Harmonized procedures lead to comparable quantification of total oxylipins across laboratories

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Abstract Oxylipins are potent lipid mediators involved in a variety of physiological processes. Their profiling has the potential to provide a wealth of information regarding human health and disease and is a promising technology for translation into clinical applications. However, results generated by independent groups are rarely comparable, which increases the need for the implementation of internationally agreed upon protocols. We performed an interlaboratory comparison for the MS-based quantitative analysis of total oxylipins. Five independent laboratories assessed the technical variability and comparability of 133 oxylipins using a harmonized and standardized protocol, common biological materials (i.e., seven quality control plasmas), standard calibration series, and analytical methods. The quantitative analysis was based on a standard calibration series with isotopically labeled internal standards. Using the standardized protocol, the technical variance was within ±15% for 73% of oxylipins; however, most epoxy fatty acids were identified as critical analytes due to high variabilities in concentrations. The comparability of concentrations determined by the laboratories was examined using consensus value estimates and unsupervised/supervised multivariate analysis (i.e., principal component analysis and partial least squares discriminant analysis). Interlaboratory variability was limited and did not interfere with our ability to distinguish the different plasmas. Moreover, all laboratories were able to identify similar differences between plasmas. 𝐉𝐥𝐫 In summary, we show that by using a standardized protocol for sample preparation, low technical variability can be achieved. Harmonization of all oxylipin extraction and analysis steps led to reliable, reproducible, and comparable oxylipin concentrations in independent laboratories, allowing the generation of biologically meaningful oxylipin patterns.

Supplementary key words eicosanoids • lipidomics • liquid chromatography • mass spectrometry • quantitation • harmonization • plasma

lipids • interlaboratory comparison • oxidized fatty acids • lipid mediators

Eicosanoids and other oxylipins are potent lipid mediators produced via the oxygenation of PUFAs. PUFAs can be oxygenated enzymatically by cyclooxygenases to form prostanoids, by lipoxygenases to form hydroperoxy fatty acids, which react further to mono- and poly-hydroxylated fatty acids, or by cytochrome P450 monooxygenases giving rise to epoxy and hydroxy fatty acids, or nonenzymatically by free radicals during autoxidation (1, 2). A major portion of circulating oxylipins (>90%) is found esterified in lipids, e.g., phospholipids, triacylglycerides, or cholesterol esters (3–5).

Oxylipins include hundreds of structurally different molecules that are involved in a variety of physiological processes, such as the regulation of blood coagulation (6), endothelial permeability (4), blood pressure, and vascular tone as well as the control of kidney function (7) and the immune system (4, 8). The function of fat tissue is also regulated by these lipid mediators, and it has been shown that oxylipins intervene in the energy homeostasis regulation of insulin and its signaling pathways (6, 9). Thus, the oxylipin pattern can provide a wealth of information regarding human health and disease and is a promising technology for translation into clinical applications. Several clinical studies have already demonstrated the utility of oxylipin profiling in the identification of potential disease biomarkers, the characterization of inflammatory and oxidative status, or the monitoring of the effects of diet or drugs (1).

Currently, the analysis of oxylipins is mainly carried out by LC coupled with MS using reversed phase columns filled with sub-2 μm particles, electrospray ionization, and triple quadrupole detectors. This provides an excellent chromatographic separation of the isomeric analytes as well as a fast detection by MS following fragmentation. Furthermore, this allows the quantification of low oxylipin

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DOI 10.1194/jlr.RA120000991

concentrations with highest sensitivity over a large dynamic range (1, 10–13). Prior to MS analysis, several steps of sample preparation are usually carried out. The samples are often pretreated with organic solvents to precipitate proteins (12, 14, 15) or extract lipids (5, 16). Moreover, when analyzing total oxylipins, quantified as the sum of free and bound oxylipins, alkaline hydrolysis is performed (5, 17, 18). Then, matrix compounds are removed and oxylipins are concentrated via solid phase extraction (SPE) (19–21). All steps of the sample preparation procedure have to be optimized to achieve good oxylipin recoveries and remove the matrix efficiently and thus minimize ion suppression (1, 21). The analysis of oxylipins is usually quantitative, which requires external calibration with internal standards (ISs). However, there are only a small number of companies that sell oxylipin standards, and the quality is not always guaranteed (22). Only a few standards are available with verified concentrations (1, 22). Other commercially available oxylipin standards show varying purities often resulting in a different actual concentration than the stated nominal concentration (22), thus leading to inconsistent results across different studies (23).

There are currently no harmonized protocols for the analysis of oxylipins, although it is well established that each analytical choice (i.e., type of biofluid, type of anticoagulant, free or esterified oxylipins, type of sample preparation protocol, type of instrument) can have a major influence on the detection and quantification of oxylipins (1). Therefore, after optimization of relevant steps of the oxylipin analysis, standardized and harmonized methods should be established to obtain reliable and comparable results. Vesper, Myers, and Miller (24) mainly recommend the following for clinical laboratory tests: *1*) the establishment of reference methods and materials, *2*) calibration using the reference system, and *3*) verification of the uniformity of method results.

Using a standardized and harmonized protocol for oxylipin quantification is a mandatory prerequisite to obtain meaningful and reproducible results, as oxylipin concentrations obtained from different laboratories are rarely comparable due to varying analytical strategies and the lack of certified analytical calibrators and reference materials (1, 10, 23). Therefore, the harmonization of oxylipin analysis can enhance the use of oxylipin profiling in clinics. Another crucial step is to assess the technical variability and interlaboratory comparability of each oxylipin quantified. This will allow the identification of potential technically critical oxylipins to appropriately power clinical studies and to guarantee the relevance of oxylipin profiling involving different laboratories. So far there are only a few studies that have investigated the comparability of targeted metabolomics across laboratories (25–28), and only one study that included oxylipins (26). In the present study, we used a standardized protocol for the quantitative analysis of total oxylipins (18) due to their higher relevance in a context of biomarker discovery (1). Five independent laboratories were involved to assess the technical

(intralaboratory) variability and comparability of 133 oxylipins following a standardized and harmonized protocol for sample preparation and MS analysis and using the same biological material [i.e., seven quality control (QC) plasmas] and standard calibration series.

MATERIALS AND METHODS

Chemicals

Acetonitrile, methanol (MeOH), *iso*-propanol (LC-MS grade), and acetic acid (Optima LC-MS grade) were purchased from Fisher Scientific. Ethyl acetate (HPLC grade) was bought from VWR and *n*-hexane (HPLC grade) was purchased from Carl Roth. The ultra-pure water with a conductivity of >18 MΩ·cm was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific. The oxylipin standards 10-HODE, 12-HODE, 15-HODE, 13-oxo-octadecatrienoic acid, 9,10,11-trihydroxyoctadecenoic acid (TriHOME), 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10,11-trihydroxyoctadecadienoic acid (TriHODE), 9,12,13-TriHODE, and 9,10,13-TriHODE were obtained from Larodan (Solna, Sweden). Other oxylipin standards and deuterated oxylipin standards used as ISs were purchased from Cayman Chemical (local distributor Biomol, Hamburg, Germany). Calcium ionophore A23187 and all other chemicals were bought from Sigma-Aldrich (Schnelldorf, Germany). Pooled human plasma was purchased from BioIVT (West Sussex, UK).

Biological samples

Different types of QC plasma having contrasted oxylipin profiles and biological significance were prepared. For QC Plasma 1 – B, human EDTA-blood was collected from 4–6 healthy male and female individuals aged between 25 and 38 years and centrifuged (10 min, 4°C, 1,200 *g*). Separated plasma was pooled, aliquoted, and stored at –80°C. To obtain n3-rich plasma (QC Plasma 2 – n3) human EDTA-blood was collected from an individual following n3-rich dietary supplementation, centrifuged (10 min, 4°C, 1,200 *g*), aliquoted, and stored at –80°C. QC Plasma 3 – S was prepared by spiking of blank plasma pool (QC Plasma 1) with oxylipin standards PGF2α, 15(S)-F2t-IsoP, 14(15)-EpETrE, 11,12-DiHETrE, 15-HETE, 14,15-DiHETE, 15-HEPE, 18-HEPE, RvD5, 17-HDHA, 12(13)-EpOME, 9,10-DiHOME at a concentration of 20 nM and LxA4, 20-HETE, 17(18)-EpETE, 19(20)-EpDPE, 16,17-DiHDPE and 13-HODE at a concentration of 50 nM in plasma. In brief, the plasma pool was gently mixed while the spiking standard in MeOH (1% v/v of plasma) was added dropwise. Prepared QC Plasma 3 was aliquoted and stored at –80°C. For QC Plasma 4 – Ca EDTA-blood was collected from one healthy individual, incubated with calcium ionophore A23187 (50 µM) for 30 min at 37°C (29), centrifuged (10 min, 4°C, 1,200 *g*), aliquoted, and stored at –80°C. To prepare plasma obtained from obese individuals, human EDTA-blood was collected from 10 obese (BMI ≥ 30 kg/m²) male and female individuals without hypertriglyceridemia and centrifuged (QC Plasma 5 – Ob-H), or human EDTA blood was collected from 7 male and female obese individuals with hypertriglyceridemia (total cholesterol > 200 mg/dl; LDL > 130 mg/dl; TG > 150 mg/dl) (QC Plasma 6 – Ob+H). The blood was then centrifuged and separated plasma was pooled, aliquoted, and stored at –80°C. A commercially obtained pooled human plasma (BioIVT, West Sussex, United Kingdom), aliquoted and stored at –80°C, was used as QC Plasma 7 – B2.

Several aliquots (100–500 µl) of each QC plasma were sent to the other laboratories to ensure that the assessment of the technical variability was independent of the biological material.

Sample preparation and LC-ESI(-)-MS/MS analysis

All laboratories used the same standardized protocol for sample preparation. Human plasma samples were extracted using SPE following protein precipitation and alkaline hydrolysis as described previously [the detailed standard operation procedure (SOP) that was provided to all laboratories can be found in the supplemental information (supplemental Tables S1–S4)] (18). In brief, to 100 µl of human plasma, 10 µl of antioxidant mixture [0.2 mg/ml BHT, 100 µM indomethacin, 100 µM $trans$-4-(-4-(3-adamantan-1-yl-ureido9-cyclohexyloxy)-benzoic acid (t-AUCB) in MeOH] and 10 µl of IS solution [100 nM of each: $^2H_4$-8-iso-PGF$_{2\alpha}$, $^2H_4$-6-keto-PGF$_{1\alpha}$, $^2H_4$-PGF$_{2\alpha}$, $^2H_{11}$-8,12-iso-iPF$_{2\alpha}$-VI, $^2H_4$-PGB$_2$, $^2H_5$-LxA4, $^2H_5$-RvD1, $^2H_5$-RvD2, $^2H_4$-LTB$_4$, $^2H_4$-9,10-DiHOME, $^2H_{11}$-11,12-DiHETrE, $^2H_4$-13-HODE, $^2H_4$-9-HODE, $^2H_6$-20-HETE, $^2H_8$-15-HETE, $^2H_8$-12-HETE, $^2H_8$-5-HETE, $^2H_4$-12(13)-EpOME, $^2H_{11}$-14(15)-EpETrE, $^2H_{11}$-8(9)-EpETrE in MeOH] were added. Following protein precipitation with iso-propanol and alkaline hydrolysis at 60°C for 30 min using 0.6 M potassium hydroxide (MeOH/water, 75/25, v/v) samples were extracted using Bond Elut Certify II SPE cartridges (200 mg, 3 ml; Agilent, Waldbronn, Germany) as described (12, 13, 18). Oxylipins were eluted into glass tubes containing 6 µl of 30% glycerol in MeOH using ethyl acetate/n-hexane/acetic acid (75/25/1, v/v/v). Samples were evaporated and the residue was reconstituted in 50 µl of MeOH. The analysis of the samples was performed using a sensitive LC-ESI(-)-MS/MS method with optimized mass spectrometric and chromatographic parameters as described (12, 13), which was provided to all participating laboratories. The quantitative analysis was based in all laboratories on the same standard calibration series comprising 133 oxylipins with isotope-labeled ISs (30), allowing the same analyte/IS assignment in the laboratories.

Study design

A comprehensive SOP (supplemental Tables S1–S4) for sample preparation and MS analysis was standardized and validated in the reference laboratory (laboratory 1). Based on the European Joint Programming Initiative Grant of the European Union, it was possible to share not only the protocol but also the QC plasmas, the standard calibration, and the extensive LC-ESI(-)-MS/MS method with the participating laboratories.

Five laboratories participated in the interlaboratory comparison. Each participating laboratory analyzed the different QC plasmas in triplicate on two consecutive days or, in case of QC plasma 1, on three consecutive days. To investigate the contribution of the instrumental LC-MS analysis to the overall variability of the results, two additional triplicates of QC plasma 1 were prepared by laboratories 2 and 5 and analyzed using the MS platforms from laboratories 1 (MS1) and 3 (MS3) (supplemental Fig. S1). Furthermore, all participants were provided a data submission template including information on the analysis and calculation of the concentrations. The oxylipin concentrations in the plasma pools were reported in nanomoles per liter. For each triplicate determination, mean and SD were calculated. If in a triplicate determination the concentration of an analyte in one sample was below the lower limit of quantification (LLOQ), the LLOQ threshold was filled in for this sample and the mean and SD were calculated. This approach was chosen, as the omission of analyte concentrations below LLOQ leads to bias of the results (31). If concentrations in two or all samples were below the LLOQ, the concentration of the analyte was set to the LLOQ. Moreover, all participants

were asked to fill out a short questionnaire with general remarks on the analysis.

Statistical analysis

Several statistical methodologies were used to assess the intra- and interlaboratory analytical variabilities. Coefficients of variation (CVs) were calculated to determine the intra- and inter-day variability for each laboratory and each QC plasma. Multivariate methods were applied to assess the interlaboratory variability. The matrix comprised 75 samples (analysis of six plasmas on two consecutive days + analysis of QC plasma 1 on three consecutive days × five laboratories) and 74 oxylipins (>LLOQ for at least one QC plasma and at least in one laboratory). First, a principal component analysis (PCA; unsupervised analysis) was built to observe the overall variability. Then, in order to specifically investigate the influence of the laboratory, a partial least squares discriminant analysis (PLS-DA; supervised analysis) was performed using the laboratory as the discriminant variable.

In order to compare the measurements from multiple laboratories, the consensus values and their associated uncertainties (u) were calculated by the median of the means (MEDM) method, as is previously described in (26). Briefly, the means and SDs of the triplicates on day 1 and day 2 (and day 3 for the QC plasma 1) for each quantified oxylipin were calculated. Then the median of the laboratories' means (i.e., MEDM consensus values) were calculated for the oxylipins quantified in at least three laboratories. The associated standard uncertainties (u) for the MEDM consensus values were calculated as $u = \sqrt{(\pi/2m)} \times 1.483 \times MAD$ where m and MAD are the number of laboratories and the median of absolute deviation of the laboratory means, respectively.

To assess the ability of laboratories to identify similar differences in the oxylipin profiles between two different QC plasmas and therefore to provide similar biological interpretation, ratios between QC plasmas 2, 3, 4, and 7 against QC plasma 1 were calculated for each laboratory as well as the ratios of QC plasma 5 (Ob-H) against QC plasma 6 (Ob+H). Additionally, to show similarities between the laboratories and between the oxylipins, hierarchical clustering analysis was performed with Euclidean distances and the Ward aggregation method.

To assess the contribution of the interlaboratory variability in the LC-MS-specific results, only a low number of samples were used; thus, no statistical analysis was performed on these specific samples and differences were assessed visually. For each quantified oxylipin and each laboratory of preparation, differences of concentration between the lower and the higher sample from MS1 (Δ intra-MS1) and MS3 (Δ intra-MS3) were calculated as well as the difference of concentration between the two closest samples from MS1-triplicate and MS3-triplicate (Δ inter-MS). When the Δ inter-MS was higher than the difference observed within the triplicate (Δ intra-MS), the MS effect was considered as consistent.

All statistical analyses were carried out using R version 3.1.3, Microsoft Excel Office version 365, and SIMCA (Umetrics AB, version 14).

RESULTS

Analytical variance of oxylipin analysis

All participating laboratories were able to simultaneously quantify 133 oxylipins in the standard calibration series using the provided LC-MS/MS method. Deviations from the SOP and analytical instruments used can be found in the

supplemental information (supplemental Table S5). Oxylipins were analyzed in seven different QC plasmas in triplicate (i.e., three different samples were prepared) on two consecutive days to determine the analytical variance of the method. In all laboratories, an average of 84 oxylipins (63%) were above the limit of quantification (LLOQ, **Table 1**).

Regarding the oxylipin pattern of QC plasmas 1–6, the determined oxylipin concentrations were in a similar range (0.16–547 nM), while in QC plasma 7 (mainly for hydroxy-PUFA and multi-hydroxylated oxylipins, as well as for linoleic acid-derived epoxy-PUFAs and oxo-PUFAs), 10- to 25-fold higher concentrations were found.

The results of the intra-day and inter-day variability of the oxylipin analysis in the reference laboratory (laboratory 1) is shown in **Fig. 1**. For this laboratory, the variabilities were assessed for 81 quantified oxylipins. Overall, low intra-day variability (CV <15%) was observed for 85% of oxylipins. The inter-day variability was also low (CV <15% for 73% of oxylipins). However, for epoxy-PUFAs as well as oxo- and trihydroxy-PUFAs a variance up to >25% was observed. This higher variability did not correlate with concentration. There was no difference between the different types of plasma.

Increased intra-day and inter-day variability for epoxy-PUFAs could also be observed in the other participating laboratories (supplemental Figs. S2–S5). For laboratories 2, 3, 4, and 5, the variabilities were assessed for 69, 73, 94, and 82 quantified oxylipins, respectively. These laboratories presented higher variabilities overall, except for laboratory 2, with low intra-day variability (CV <15%) for 81% of oxylipins for day 2 and low inter-day variability (CV <15%) for 72% of oxylipins (supplemental Fig. S2), which is consistent with laboratory 1. Notably, for laboratories 2–5, the intra-day variability for day 1 was higher than for day 2, which impacted the inter-day variability (supplemental Figs. S2–S5). For example, laboratory 3 (supplemental Fig. S3) showed the highest inter-day variability (only 39% of oxylipins with a CV <15%), which is due to the very high intra-day variability for day 1 (CV <15% for 36% of oxylipins) compared with day 2 (CV <15% for 61% of oxylipins). For laboratories 4 and 5, the inter-day variability was also high with a CV <15% only for 52% and 44% of the oxylipins, respectively.

Laboratory comparison

The quantified oxylipin concentrations in all QC plasmas by all laboratories can be found in the supplemental information (supplemental Table S6).

Unsupervised multivariate analysis (i.e., PCA) was first performed to assess the variability of the overall oxylipin profiles obtained in each laboratory and its major determinant (i.e., type of QC plasma, laboratory). The initial PCA shows that the main variability (40.5% on the first component) is related to the type of QC plasma with QC plasma 7 (B2) being different from the others. The variability on the second component is much lower (i.e., 18.6%) and mainly driven by laboratory 4 (supplemental Fig. S6A). The loading plot shows that the discrimination of laboratory 4 is based mainly on epoxy-PUFAs (supplemental Fig. S6B). Epoxy-PUFAs have already been noted for their high technical variability. Moreover, in laboratory 4, the samples could either not be measured directly or they had to be injected several times due to technical issues (as described in supplemental Table S5), the latter of which results in increased concentrations of epoxy-PUFAs (supplemental Fig. S7). Therefore, we speculated that including the epoxy-PUFAs in the PCA model might have artificially driven the discrimination of laboratory 4. To exclude this possibility and to precisely determine the influence of the laboratory on the variability of the oxylipin profiles, the 12 epoxy-PUFAs were excluded from further biostatistical analysis.

