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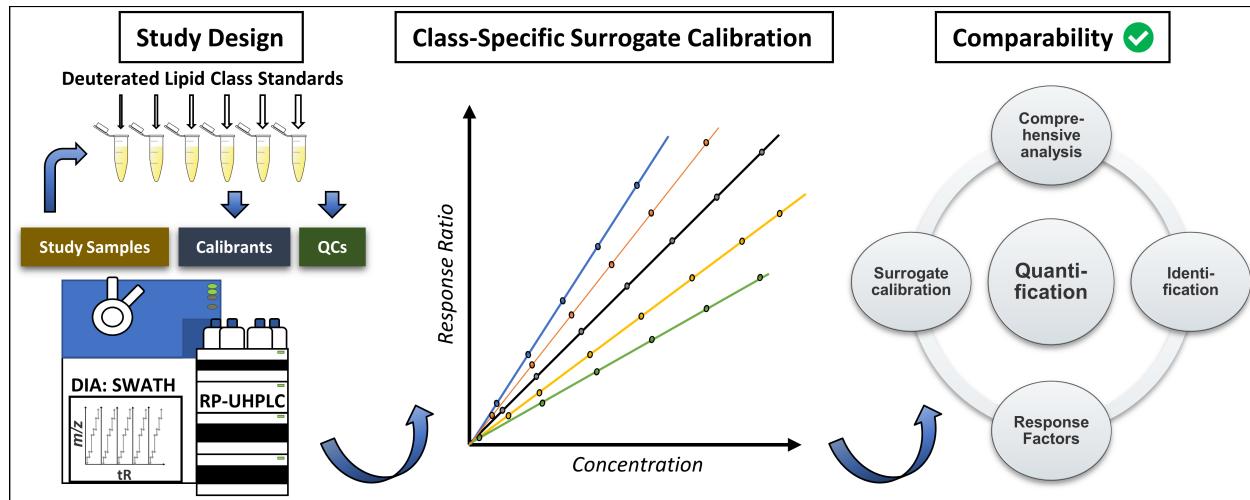
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1 **Comprehensive Lipidomics of Mouse Plasma using Class-Specific Surrogate
2 Calibrants and SWATH Acquisition for Large-Scale Lipid Quantification in
3 Untargeted Analysis**

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25

26 **Abbreviations:**

27 *BK*, voltage- and Ca^{2+} -activated K^+ channel of big conductance (BK); BW, body weight; CE,
28 Cholestryl ester; CD, Control diet; COD, Coefficient of dispersion; DAG, Diacylglycerol; DIA,
29 Data-Independent Acquisition; EIC, Extracted ion chromatogram; IS, Internal standard; FA,
30 Fatty acid; HFD, High fat diet; IDA, Information-Dependent Acquisition (synonymous with
31 DDA, Data-dependent Acquisition); ITC, Ion transmission control; IPA: Isopropanol; LPC,
32 Lysophosphatidylcholine; MAG, Monoacylglycerol; MeCN, Acetonitril; MEDM, Median of
33 means; MeOH, Methanol; MRM, Multiple reaction monitoring; PA, Phosphatidic acid; PC,
34 Phosphatidylcholine; PCA, Principal component analysis; PE, Phosphatidylethanolamine; PG,
35 Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; SIL stable isotope
36 labeled; SM, Sphingomyelin; SWATH, Sequential Window Acquisition of all Theoretical
37 Fragment Ion Mass Spectra; TAG, Triacylglycerol; TIC, Total ion chromatogram; t_R , Retention
38 time.

39

40 **ABSTRACT**

41 Lipidomics has gained rising attention in recent years. Several strategies for lipidomic profiling
42 have been developed, with targeted analysis of selected lipid species, typically utilized for lipid
43 quantification by low-resolution triple quadrupole MS/MS, and untargeted analysis by high-
44 resolution MS instruments, focusing on hypothesis generation for prognostic, diagnostic and/or
45 disease-relevant biomarker discovery. The latter methodologies generally yield relative
46 quantification data with limited inter-assay comparability. In this work we aimed to combine
47 untargeted analysis and absolute quantification to enhance data quality and to obtain independent
48 results for optimum comparability to previous studies or database entries. For the lipidomic
49 analysis of mouse plasma, RP-UHPLC hyphenated to a high-resolution quadrupole TOF mass
50 spectrometer in comprehensive data-independent SWATH acquisition mode was employed. This
51 way, quantifiable data on the MS and the MS/MS level were recorded, which increases assay
52 specificity and quantitative performance. Due to the lack of an appropriate blank matrix for
53 untargeted lipidomics, we herein established a sophisticated strategy for lipid class-specific
54 calibration with stable isotope labeled standards (surrogate calibrants). LLOQs were in the range
55 between 10-50 ng mL⁻¹ for LPC, LPE, PI, PS, PG, SM, PC, PE, DAG) or 100-700 ng mL⁻¹
56 (MAG, TAG), except for cholesterol and CE (1-20 µg mL⁻¹). Acceptable values for accuracy
57 and precision well below ±15 % bias were reached for the majority of surrogate calibrants.
58 However, to achieve sufficient accuracy for target lipids, response factors to corresponding
59 surrogate calibrants are required. An approach to estimate response factors via a standard
60 reference material (NIST SRM 1950) was therefore conducted. Furthermore, a useful workflow
61 for post-acquisition re-calibration, involving response factor determination and iteratively built
62 libraries, is suggested. In comparison to single-point calibration, the presented surrogate calibrant
63 method was shown to yield results with improved accuracy that are largely in accordance with
64 standard addition. Quantitative results of real samples (high-fat diet vs control diet) were then
65 compared to two previously published dietary mouse plasma studies that provided absolute lipid
66 levels and showed similar trends.

67

68 **1. INTRODUCTION**

69 Due to emerging insights in biological pathways of endogenous lipids and their importance in
70 disease progression,[1] lipidomic profiling and related analytical methods have evolved to
71 become a key field in analytical chemistry. With the steady development of sophisticated mass
72 spectrometric methods, the number of published articles covering lipidomics has been
73 continuously increasing in the recent years.[2] Many advances in lipidomic biomarker discovery
74 have been made,[3-5] but also methodological progress regarding study design,[6] databases,[7,
75 8] software applications and data processing[9] has been steadily deployed. Studies aiming for
76 new results in hypothesis generation of potential biomarkers typically utilize high-resolution
77 mass spectrometry for untargeted data acquisition and focus on the observation of relative fold-
78 changes of detected compounds between distinct study groups e.g. diseased versus control.
79 These metrics of relative quantification, however, limit inter-study, inter-batch or even inter-
80 sequence comparability and do not allow the comparison of found biomarker levels to reference
81 levels that are covered in databases, such as the Human Metabolome Database.[10] The ultimate
82 approach to overcome these limitations is absolute quantification of compounds of interest.
83 Although accurate determination of absolute levels for hundreds or thousands of features in
84 complex matrices is difficult to accomplish, currently established approaches towards
85 quantitative, untargeted lipidomics must be improved. In this context, various difficulties
86 concerning calibration, normalization via internal standards (ISs), control of matrix effects and
87 requirements for validation arise.

88 Most approaches towards quantification in untargeted lipidomics employed shotgun analysis
89 methods in combination with high-resolution instruments.[11-15] Its main advantage is the
90 simultaneous ionization of lipids with added ISs for optimum normalization, yet, the enhanced
91 concurring ionization processes can lead to ion suppression, which results in decreased
92 sensitivity and impeded detection of low abundant lipids. Moreover, direct infusion adds an
93 increased risk for compromised assay specificity by in-source fragmentation and higher
94 probability for signal-interferences from isomers and isobars.[16] Other promising results have
95 already been achieved by using SFC-MS[17] or HILIC-MS[18]. Here, lipids are separated
96 according to lipid class polarities and class-specific ISs are (largely) co-eluted. Efforts to utilize
97 lipid species separation via RP-LC-MS for quantitative purposes are mainly limited to a reduced

98 number of target compounds[19-22] mainly using low resolution triple quadrupole
99 instruments.[23, 24], or require global lipid labeling.[15] Due to the study design of many
100 untargeted methods with single-point calibration by class-specific ISs,[25, 26] results are
101 typically semi-quantitative[27] as absolute quantification in accordance to quantification
102 guidelines (e.g. FDA guidelines for bioanalytical method validation[28]) requires multi-level
103 calibration for each analyte. On the other hand, following strictly the existing guidelines is not
104 always possible for the untargeted analysis of endogenous compounds, and no comparable
105 instructions exist for this purpose. Alternative approaches that comply as far as achievable thus
106 need to be developed and investigated.

107 In this work, an approach to achieve absolute quantification of selected lipid classes in an
108 untargeted lipidomic RP-LC-MS assay is presented. By using stable isotope labeled lipids of
109 various classes for matrix-matched surrogate calibration, lipid species quantification of
110 compounds of interest by class-specific surrogate calibrants can be executed retrospectively post-
111 analysis. Via SWATH acquisition (sequential window acquisition of all theoretical fragment ion
112 mass spectra),[29] comprehensive lipid analysis could be achieved, SWATH is a data-
113 independent acquisition technique that offers many advantages like full coverage of MS/MS
114 fragments for enhanced selectivity and higher identification rates than data-dependent
115 acquisition. In addition, it provides high sensitivity that is comparable to that of multiple reaction
116 monitoring (MRM) with triple-quadrupole instruments. It allows extraction of chromatograms
117 not only on MS level (as in data dependent acquisition mode) but due to comprehensive MS/MS
118 data also at the MS/MS level, which enables quantification on TOF-MS or SWATH-MS/MS
119 level (using precursor or product ions).

120 However, an additional determination and application of response factors between target analytes
121 and surrogate calibrants as well as knowledge about linear ranges is required to achieve true
122 absolute quantification.[18] Thus, in this work the performance of surrogate calibration was
123 compared to single-point (i.e. 1-point) calibration and standard addition for selected examples.
124 Furthermore, method-specific response factors for various lipid species were calculated using
125 consensus values obtained from certified reference material for human plasma (NIST SRM
126 1950).The application of the above surrogate calibration and those lipid-species specific response
127 factors should lead to improved quality of large scale lipid quantification, even if it does not

128 achieve the quality of accurate targeted quantification with authentic standards and isotope-
129 labelled IS for each lipid species which is not possible in global lipidomics profiling.

