvent blank (N=5), though some minor contaminations of common natural (non-labeled) TAG and cardiolipins were observed. In order to ensure lipid identification in the ^{13}C enriched material, we compared endogenous yeast samples (N=3) and isotopically enriched yeast extract (N=3). The final lipid list comprise For the absolute quantification a PRM list of exact monoisotopic masses for ^{12}C and ^{13}C analogs (PA 34:1- ^{12}C= 673.4814, ^{13}C=710.6055; PC 34:1- ^{12}C=804.5760, 13C=846.7169; PE 34:2- ^{12}C=714.5079, ^{13}C=753.6388; PI 34:1- ^{12}C= 835.5342, 878.6785; PS 34:1- ^{12}C=760.5134, ^{13}C=800.6476)d of ^{13}C lipids observed in isotopically enriched *Pichia pastoris* extract with endogenous lipid analogs identified in the non-labeled yeast material. For the relative and absolute quantification experiments, all chromatographic spectra were imported into Thermo Tracefinder (Version 3.3.350.0) and quantified on areas extracted on MS1 or MS2 level (depending on the experimental conditions). MS2 information was used for species confirmation or quantification on PRM level using the specific fragments present in the analyzed glycerophospholipids.

Labeling degree determination

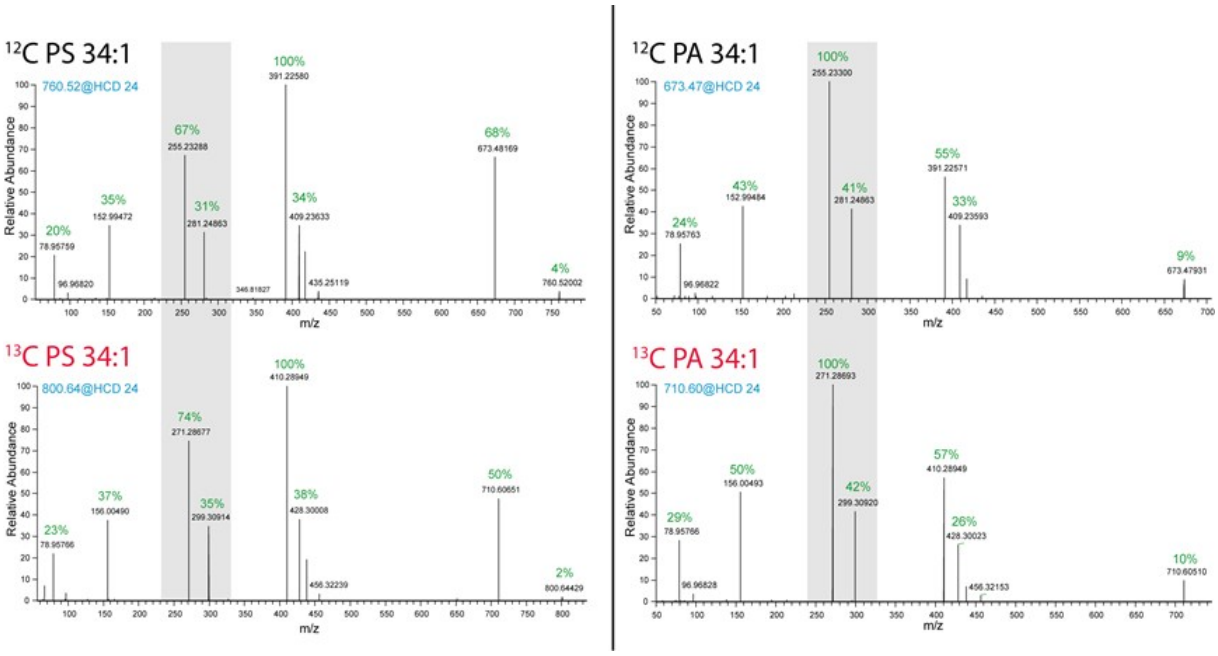
The labeling degree was assessed by using a high resolution mass spectrometry (Q Exactive plus, Thermo Fisher Scientific). Isotope pattern calculation was based on absolute intensities followed interpretation.