A second PCA model was built without the epoxy-PUFAs (matrix with 75 samples and 62 oxylipins). The score plot (**Fig. 2A**) confirms that the variability on the first component (46.8%) is related to the type of plasma with QC plasma 7 (commercial plasma rich in hydroxy-PUFAs) being clearly different from the others. In this second model, the variability on the second component (12.3%) is also related to the type of QC plasma with QC plasma 3 (plasma spiked with $PGF_{2\alpha}$, 15(S)-F_{2t}-IsoP, 14(15)-EpETrE, 11,12-DiHETrE, 15-HETE, 14,15-DiHETE, 15-HEPE, 18-HEPE, RvD5, 17-HDHA, 12(13)-EpOME, 9,10-DiHOME, LxA4, 20-HETE, 17(18)-EpETE, 19(20)-EpDPE, 16,17-DiHDPE, and 13-HODE) being different from the others. The loading plot (Fig. 2B) shows that the oxylipins that contribute the most to the discrimination of QC plasmas 7 and 3 are consistent with the highly concentrated oxylipins in the profiles of the plasma, i.e., enriched with hydroxy-PUFAs and spiked with specific oxylipins, respectively.

In order to go further in the assessment of the laboratory influence, a supervised multivariate method (i.e., PLS-DA) using the laboratory as discriminant variable was applied. For this, we again excluded the 12 epoxy-PUFAs from the PCA model as well as samples from QC plasma 7 (i.e., plasma with a very distinct oxylipin profile, 10 samples excluded) to highlight the laboratory effect. This allowed the generation of a PLS-DA model with good discrimination performances (R^2Y = 0.78 and Q^2 = 0.729). On the first

TABLE 1. Number of quantified oxylipins

	QC Plasma 1 (B)	QC Plasma 2 (n3)	QC Plasma 3 (S)	QC Plasma 4 (Ca)	QC Plasma 5 (Ob-H)	QC Plasma 6 (Ob+H)	QC Plasma 7 (B2)
Laboratory 1	80	82	90	84	85	92	103
Laboratory 2	68	71	75	75	69	71	89
Laboratory 3	71	76	79	80	71	70	97
Laboratory 4	93	88	96	95	92	95	104
Laboratory 5	83	79	89	86	84	84	91

Seven plasma pools were analyzed in five independent laboratories according to the SOP (including 133 oxylipins). Shown is the number of oxylipins above LLOQ quantified in each laboratory for each plasma pool.
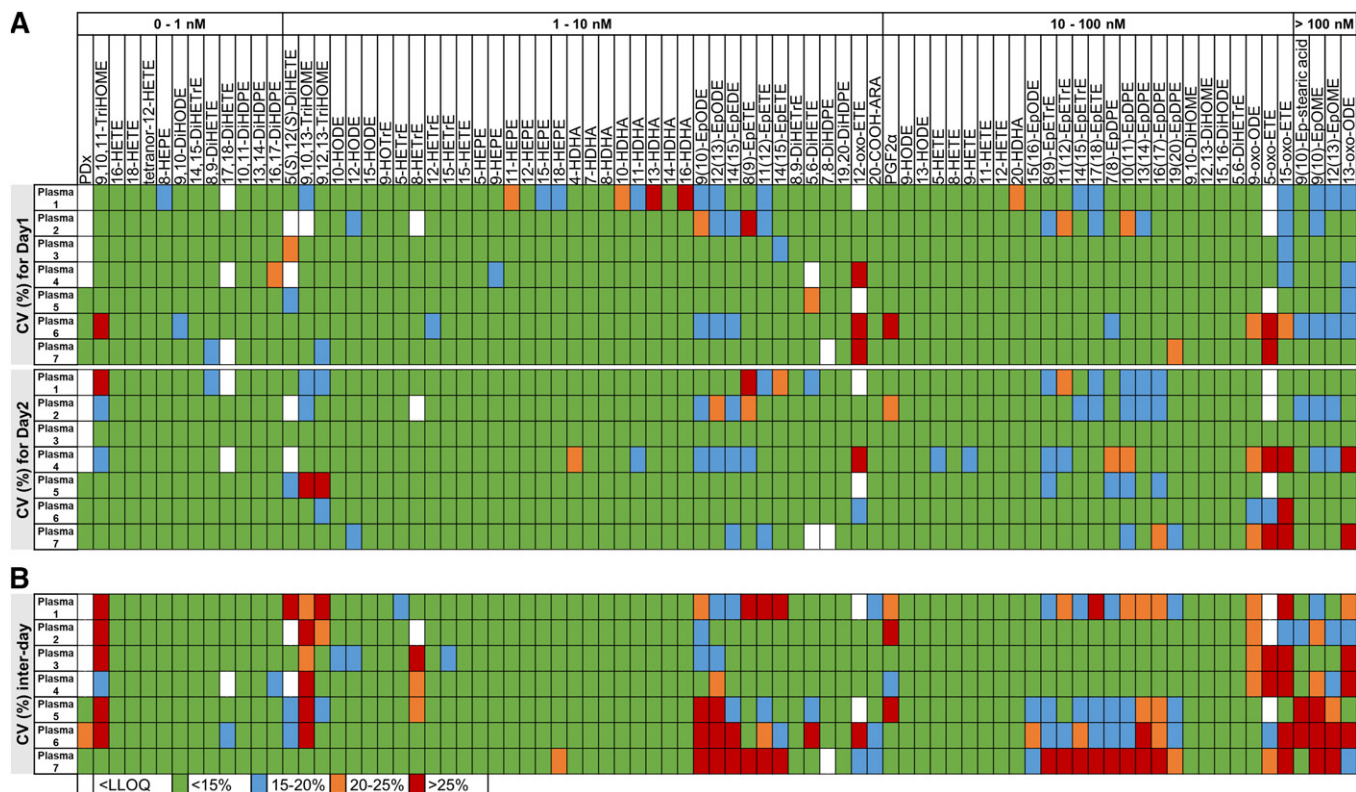
Fig. 1. Intra-day (A) and inter-day (B) variability of the oxylipin analysis in laboratory 1. Oxylipin analysis in the seven QC plasmas was carried out in triplicate on two consecutive days in the reference laboratory (laboratory 1) and the variability within each day as well as the inter-day variability were determined. The CVs were calculated using the mean concentration and SD $\left(CV = \frac{SD}{mean} \times 100\right)$. Shown are the CVs of 81 quantified oxylipins in the seven plasmas in laboratory 1 displayed in four different colors.

component of the cross-validated score plot (CV score plot) bringing 42.7% of variability (**Fig. 3A**), laboratory 4 clearly distinguishes itself. The loading plot (Fig. 3B) shows that this difference is driven by the hydroxy-PUFAs. On the second component, the variability is much lower (13.1%). However, it shows that laboratory 5 is different from the others. Concerning the other three laboratories, no separation could be achieved with the model, suggesting very similar oxylipin profiles.

The consensus values were evaluated using the MEDM approach to assess the interlaboratory comparability. The consensus values were deemed acceptable when the coefficient of dispersion (COD) (COD = 100 × u/MEDM) was less than 40% as described in (26). The mean ± SD for each laboratory and MEDM consensus values ± u were plotted for oxylipins with an acceptable consensus value. The MEDM ± u and COD for oxylipins quantified in all QC plasmas can be found in the supplemental information (supplemental Tables S7, S8; supplemental Figs. S9–S14).

In total, 78 oxylipins were reported for QC plasma 1 in all laboratories, with an acceptable consensus value for 17 oxylipins (22%) (**Fig. 4**). Notably, the same analysis was performed without laboratory 4 (due to the issues encountered during sample analysis) and shows that an acceptable consensus value is obtained for 73% of the oxylipins (47 out of 64 quantified oxylipins) (supplemental Fig. S8). In QC plasmas 2, 3, 4, 5, 6, and 7, the consensus values were acceptable for 19, 29, 29, 21, 23, and 60 oxylipins, respectively.

Identification of differences between plasma pools

To assess the ability of the laboratories to identify similar differences (in magnitude and direction) between two plasmas, we compared the ratios calculated between different QC plasmas by each laboratory. For this purpose, the concentration of each oxylipin for a given QC plasma was divided by the oxylipin concentration obtained for another QC plasma.

First, we compared the differences observed between two very contrasted QC plasmas, i.e., QC plasma 7 (commercial plasma rich in hydroxy-PUFAs and linoleic acid metabolites) versus QC plasma 1 (plasma prepared from fresh EDTA-blood of healthy donors immediately stored at −80°C). For 98% of the oxylipins (matrix consisting of 60 oxylipins), the ratio between QC plasma 7 and QC plasma 1 was similar for the five laboratories. The only noticeable difference in concentration ratios of compared QC plasmas occurred between laboratory 2 and laboratory 3 for 13-oxo-ODE (concentration ratio of 5–15 and <0.067, respectively), whereas the laboratories 1, 4, and 5 obtained very similar ratios between compared plasmas (concentration ratio 1.33–5; **Fig. 5A**).

A second laboratory comparison was made with the ratios calculated between the QC plasmas obtained in obese individuals with or without hypertriglyceridemia (QC plasma 5 vs. QC plasma 6). For 92% of the oxylipins (matrix consisting of 61 oxylipins), the ratios were very similar between the five laboratories. However, ratios in opposite directions were obtained for 9,10,11-TriHOME, 11,12-DiHETE, 9-HODE, 13-HODE, and 15-oxoETE (Fig. 5B).
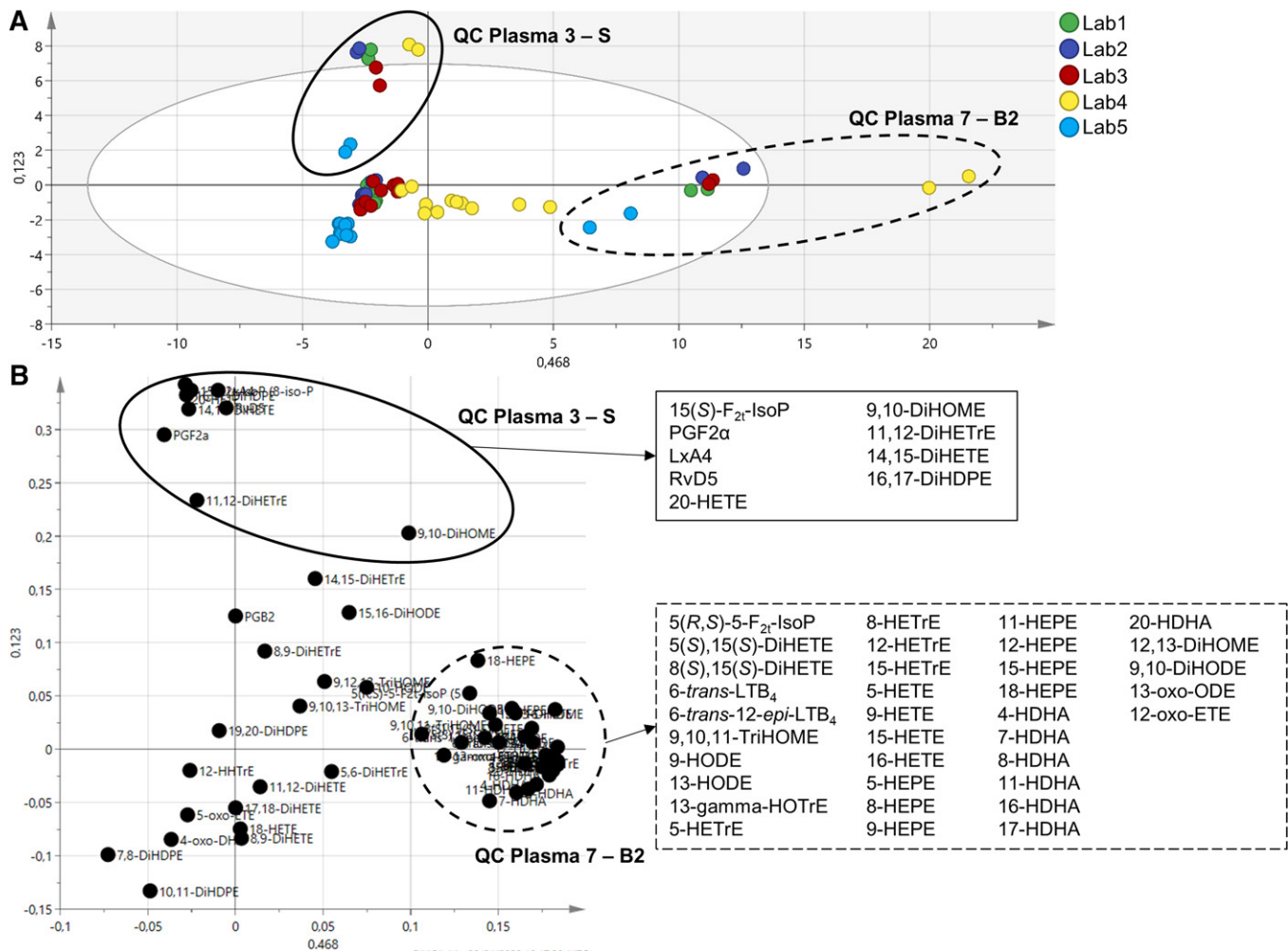
Fig. 2. Principal components analysis (PCA) model without epoxy-PUFAs. The model was built with 75 samples and 62 oxylipins (R^2X = 0.592 and Q^2 = 0.544). A: The score plot shows that the main variability is related to the type of plasma: QC plasma 7 (B2) (dotted circle) and QC plasma 3 (S) (solid circle). B: The loading plot displays oxylpins contributing to the discrimination of QC plasmas 7 (dotted circle) and 3 (solid circle). Lists of these oxylipins are depicted in respective boxes.

Interlaboratory variability in the LC-MS/MS-specific results

Knowing that the different laboratories use different mass spectrometers, the contribution of this factor on the overall variability of the oxylipin profiles was assessed (i.e., 42 oxylipins that were detected at a concentration above the LLOQ in all laboratories). The oxylipin profiles were generated from samples prepared in two different laboratories (laboratory 2 and laboratory 5), and the prepared samples were analyzed on either MS platform 1 or 3. The described approach should make it possible to separate the laboratory specific-variance from the instrument-specific variance. For samples prepared in laboratory 2, seven oxylipins (i.e., 9,10,13-TriHOME, 9,12,13-TriHOME, 10-HODE, 12-HETrE, 12-HEPE, 7-HDHA, and 11(12)-EpETrE) had different concentrations depending on where the LC-MS analysis was performed (MS1 or MS3), whereas in samples prepared in laboratory 5, only one oxylipin (i.e., 12-HETrE) had a different concentration depending on the MS platform (supplemental Table S9, supplemental Fig, S18). Notably, the intra-MS variabilities (i.e., variability within each independent triplicate) were generally higher for the samples prepared in laboratory 5, which impairs the detection of valid differences between the two sets of triplicate analyses.

DISCUSSION

The quantitative analysis of eicosanoids and other oxylipins has gained a lot of attention, especially with regard to the discovery of new biomarkers in health and disease (1). However, the analysis of oxylipins is complex and can be influenced by many factors, such as sample preparation and LC-MS-based quantification. For this reason, standardized procedures are required to achieve comparable and reproducible results. This was assured in this study by providing a detailed SOP (supplemental information), calibration series, and IS solutions to all the laboratories. This harmonization allows the extent and sources of technical variability to be assessed and investigation of which oxylipins can be robustly quantified as reliable potential biomarkers.
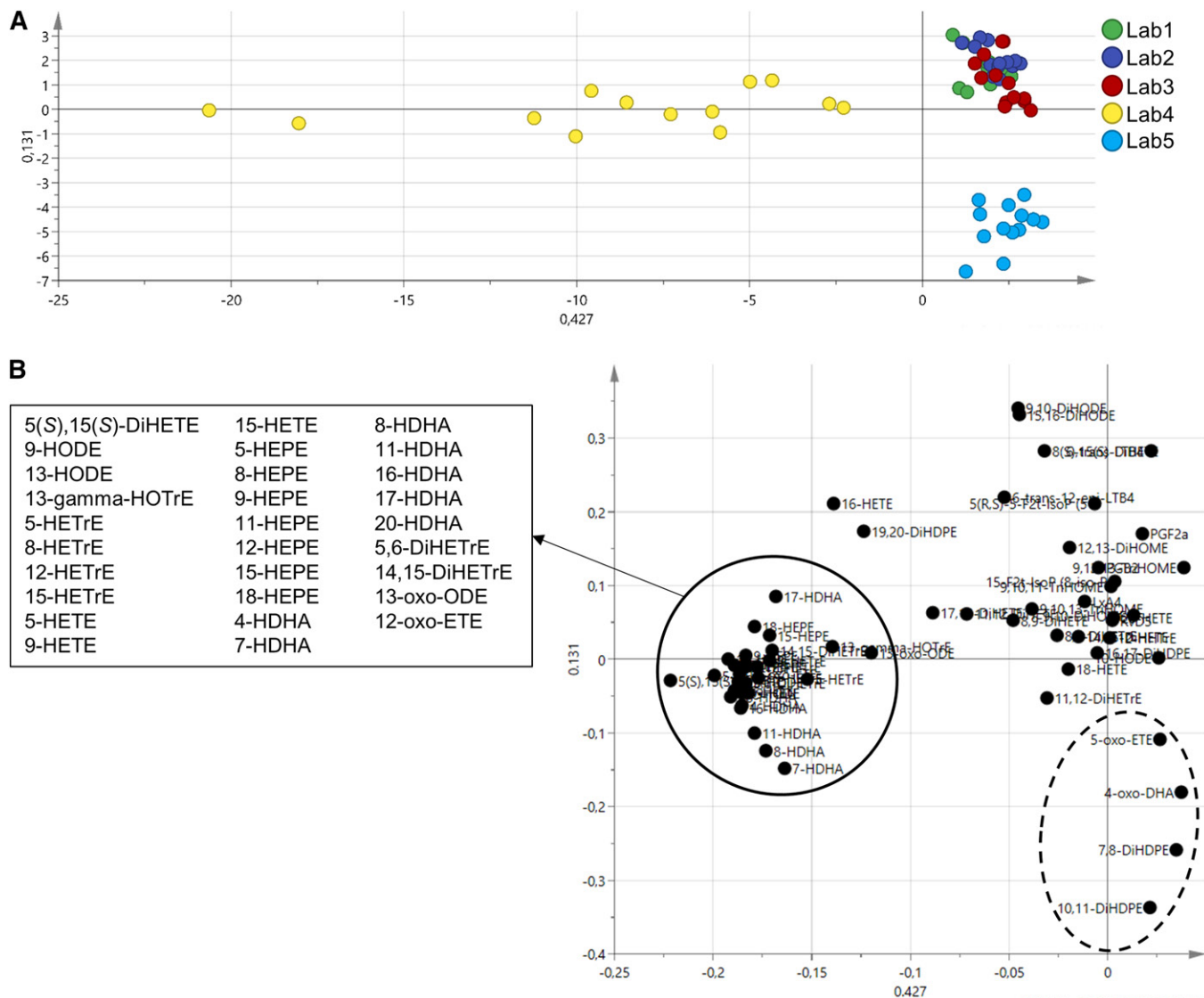
Fig. 3. PLS-DA. The model was built with 65 samples and 62 oxylipins ($R^2Y = 0.78$ and $Q^2 = 0.729$). A: The cross-validated score plot shows that laboratories 4 and 5 distinguish themselves from the others. B: The loading plot shows the oxylipins contributing to the discrimination of laboratory 4 (solid circle); the list of these oxylipins indicates that this discrimination is driven by hydroxy-PUFAs. With less variability, four oxylipins (dotted circle) contribute to the discrimination of laboratory 5.