130 **2. EXPERIMENTAL SECTION**

131 **2.1 Materials.**

132 Acetonitrile (MeCN, Ultra LC-MS grade), methanol (MeOH, Ultra LC-MS grade), 2-propanol
133 (IPA, Ultra LC-MS grade) and formic acid (98 %, w/v, ACS grade) were supplied by Carl Roth
134 (Karlsruhe, Germany). Ammonium formate was purchased from Sigma–Aldrich (Saint Louis,
135 MO, USA). SPLASH LipidoMIX (Lipidomix), 14:0-14:0 phosphatidylcholine (PC), 16:0-16:0
136 PC, 18:0-18:0 PC, 17:1 lysophosphatidylcholine (LPC) and 20:0 LPC were purchased from
137 Avanti Polar Lipids (Alabaster, AL, USA). Arachidonic-acid(d8), α -linolenic-acid(d14) and
138 linoleic-acid(d4) were acquired from Cayman Chemical (Ann Arbor, MI, USA). Oleoylethanolamide was acquired from abcr GmbH (Karlsruhe, Germany). Type I purity water
139 was obtained from a Purelab Ultra purification system (ELGA LabWater, Celle, Germany). Standard reference material (SRM) for human plasma (SRM 1950) for response factor evaluation
140 of lipids was acquired from the National Institute of Standards and Technology (NIST,
141 Gaithersburg, MD, USA). Plasma samples of mice were collected during a previous study[30]
142 with permission of the local authorities and conducted in accordance with the German legislation
143 on the protection of animals.

144 **2.2 Sample preparation.** Blood was collected by cardiac puncture from experimental mice after
145 18 weeks of dietary feeding under deep terminal anaesthesia induced by xylazine (10 μ g/g body
146 weight (BW)), ketamine (80 μ g/g BW) and a 2 - 4 % isoflurane in oxygen inhalation. Upon
147 disposal of the 27-gauge needle, blood was transferred from the syringe into 0.5 mL EDTA
148 coated tubes that were chilled on ice and gently mixed. Samples were centrifuged for 15 min at
149 3,000 rpm at 4 °C and stored as aliquots of 50 μ L plasma per individual at -80 °C upon further
150 processing. After thawing on ice, aliquots of 25 μ L were used for sample preparation,
151 respectively, whereas residual volumes were pooled for the preparation of calibration and QC
152 samples (see section below). IPA-based protein precipitation for untargeted lipid extraction[31,
153 32] was performed by addition of 55 μ L IPA and 20 μ L MeOH to the individual plasma aliquots.
154 This ratio was chosen to achieve uniform solvent composition in all samples (see preparation of
155 calibration and QC samples). After precipitation and subsequent vortexing, the samples were
156

158 centrifuged for 10 min at 15,000 × g and 4 °C with a 5415R microcentrifuge (Eppendorf,
159 Hamburg, Germany). The supernatant was transferred to a 250 µL conical glass insert in a 1.5
160 mL glass vial, which was immediately sealed with a crimp cap and stored at 4 °C in the
161 autosampler for the time of analysis. Samples were analyzed as soon as possible after preparation
162 and the analytical sequence was started within 2 h after the final centrifugation.

163 In order to perform 1-point calibration, Lipidomix and labeled fatty acids (arachidonic-acid(d8),
164 α-linolenic-acid(d14), linoleic-acid(d4)) were spiked into the methanolic portion of the
165 precipitation solvent. Addition of 1-point calibrants also enabled the application of various IS-
166 based normalization techniques for untargeted data processing. Final concentrations of spiked
167 standards in study samples are listed in Table S-1.

168 **2.3 Preparation of calibration and QC samples - Quantitative study design.**

169 In accordance to regular targeted, quantitative assays, calibration and QC samples were prepared
170 to assess linear dynamic range, precision and accuracy. For matrix-matched calibration, labeled
171 lipid standards (Lipidomix and fatty acids) were spiked into a mouse plasma pool (prepared from
172 aliquots of all study samples) in differing concentrations to serve as class-specific surrogate
173 calibrants.[33-35]

174 The Lipidomix contains quantitative amounts of deuterated lipids to relatively reflect the ratios
175 in human plasma. The following lipids are covered: 18:1(d7) cholesteryl ester (CE), 15:0-
176 18:1(d7)diacylglycerol (DAG), 18:1(d7) lysophosphatidylcholine (LPC), 18:1(d7)
177 lysophosphatidylethanolamine (LPE), 18:1(d7) monoacylglycerol (MAG), 15:0-18:1(d7)
178 phosphatidylcholine (PC), 15:0-18-1(d7) phosphatidylethanolamine (PE), 15:0-
179 18:1(d7)phosphatidylglycerol (PG), 15:0-18:1(d7) phosphatidylinositol (PI), 15:0-18:1(d7)
180 phosphatidylserine (PS), d18:1-18:1(d9) sphingomyelin (SM), 15:0-18:1(d7)-15:0 triacylglycerol
181 (TAG) as well as cholesterol(d7) (see Table S-1). In addition, also 15:0-18:1(d7) phosphatidic
182 acid is present in the Lipidomix, but was not further considered for this study due to its poor
183 peak shape.

184 As the concentrations of the individual lipids in the Lipidomix could not be altered and since
185 detection sensitivity for each lipid class representative is distinct, regular 6-point-calibration
186 (minimally required by international guidelines) would not have been suitable for universal
187 coverage of the linear ranges of each surrogate calibrant. Therefore, 11 calibration samples (plus

188 an additional true matrix blank, i.e. unspiked mouse plasma pool) were prepared by serial
189 dilution of the spiked methanolic portion of the extraction solvent (see sample preparation). The
190 specified content of MeOH in the precipitation mix (IPA:MeOH, 2.75:1, v/v) was selected, as the
191 Lipidomix is provided in a methanolic solution by the manufacturer. Consequently, sample
192 preparation was adjusted to the composition of the highest calibration sample (calibration 11: 25
193 μ L plasma, 55 μ L IPA, 20 μ L Lipidomix and SIL fatty acids in MeOH). To evaluate and control
194 for intra-sequence (i.e. intra-assay) precision and accuracy, 5 quantitative QCs (QC_{quant}), which
195 were spiked to yield concentrations at 1 %, 5 %, 25 %, 50 % and 80 % of the highest calibration
196 sample, were established. For internal standardization, 80 ng mL⁻¹ of 17:1 LPC were spiked to
197 each sample before sample preparation. Surrogate calibrant concentrations in calibration and QC
198 samples are listed in Table S-2 and Table S-3.

199 The sequence of analysis was designed to cover calibration and QC_{quant} samples at the beginning,
200 middle and end of the batch. In-between, study samples with embedded system QCs (QC_{syst} ;
201 after each block of 5 samples) were incorporated in a randomized manner. QC_{syst} samples were
202 independently prepared but had identical surrogate calibrant concentrations to QC_{quant} level 3.
203 This way, besides being used for monitoring of instrument stability and normalization (e.g.
204 LOWESS[36]), QC_{syst} samples were also used to control for stability of quantitative performance
205 throughout the sequence. The principal scheme for the analytical batch can be seen in Figure 1
206 (for more details see Table S-4).

207 **2.4 LC-method.** Instrumental analysis was performed based on the method of Tsugawa et. al.[9]
208 Chromatography was carried out on a 1290 Infinity UHPLC system (Agilent Technologies,
209 Waldbronn, Germany) via an Acquity UPLC CSH C18 column (100 mm \times 2.1 mm, 1.7 μ m, 130
210 \AA) with a VanGuard Acquity UPLC CSH C18 pre-column (5 mm \times 2.1 mm, 1.7 μ m, 130 \AA)
211 (Waters, Milford, MA, USA). Mobile phase A consisted of 60:40 MeCN:H₂O (v/v) with 0.1 %
212 formic acid (v/v) and 10 mM ammonium formate. Mobile phase B consisted of 90:9:1
213 IPA:MeCN:H₂O (v/v/v) with 0.1 % formic acid (v/v) and 10 mM ammonium formate. The
214 gradient (0.0 min, 15 % B; 2.0 min, 30 % B; 2.5 min, 48 % B; 11.00 min, 82 % B; 11.50 min, 99
215 % B; 12.00 min, 99 % B; 12.10 min, 15 % B, 15.00 min, 15 % B) was operated at a flowrate of
216 0.6 mL min⁻¹ and a constant oven temperature of 65 °C. Injection volume of a connected PAL
217 HTC-xt autosampler (CTC Analytics, Zwingen, Switzerland) was set to 3 μ L in positive and 5

218 μL in negative ionization mode (to increase feature detection due to generally lower sensitivity
 219 in negative mode).

220 **2.5 MS-method.** The chromatographic system was hyphenated to a TripleTOF 5600+ mass
 221 spectrometer and operated with the ESI-probe of a DuoSpray source (Sciex, Framingham, MA,
 222 USA). Ion source parameters were as follows: curtain gas (N_2) 35 psi; nebulizer gas (N_2) 60 psi;
 223 heater gas (N_2) 60 psi, ion source voltage floating +5,500 V (positive mode) and -4,500 V
 224 (negative mode), declustering potential: ± 80 V, source temperature 350 °C. For fragmentation in
 225 untargeted screening, collision energy was set to 35 V with a spread of ± 15 V for each SWATH-
 226 MS/MS experiment, respectively. An accumulation time of 200 ms was assigned to the TOF-MS
 227 experiment for precursor detection in the mass range of m/z 50 – 1,250. Every ionization mode-
 228 specific method covered 25 SWATH-MS/MS experiments with a respective accumulation time
 229 of 20 ms. Resolving power on TOF-MS level was over 30,000 (FWHM @ m/z 829.5393) and
 230 over 15,000 (FWHM @ m/z 397.2122) on SWATH-MS/MS level in high sensitivity mode. Total
 231 cycle time summed up to 750 ms, which yielded a minimum of 10 points per peak for an average
 232 peak width at base of 8 s. Ionization mode-dependent selection of SWATH window widths was
 233 done using swathTUNER.[37] and the initial input data were generated from a QC_{syst} sample that
 234 was analysed with a preliminary TOF-MS method that was operated in an information-dependent
 235 acquisition (IDA) mode. Final SWATH window settings are listed in Table S-5. Samples were
 236 first analyzed in positive and subsequently in negative mode. Mass calibration was achieved via
 237 infusion of sodium acetate (0.1 mg mL⁻¹ in MeCN:H₂O, 1:1, v/v) every 10th injection. The
 238 analytical system was controlled by Analyst 1.7 TF software (Sciex).