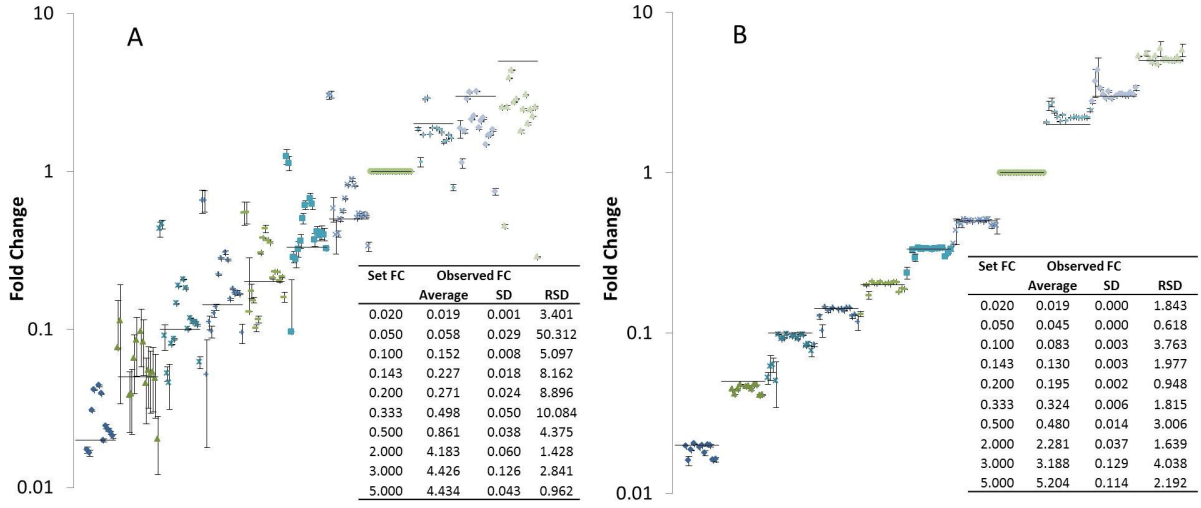
Supporting Figure S1. RP-LC-MS elution profile of four isotopically labeled lipids and their natural analogues: A) Chromatographic peak of uniformly ^{13}C labeled phosphatidic acid PA 34:1 recorded in negative MS mode; B) Chromatographic peak of uniformly ^{13}C labeled and natural abundant inositolphosphorylceramide IPC 42:0,4 recorded in negative MS mode; C) Chromatographic peak of uniformly ^{13}C labeled diacylglycerol DAG 34:1 recorded in positive MS mode; D) Chromatographic peak of uniformly ^{13}C labeled ceramide Cer 36:0 recorded in positive MS mode. All chromatograms were extracted within the range of ± 3 ppm of the theoretically calculated mass.



Supporting Figure S2. Comparison of A) the theoretical ^{13}C isotopic enrichment of 99.8% calculated by Isopro[5] and B) the observed isotopic pattern (exported from Xcalibur, Thermo) by high resolution LC-MS exemplary shown on the example of PE 34:1.