Technical variability of oxylipins

In each participating laboratory, the precision and reproducibility of the analytical method was determined using seven QC plasmas. In the reference laboratory (laboratory 1), the lowest inter-day variance (inter-day) was observed (<15% for 73% of oxylipins, Fig. 1). The SOP and LC-MS method have been developed here, and thus this laboratory is best trained in the procedures. The achieved analytical variances meet the criteria of international guidelines, i.e., analytes above the LLOQ should have a precision of <15% (32, 33). Low inter-day variances for 72% of oxylipins (<15%, supplemental Fig. S2) were also observed in laboratory 2, whose personnel were trained in the reference laboratory. In laboratories where the analysis was carried out only based on the SOP, slightly higher variances (>20%, supplemental Figs. S3–S5) were obtained for 38–56% of oxylipins. Although all participating laboratories

are experienced in the field of oxylipin analysis, they have established methods (1) that slightly differ from the SOP used in this interlaboratory comparison. Thus, the personnel were not used to the detailed procedures, and a training effect could be observed with lower variance on day 2 of analysis than on day 1. It is quite possible that a slight difference in the handling of samples could lead to higher variances, as oxylipins can be easily formed or degraded (30). Training of technical skills for accurate sample preparation has been found to be of general importance for quantitative analysis, as discussed by Percy et al. (34) and Siskos et al. (25). Therefore, our results show that not only is the use of the same protocol important to achieve comparability between the laboratories but also personnel that have been trained in the technical skills required for the sample preparation procedure.

It should be noted that the observed CVs in reference laboratory 1 were comparable to those described by
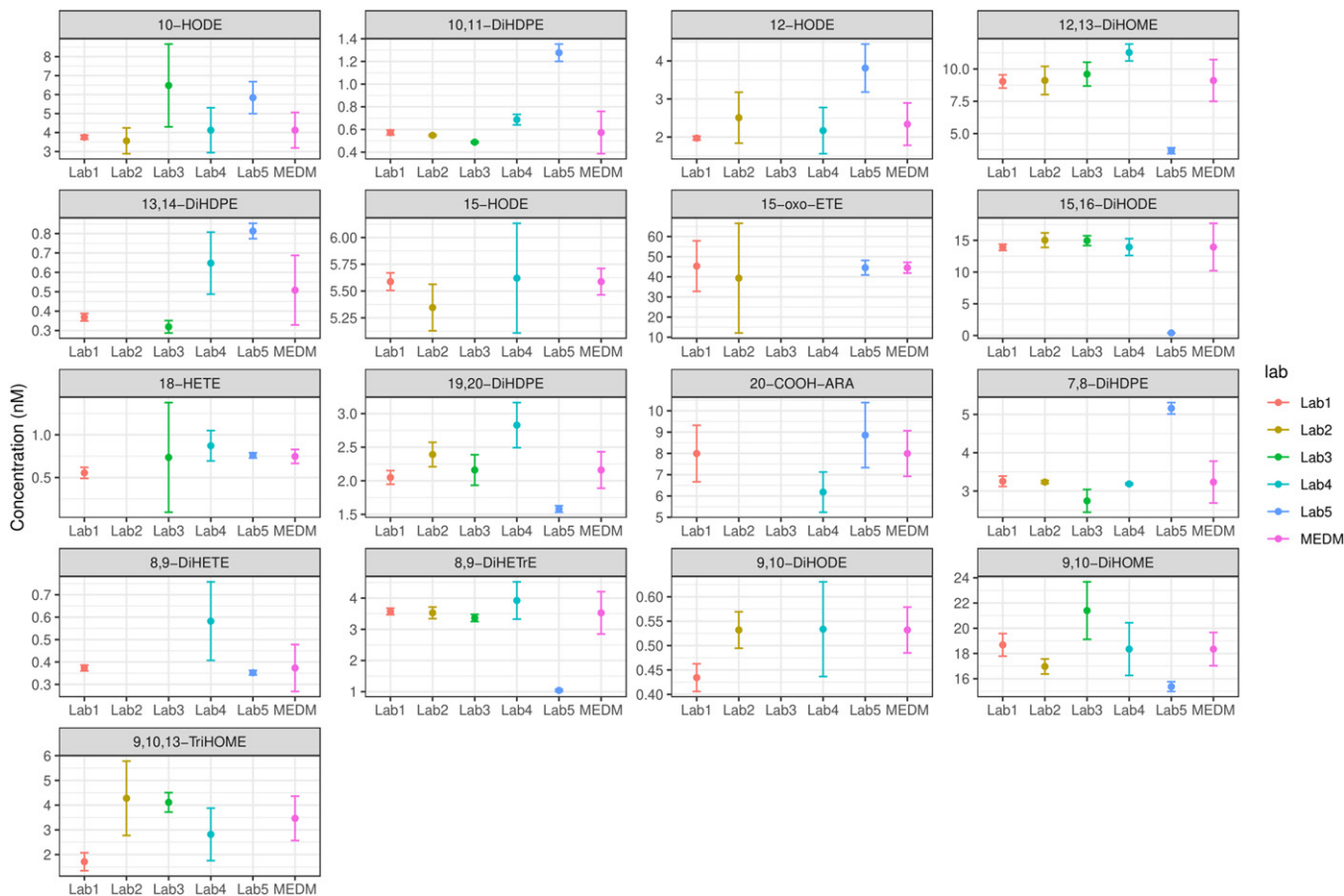
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Fig. 4. MEDM location plots. Plots were created for oxylipins quantified in QC plasma 1 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and the MEDM ± u for 17 oxylipins with acceptable consensus value (COD <40%).

Ostermann et al. (18) (CV <20% using the same sample preparation protocol). However, when comparing with the independent method for the quantitation of total oxylipins described by Quehenberger et al. (17) (CV 5–20%), the observed CVs are slightly higher, indicating further potential of optimizing the protocol.

Notably, in all laboratories, higher CVs were observed for epoxy- and oxo-PUFAs as well as for TriHOMEs (up to ≥25%). We believe that this higher variance may be caused by deviating from the SOP as different steps of sample preparation can influence the quantified oxylipin concentration. Especially, epoxy-PUFAs can be artificially formed during sample preparation. Ostermann et al. (18) described the drying of samples on the SPE cartridges as a particularly critical step during sample preparation. During the SPE, the PUFAs released in large quantities during hydrolysis can adsorb on the phase material (mainly on nonendcapped silica groups) and be oxidized by atmospheric oxygen, mainly to epoxy-PUFAs (18, 35, 36). In addition, the analysis of the samples in laboratory 5 was carried out by two operators, probably resulting in increased variability. Using the presented sample preparation protocol, the inter-operator variability for most oxylipins is ≤21% (18).

Furthermore, reinjection of lipid extracts into the LC-MS instrument due to technical issues led to increased concentrations of epoxy-PUFAs. The fact that multiple injections of the same extract lead to an increased variability (up to 24% higher) was earlier described by Reinicke et al. (37) for arachidonic acid-derived eicosanoids. Reinicke et al. (37) examined the within-run (reinjection of the same sample 10 times within one analytical run) and between-run (reinjection of the same sample on 10 consecutive days) variability of oxylipins, whereby the variability was in a range of 1–24% in each case.

Although the individual laboratories have complied with the SOP as far as possible, the laboratories are nevertheless equipped with different analytical instruments. For this reason, we aimed to investigate the presence of confounding effects across laboratories by preparing and analyzing the samples in different laboratories. Overall, the observed influence of the MS instrument used is negligible. However, the differences in results obtained when analyzing sample extracts on two different MS platforms were interpreted as inherent acquisition platform variability. Whereby, this variability can be caused by various factors, such as variability in chromatographic performance, peak integrations, or hardware-associated sensitivity or specificity. Although different concentrations were obtained for 7 out of 42 quantified oxylipins in plasma extracts from laboratory 2, a clear MS platform effect could only be observed for 12-HETrE. This is consistent with the results from Percy
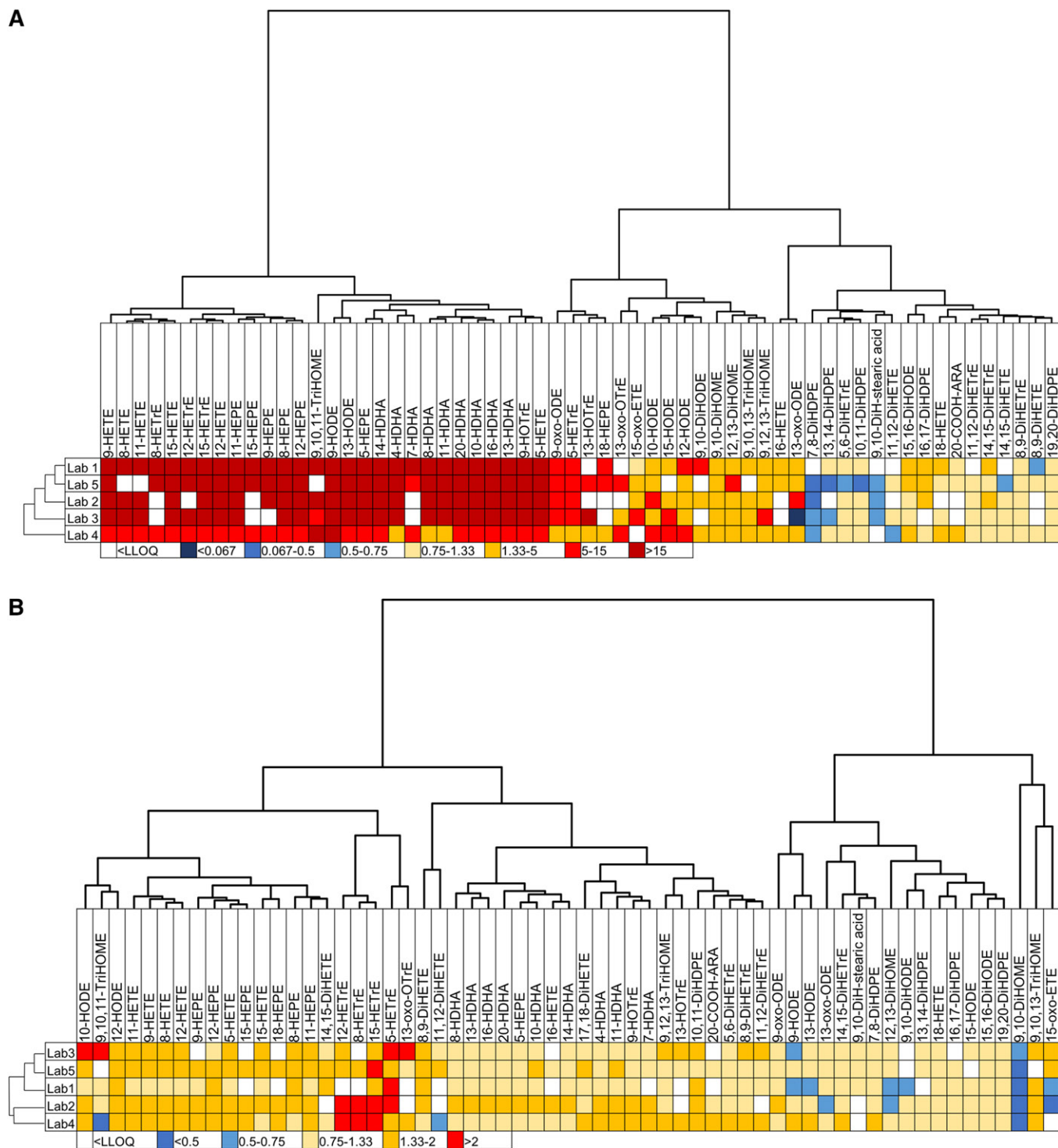
Fig. 5. The ability of laboratories to identify similar differences in oxylipin profiles. Shown are heatmaps with hierarchical clustering (Euclidean distances and Ward aggregation method) for laboratories and oxylipins. A: Heatmap of ratio between QC plasma 7 (B2) (rich in hydroxy-PUFAs) and QC plasma 1 (B). The range of ratio goes from <0.067 to more than 15. B: Heatmap of ratio between QC plasma 5 (Ob-H) and QC plasma 6 (Ob+H). The range of ratio goes from <0.5 to more than 2.

et al. (34), indicating that the accuracy of the MS platform depends mostly on user handling. The quantification was carried out by an external calibration series with isotopically labeled standards (analyte/IS peak area ratio). Thus, sensitivity of the instruments impacts the number of analytes detected but not the determined concentrations. Overall, our results for the quantification of oxylipins show

that comparable results are obtained if different MS instruments are used, which may differ in sensitivity.

Interlaboratory comparability of the total oxylipin profiles

A large number of laboratories deal with the analysis of oxylipins. Each laboratory has developed its own methods for this purpose, whereby an impressive number of oxylipins

are covered by todays methods (1). However, methods differ greatly, which generates technical variance (1) and makes it difficult to assess the real interlaboratory comparability of oxylipin profiles. There are only a very small number of interlaboratory comparisons that investigate the comparability of lipidomic approaches (25–28) and even fewer studies that explicitly address the precision, reproducibility, and comparability of oxylipin analysis using a standardized and harmonized protocol. The few existing studies that address these points therefore show poor comparability (26, 38, 39).

The procedures for investigating interlaboratory comparability and reproducibility differ. A direct comparison of absolute concentrations is hardly possible and results in very large deviations, whereby the variability among laboratories can be caused mainly by the quality of the analytical standards used. When comparing clinical studies, it becomes apparent that the quality of the analytical standards used for quantification is a critical parameter, which may lead to noncomparable results (23). In order to solve this issue Hartung et al. (22) recently described a strategy to verify the concentration of commercially available standards. Because this strategy was not used in previous studies, interlaboratory comparisons can today only be evaluated under consideration of relative results. A common evaluation strategy in lipidomic studies is the normalization to a standard material, such as the National Institute of Standards and Technology standard reference material (NIST SRM) plasma. This normalization significantly improves variance and reproducibility (25, 27). In our study, we show that by normalization (the QC plasmas were normalized to QC plasma 1) and subsequent evaluation of relative results, no differences between laboratories are identified (Fig. 5, supplemental Figs. S15–S17).

The major field of application of targeted oxylipin metabolomics is biological studies aiming to provide a relative comparison of results, such as case and control groups (40–42). When comparing the two QC plasmas of obese subjects with and without hypertriglyceridemia in this study, for most oxylipins comparable ratios were identified, while for only 8% of the oxylipins a trend in the opposite direction was observed (Fig. 5B). Thus, our data clearly demonstrate that LC-MS oxylipin quantification is suitable to characterize differences in biological samples, e.g., in clinical studies.

The comparability of interlaboratory results after standardization to a reference material leads to the fact that a harmonization of methods to a standard material is increasingly desirable. Many describe the importance of standard reference materials, such as the NIST SRM plasma, to ensure interlaboratory comparability and to harmonize data sets (10, 24, 25, 27, 43). However, the introduction of a common standard material does not solve the problem that absolute concentrations are not comparable. This is most clearly shown by the comparison of two independent studies by Bowden et al. (26) and Quehenberger et al. (43) where the NIST SRM plasma was solely analyzed. When comparing the results of Bowden et al. (26) with the results of Quehenberger et al. (43), clear differences in the abso-

lute oxylipin concentrations become apparent. Bowden et al. (26) quantified 10, 6.8, and 2.4 nM for 5-HETE, 12-HETE, and 15-HETE, respectively. While Quehenberger et al. (43) quantified the concentrations for these oxylipins at 11.9, 4.22, and 0.8 nM. Thus, the deviations in the quantified oxylipins 5-, 12-, and 15-HETE are respectively 14, 61, and 199% (26, 38, 43).

Although a relative quantification based on a reference standard is often used, this approach is limited and cannot replace a true quantification of concentrations, i.e., moles per amount per volume or gram sample. In order to compare molar concentration determined in independent laboratories, the consensus value is often evaluated. There are different methods whose advantages and disadvantages should be weighed beforehand (38). In the laboratory comparison by Bowden et al. (26), the MEDM approach was used to compare the results and the COD was calculated. This method is especially robust against outliers (independent of the intra-laboratory variance), as the participating laboratories processed the used NIST SRM plasma applying their own protocols and analytical methods, and the results varied considerably between laboratories (26). The disadvantage of the MEDM method, however, is that it does not take into account intra-laboratory variance, as all laboratories are equally weighted (38, 44). In the study by Bowden et al. (26), participating laboratories quantified all possible lipid classes; however, a large number of oxylipins (143 oxylipins) were quantified in only one laboratory. Further, only 5-HETE, 12-HETE, and 15-HETE were quantified in a sufficient number of laboratories to calculate a consensus value, with the COD being 13, 23, and 27%, respectively. However, these results show that the different procedures result in different analytical concentrations, so that the method used can be a limiting factor.

Using standardized and harmonized protocols for oxylipin quantification can greatly reduce variance and promote the generation of meaningful and reproducible results when data are to be compared across multiple laboratories. In the absence of such harmonization efforts, robust performance-based quality assurance protocols must be implemented to allow the harmonization of data sets processed using discrete protocols and will be critical to enhance the use of oxylipin profiling in clinics. Our study is the first where harmonization of all procedures has been carried out allowing a comparison of absolute concentrations of oxylipins determined in five different laboratories. This comparison is possible because, in the present study, a standardized protocol for sample preparation was used, and the standard calibration series (30) as well as the LC-MS method were established in all participating laboratories in order to reduce as many factors responsible for variability as possible. The concentration of the standard calibration series used was characterized as described by Hartung et al. (22) based on standards with verified concentrations. Based on the standard calibration series, all laboratories determined the limit of detection and LLOQ using a provided protocol, which takes into account the validation criteria of the European Medicines Agency guideline on bioanalytical method validation (limit of detection

set to concentration with signal-to-noise ratio ≥3; LLOQ set to concentration with signal-to-noise ratio ≥5 and accuracy within ±20% of nominal concentration) (32). Sample preparation was carried out according to the same optimized protocol in all laboratories, also taking into account the use of materials from the same manufacturers, as different sample preparation procedures can influence the amount of quantified oxylipins (1, 23). Moreover, the used protocol for sample preparation was optimized to yield high reproducibility following the evaluation of various saponification techniques (1, 18). Furthermore, the integration of peaks was manually validated to ensure optimum precision, as automatic peak integration may lead to incorrect peak integration. Especially, the integration of peaks close to LLOQ or with unusual peak shapes using automatic integration may lead to deviating results.