239 **2.6 Validation.**

240 In general, it was pursued to follow existing guidelines for bioanalytical method validation as far
 241 as possible. Therefore, selectivity of surrogate calibrants was assessed via the analysis of 6
 242 individual blank mouse plasma samples in positive and negative mode.

243 Furthermore, ≥ 6 -point calibration and multi-level QCs were established for each surrogate
 244 calibrant to evaluate the linear range, precision and accuracy. The acceptance criteria for the
 245 inclusion of surrogate calibration samples were adopted from the FDA guidelines, i.e. non-zero
 246 calibrants were ± 15 % of the nominal concentrations (except for LLOQ where ± 20 % were
 247 accepted) and ≥ 75 % of the included surrogate calibrants met the criteria. For the linear ranges,

248 high similarities between surrogate calibrants and corresponding target analytes must be assumed
 249 for correct quantification. If this assumption is in doubt, its validity can be verified post-
 250 acquisition via standard addition of the target analyte of interest to a pooled plasma sample.
 251 Matrix effects were elaborated by continuous post-column infusion [44] of surrogate calibrants
 252 into the regular analytical flow of blank mouse plasma samples (i.e. devoid of deuterated
 253 surrogate calibrants) via a T-piece. This way, matrix effects could be monitored and estimated
 254 across the whole retention time interval of the class, instead of just for one peak as for post-
 255 extraction spiking experiments.[43] In contrast to post-column infusion, these experiments would
 256 have also required an exceeding volume of the mouse plasma samples which was not available.
 257 In this approach, potential matrix compounds, that are causing an increase (ion enhancement) or
 258 decrease (ion suppression) in the extracted ion chromatograms (EICs) of the surrogate calibrants,
 259 are exhibited. To verify the absence of matrix effects, surrogate calibrant EICs should show a
 260 constant signal during relevant retention time (t_R) intervals of corresponding lipid classes and
 261 lipid species, respectively. Here, it is assumed that lipid species are exposed to similar behavior
 262 in terms of matrix effects within a lipid class during post-column infusion experiments and that
 263 deviations in chain length and saturation lead to identical results. This assumption was also
 264 investigated by post-column infusion (see Figure S-6). The real matrix effect of the analytes can
 265 then be estimated from the position of elution of the respective lipid species.

266 **2.7 Data processing.**

267 The LC-MS setup enabled the usage of a well-established workflow for lipidomic analysis[38]
 268 with rapid data processing via MS-DIAL[9] (version 3.20), which offers tools for peak finding,
 269 alignment, deconvolution, identification (score-based on t_R , precursor ion, isotope pattern and
 270 MS/MS similarity to the LipidBlast library[8]), and normalization of SWATH data. After
 271 conversion of the recorded raw data (.wiff extension) into Analysis Base Files (.abf extension)
 272 via the ABF converter (Reifycs, Tokys, Japan), MS-DIAL projects were created separately for
 273 each ionization mode. Processing parameters were adjusted to the following settings: peak
 274 finding between 0.3 – 13 min; precursor m/z range from 50 – 1,250; TOF-MS tolerance: m/z
 275 0.01; SWATH-MS/MS tolerance: m/z 0.025; smoothing level: 2; minimum number of points per
 276 peak: 5; minimum peak height: 500 cps; t_R tolerance for LipidBlast[8] based identification: 1.0
 277 min; identification score cut-off: 80 %. Peak alignment was based on the 3rd QC_{syst} sample with a

278 t_R tolerance of 0.1 min, TOF-MS tolerance of *m/z* 0.02 and a detection frequency of at least 70 %
 279 in one group. Blank subtraction was exerted for signals that had a foldchange <5 in the average
 280 samples compared to the average blank signals. The final alignment files covered the following
 281 feature counts: positive mode, 2083 features after blank subtraction including 529 identified
 282 lipids; negative mode, 1103 features after blank subtraction including 179 identified lipids.
 283 For quantitative data processing, PeakView 2.2 (Sciex) and MultiQuant 3.0 (Sciex) were utilized.
 284 Here, peak areas were extracted with a \pm 10 mDa mass window in the associated mass
 285 spectrometric experiment. Further settings were automated integration by a MQIII algorithm,
 286 Gaussian smoothing (width: 2 data points), noise percentage of 90 %, baseline subtraction
 287 window of 0.1 min and a peak splitting factor of 2. Moreover, Excel 2019 (Microsoft, Redmond,
 288 WA, USA), SPSS Statistics 23 (IBM, Armonk, NY, USA) and Origin 2019 (OriginLab,
 289 Northampton, MA, USA) were used for additional data evaluation.

290 **2.8 Study samples.**

291 Plasma samples were derived from control mice and mice lacking *BK* in various adipocyte
 292 populations (both on a C57Bl/6N strain background). Adipocyte-specific controls (genotype:
 293 *adiponectin-CreERT2*^{tg/+}; *BK*^{+/L2} (CTR group)) and pre-mutant *BK* animals (genotype:
 294 *adiponectin-CreERT2*^{tg/+}; *BK*^{L1/L2} (KO group)) were generated as previously described.[30, 39,
 295 40]

296 Dietary feeding protocols were performed with *adipoqBK-CTR* and *adipoqBK-KO* mice that
 297 either received a high-fat-diet (HFD) or a control diet (CD) for 18 weeks.[39] To avoid sex-
 298 dependent effects only male mice were designated to the dietary feeding at an age of 10 weeks.
 299 Body weight gain, fat masses and non-fat components of the body, food intake, body core
 300 temperature and numerous other parameters of the CD- and HFD-exposed *adipoqBK-CTR* and
 301 *adipoqBK-KO* mice were reported by Illison et al.[30]

302 **3. RESULTS AND DISCUSSIONS**

303 **3.1 Method characteristics.**

304 The employed RPLC-MS/MS assay is a lipid-species separation method. It allows separation of
 305 potential isotopic interferences ($M+2$ isotopologues of lipids with 1 additional double bond[41]),
 306 of many isomeric lipid species and leads to reasonable spread of the lipids over the
 307 chromatogram as to minimize matrix effects. Data-independent acquisition with SWATH was

308 utilized for data generation. It results in comprehensive MS and MS/MS data over the entire
 309 chromatogram and across all samples with the benefit that EICs for quantitative analysis can be
 310 retrieved post-acquisition from MS and MS/MS data, whichever is more selective or more
 311 sensitive. Other selective ion traces can then serve for intra-assay cross validation of assay
 312 specificity. Lipidomix standards added to the samples before preparation are usually used as ISs
 313 for single point calibration. This standard mix has the advantage that the incorporated multiply
 314 deuterated lipids are not endogenous and elute in the middle of the lipid species distribution so
 315 that all lipids quantified with this single lipid class specific standard elute relatively close-by.
 316 However, herein we test a complementary calibration approach using the Lipidomix as calibrant
 317 series for matrix-matched surrogate calibration. Its performance was then compared with single
 318 point lipid class specific calibration in terms of assay accuracy.

319 **3.2 Selectivity and IS selection.**

320 After analysis of 6 individual blank mouse plasma samples, no interfering peaks were detected
 321 for surrogate calibrant mass traces in relevant t_R intervals (see Table S-1 and Table S-6).
 322 For optimum internal standardization, addition of a complementary set of labeled lipids (with a
 323 mass shift of ≥ 3 Da to surrogate calibrants and unlabeled analytes) would have been
 324 advantageous. However, commercial availability of such standards is limited or not given. As an
 325 alternative, odd-chain lipid species, which were shown to be of explicitly lower content in human
 326 plasma than even-chain lipids,[42] can be suitable as ISs as long as endogenous concentrations
 327 are below detectable levels in study samples. Therefore, the measured blank plasma samples
 328 were screened for potentially suitable odd-chain lipid ISs by checking endogenous background
 329 signals for mass traces of odd-chain lipid standards. The odd-chain lipid species that were
 330 detected in these samples are shown in Table S-11. Eventually, only one odd-chain lipid species
 331 that was available at short notice in our lab, 17:1 LPC, was found acceptable and could
 332 subsequently be used as a single IS (IS_{quant}) for surrogate calibration.

333 **3.3 Linear ranges and intra-assay precision and accuracy.**

334 Following the scheme in Figure 1, intra-sequence (i.e. intra-assay) precision and accuracy were
 335 determined for independent QC_{quant} ($n = 3$ per level) and QC_{syst} ($n = 11$) samples. Results were
 336 obtained in positive and negative mode for the most sensitive and interference-free adduct of
 337 each surrogate calibrant (see Table S-1). A chromatogram of all surrogate calibrants is presented

338 in Figure 2. Due to the comprehensive nature of SWATH acquisition, fully quantifiable EICs on
 339 MS/MS level could also be obtained for the evaluation of precision and accuracy, provided that
 340 selective fragments in the corresponding SWATH windows are present.