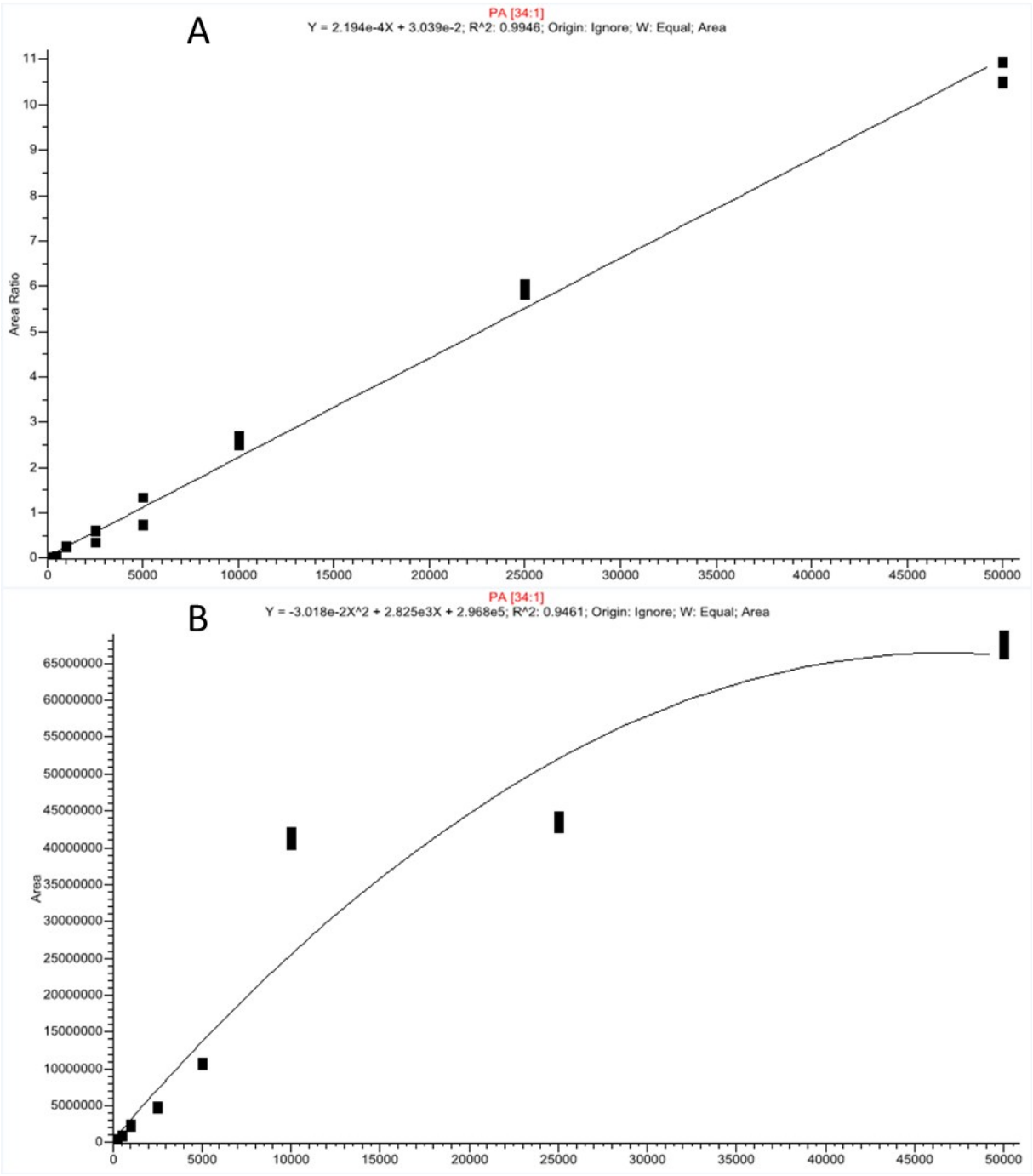
Supporting Figure S3: MS2 fragmentation pattern of labeled and unlabeled PA 34:1 and PS 34:1 using high collision dissociation (normalized collision energy: 24) measured with a Q Exactive plus. Comparable fragmentation is observed for both ^{13}C-enriched and unlabeled lipids



Set FC	Observed FC		
	Average	SD	RSD
0.020	0.019	0.001	3.401
0.050	0.058	0.029	50.312
0.100	0.152	0.008	5.097
0.143	0.227	0.018	8.162
0.200	0.271	0.024	8.896
0.333	0.498	0.050	10.084
0.500	0.861	0.038	4.375
2.000	4.183	0.060	1.428
3.000	4.426	0.126	2.841
5.000	4.434	0.043	0.962

Set FC	Observed FC		
	Average	SD	RSD
0.020	0.019	0.000	1.843
0.050	0.045	0.000	0.618
0.100	0.083	0.003	3.763
0.143	0.130	0.003	1.977
0.200	0.195	0.002	0.948
0.333	0.324	0.006	1.815
0.500	0.480	0.014	3.006
2.000	2.281	0.037	1.639
3.000	3.188	0.129	4.038
5.000	5.204	0.114	2.192

Supporting Figure S4: Trueness of observed fold changes in a dilution experiment on *Pichia pastoris* lipid extract, shown for 24 lipids analyzed by a RP-LC-MS method based on Full-MS quantification. Black bars indicate the true fold change set by the relative spike concentrations, table inserts reflect the average across all lipids investigated. A) Fold change (FC) calculated based on raw peak areas (no normalization), B) Fold change (FC) after lipid specific peak area normalization using the respective ^{13}C labeled lipid analog.



Supporting Figure S5. Exemplary calibration curves (1 nM to 50 µM) for absolute quantification PA 34:1 in *Pichia pastoris* A) with and B) without internal standardization. With internal standardization an increased linear dynamic range over more than four orders of magnitude was observed in raw data exported in Tracefinder (Thermo).

SI References

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