With our study, we show that the harmonization of parameters that can cause technical variability leads to comparable absolute oxylipin concentrations obtained in different laboratories. If the quantified oxylipin concentrations for all plasma pools in all laboratories are presented in a PCA model, for three of five laboratories, no differences between the results can be observed (Fig. 2). The analysis instead reveals differences between the plasma pools, indicating that biological differences could be detected in batch samples analyzed in different laboratories. Notably, laboratory 4, showing the highest variability and most extreme deviation from the other results, experienced a MS/MS turbo pump failure mid analysis leading to a prolonged delay between sample processing and data acquisition, representing a serious deviation from the described protocols. To better reveal a difference between laboratories, the analysis tool can be forced to display such differences, as shown by the PLS-DA model (Fig. 3). If the results from laboratory 4 were excluded, the consensus value estimates were acceptable for 73% of the oxylipins in QC plasma 1. In general, excluding laboratory 4 increased the number of acceptable consensus values (supplemental Tables S7, S8). It is particularly enlightening that the long post extraction acquisition delays and repeated injections led to higher concentrations of epoxy-PUFAs in these samples (supplemental Figs. S7, S8). Furthermore, excluding the epoxy-PUFAs from the analysis, higher levels and variance in hydroxy-PUFAs were also observed in laboratory 4 data (Fig. 3). Therefore, extreme caution is generally suggested in the timing of post preparation delays in data acquisition.

Oxylipins have the potential to provide a wealth of information regarding human health and disease and are a promising technology for translation into clinical applications. The changes in the oxylipin profile are of particular importance, as physiological effects are not attributed to a single oxylipin but to an interplay of many oxylipins or a general shift of the oxylipin profile (1, 45). In addition, the oxylipins in biological samples come in a concentration range of several orders of magnitude (>4) with differences in polarity and stability. Therefore, the targeted metabolomic analysis of these potent lipid mediators requires sensitive and precise methods to detect as many different oxylipins as possible and to detect even the smallest concentration differences (1, 46–48).

The presented study is unique in evaluating comparability and reproducibility of oxylipin analysis. We are able to show for the first time that the standardization and harmonization of the processing protocol as well as the analysis allows an interlaboratory comparison not only in terms of relative results, but also in the absolute concentrations obtained. In the lipidomics community, there is an increasing demand for standardized methods (1, 10). Our study could be used as the first step for the development of internationally agreed upon oxylipin quantification procedures and benchmarks. Moreover, the standardization of routine performances will allow direct comparisons of data sets generated at various laboratories.

CONCLUSIONS

Our study is the first to investigate the technical variability and interlaboratory comparability of the targeted metabolomics analysis of total oxylipins. Epoxy- and oxo-PUFAs appear to be particularly sensitive to analytical sample handling, and delayed postprocessing analyses are to be avoided. In addition, when analyzing total oxylipins, special care should be taken during the drying steps when using non-endcapped silica materials. However, our findings show that reproducible results with low variability can be obtained using standardized protocols for sample preparation and analysis, and that specific training of personnel in these complex protocols reduces variability. This will be crucial to appropriately power experimental designs and to enhance the identification of reliable and relevant biomarkers of disease.

Overall, we could show that with appropriate standardization, a direct comparison of absolute concentrations obtained in different laboratories is possible, which opens a new door for the quantitative analysis of oxylipins and into clinical applications.

Limitations of this study

The small number of participating laboratories in this study is a significant limitation. However, the five participating laboratories represent three countries and laboratories in academic, governmental, and industrial environments, arguing that the findings have broad implications. The lack of a commercially available certified reference material for total oxylipins also limits future direct comparisons to the data reported here. Future efforts in this area should expand the number of laboratories to no less than 7 and include a commercially available reference plasma (e.g., NIST SRM 1950–Metabolites in Human Plasma) allowing the development of consensus values for the total oxylipin content of this material.

Data availability

All data are contained within the article or supplemental data.▮▮

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

COD, coefficient of dispersion; CV, coefficient of variation; DiHDPE, dihydroxydocosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; DiHETrE, dihydroxyeicosatrienoic acid; DiHOME, dihydroxyoctadecenoic acid; EpDPE, epoxydocosapentaenoic acid; EpETE, epoxyeicosatetraenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxyoctadecenoic acid; HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; IS, internal standard; LLOQ, lower limit of quantification; Lx, lipoxine; MEDM, median of the means; MeOH, methanol; NIST SRM, National Institute of Standards and Technology standard reference material; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; QC, quality control; Rv, resolvin; SOP, standard operation procedure; SPE, solid phase extraction; TriHODE, trihydroxyoctadecadienoic acid; TriHOME, trihydroxyoctadecenoic acid.

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Supplementary Information

Harmonized procedures lead to comparable quantification of total oxylipins across laboratories

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Standard operation procedure (SOP) for extraction and hydrolysis of total oxylipins from human plasma

The sample preparation is carried out according to Ostermann et al. 2020 (Prostaglandins Other Lipid Mediat 14, 106384).

Reference values for acceptable variations in oxylipin concentrations using this SPE protocol

It should be noted that the variation of oxylipin concentrations in general depends on the analyte level, the individual analyte and the operator. In our experience, when one well-trained operator is preparing the samples, the general relative intra- and inter-day variance is ≤15% for epoxy-PUFA, hydroxy-PUFA, dihydroxy-PUFA and 5(*R,S*)-5-F$_{2t}$-IsoP, while inter-day variance is up to 30% when different operators are preparing the samples .

The analysis is considered sufficient if an intra- and inter-day variability below 20% is achieved.

Abbreviations: EA = ethyl acetate; *n*Hex = *n*-hexane; HAc = acetic acid; MeOH = methanol

Before starting with sample preparation

- We recommend using solvents, solids and materials specified in the "materials" table for the SPE since comparable results cannot be guaranteed using different materials.
  - ➔ If other products are used, their suitability (e.g. contamination with oxidized fatty acids) should be checked in each lab
- Check if enough material for sample preparation is available in the lab (cartridges, inserts, vials etc.)
- Prepare enough buffer, eluent and washing solutions
- Cool centrifuge to 4 °C
- Get enough ice to cool the samples during preparation
- Cool *iso*-propanol to -30°C
- Pre-heat sample shaker to 60°C
- Pre-cool two sample racks to -80 °C

Sample preparation

Work on ice if possible and store samples on ice in case of delays.

1. Thaw human plasma at room temperature, check and vortex sample every few minutes and put them on ice when thawed
2. Transfer 100 µL plasma into 1.5 mL sample tube with an accurate microliter pipette (e.g. Eppendorf pipette or different brand)
   - CAVE: Only use the reaction tubes specified in the "materials" table (see below)
3. Add 10 µL "Antiox-Mix" (=BHT (0.2 mg/mL) with sEHi (*t*-AUCB) and Indomethacin (100 µM each) in MeOH) using a microliter pipette
4. Add 10 µL of internal standard solution with a Hamilton-Repeater (use 500 µL syringe; see "materials" table for product details)

- o Moisten Hamilton syringe before use 1-2 times with MeOH
- o Get rid of any residual MeOH visible in the syringe by rapidly moving plunger up and down a few times
- o Hold syringe tip to the wall of the reaction tube while adding the IS or wipe tip on the wall after adding the IS in order to make sure it is quantitatively transferred to the tube
- o Clean syringe after use 3-5 times with MeOH

5. Vortex samples
6. Add 400 µL ice cold *iso*-propanol (-30 °C) with a microliter pipette
    - o Moisten pipette tip once before use
7. Vortex samples
8. Freeze samples at -80 °C for at least 30 min using a pre-cooled (-80 °C) sample rack
9. When samples are taken from -80 °C freezer, leave them at room temperature for 1-2 min, then vortex samples briefly
10. Centrifuge samples (20 000 x *g*, 10 min, 4 °C)
11. Transfer supernatant into 1.5 mL sample tube
12. Add 100 µL 0.6 M KOH in MeOH/H_2O (75/25, *v/v*)
13. Vortex samples
14. Hydrolyze samples for 30 min at 60 °C using the pre-heated shaker
15. After hydrolysis, cool samples quickly by putting them in a pre-cooled sample rack (-80 °C)
16. Add 20 µL 25% acetic acid
17. Vortex sample
18. Centrifuge samples very briefly in order collect sample on the bottom of the reaction tube
19. Transfer samples to prepared cartridges (see SPE Procedure, Point 4)

SPE Procedure

If not indicated otherwise, all elution steps are conducted by gravity.

1. Wash cartridge (Bond Elut Certify II, 200 mg, 3 mL, Agilent) with
    - o one column volume EA/*n*Hex (75/25, *v/v*) with 1% HAc
    - o one column volume MeOH (LC-MS grade)
2. Condition cartridges with one column volume 0.1 M Na_2HPO_4/HAc (pH=6.0) in H_2O/MeOH (95/5, *v/v*)
    - o Close valve when solution is 2-3 mm above stationary phase
3. Add 2000 µL 0.1 M Na_2HPO_4/HAc (pH=6.0) to cartridges
4. Transfer samples with a pasteur pipette and mix thoroughly with the buffer
    - o Check pH in one sample per set using pH stripes with a 5.1-7.2 scale
    - o Only if necessary: Carefully adjust pH to pH=6 with diluted HAc (if HAc has to be added, only few µL are needed)
5. Open valves and let samples run by gravity until completely sunk into stationary phase
    - o If one or two samples run very slowly, the pasteur pipette cone can be used to create low positive pressure for faster elution
6. Wash samples with
    - o One column volume water (ultrapure, 18 MΩ)
    - o One column volume MeOH/H_2O (50/50, *v/v*)
    - o CAVE: Let solvents sink into stationary phase completely
7. Dry samples with vacuum:

- Close valves of all cartridges and create stable -200 mbar negative pressure within the manifold
- Open valves of three samples and test if cartridges are dried by putting a pasteur pipette cone on top of the cartridge → cone should tighten
- Close valves after 30 seconds (it is not critical if samples dry a few seconds longer, however, do not dry them longer than 1 min) and repeat drying step for all samples

8. Elute samples by gravity into glass reaction tubes containing 6 μL 30% Glycerol in MeOH using 2 mL EA/*n*Hex (75/25, *v/v*) with 1% HAc
    - A dispenser resistant to organic solvents can be used to measure the eluent
    - If the solvent is not eluting after 5 min, a pasteur pipette cone can be used to create low positive pressure in order to fasten up elution. Stop with the positive pressure when the first drop of solvent elutes
    - Remove last drops of eluent from stationary phase by applying positive pressure with the pasteur pipette cone
9. Evaporate solvent to dryness using a vacuum centrifuge (1 mbar, 30 °C, ~60 min)
10. Freeze dried samples at -80 °C for at least 30 min

Possible break for overnight: Leave samples in -80 °C freezer
Samples can be left in -80 °C freezer for up to 7 days with only slight changes in the oxylipin pattern (< 20% for most analytes, CAVE: quantification of isoP might be impaired)

11. Reconstitute samples in 50 μL MeOH using a Hamilton-Repeater (use 2.5 mL syringe, see above)
    - Dissolve samples by sonication and vortexing
12. Transfer samples completely into 1.5 mL sample tubes
13. Centrifuge samples (20 000 x *g*, 10 min, 4 °C)
14. Transfer clear (!) supernatant into vial with insert
    - Centrifuge sample again if supernatant is not completely clear

Possible break: Reconstituted samples can be stored for up to 2 months in -80°C freezer with only slight changes in the oxylipin pattern (< 20% for most analytes, CAVE: quantification of isoprostanes might be impaired)

15. LC-MS/MS analysis
    - Cool autosampler to 5 °C
    - Transfer samples to -30/-80 °C for post-analysis storage after a maximum of 24 h in the autosampler
    - Change screw cap of the vials before storage

Preparation of Antiox-Mix, eluent, buffers, etc.

- Antiox-Mix
  BHT (0.2 mg/mL) with sEHi (*t*-AUCB) and Indomethacin (100 μM each) in MeOH
  a) **BHT** (0.2 mg/mL): Dissolve 10 mg BHT in 20 mL MeOH (LC-MS grade)
  b) *t*-**AUCB:** Dissolve 4.13 mg *t*-AUCB / 1 mL DMSO for a 10 mM stock
  c) **Indomethacin:** Dissolve 3.58 mg Indomethacin / 1 mL DMSO for a 10 mM Stock
  → Add inhibitors Indomethacin und *t*-AUCB to BHT solution (final inhibitor concentration: 100 μM):
  + 20 μL 10 mM *t*-AUCB

+ 20 µL 10 mM Indomethacin

+ 1960 µL BHT (0.2 mg/mL)

- M Na_2HPO_4/HAc (pH 6.0) in H_2O/MeOH (95/5, *v/v*) and in H_2O

  Fill and dissolve 1.78 g disodium phosphate dihydrate to ~95 mL with H_2O/MeOH (95/5, *v/v*), add 450 µL concentrated HAc and fill to 100 mL with H_2O/MeOH (95/5, *v/v*). Adjust pH carefully to 6.0 with concentrated HAc. Use ultrapure 18 MΩ*cm water and LC-MS grade MeOH for H_2O/MeOH (95/5, *v/v*).

- M Na_2HPO_4/HAc (pH 6.0) in H_2O
  Same procedure as above, but dissolve disodium phosphate dihydrate in H_2O.

- EA/nHex (75/25, *v/v*) with 1% HAc
  Mix 750 mL ethyl acetate with 250 mL *n*-hexane, discard 10 mL (measuring cylinder) and add 10 mL HAc (LC-MS grade). Use solvents specified in the 'materials' table for EA/*n*Hex.

- 0.6 M KOH in MeOH/H_2O (75/25, *v/v*)
  Add ~20 mL MeOH/H_2O (75/25, *v/v*) to 1.98 g potassium hydroxide (85%) and dissolve (CAVE: exothermic reaction, work on ice). Following complete dissolution, fill up to 50 mL with MeOH/H_2O (75/25, *v/v*). Use ultrapure 18 MΩ*cm water and LC-MS grade MeOH for MeOH/H_2O (75/25, *v/v*).

- MeOH/H_2O (50/50, *v/v*)
  Use LC-MS grade MeOH and ultrapure 18 MΩ*cm water.

Table S1: List of materials needed for sample preparation

Article	Supplier	Article No.
Materials		
Autosampler vial (brown) (1000/pk)	IVA Analysentechnik (Meerbusch, Germany)	70911302
Glass test tubes (70 x 10 mm, 100/pk)	LAT (Labor und Analysentechnik) (Garbsen, Germany)	11 18 14004
Inserts for autosampler vial (1000/pk)	IVA Analysentechnik	70906500
Hamilton syringe 500 µL (gastight, 22/51mm/pst.3)		1750 LTN [Hamilton]
Hamilton syringe 2.5 mL (gastight, 22/51mm/pst.3)		1002 LTN [Hamilton]
Hamilton Repeating Dispenser		PB600-1 [Hamilton]
Screw caps for autosampler vial (1000/pk)	IVA Analysentechnik	71509342
Pasteur pipettes (1000/pk)	Carl Roth (Karlsruhe, Germany)	4518.1
Alternative product	*VWR (Darmstadt, Germany)*	*612-1701*
Pipette Tips (20 µL)	StarLab (Hamburg, Germany)	S1110-3000
Pipette Tips (200 µL)	StarLab	S1113-1006
Pipette Tips (1000 µL)	StarLab	S1111-6000
Alternative products: 20 µl	*Sarstedt (Nümbrecht, Germany)*	*70.1114*
300 µl	*Sarstedt*	*70.765*
1000 µl	*Sarstedt*	*70.762*
Reaction tube 1,5 mL (2000/carton)	Sarstedt	72.706
Alternative product	*Sarstedt*	*72.690*
SPE Cartridges Bond Elut Certify II 200mg 3ml, 50/pk	Agilent (Waldbronn, Germany	12102080
pH Sticks (pH-Fix 5.1-7.2, 100/pk)	Macherey-Nagel (Dueren, Germany)	92140
Chemicals		
Acetic Acid (99.7%, ACS reagent, 500 mL, Acros)	Fisher Scientific (Langenselbold, Germany)	423225000
Butylated hydroxytoluene (BHT, ≥ 99%, 1kg)	Sigma Aldrich (Schnelldorf, Germany)	W218405
Disodium hydrogen phosphate dihydrate (≥ 99,5 %, p.a., 1 kg)	Carl Roth	4984,1
Ethyl acetate (CHROMASOLV®, for HPLC, ≥ 99.7%, 2.5 L)	Sigma Aldrich	34858
Alternative product: Ethyl acetate (HPLC grade, 2.5 L)	*Fisher Scientific*	*E/0906/17*
Glycerol (98%, water free, 1L)	Sigma Aldrich	G7893
Indomethacin (≥ 99%, 5 g)	Sigma Aldrich	I7378
Isopropanol (Optima™ LC/MS Grade, 2.5 L)	Fisher Scientific	A461-212
Methanol (Optima™ LC/MS Grade, 4L)	Fisher Scientific	10402824
n-Hexane (Rotisolv HPLC, 2.5 L)	Carl Roth	7339.1
Alternative product: n-Hexane (HPLC grade, 2.5 L)	*Fisher Scientific*	*H/0406/17*
Potassium Hydroxide (85%, for analytical purposes, 1 kg)	Grüssing GmbH (Filsum, Germany)	120381000
t-AUCB (≥ 90%, 10 mg)	Cayman Chemical (local distributor Biomol, Hamburg, Germany)	Cay16568-1

Table S2: Critical separation pairs that require chromatographic separation.

Interference	Comment / qualitative description if interference
12,13-DiHODE causes signal on 15,16-DiHODE transition	chromatographic separation required
ω and (ω-1) hydroxy fatty acids of EPA and DHA	chromatographic separation required
8(9)-EpETE causes signal on 11(12)-EpETE transition	chromatographic separation required
11(12)-EpETE causes signal on 14(15)-EpETE transition	chromatographic separation required
8(9)-EpETrE causes signal on 11(12)-EpETrE transition	chromatographic separation required
PGB_2 causes signal on PGJ_2 transition (PGJ_2 causes only minimal signal on PGB_2 transition)	chromatographic separation required
7-HDHA causes signal on 11-HDHA Transition	chromatographic separation required

Table S3: Interfering analytes.

Interference	Separate Integration possible?	Comment / qualitative description if interference
4-HDHA causes signal on 19(20)-EpDPE transition	(Y)	high concentrations of 4-HDHA interfere with the integration of 19(20)-EpDPE
14-HDHA causes signal on 10-HDHA Transition	Y	coelution, interference in 10-HDHA quantification by 14-HDHA ~1%
HDHA isomers	Y	most analytes chromatographically separated, but in part small "feet" at the base of the peaks visible
EpDPE isomers	Y	most analytes chromatographically separated, but in part small "feet" at the base of the peaks visible
LxA_4 and $15(R)$-LxA_4	Y	only little chromatographic separation, $15(R)$-LxA_4 not included in calibration
MaR1 and RvD5	(Y)	coelution; interferences <5%; do not quantify RvD5 via 359>141 if MaR1 is present in sample (Interference <10%)
MaR1 and PDx	Y	in part chromatographically separated; PDx causes signal on MaR1 359>221 Transition
RvD5 and PDx	Y	in part chromatographically separated; PDx causes signal on RvD5 359>199 with low intensity

(Y) - integration possible; at high analyte concentrations interferences might occur.

Table S4: Internal standard assignment to analytes.