341 Linear ranges, coefficients of determination of calibration functions (R^2) and estimated LODs for
 342 the mode of favourable performance are shown in Figure 3 or are presented in more detail for all
 343 modes in Table S-8. Results for precision and accuracy are listed in Table 1. They reveal that
 344 thresholds of $\pm 15\%$ bias, which are generally accepted for targeted assays, can be reached for
 345 the majority of surrogate calibrants. Overall, good precision, with most values being below 15 %
 346 for the favourable modes of the respective lipids, is achieved. Accuracy is in acceptable ranges
 347 as well and only a limited number exceeds $\pm 15\%$ bias for certain QC levels in the best-
 348 performing modes. For poor-performing calibrants that did not pass the acceptance criteria
 349 according to the FDA guidelines, the reason could be the lack of suitable ISs, as one early eluting
 350 IS might not be able to sufficiently reflect analytical behavior of all lipid classes throughout the
 351 run. With an upgraded IS design, if suitable standards become available, further improved results
 352 can be expected for future studies. Due to the comprehensive design of the MS method, general
 353 settings of declustering potential and collision energy were chosen. Enhanced and refined results
 354 for lipids or lipid classes of special interest can therefore certainly be reached when working with
 355 optimized MS and MS/MS parameters.

356 **3.4 Evaluation of matrix effects via post-column infusion.**

357 Post-column infusion of surrogate calibrant solution and injection of a mouse plasma pool can
 358 indicate regions in the chromatogram where matrix effects can impair assay accuracy. Data for
 359 positive mode TOF-MS traces are shown in Figure 4. Here, for most surrogate calibrants no or
 360 only negligible matrix effects were detected. Nevertheless, for SM(d9) (Figure 4F) and PC(d7)
 361 (Figure 4H) minor ion suppression intervals can be spotted. These ion suppression effects seem
 362 to be of lesser extent on the positive mode SWATH-MS/MS level (see Figure S-1).
 363 Complementing results for matrix effects in negative mode can be seen in the Supplementary
 364 Material (TOF-MS: Figure S-2; SWATH-MS/MS: Figure S-3). Ion suppression seems to be
 365 much more drastic in negative TOF-MS, especially for PS(d7), PG(d7), SM(d9), PC(d7) and
 366 PE(d7) (see Figure S-2F - Figure S-2J). This effect is likely to be caused by the generally high
 367 abundant PCs in plasma and the elevated injection volume in negative mode. To resolve this

368 issue, adjustments in sample preparation, injection volume or chromatographic separation would
 369 be needed. However, this repeatedly underlines the need for proper IS coverage across the
 370 chromatogram, as densely distributed stable isotope labeled ISs in critical t_R regions would be
 371 able to control for matrix effects.

372 Interestingly, matrix effects again appear to be reduced on SWATH-MS/MS level (Figure S-3)
 373 and can only be significantly detected for PC(d7) (Figure S-3G). This finding seems paradox
 374 since similar extents of matrix effects are expected for TOF-MS and SWATH-MS/MS
 375 experiments as ion suppression occurs during ionization in the ion source. A contributing factor
 376 for this finding could be the gated ion transmission control (ITC), which is usually applied in
 377 TOF-MS to avoid detector saturation in case of high ion load.[45] Depending on total ion current
 378 and individual peak intensities, ion transmission is modulated cycle-by-cycle and a correction
 379 factor is applied to the output signal to account for ITC fluctuations. Whereas ITC is steadily
 380 regulated in TOF-MS, ion transmission is permanently set to 100 % for SWATH-MS/MS
 381 experiments due to the significantly lower ion load after precursor isolation and the greatly
 382 reduced risk for detector saturation. Total ion chromatograms (TICs) and ITC progression from
 383 the same experiments for positive and negative mode TOF-MS are shown in Figure S-4. Plots for
 384 comparison of TOF-MS and SWATH-MS/MS matrix effects for exemplary surrogate calibrants
 385 are displayed in Figure S-5.

386 Ultimately, special care has to be taken for lipid classes affected by matrix effects, in particular
 387 when surrogate calibrant and target analyte do not underlyingly identical degrees of matrix effects.
 388 For critical lipid classes, the number of surrogate calibrants and ISs should be maximized.
 389 Concerning the already discussed lack of suitable lipid standards, another approach could be the
 390 determination of response factors in representative matrix like a QC sample. However, enhanced
 391 uncertainty of results is given if high inter-sample variability of matrix effects is observed, since
 392 response factors are dependent on the underlying matrix and may change from lot-to-lot.

393 **3.5 Response factors of target analytes related to surrogate calibrants.**

394 Most studies dealing with class-specific quantification of lipids are based on shotgun lipidomics
 395 approaches, [11-13] which require high resolution instruments with resolving power >100,000 to
 396 rule out signal interferences of co-ionized compounds with minor mass differences. Yet, some
 397 isomeric lipids cannot be distinguished in shotgun lipidomics and there is a certain risk for

misannotations due to in-source fragmentation (e.g. LPC can decompose to FA or LPE).[46, 47] For shotgun assays, instrument responses of lipid species within polar lipid classes were reported to be mostly identical due to the main effect of the polar head group on the ionization efficiency.[19] For the other lipid classes, responses between lipid species are dependent on chain length and the degree of saturation.[12] Other factors affecting detector response were total lipid concentration, instrument settings and solvent composition. Furthermore, studies utilizing surrogate calibrants found differing ionization efficiencies between stable isotope labeled compounds and unlabeled target analytes.[33, 34] For lipidomics based on lipid species separation by RPLC, distinct ionization conditions (due to gradient composition or matrix effects) may also contribute to inaccuracy. Accordingly, the determination of response factors between surrogate calibrants and corresponding target analytes is essential to ensure accurate quantification. To take matrix effects (and recovery) into account, response factors are ideally determined in representative sample matrix and after the analytes have underwent the identical sample preparation. Owing to limited sample volume in the current study with mouse plasma, evaluation of response factors in neat solution was assumed to be an acceptable compromise for this general global profiling method aiming primarily at new hypothesis generation.

For proof-of-principle, several lipid standards (14:0-14:0 PC, 16: 16:0 PC, 18:0-18:0 PC, 20:0 LPC) were acquired. Standards, surrogate calibrants and 17:1 LPC (IS_{quant}) were spiked into MeOH, followed by serial dilution (with IS_{quant} spiked MeOH) to 5 different concentration levels. These samples were analyzed with the identical method that was used for previous study measurements. Response factors were calculated via the slopes ($slope_{lipid\ standard} / slope_{surrogate\ calibrant}$) and results are shown in Table 2. It can be seen that for the majority of lipids the response factors in MS are close to 1, while in MS/MS level they are significantly deviating from 1, as expected. For PC 18:0-18:0, response factors are significantly lower (<1). It can be due to matrix effects, but also due to a poor quality of the standard. For shotgun approaches, linear relationships between acyl chain length and response factors could be observed for PCs.[12] Here, neither using mass concentrations nor molarities, an apparent relationship between chain length and response factor could be constructed. This implies that for gradient elution in RP chromatography, which results in differing retention times for lipid species, estimation of response factors is aggravated due to the changing solvent composition. A simple extrapolation

428 factor that is depending on structural characteristics can therefore not be readily determined. The
 429 same applies to SWATH-MS/MS data, in which the additional factor of fragmentation efficiency
 430 is further complicating the matter.

431 Assessment of response factors for numerous lipid species is labor intensive and associated to
 432 high costs for standard compounds. Yet, method-specific response factor libraries could be
 433 iteratively established, as the stability of response factors has been previously demonstrated for
 434 other mass spectrometric instruments.[18] A productive workflow for future studies could be to
 435 acquire standards for compounds of interest after final data processing and to subsequently
 436 determine their response factors to the corresponding surrogate calibrants. This way,
 437 significantly regulated lipids and/or potential biomarkers can be quantified post-acquisition for
 438 universal comparability. This workflow (i.e. post-acquisition re-calibration) can be of special
 439 interest when sample volume of individual samples is limited and standard addition is hence not
 440 applicable.

441 **3.6 Comparison of quantification between standard addition, surrogate calibration and 1-
 442 point calibration.**

443 In general, standard addition has several drawbacks which limit its routine implementation into
 444 untargeted studies: (i) it is not suitable if the concentration in the sample is close to the upper
 445 limit of the linear range, since additional spiking will result in a nonlinear increase of the signal;
 446 (ii) it requires a significant amount of additional laboratory work and analysis time for each
 447 sample; (iii) it is not applicable if the sample volume is limited because several aliquots of the
 448 sample are required. However, since it is accepted as a valid approach for quantification, it was
 449 used herein as a reference method for the comparison of surrogate calibration and 1-point
 450 calibration on a limited number of examples.

451 Given the linear calibration functions of the respective surrogate calibrant (see Table S-8) and
 452 the response factor of the target lipid species (Table 2), absolute quantification via surrogate
 453 calibration can be executed. For cross-validation purposes, a 5-level standard addition of 14:0-
 454 14:0 PC, 16:0-16:0 PC, 18:0-18:0 PC and 20:0 LPC into pooled QC samples was conducted (see
 455 Table S-7). The results for standard addition were considered as the most accurate; thus, they
 456 were compared to results obtained from surrogate calibration (established via post-acquisition re-
 457 calibration with determined response factors) and common 1-point calibration (Table S-1 and

458 Table S-3) of the QC pool. Precision for standard addition was calculated via the error of the y-
 459 intercept. Accuracy was determined via the unspiked sample, after adjusting for the endogenous
 460 lipid concentration calculated by extrapolation of the standard addition curve. For surrogate
 461 calibration and 1-point calibration, precision was determined via the 14 replicates of $\text{QC}_{\text{syst}} +$
 462 QC_{quant} 3 samples. Response factors are generally mandatory for 1-point calibration, too.
 463 Accordingly they were also applied to this method. A comparison of the three quantification
 464 methods is given in Table 3.

465 Except for 14:0-14:0 PC, acceptable agreement between standard addition and surrogate
 466 calibration was obtained in positive TOF-MS mode by this post-acquisition re-calibration.
 467 Relatively high deviations in 14:0-14:0 PC quantification could be due to the neglected matrix
 468 effects (Figure 4H and Figure S-2I) (note that the response factor was determined in MeOH), or
 469 the relatively low concentration close to the lower end of the linear range of the surrogate
 470 calibrant 15:0-18:1(d7) PC (LLOQ: 25.11 ng mL^{-1}). Quality of standards is also a factor which
 471 can contribute significantly to inaccuracies. Excellent results were achieved for 20:0 LPC
 472 throughout all modes for surrogate calibration. This finding could furtherly imply the need for
 473 improved internal standardization as 20:0 LPC was quantified with a class-specific IS (IS_{quant}
 474 17:1 LPC). In most cases, 1-point calibration led to overestimation of target lipid concentrations
 475 and to systematically higher concentration values than for surrogate calibration. Major
 476 drawbacks, which are resulting in increased uncertainty for 1-point calibration, are: (i) the
 477 defective calibration function that is automatically forced through the origin; (ii) the inability to
 478 apply weighted regression; (ii) the need for the calibrant concentration to be in the linear range,
 479 which requires preliminary experiments. In contrast to the partially unacceptable results obtained
 480 from 1-point calibration (despite considering response factors), surrogate calibration involving
 481 post-acquisition re-calibration yielded absolute concentration values comparable to standard
 482 addition results. By assessment of matrix-matched response factors and proper internal
 483 standardization, performance of surrogate calibration is likely to be further improved.

484 **3.7 Estimation of (matrix-matched) response factors using human plasma NIST SRM 1950.**

485 To purchase a large number of lipid standards for response factor determination is usually not
 486 possible. Instead, response factors for individual lipids could be determined with certified
 487 reference materials with specified lipid concentration values. Currently, such certified reference

material is not available but NIST SRM 1950 provides consensus values which can be and have been used herein for this purpose. These consensus values should be reliable enough as long as there are no better options (a brief description of the NIST SRM 1950 standard reference material and further information about associated consensus values can be found in the Supplementary Material Text-S1). Thus, for the large-scale response factor determination, surrogate calibrants and 17:1 LPC (IS_{quant}) were spiked to NIST SRM 1950 plasma. The samples were subsequently prepared with the identical protocol that was also used for the experimental mouse plasma samples. The supernatant was then diluted with IS_{quant} -spiked precipitation solvent to yield a 5-point dilution series. In order to avoid bias by underestimation of matrix effects, only moderate dilutions of the supernatant were prepared (1:1.33, 1:2, 1:4, 1:8; v:v). Equivalent to the above described response factor evaluation procedure for standard reference analytes (i.e. post-acquisition re-calibration approach), slopes of SRM 1950 lipids were divided by the slope of the corresponding surrogate calibrant. Only lipids with a COD <30 % (see Text S-1) that could be detected free of interference, were considered for response factor evaluation. As lipid concentrations were reported for total sum compositions (e.g. PC 36:0), it had to be assumed that different isomer lipid species (e.g. PC 18:0-18:0 and PC 16:0-20:0) contribute equally to the resulting response factor. If individual species should be quantified, a specific MS/MS signal has to be used for calibration, response factor determination and re-calibration. Results are listed in Table S-9 and the distribution of response factors in positive and negative mode are visualized in Figure S-7.

To achieve reliable results, peak integration has to be consistent throughout the respective lipid class. As some lipid signals are close to detector saturation and others are close to LOQ, further care has to be taken to verify working in correct linear ranges. Although high similarities in the matrix composition of mouse and human plasma can be expected, potential response factor inaccuracies should be kept in mind due to the usage of plasma from distinct species. One major drawback of this approach is the partially high uncertainty associated with employed consensus values and variance of inter-laboratory results, and the observed discrepancies to values determined by the LIPID MAPS consortium (Table S-9). Nevertheless, reasonable results, especially for LPCs, for which response factors are closely distributed around 1, could be obtained. As LPCs are one of the most polar lipid classes, instrument response is less affected by

518 differences in chain length or saturation. Also matrix effects were shown to be of minor extent or
 519 at least uniform throughout the elution interval for LPCs (see Figure 4 and Figure S-2). This
 520 approach of post-acquisition recalibration with response factors determined from consensus
 521 values of NIST SRM 1950 should lead to improved accuracies of large-scale quantitation in
 522 untargeted RP-UHPLC-MS/MS lipidomics and is supposed to provide further improvements
 523 once true certified reference values become available.

524 **3.8 Application and inter-study comparison.**

525 The comparison of lipidomic profiles of rodents that received a high fat diet versus rodents that
 526 received a standard (control) diet has been described in the literature. For two dietary mouse
 527 studies,[48, 49] absolute concentration values of various lipid species were reported. These
 528 results were compared to the quantitative results in this study, which were obtained via surrogate
 529 calibration. Due to differences like genetic background, age, diet composition, duration of
 530 feeding and other factors, which can highly influence the plasma lipidome (see Table S-10),
 531 unrestricted inter-study comparability is not possible and perfect match of lipid levels cannot be
 532 expected. Nevertheless, quantitative results allow a relative comparison of lipid plasma levels
 533 and to identify lipid species unaffected by diet. Comprehensive results for inter-study
 534 comparison are listed in Table S-10. For instance, many LPC species seemed to be altered
 535 between the groups with differing diet. The main findings for this lipid class are shown in Figure
 536 S-8.

537 In conclusion, levels of 11 lipid species showed less than 30 % deviation when compared to
 538 results of CD-fed mice from Eisinger et al.[49] (for HFD-fed mice 5 lipids showed less than 30
 539 % deviation). Compared to results from Barber et al.,[48] 4 lipids in CD-fed mice and only 3
 540 lipids in HFD-fed mice had lower than 30 % deviation. Furthermore, when comparing the two
 541 previously published studies, also only 5 lipids in CD-fed mice and 6 lipids in HFD-fed mice
 542 showed a plasma level deviation below 30 %. Overall, the majority of lipids were elevated,
 543 compared to the previously published studies (72.2 % of all lipids for Barber et al.[48], 83.8% of
 544 all lipids for Eisinger et al.[49]), which might be related to the time point of sampling or the
 545 duration of the feeding (Table S-10). It should also be noted that 32.4 % of the obtained results
 546 from surrogate calibration exceeded the linear range and are likely to be overestimated.
 547 However, the study design enables the choice of a less sensitive polarity (e.g. ESI⁻ instead of

548 ESI⁺) or MS/MS level quantification with a less sensitive fragment ion, which can yield an
 549 enhanced linear range for improved quantification.

550 **4. CONCLUSIONS**

551 With the presented work, an alternative approach towards class-specific quantification in an
 552 untargeted RP-LC-MS lipidomic assay is suggested. Surrogate calibrant methods have been
 553 shown highly suitable for quantification of endogenous analytes, especially when no true blank
 554 matrix is available. This principle has been transferred to untargeted high-resolution MS in
 555 combination with comprehensive, quantifiable SWATH-acquisition, which offers potential
 556 improvements regarding selectivity when compared to TOF. Moreover, SWATH is able to
 557 generate enhanced sensitivity, as MS/MS generally yields beneficial signal-to-noise ratios.
 558 Nevertheless, SWATH did not automatically provide the best results for all lipid classes and
 559 several surrogate calibrants showed superior performance on the TOF-level. This might be due to
 560 the lack of specific and sensitive fragments, yet, MS parameters like collision energy can be
 561 individually tailored for each SWATH experiment to optimize the sensitivity. After all, it was
 562 demonstrated that sufficient precision and accuracy can be obtained with SWATH (and TOF)
 563 and that previously determined response factors are needed for accurate quantification.

564 For the majority of surrogate calibrants, sufficient results for precision and accuracy, complying
 565 with proposed thresholds for targeted assays, were obtained. The presented approach was shown
 566 to yield improved results compared to 1-point calibration and was in acceptable agreement with
 567 standard addition for tested lipids. Moreover, issues concerning response factors, which were
 568 shown to be essential for accurate quantification, were addressed by post-acquisition re-
 569 calibration via analysis of authentic standards (in methanolic solution) and NIST SRM 1950
 570 (matrix-matched but species mismatched). More powerful workflows, which comprise response
 571 factor determination for analytes of interest post-analysis, or method-specific response factor
 572 libraries could result by this approach with doable extra expenditure, yet significant
 573 improvement in data quality.

574 Ultimately, the target goal to obtain quantitative results that enhance inter-study, inter-batch or
 575 database comparability was demonstrated. Yet, future challenges remain, primarily with the
 576 persistent lack of suitable ISs. An alternative approach to account for this issue could be the use
 577 of low abundant odd-chain lipid species for surrogate calibration in combination with stable

578 isotope labeled lipids as ISs for interference-free normalization. Regarding the potential
579 advantages, further studies addressing absolute quantification are anticipated as they can aid to
580 maximize the extent and quality of the information that can be extracted from untargeted
581 lipidomic assays.

582

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588

589 **AUTHOR INFORMATION**

590 **Author Contributions.** M.L, R.L and B.D. conceived and designed the study. J.I. was
591 responsible for handling of mice and sample collection. B.D. performed the experiments and
592 measurements. B:D and J.S. established the data processing workflow. The manuscript was
593 written through contributions of all authors.

594 **Notes.** The authors declare no competing financial interest.

595

596 **ASSOCIATED CONTENT**

597 **Supporting Information.** The Supporting Information is available free of charge on the
598 Journal website.