Table S5: Comments of participating laboratories on sample preparation.

	laboratory 1	laboratory 2	laboratory 3	laboratory 4	laboratory 5
deviations from SOP	None *Number of operators:* one	Centrifugation: 18 000 x *g*/10 min/4°C *Sample Preparation*: Step 6: use of -20°C *iso*-propanol *SPE:* Step 4: sample plasma 2 - replicate 3 was dried for 1 min *Number of operators:* one	Centrifugation: 16 602 x *g*/20 min/4 °C *SPE:* Step 9: evaporation under N_2 *Number of operators:* one	*Sample Preparation*: Step 6: use of -20°C *iso*-propanol *SPE:* Step 8: 9µL of 20% Glycerol Step 10: Pool B, Day 1 samples were stored at -80°C overnight. *Number of operators:* one	*SPE:* Step 9: evaporation under N_2 *Number of operators:* two
unexpected occurrences during sample preparation	None	none	none	*Day 1/Day 2 sample acquisition*: leak developed overnight, compromising the calibration level 16 and the following 13 sample injections. The issue was located and corrected and a partial curve was run prior to running samples again. Fixing the first leak altered retention times between the first curve (pre-leaks) and following curves (post-leaks) by almost 30 seconds, which was caught after a few injections. A partial curve was run a third time, and then all samples were successfully acquired or re-acquired. *Day 3 sample acquisition*: During acquisition of Day 3 samples, a buildup on the pre-filter of the column resulted in the UPLC rapidly over-pressuring across 6 injections with steadily degrading retention time performance, and erring out on the sixth injection, around 11am local time. The issue was caught almost immediately and we had corrected it by 2pm. A single calibration point was run to confirm retention times, and then the last 8 samples were acquired successfully.	none
analytical	*LC*: Agilent 1260 Infinity II *MS*: SCIEX API 5500 QTRAP	Analysis of samples in laboratory 3	*LC*: Agilent 1290 Infinity *MS*: Agilent 6460 triple quadrupole	*LC*: Shimadzu Nexera X2 series *MS*: SCIEX API 6500 QTRAP	*LC*: Agilent 1200 *MS*: Agilent 6460 Triple Quad

Table S6: Analyte concentrations of total oxylipins in seven QC plasmas. Shown are mean ± SD concentrations quantified in all laboratories. When analyte concentration was <LLOQ in more than 50% of the samples the LLOQ (highlighted in red) is shown instead of mean.

n.a.: analyte was not analyzed

ISTD issue: the respective internal standard could not be detected, and a different internal standard was assigned

Table S7: Consensus value estimates for oxylipins quantified in seven QC plasmas. Shown are MEDM, u and COD for oxylipins with acceptable consensus value.

Table S8: Overview of consensus value estimates for oxylipins quantified in seven QC plasmas. Shown are number of quantified oxylipins in each QC plasma and number of oxylipins with an acceptable consensus value quantified in all laboratories (top) and without consideration of laboratory 4 (bottom).

Table S9: Variability of the LC-MS/MS platform. QC Plasma 1 – B extracts were prepared in Lab 2 and 5 (Prep lab 2 and Prep lab 5) and analyzed by means of LC-MS/MS in Lab 1 and 3 (MS1 and MS3). 42 oxylipins above LLOQ were analyzed. Δ inter-MS: difference of concentration between the two closest sample from each triplicate from MS1 and MS3, respectively; Δ intra-MS1: difference of concentration between the lower and the higher

	Laboratory of preparation 2				Laboratory of preparation 5				MS-platform effect
	Δ inter-MS	Δ intra-MS1	Δ intra-MS3	Δ inter-MS > Δ intra-MS1 & intra-MS3	Δ inter-MS	Δ intra-MS1	Δ intra-MS3	Δ inter-MS > Δ intra-MS1 et intra-MS3	
9,10,13-TriHOME	2.16	0.21	0.68	yes	0.50	1.23	0.69	-	-
9,12,13-TriHOME	5.35	0.83	3.77	yes	1.83	3.57	0.88	-	-
9-HODE	0.21	2.83	7.41	-	15.52	23.79	18.99	-	-
10-HODE	0.81	0.25	0.26	yes	1.13	5.53	1.85	-	-
13-HODE	1.56	6.76	6.64	-	9.24	32.23	61.08	-	-
5-HETrE	0.05	0.27	0.73	-	0.52	0.34	1.60	-	-
12-HETrE	1.13	0.43	0.50	yes	16.48	1.31	14.68	yes	yes
15-HETrE	0.09	0.21	0.94	-	0.18	1.50	1.46	-	-
5-HETE	2.52	1.22	4.20	-	0.35	5.40	3.63	-	-
9-HETE	0.95	0.32	2.39	-	0.57	5.38	2.61	-	-
5-HEPE	0.23	0.04	0.62	-	0.12	0.50	0.38	-	-
8-HEPE	0.24	0.06	0.42	-	0.46	0.51	0.89	-	-
11-HEPE	0.08	0.02	0.22	-	0.72	0.51	1.26	-	-
12-HEPE	0.82	0.09	0.42	yes	0.31	0.93	0.12	-	-
4-HDHA	0.69	0.66	1.30	-	2.30	3.44	2.74	-	-
7-HDHA	0.85	0.22	0.45	yes	1.25	1.52	1.13	-	-
8-HDHA	0.14	0.27	0.51	-	0.69	1.54	0.66	-	-
11-HDHA	0.17	0.48	1.79	-	1.21	2.85	2.50	-	-
16-HDHA	0.48	0.20	1.02	-	0.74	2.08	2.21	-	-
20-HDHA	0.24	0.08	1.74	-	1.00	4.41	2.62	-	-
14(15)-EpEDE	0.37	0.44	2.13	-	1.47	2.09	3.04	-	-
8(9)-EpETrE	0.14	1.84	3.90	-	7.15	6.18	9.72	-	-
11(12)-EpETrE	5.16	3.26	4.95	yes	2.67	9.06	8.85	-	-
14(15)-EpETrE	3.08	1.75	9.93	-	0.76	11.45	11.84	-	-
11(12)-EpETE	0.25	0.27	0.54	-	0.52	1.12	1.87	-	-
7(8)-EpDPE	0.97	1.01	2.71	-	1.48	2.75	2.30	-	-

10(11)-EpDPE	1.24	0.79	1.61	-	2.25	3.32	1.68	-	-
13(14)-EpDPE	1.30	0.54	1.31	-	0.29	2.57	2.05	-	-
16(17)-EpDPE	1.13	0.43	1.95	-	3.49	2.69	4.33	-	-
19(20)-EpDPE	1.04	1.15	3.54	-	2.92	4.25	6.05	-	-
9,10-DiHOME	2.69	1.22	2.77	-	0.71	0.75	1.80	-	-
12,13-DiHOME	2.15	0.37	3.19	-	2.77	0.29	2.21	-	-
15,16-DiHODE	0.09	1.15	3.40	-	0.86	0.41	2.15	-	-
5,6-DiHETrE	1.34	0.79	3.66	-	0.05	0.33	2.16	-	-
8,9-DiHETrE	0.32	0.02	1.39	-	0.06	0.14	0.54	-	-
11,12-DiHETrE	0.06	0.06	0.34	-	0.11	0.05	0.19	-	-
14,15-DiHETrE	0.08	0.02	0.26	-	0.0002	0.05	0.08	-	-
7,8-DiHDPE	0.52	0.36	1.39	-	0.39	0.25	1.23	-	-
11,12-DiHDPE	0.12	0.02	0.17	-	0.002	0.08	0.04	-	-
13,14-DiHDPE	0.01	0.03	0.07	-	0.15	0.01	0.04	-	-
19,20-DiHDPE	0.25	0.26	0.80	-	0.26	0.04	0.02	-	-
13-oxo-ODE	21.24	30.04	36.09	-	31.53	41.99	46.88	-	-
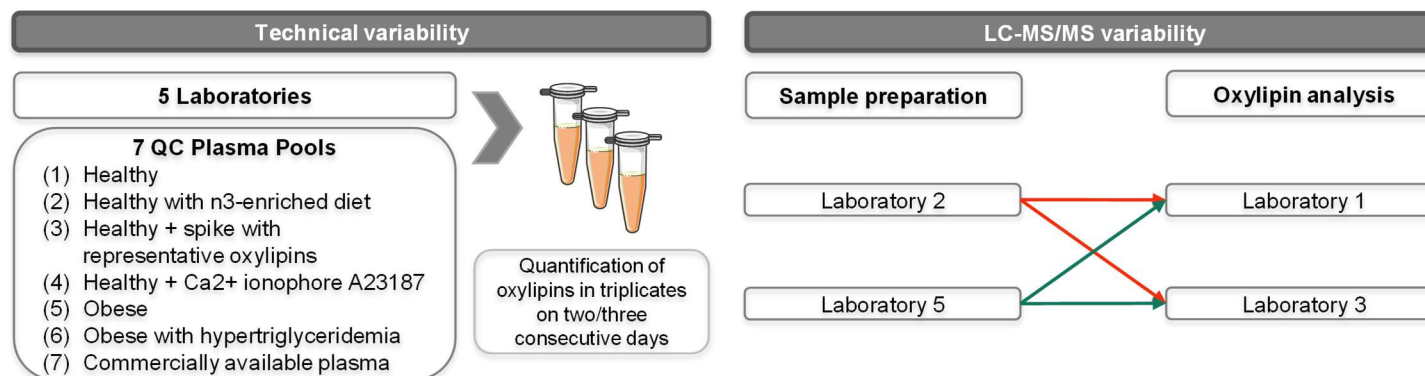
Figure S1: Study design of the interlaboratory comparison.

Left: For the assessment of the technical variability seven QC plasmas were prepared in triplicates on two consecutive days (in case of plasma 1 on three consecutive days) in the five participating laboratories applying same standardized protocol for sample preparation and LC-MS/MS analysis as well as same standard calibration series for the quantification of total oxylipins. **Right:** To investigate the variability of the LC-MS/MS platform, additional QC plasma 1 extracts were prepared in laboratories 2 and 5 and send to laboratories 1 and 3 for the LC-MS/MS analysis.
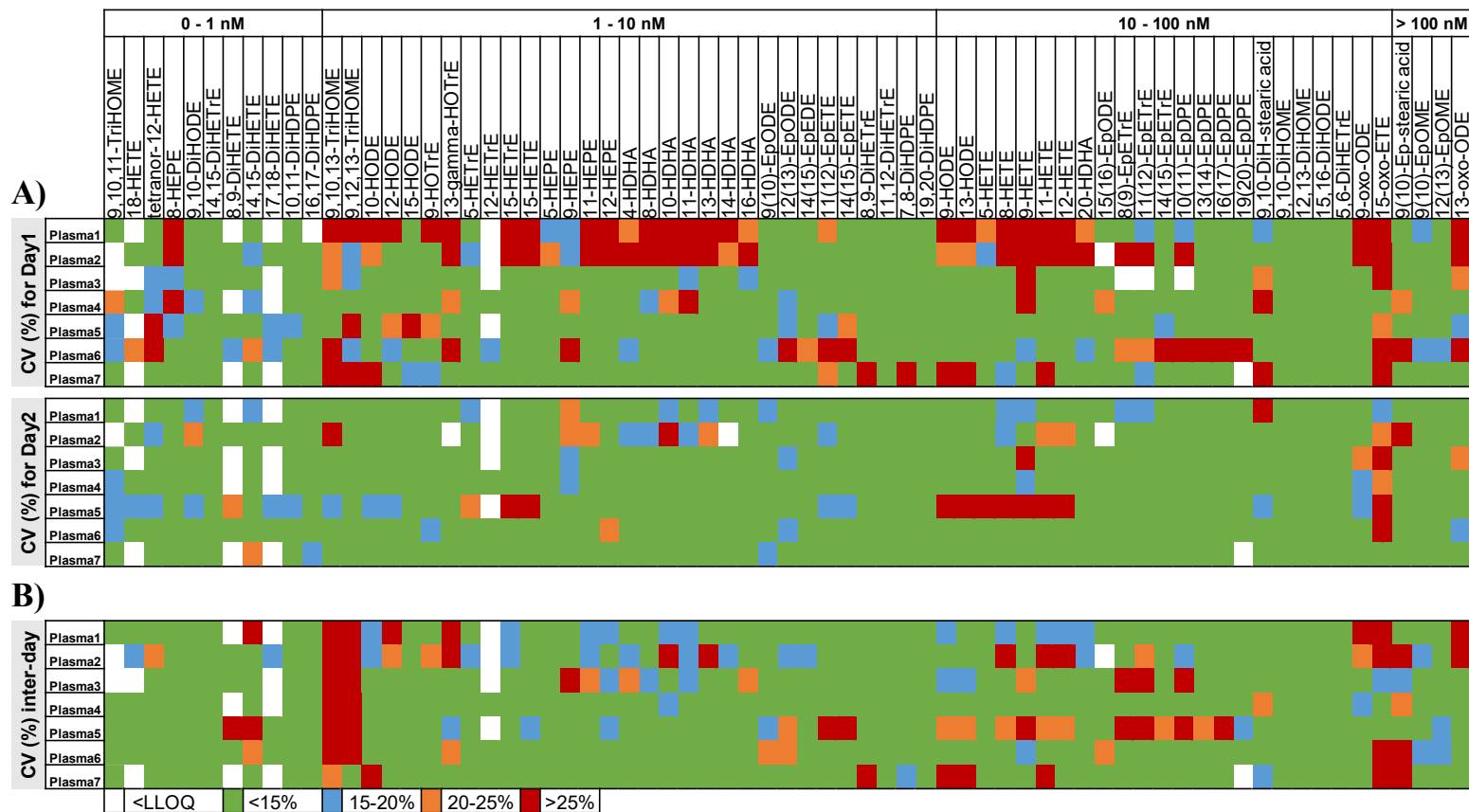
Figure S2: Intra-day (A) and inter-day (B) variability of the oxylipin analysis in laboratory 2. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD (CV = $\frac{SD}{mean} * 100$). Shown are the CVs of 69 oxylipins analyzed in the seven QC plasmas in laboratory 2 displayed in four different colors.
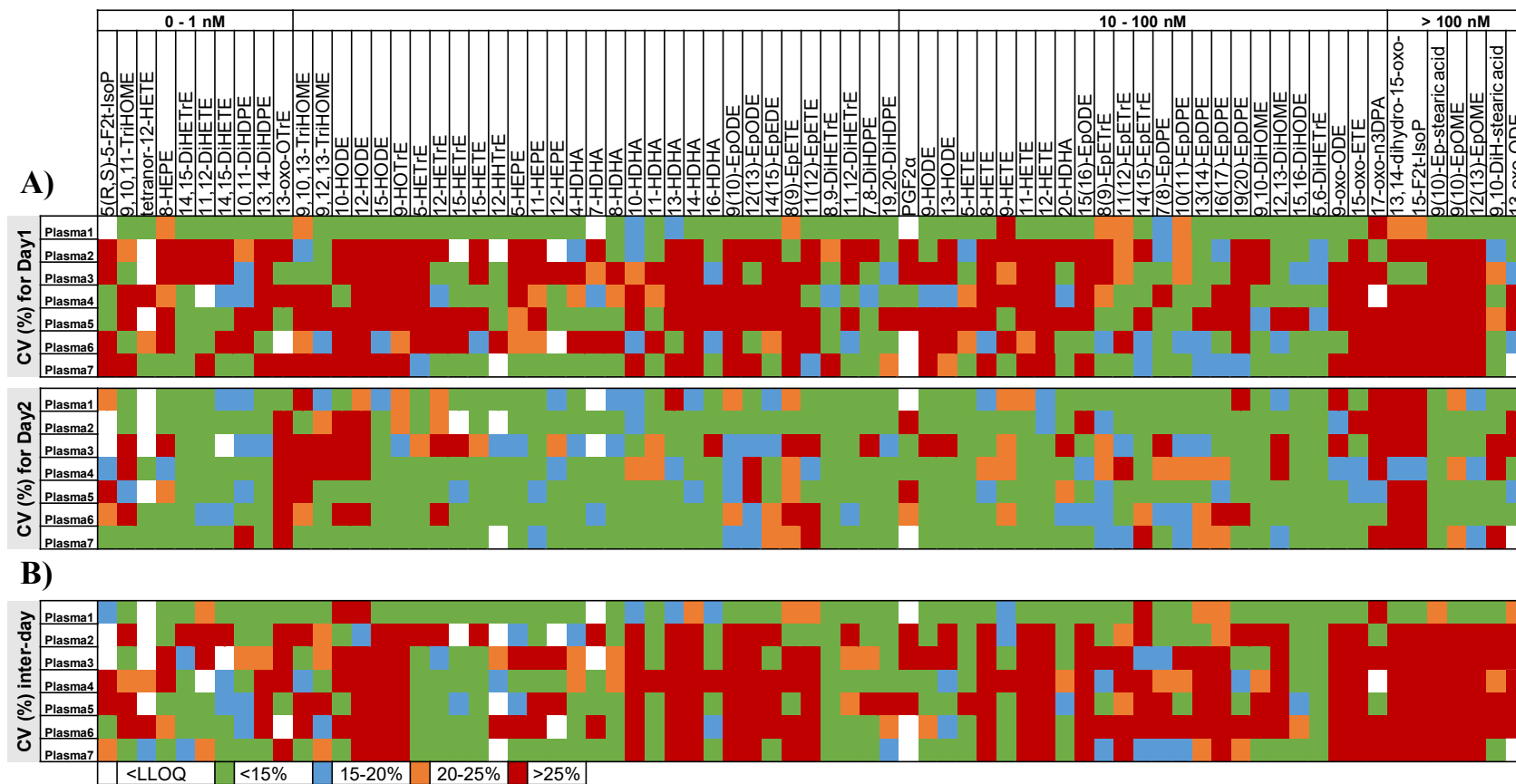
Figure S3: Intra-day (A) and inter-day (B) variability of the oxylipin analysis in laboratory 3. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD (CV = $\frac{SD}{mean} * 100$). Shown are the CVs of 73 oxylipins analyzed in the seven QC plasmas in laboratory 3 displayed in four different colors.
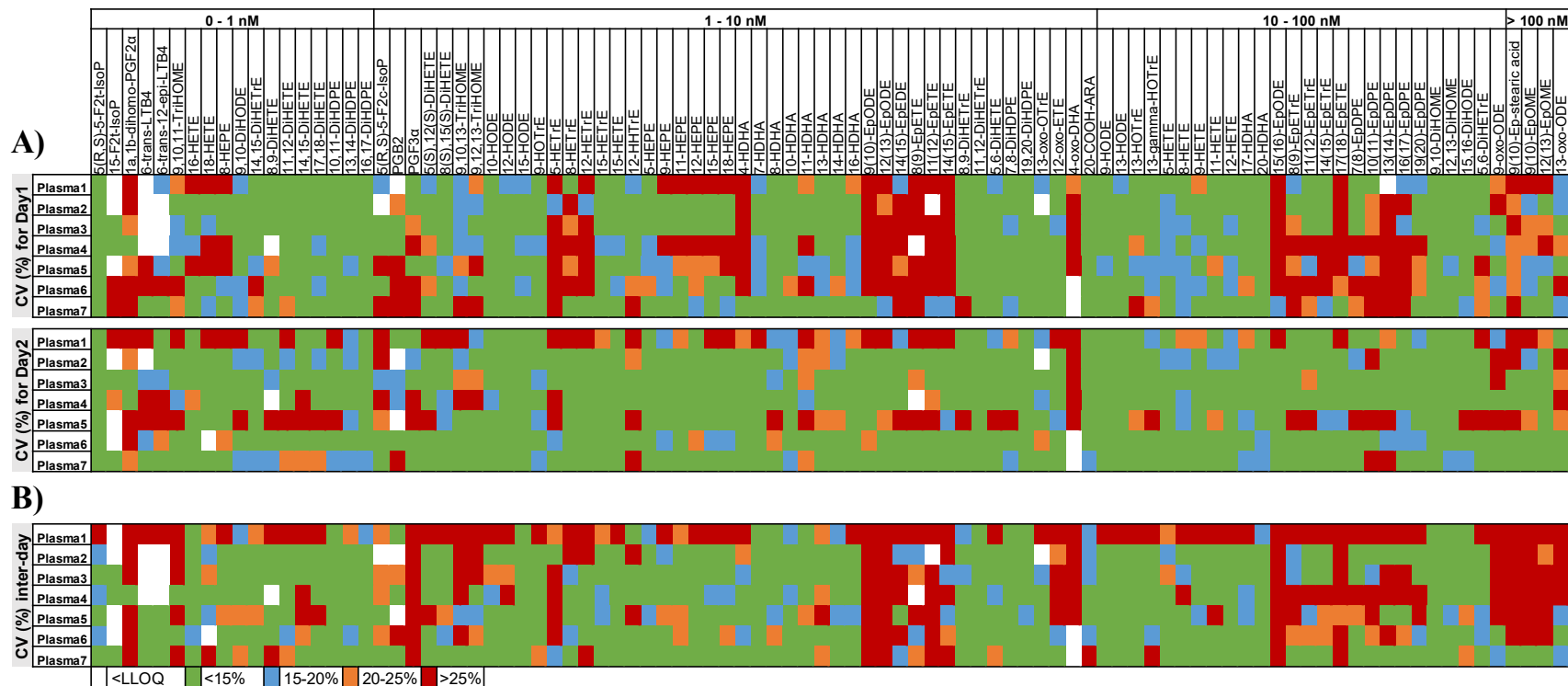
Figure S4: Intra-day (A) and inter-day (B) variability of the oxylipin analysis in laboratory 4. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD ($CV = \frac{SD}{mean} * 100$). Shown are the CVs of 94 oxylipins analyzed in the seven QC plasmas in laboratory 4 displayed in four different colors.
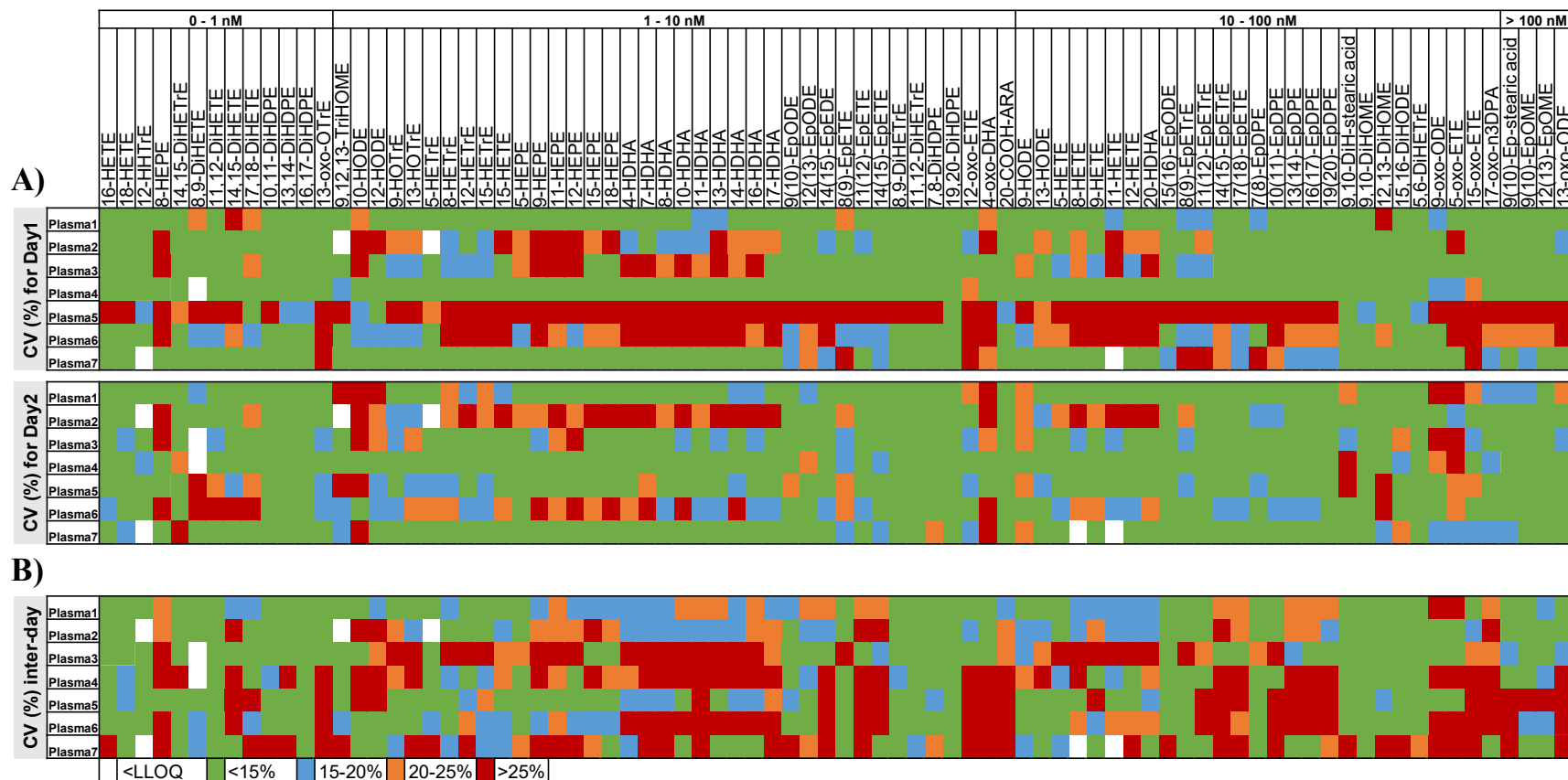
Figure S5: Intra-day (A) and inter-day (B) variability of the oxylipin analysis in laboratory 5. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD (CV = $\frac{SD}{mean} * 100$). Shown are the CVs of 82 oxylipins analyzed in the seven QC plasmas in laboratory 5 displayed in four different colors.
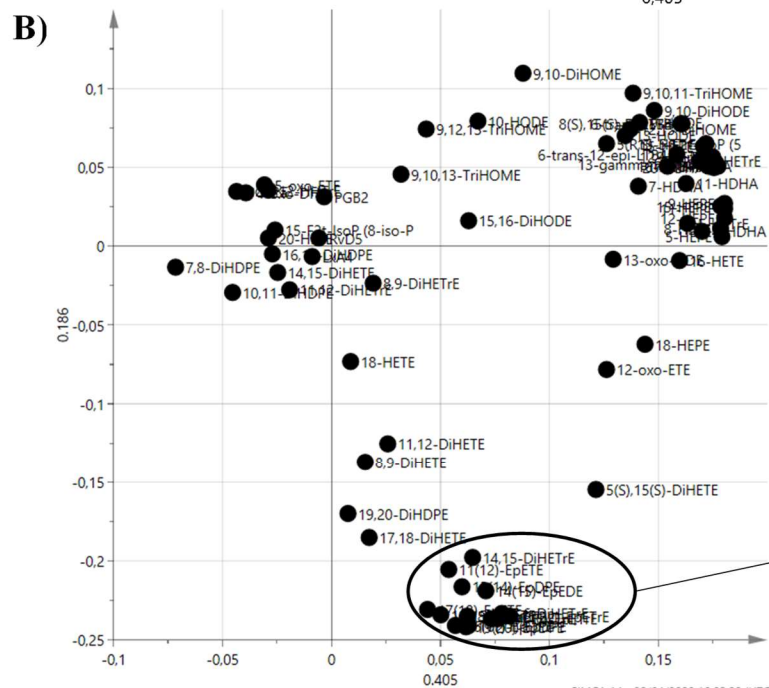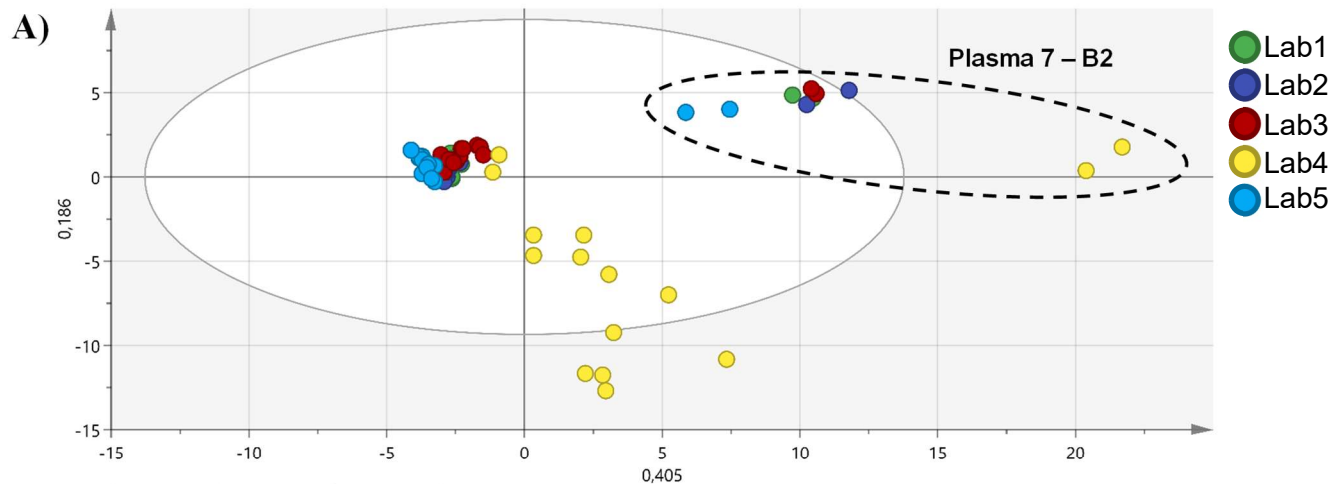
Figure S6: Principal components analysis (PCA) including epoxy-PUFA. The model was built with 75 samples and 74 oxylipins ($R^2X=0.695$ and $Q^2=0.633$). **A)** The score plot shows that the main variability is related to the type of plasma, QC plasma 7 – B2 (dotted circle). In the 2nd component, laboratory 4 distinguishes itself. **B)** The loading plot shows that the discrimination of laboratory 4 is related to epoxy-PUFA (solid circle).