599

600

601 **TABLES AND FIGURES**602 **Table 1.** Results for precision and accuracy of surrogate calibrants in mouse plasma (pooled QC).^a

Surrogate Calibrant	Experiment	QC _{quant 1}		QC _{quant 2}		QC _{quant 3}		QC _{quant 4}		QC _{quant 5}	
		Prec. [%]	Acc. [%]								
LPC(d7)	*TOF ⁺	8.7	88.7	2.7	88.5	5.5	93.8	0.6	113.8	1.8	97.5
	SWATH ⁺	30.9	81.9	6.7	90.7	7.4	92.4	1.7	116.2	2.2	101.7
	TOF ⁻	11.1	93.0	4.5	88.8	2.4	92.6	5.4	116.8	7.7	108.6
	SWATH ⁻	2.6	92.8	9.7	91.0	6.5	86.5	9.1	102.2	0.8	85.2
LPE(d7)	TOF ⁺	22.6	92.2	12.9	87.2	6.3	93.8	3.7	113.3	3.5	92.0
	SWATH ⁺	2.1	113.0	9.8	98.3	7.6	96.5	2.6	114.5	4.9	99.3
	*TOF ⁻	11.9	100.1	1.8	87.0	6.2	93.4	0.8	114.0	0.4	99.1
	SWATH ⁻	-	-	-	-	1.5	86.8	16.3	83.9	8.6	86.4
MAG(d7)	TOF ⁺	-	-	-	-	-	-	-	-	-	-
	SWATH ⁺	-	-	-	-	17.1	95.6	13.1	113.4	5.2	109.1
	*TOF ⁻	-	-	-	-	16.8	90.5	6.9	110.8	6.3	92.0
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
PI(d7)	TOF ⁺	-	-	7.2	89.4	7.4	90.1	4.8	114.4	1.0	98.2
	*SWATH ⁺	18.0	88.0	2.5	85.8	13.6	93.2	1.8	115.2	5.6	101.5
	TOF ⁻	-	-	-	-	-	-	-	-	-	-
	SWATH ⁻	-	-	-	-	9.8	82.3	7.0	108.6	4.8	97.6
PS(d7)	TOF ⁺	-	-	-	-	7.4	97.8	1.0	115.0	2.9	103.7
	SWATH ⁺	-	-	17.3	73.5	14.6	80.9	13.2	96.6	7.2	81.4
	TOF ⁻	10.6	108.4	7.7	86.6	3.7	87.6	4.5	113.8	1.0	98.4
	*SWATH ⁻	8.0	105.2	12.9	90.1	10.3	91.4	8.9	99.0	4.4	90.3
PG(d7)	TOF ⁺	-	-	-	-	-	-	6.0	108.6	4.8	79.0
	SWATH ⁺	9.3	99.9	4.6	93.7	8.4	101.8	5.1	97.2	-	-
	*TOF ⁻	7.0	85.9	5.9	81.0	8.7	98.0	1.2	114.3	4.9	101.9
	SWATH ⁻	-	-	20.9	89.4	12.4	92.7	10.5	119.4	14.6	101.5
SM(d9)	*TOF ⁺	4.9	87.2	3.3	75.1	11.0	88.3	2.7	106.9	3.8	87.8
	SWATH ⁺	4.7	101.7	8.2	95.0	7.7	90.6	3.9	108.9	2.7	92.4
	TOF ⁻	23.7	71.7	10.8	75.0	8.6	88.9	6.6	121.6	4.5	105.2
	SWATH ⁻	4.2	96.1	20.6	103.4	7.0	98.7	6.8	128.7	10.8	107.2
Cholesterol(d7)	TOF ⁺	-	-	-	-	-	-	-	-	-	-

	[*] SWATH ⁺	-	-	20.1	84.1	10.6	96.8	7.3	104.0	1.4	103.0
	TOF ⁻	-	-	-	-	-	-	-	-	-	-
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
PC(d7)	TOF ⁺	3.5	84.8	2.3	73.9	7.7	84.0	3.4	120.7	2.7	94.8
	SWATH ⁺	7.9	94.4	4.0	79.7	11.4	92.1	4.4	110.1	3.2	103.1
	[*] TOF ⁻	6.6	84.4	3.0	75.0	5.9	87.7	6.1	123.9	9.4	100.6
	SWATH ⁻	15.4	70.7	22.7	85.3	9.0	81.1	10.7	100.8	-	-
PE(d7)	TOF ⁺	22.0	88.1	4.9	79.7	4.7	89.6	0.9	115.6	1.7	98.1
	[*] SWATH ⁺	0.7	100.6	1.7	89.0	9.6	96.5	6.8	114.4	8.8	95.4
	TOF ⁻	12.6	94.7	3.3	75.7	4.3	89.2	4.8	117.2	2.2	101.2
	SWATH ⁻	-	-	50.0	83.0	6.6	83.6	8.0	115.0	5.9	99.1
DAG(d7)	TOF ⁺	7.3	111.6	2.7	74.4	7.4	85.4	4.5	119.4	3.8	103.3
	[*] SWATH ⁺	11.8	84.8	10.4	73.8	10.6	87.1	7.3	126.7	6.0	112.2
	TOF ⁻	-	-	-	-	22.8	74.0	21.3	70.8	24.1	95.8
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
TAG(d7)	TOF ⁺	-	-	-	-	-	-	-	-	-	-
	[*] SWATH ⁺	-	-	15.6	84.5	7.8	86.7	1.8	122.0	9.9	93.6
	TOF ⁻	-	-	-	-	-	-	-	-	-	-
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
CE(d7)	[*] TOF ⁺	-	-	-	-	25.8	114.1	17.9	150.8	35.7	110.2
	SWATH ⁺	-	-	-	-	-	-	-	-	-	-
	TOF ⁻	-	-	-	-	-	-	-	-	-	-
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
Arachidonic acid(d8)	TOF ⁺	-	-	-	-	-	-	-	-	-	-
	SWATH ⁺	-	-	-	-	-	-	-	-	-	-
	[*] TOF ⁻	3.3	58.6	3.8	56.7	5.0	60.9	6.7	132.6	4.8	112.8
	SWATH ⁻	-	-	28.9	102.6	17.9	57.4	13.4	89.8	6.9	93.3
α -Linolenic acid(d14)	TOF ⁺	-	-	-	-	-	-	-	-	-	-
	SWATH ⁺	-	-	-	-	-	-	-	-	-	-
	[*] TOF ⁻	-	-	-	-	9.4	57.6	4.8	122.9	3.2	102.5
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-
Linoleic acid(d4)	TOF ⁺	-	-	-	-	-	-	-	-	-	-
	SWATH ⁺	-	-	-	-	-	-	-	-	-	-
	[*] TOF ⁻	-	-	-	-	6.0	54.1	1.5	102.4	2.4	85.9
	SWATH ⁻	-	-	-	-	-	-	-	-	-	-

603 ^aResults for QC_{quant} samples were obtained from 3 technical replicates except for QC_{quant} 3. Here results were combined from 3
604 technical replicates of a QC_{quant} 3 sample and 11 technical replicates of a QC_{syst} sample. Concentrations for calibrants and QCs are
605 listed in Table S-2 and Table S-3. The mode that shows favourable performance in terms of linear range, precision and accuracy is
606 marked with *.

607

608 **Table 2.** Response factors of lipid standards to surrogate calibrants in neat solution.^b

		14:0-14:0 PC	16:0-16:0 PC	18:0-18:0 PC	20:0 LPC
TOF-MS ⁺	mass	1.013	1.022	0.665	0.966
	molarity	0.912	0.996	0.698	1.008
SWATH- MS/MS ⁺	mass	2.383	0.990	0.724	0.849
	molarity	2.145	0.965	0.760	0.886
TOF-MS ⁻	mass	1.127	1.093	0.612	1.058
	molarity	1.015	1.065	0.642	1.104
SWATH- MS/MS ⁻	mass	1.934	1.789	0.848	0.922
	molarity	1.741	1.744	0.890	0.962
<i>t_R [min]</i>		4.32	5.61	7.02	2.88

609 ^bResponse factors were calculated on the basis of mass concentration and molarity. Results are
 610 based on peak area as this parameter was also used for quantification. However, also peak height
 611 did not reveal any linear relationship between carbon chain length of lipid species and response
 612 factors. 17:1 LPC was used as IS for all compounds.

613 **Table 3.** Results of standard addition, surrogate calibration and 1-point calibration of mouse plasma samples (pooled QC).^c

Method	14:0-14:0 PC			16:0-16:0 PC			18:0-18:0 PC			20:0 LPC		
	Conc. [ng mL ⁻¹]	Acc. [%]	Prec. [%]	Conc. [ng mL ⁻¹]	Acc. [%]	Prec. [%]	Conc. [ng mL ⁻¹]	Acc. [%]	Prec. [%]	Conc. [ng mL ⁻¹]	Acc. [%]	Prec. [%]
TOF-MS - positive mode												
Standard addition	87.6	102.9	2.6	13,288	102.3	3.0	789.1	99.0	3.5	1,298.4	103.5	0.8
Surrogate calibration	125.2	142.9	6.0	14,701	110.6	8.0	813.6	103.1	6.7	1,468.9	113.1	4.8
1-point calibration	148.7	169.7	9.9	17,534	132.0	2.2	973.1	123.3	8.5	1,571.1	121.0	7.7
SWATH-MS/MS - positive mode												
Standard addition	-	-	-	15,910	90.9	3.0	-	-	-	1,543.3	98.0	2.1
Surrogate calibration	-	-	-	12,689	79.8	6.1	-	-	-	1,720.1	111.5	9.9
1-point calibration	-	-	-	13,644	85.8	12.9	-	-	-	1,864.2	120.8	12.4
TOF-MS - negative mode												
Standard addition	96.8	100.3	5.2	14,420	91.1	0.4	759.9	102.0	5.4	1,404.9	100.5	0.9
Surrogate calibration	140.5	145.1	9.3	12,398	86.0	5.8	595.5	78.4	2.3	1,417.6	100.9	3.5
1-point calibration	161.6	166.9	11.8	14,013	97.2	9.9	624.4	82.2	7.7	1,511.3	107.6	4.1
SWATH-MS/MS - negative mode												
Standard addition	-	-	-	-*	-*	-*	677.6	98.1	3.2	1,324.9	95.2	2.6
Surrogate calibration	-	-	-	-*	-*	-*	717.1	105.8	11.2	1,472.4	111.1	6.4
1-point calibration	-	-	-	-*	-*	-*	1,180.5	174.2	13.5	1,680.0	126.8	8.7

614 ^cCells marked with * indicate a peak interference in the respective mode, leading to inaccurate integration and quantification.

615 **Figure Captions:**

616 **Figure 1.** Measurement scheme of the analytical sequence.^a

617 ^aQC_{syst} samples were repeatedly analyzed after each 5 real sample measurements.