14(15)-EpEDE	17(18)-EpETE
8(9)-EpETrE	10(11)-EpDPE
11(12)-EpETrE	13(14)-EpDPE
14(15)-EpETrE	16(17)-EpDPE
8(9)-EpETE	19(20)-EpDPE
11(12)-EpETE	5,6-DiHETrE
14(15)-EpETE	14,15-DiHETrE
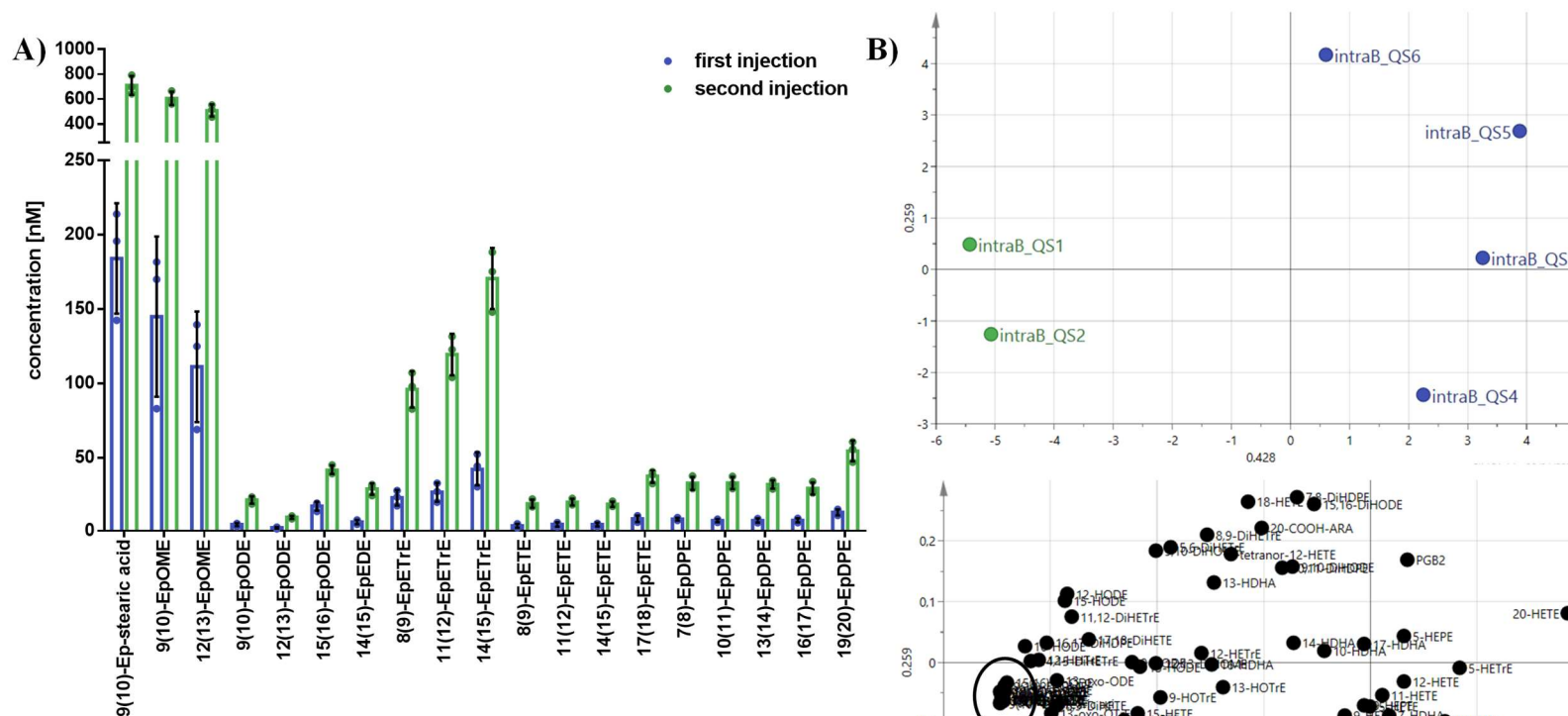
Figure S7: Effects of sample re-injection on the concentration of epoxy-PUFA.

A) Apparent concentrations of epoxy-PUFA in plasma 1. Concentrations derived from re-injected samples are marked in green and once injected samples are depicted in blue. **B)** Partial least square discriminant analysis (PLS-DA with 6 samples and 79 oxylipins, $R^2Y=0.984$ and $Q^2=0.86$). The cross-validated score plot (on the top) shows a strong discrimination according to the number of injections (first injection in blue and second injection in green). On the bottom, the loading plot highlights that epoxy-PUFAs drive the model.
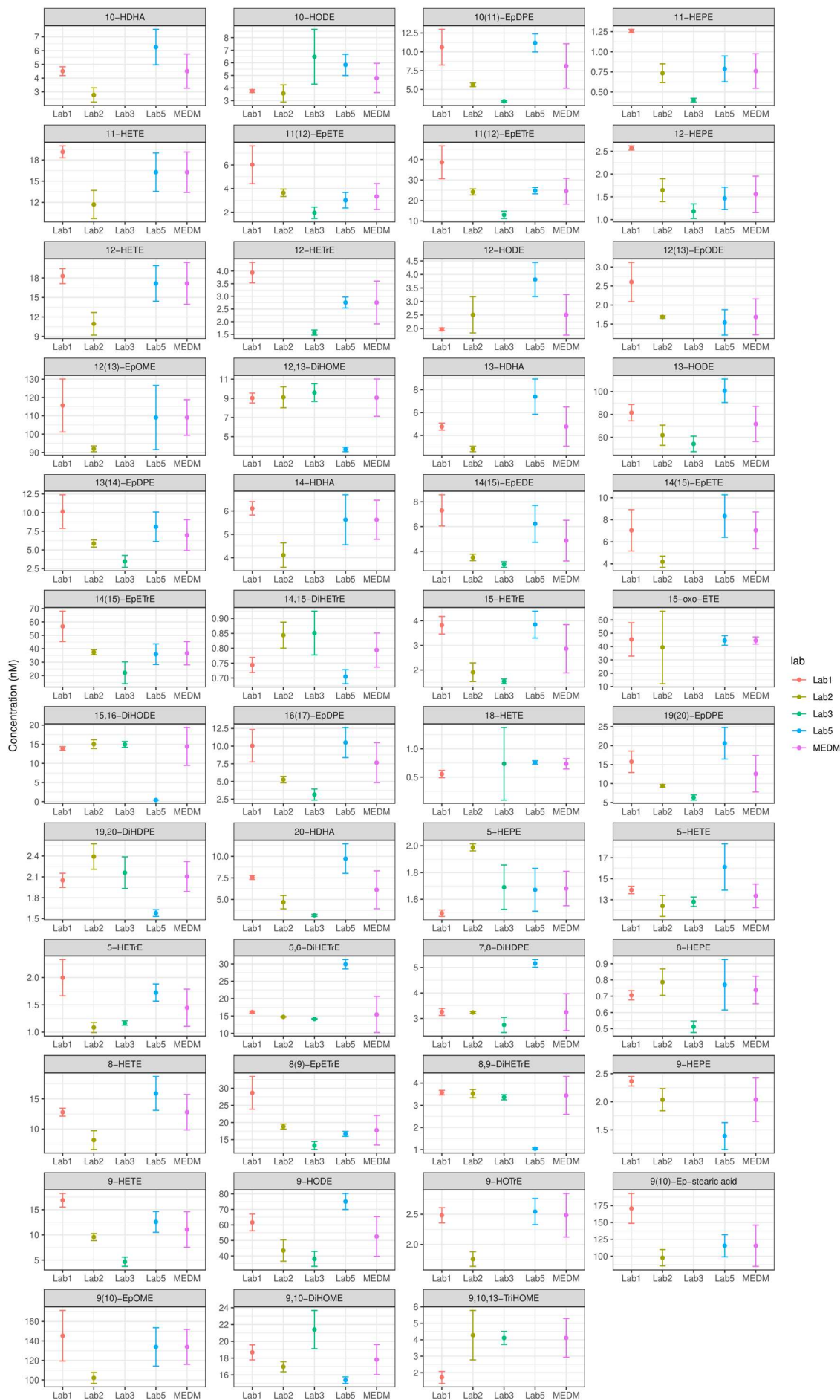
Figure S8: MEDM location plots for QC plasma 1. Plots were created for oxylipins quantified in QC plasma 1 by laboratories 1, 2, 3 and 5. Laboratory 4 was excluded from the consensus value evaluation based on the results of the PLS-DA model (43% of variability on the 1st component). Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 47 oxylipins with acceptable consensus value (COD < 40%).
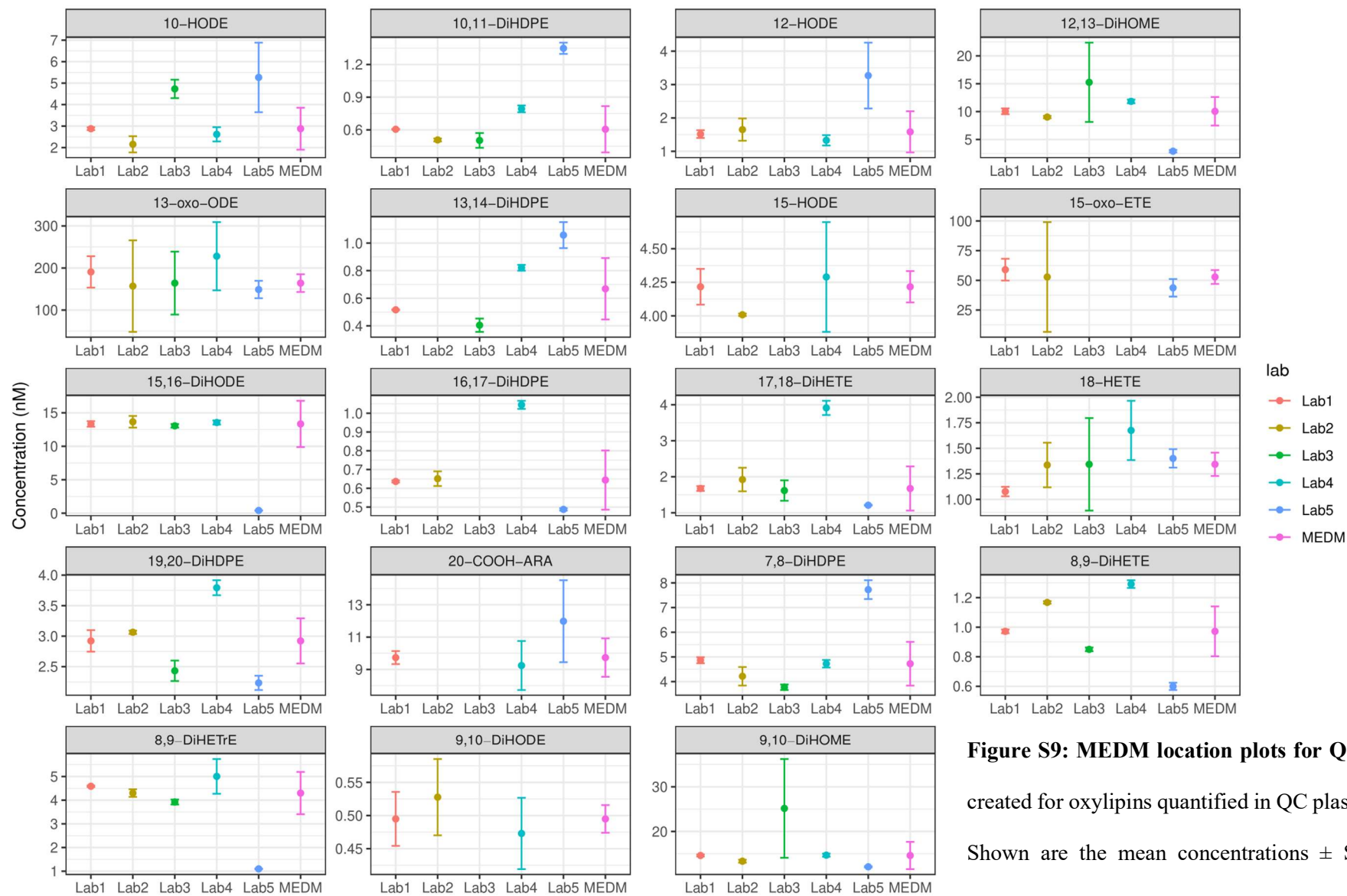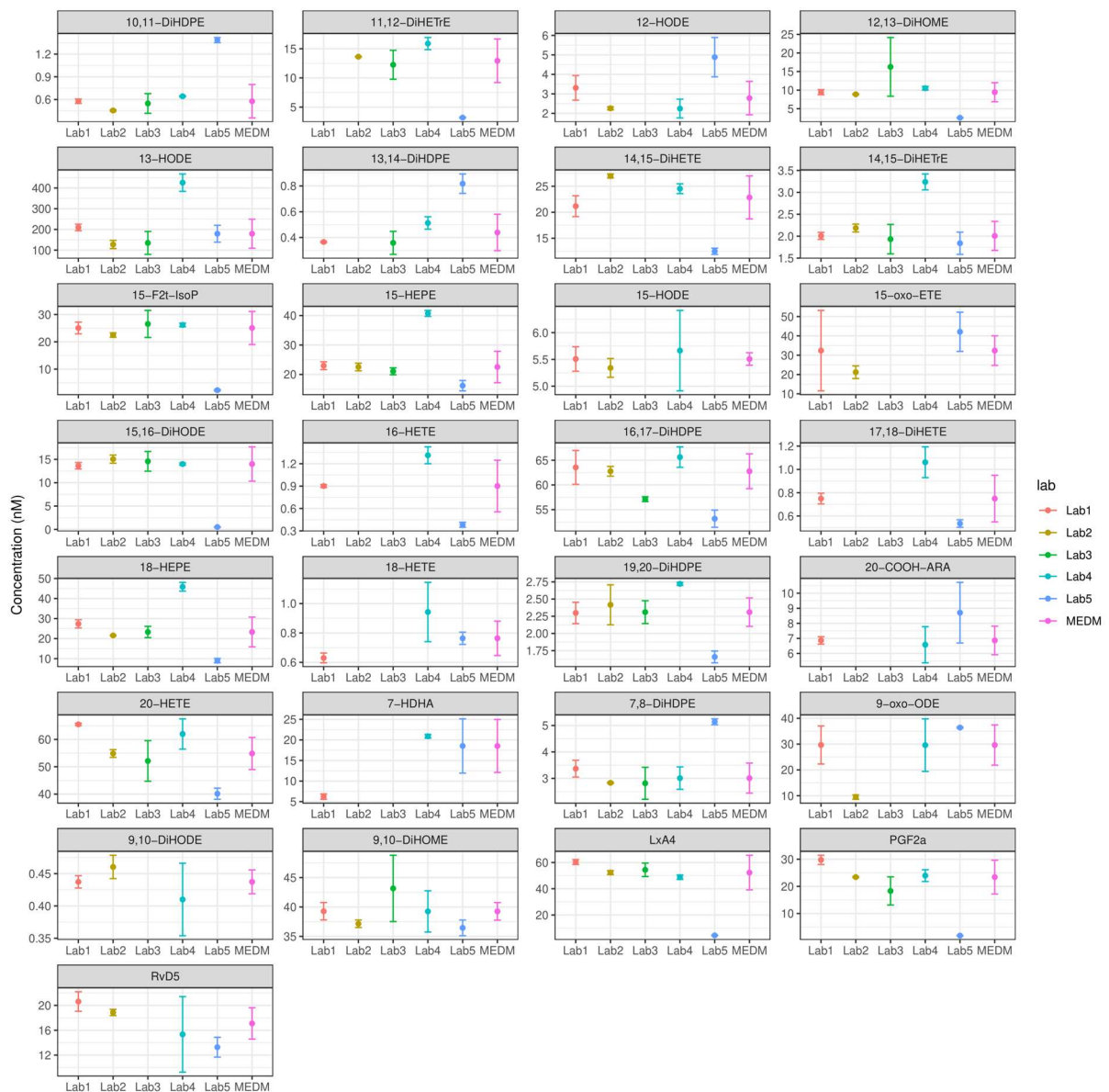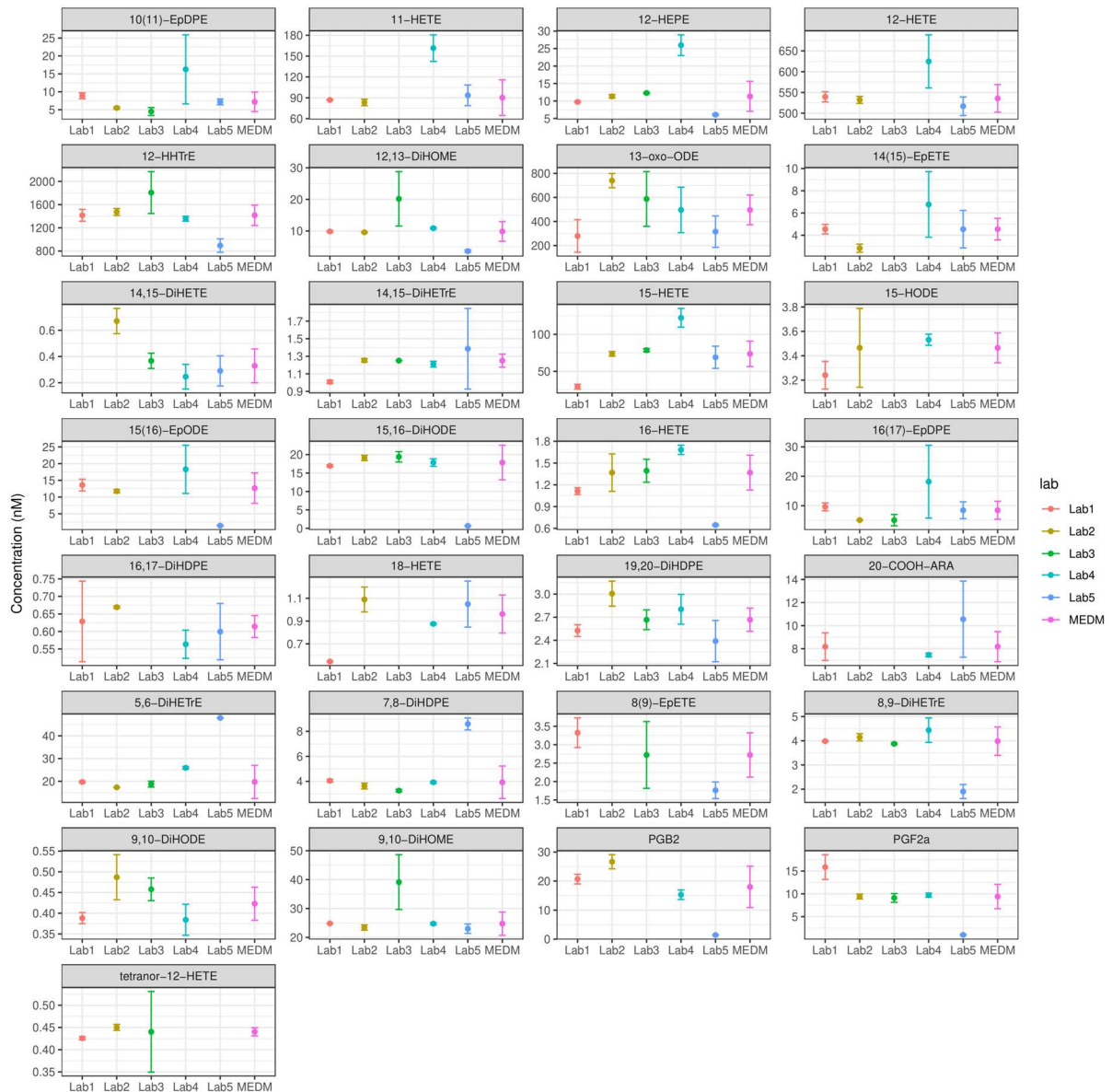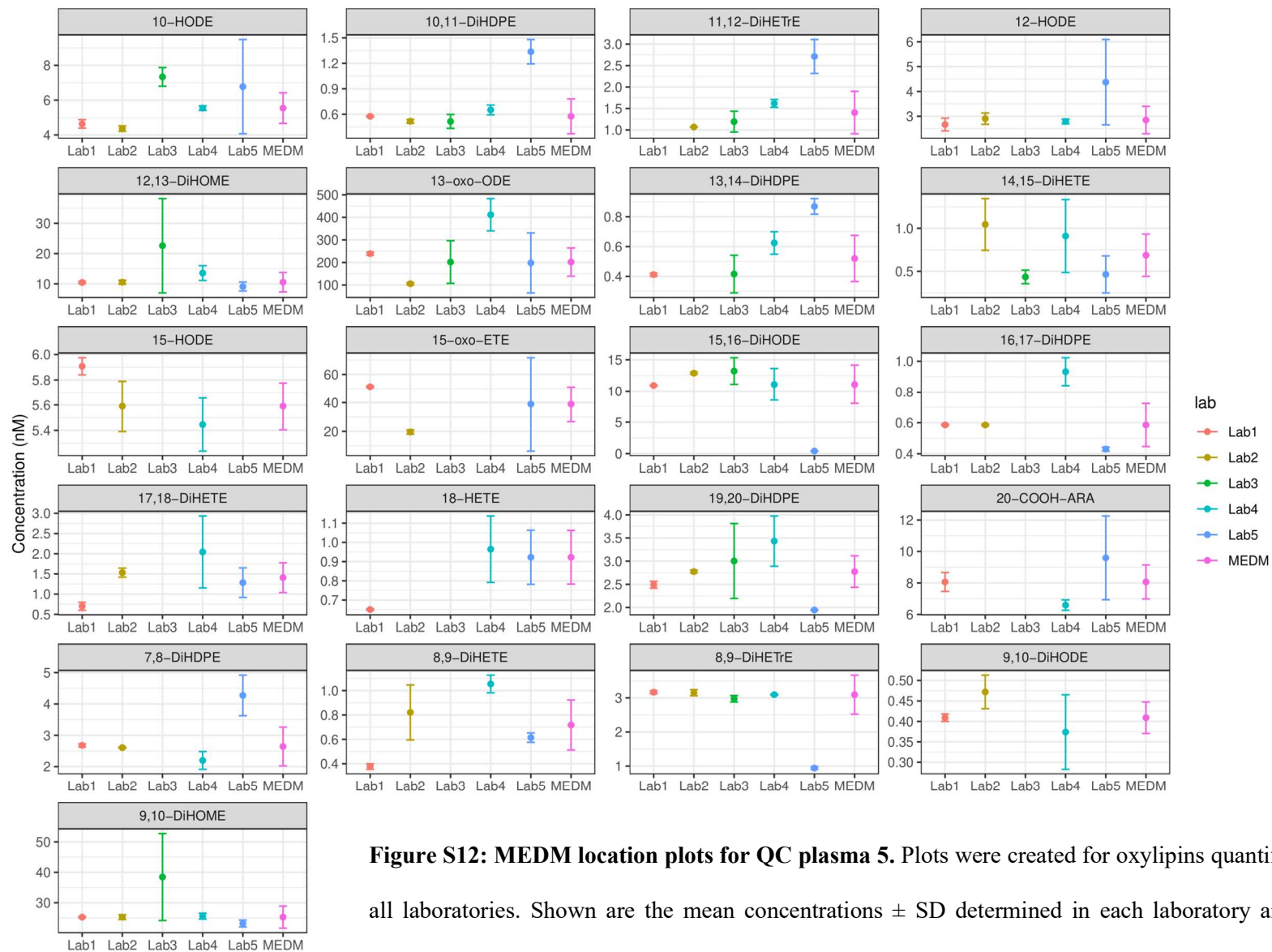
Figure S9: MEDM location plots for QC plasma 2. Plots were created for oxylipins quantified in QC plasma 2 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 19 oxylipins with acceptable consensus value (COD < 40%).
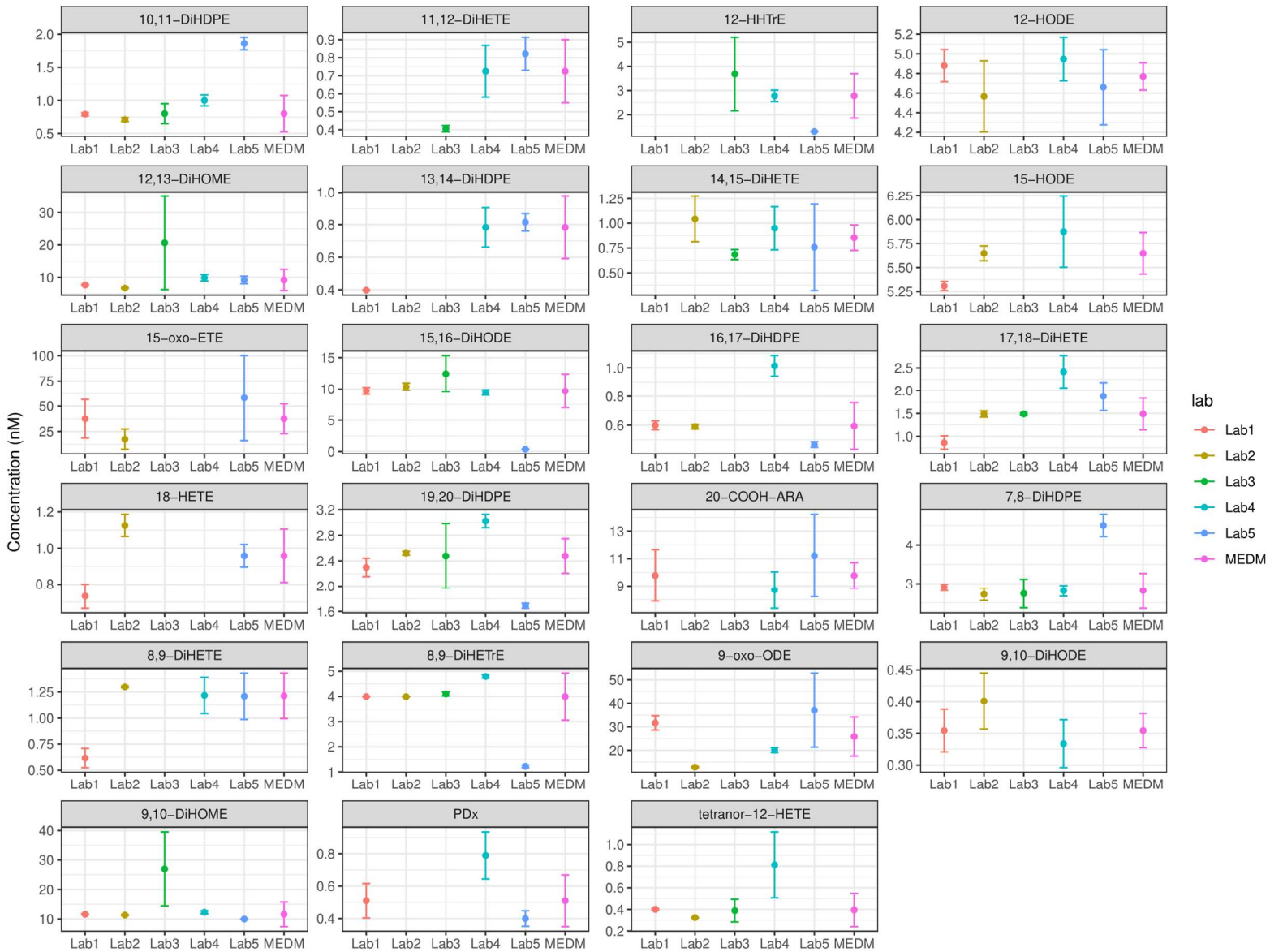
Figure S10: MEDM location plots for QC plasma 3. Plots were created for oxylipins quantified in QC plasma 3 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 29 oxylipins with acceptable consensus value (COD < 40%).