618

619

620 **Figure 2.** Chromatogram of surrogate calibrants.^b

621 ^bResults from calibration 11 sample in mode of favourable performance (see Table 1 or the
622 marked mode of favourable performance in Figure 3.) is shown. The specific *m/z* values of the
623 presented EICs are presented in Table S-6. In addition, the positive TOF-MS signal of 17:1 LPC
624 (IS_{quant}) is drawn.

625

626

627 **Figure 3.** Linear ranges of surrogate calibrants.^c

628 ^cPolarity that showed favourable performance, concerning linear range, precision and accuracy,
629 is marked with a *. The MS level of best performance, as well R² values derived from calibration
630 results are listed in the table. For more detailed results of all MS modes see Table S-8. LOD
631 results are estimated via results for signal-to-noise ratio of ≈ 3.

632

633

634 **Figure 4.** Matrix effect evaluation by post-column infusion – positive mode TOF-MS.^d

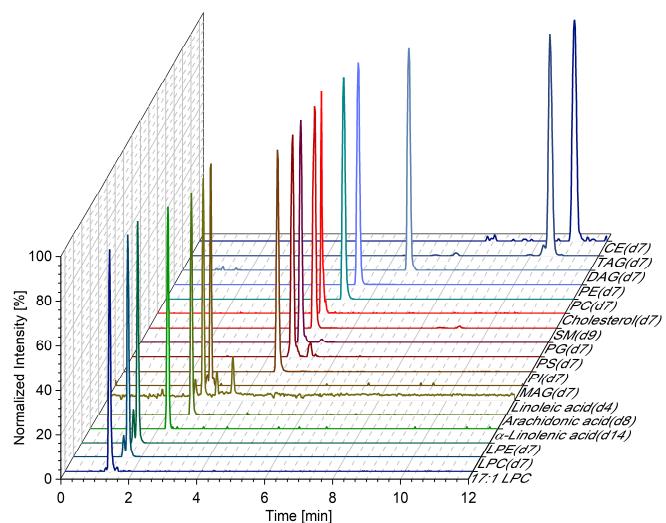
635 ^dTOF-MS mass traces of surrogate calibrants in positive mode during analysis of blank plasma
636 samples and simultaneous post-column infusion of surrogate calibrants via a T-piece. Shaded
637 areas indicate t_R intervals in which species of corresponding lipid classes were detected and
638 identified. For PG(d7) (panel E) the increasing signal does not indicate ion enhancement but is
639 rather caused by a closely co-eluting peak interference. A: LPC(d7), B: LPE(d7), C: PI(d7), D:
640 PS(d7), E: PG(d7), F: SM(d9), G: Cholesterol(d7), H: PC(d7), I: PE(d7), J: DAG(d7), K:
641 TAG(d7), L: CE(d7). Ion suppression intervals can be observed for SM(d9) (Figure 4F) and
642 PC(d7) (Figure 4H).

643



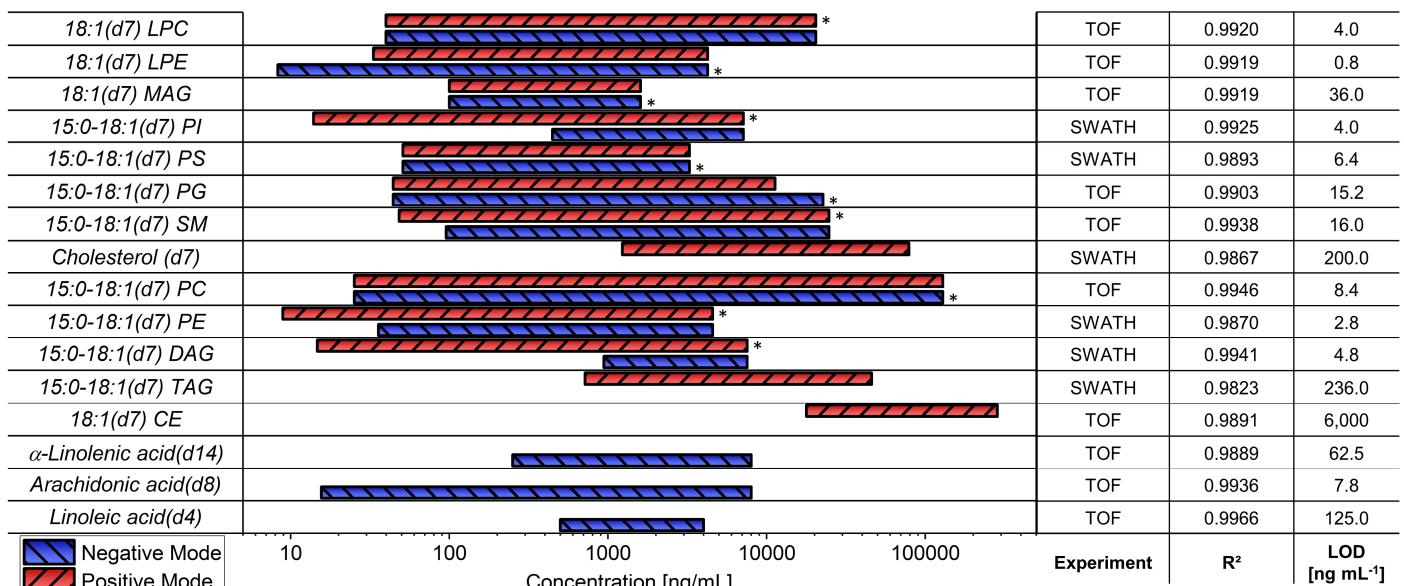
644 **Figure 1.** Measurement scheme of the analytical sequence.^a

645

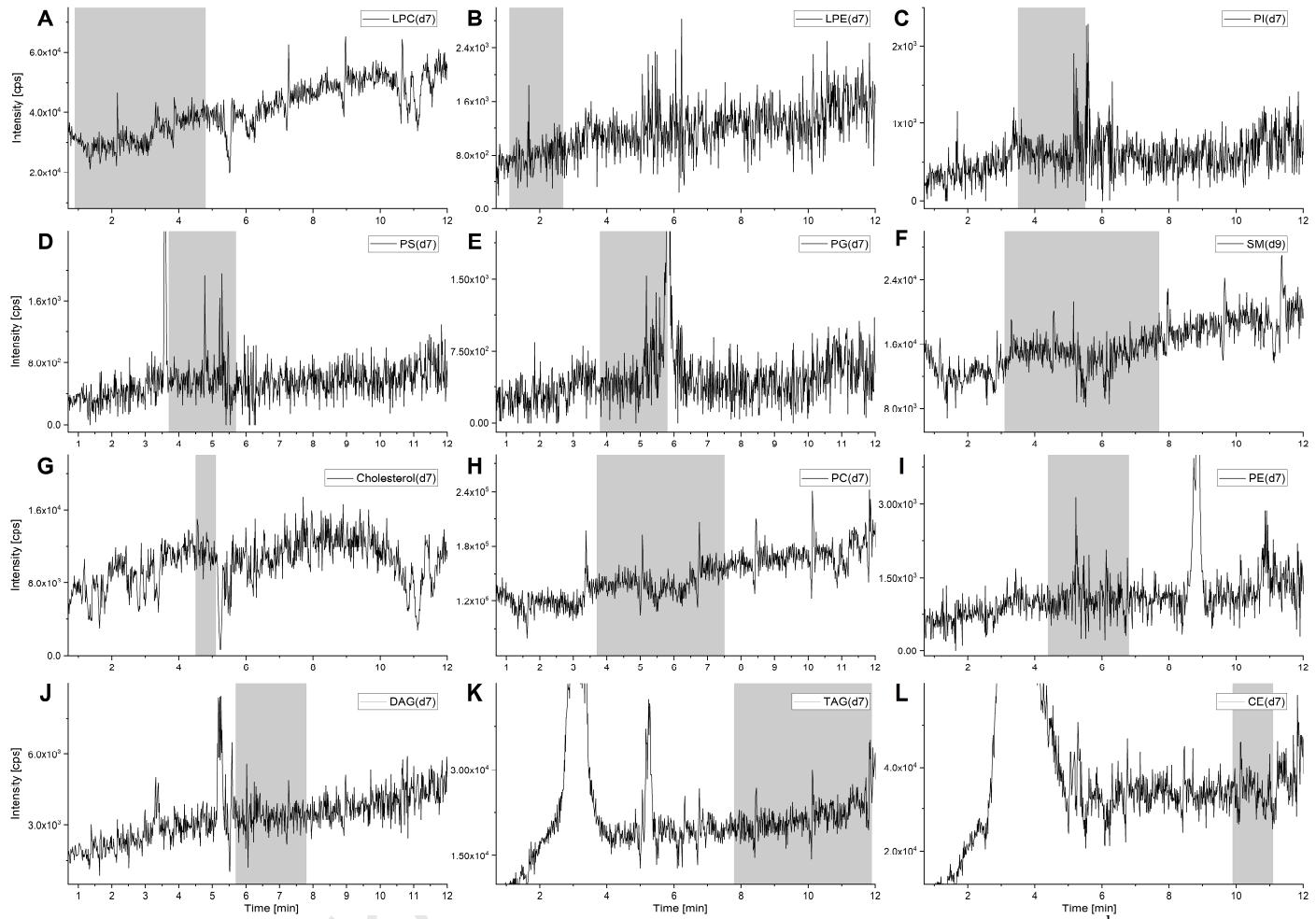


646 **Figure 2.** Chromatogram of surrogate calibrants in mouse plasma.^b

647

648 **Figure 3.** Linear ranges of surrogate calibrants in mouse plasma (pooled QC).^c

649



650 **Figure 4.** Matrix effect evaluation by post-column infusion – positive mode TOF-MS.^d