Figure S11: MEDM location plots for QC plasma 4. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 29 oxylipins with acceptable consensus value (COD < 40%).
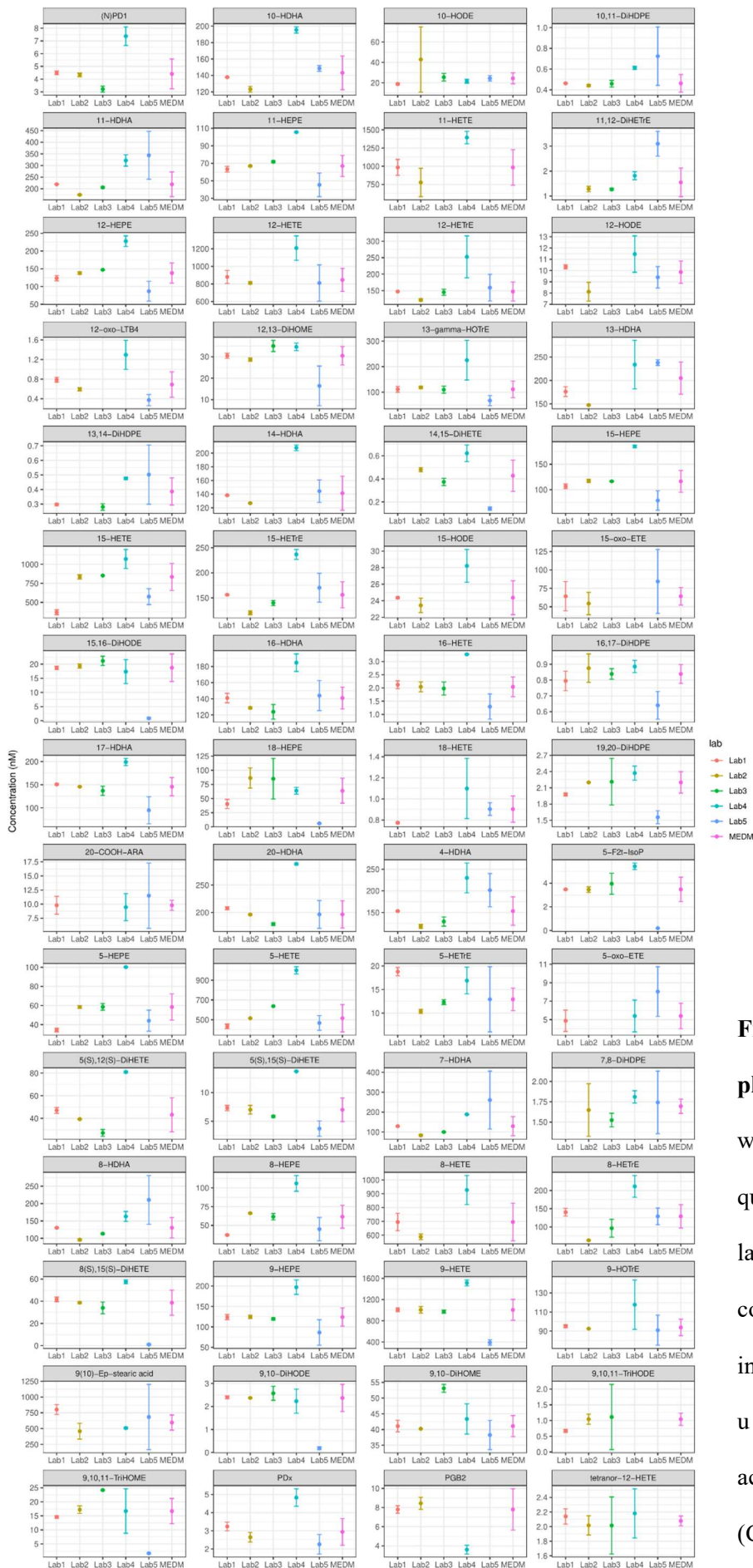
Figure S12: MEDM location plots for QC plasma 5. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 21 oxylipins with acceptable consensus value (COD < 40%).

Figure S13: MEDM location plots for QC plasma 6. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 23 oxylipins with acceptable consensus value (COD < 40%).

Figure S14: MEDM location plots for QC plasma 7. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± u for 60 oxylipins with acceptable consensus value (COD < 40%).
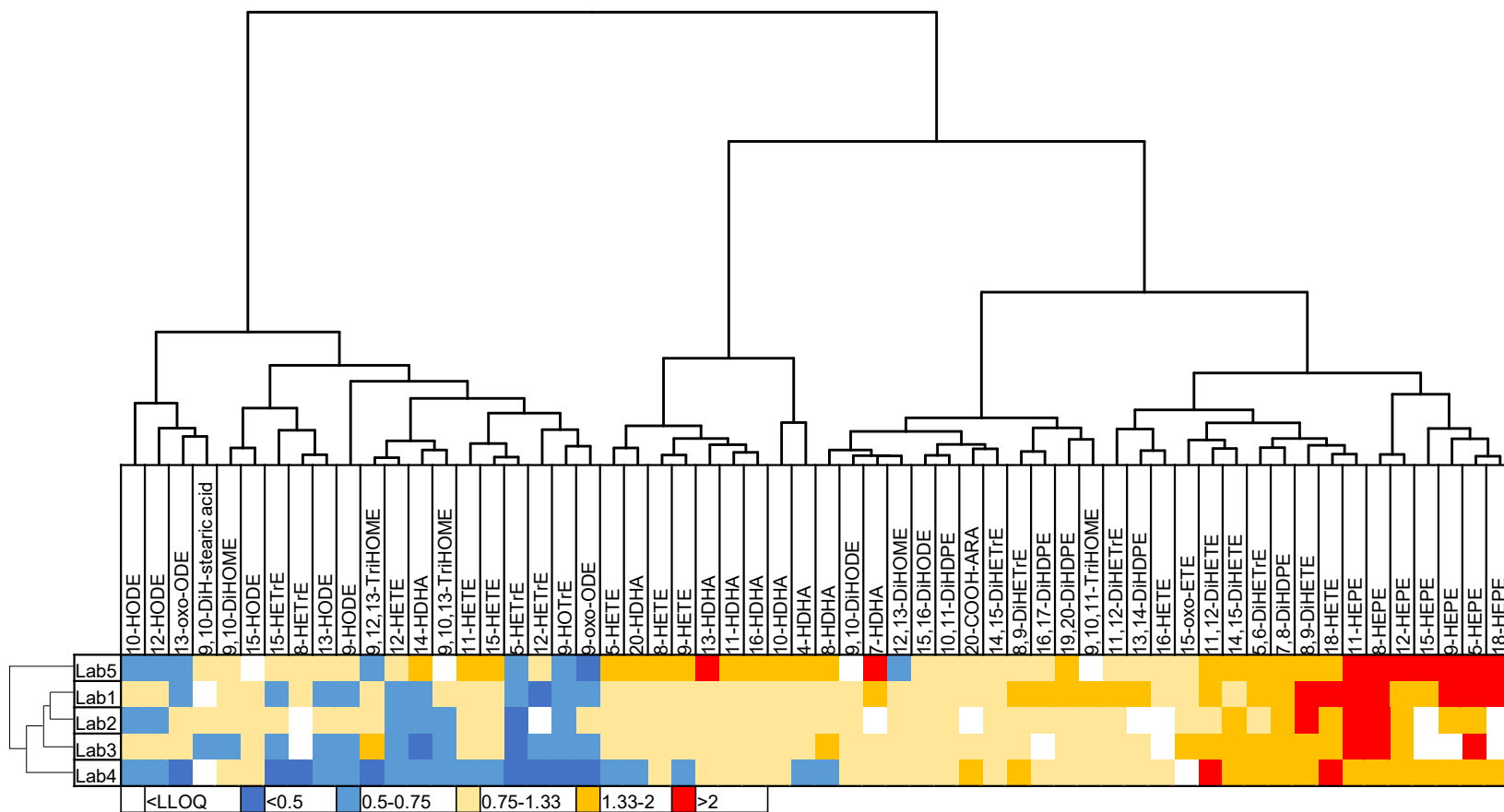
Figure S15: Heatmap of ratios between the QC plasma 2 – n3 against QC plasma 1 - B. The heatmap is displayed in six different colors according the range of ratio. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.
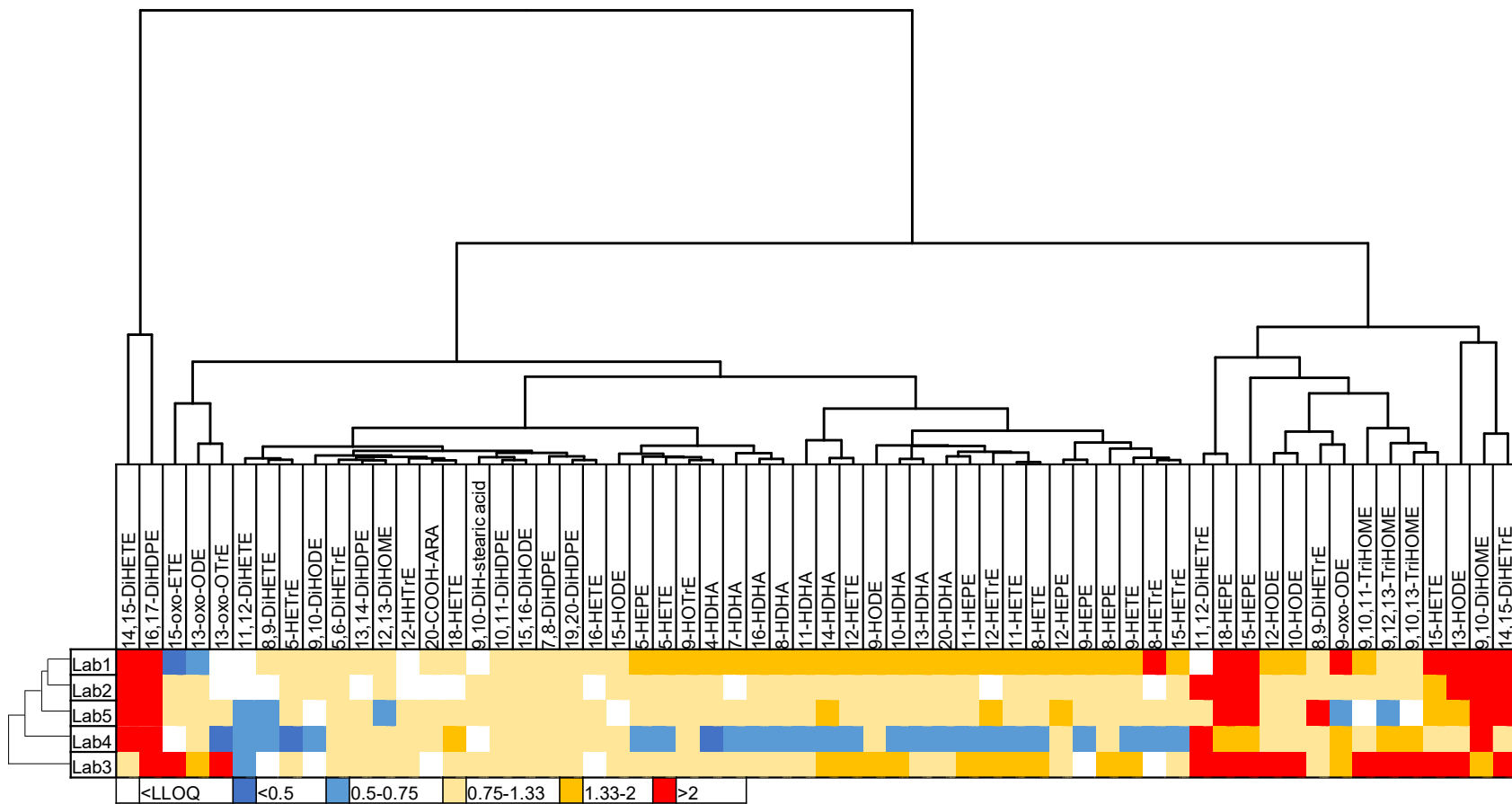
Figure S16: Heatmap of ratios between the QC plasma 3 − S against QC plasma 1 - B. The heatmap is displayed in six different colors according the range of ratios. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.
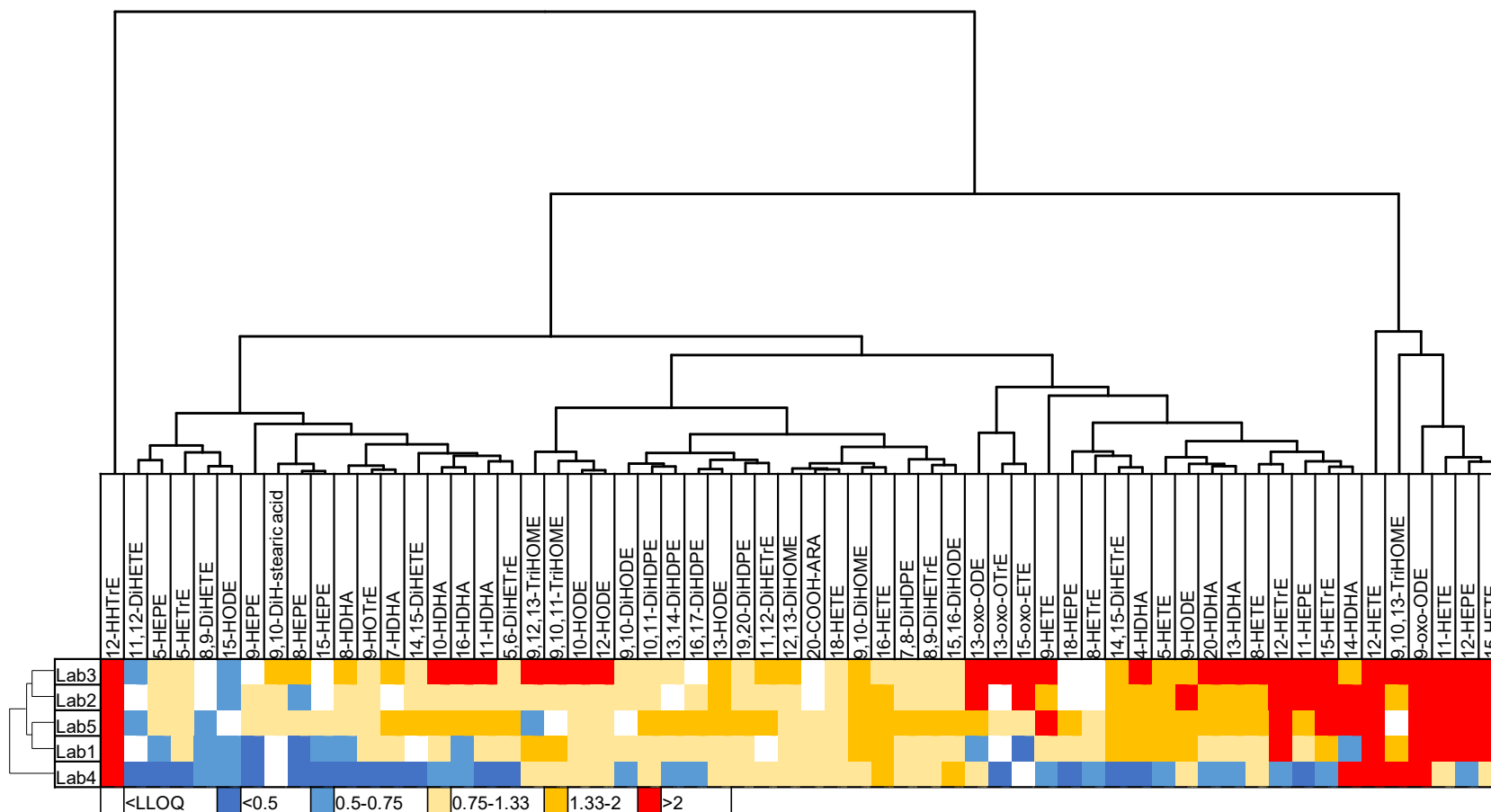
Figure S17: Heatmap of ratios between the QC plasma 4 – Ca against QC plasma 1 - B. The heatmap is displayed in six different colors according the range of ratio. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.
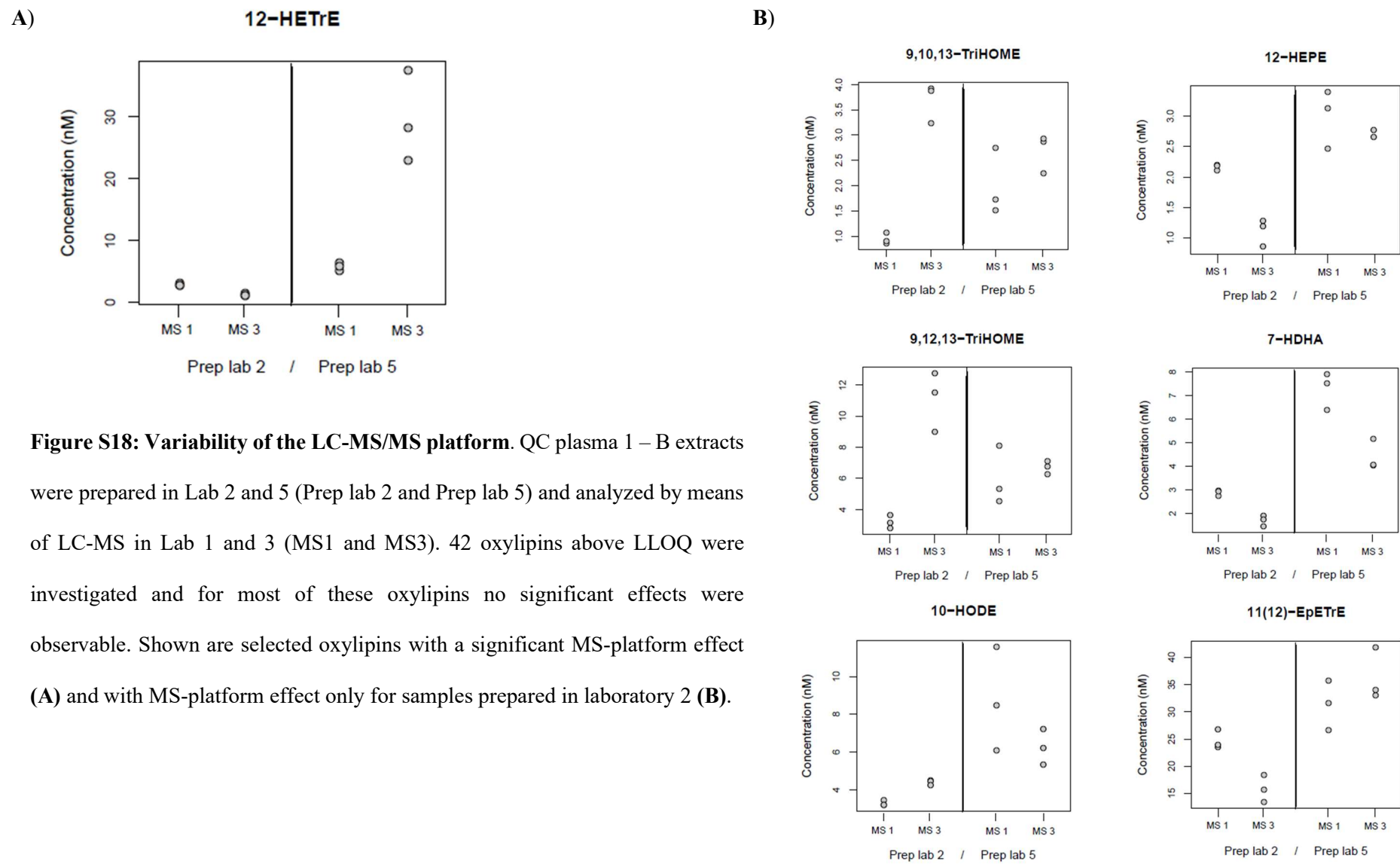
A)

12-HETrE

B)

9,10,13-TriHOME

12-HEPE

9,12,13-TriHOME

7-HDHA

10-HODE

11(12)-EpETrE

Figure S18: Variability of the LC-MS/MS platform. QC plasma 1 – B extracts were prepared in Lab 2 and 5 (Prep lab 2 and Prep lab 5) and analyzed by means of LC-MS in Lab 1 and 3 (MS1 and MS3). 42 oxylipins above LLOQ were investigated and for most of these oxylipins no significant effects were observable. Shown are selected oxylipins with a significant MS-platform effect **(A)** and with MS-platform effect only for samples prepared in laboratory 2 **(B)**.