651

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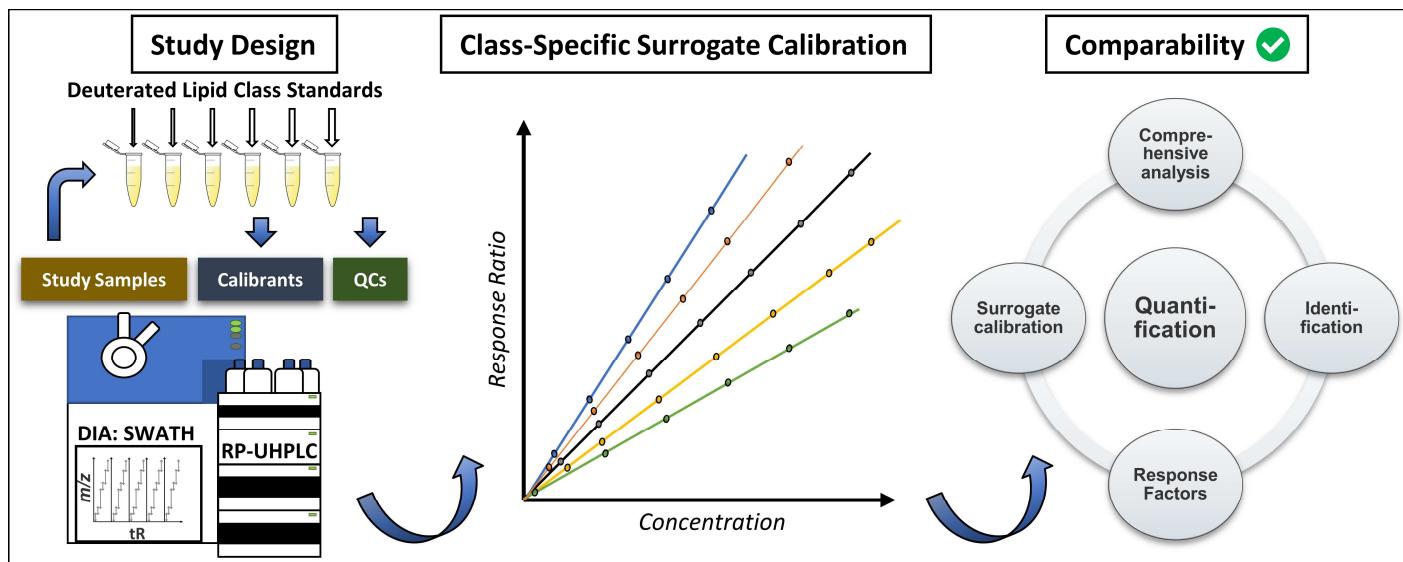
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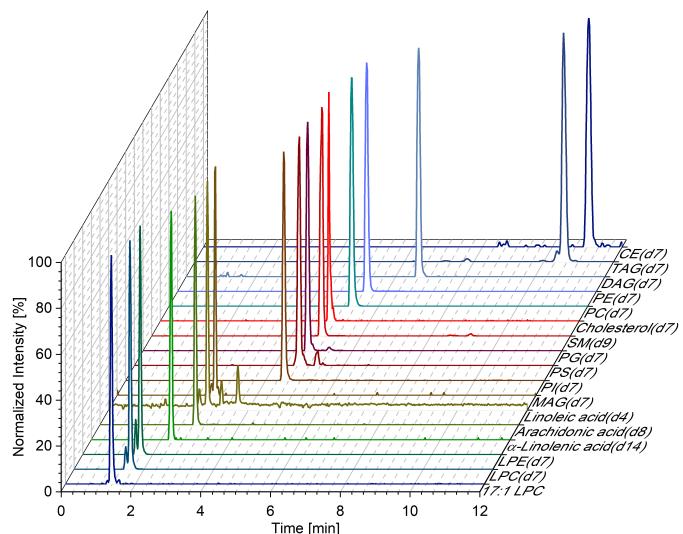
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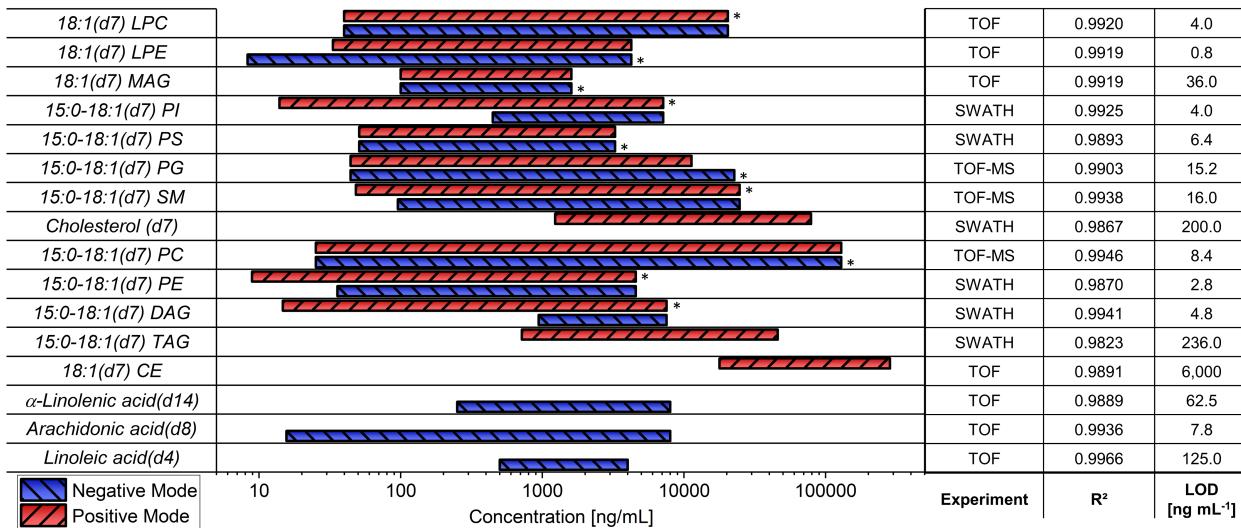
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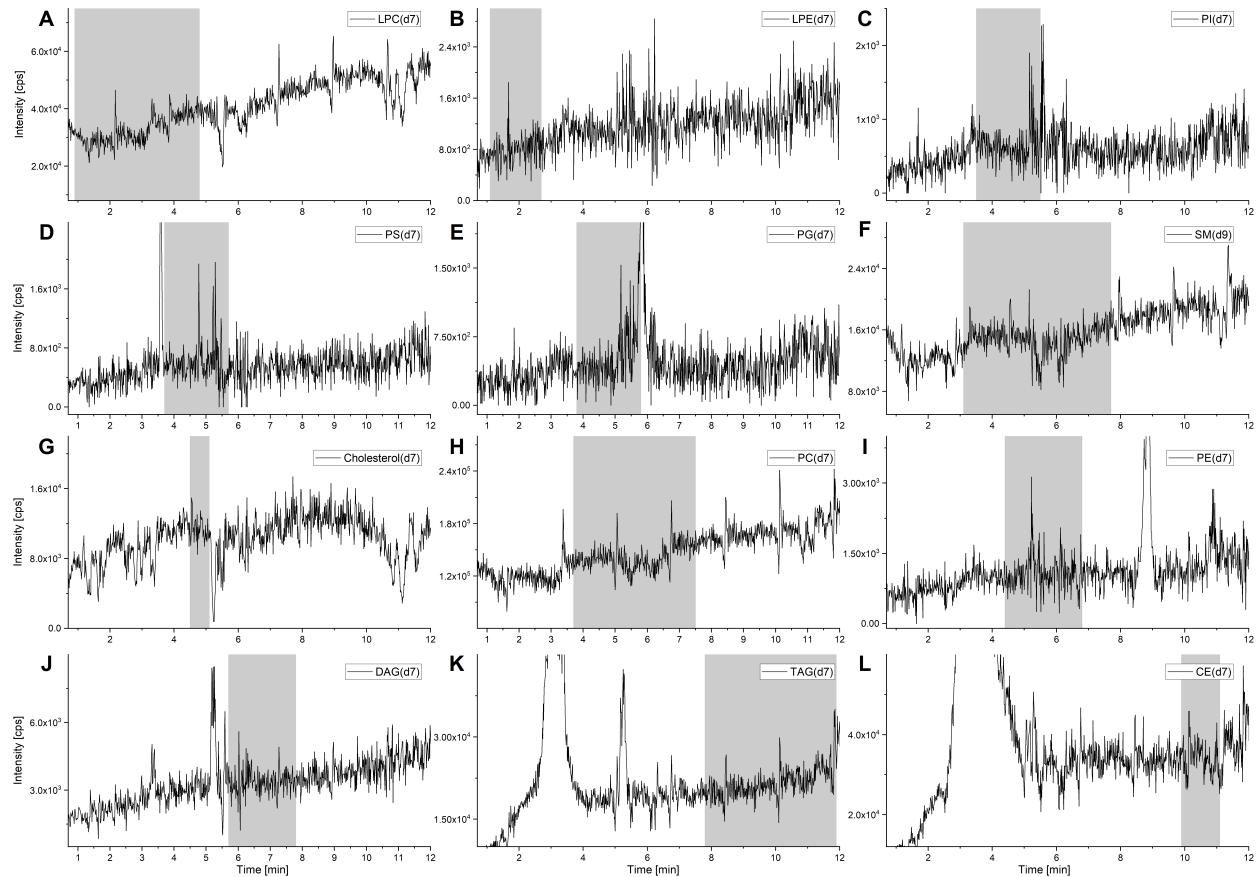


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Highlights

- Comprehensive lipidomic analysis of mouse plasma using high-resolution LC-MS with data-independent SWATH acquisition
- Surrogate calibration with quantifiable data on TOF-MS and SWATH-MS/MS level for optimized assay-specificity and linearity
- Response factor evaluation of lipid species to surrogate calibrants via standard reference plasma NIST SRM 1950
- Post-acquisition re-calibration of target analytes by response factor adjustment
- Inter-study comparison of absolute lipid concentrations



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Tübingen, June 6, 2019

Submission of a manuscript to Anal Chim Acta

Dear editor,

we refer to the manuscript entitled "**Comprehensive Lipidomics of Mouse Plasma using Class-Specific Surrogate Calibrants and SWATH Acquisition for Untargeted Quantification**" by B. Drotleff et al. for publication in Anal. Chim. Acta.

The authors declare no conflict of interest.

With kind regards

Michael Lämmerhofer

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

