HARMONIZATION OF LC-MS OXYLIPIN ANALYSIS TO INVESTIGATE LIPID MEDIATOR FORMATION AND SIGNALING IN IMMUNE CELLS

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Für meine Familie

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Chapter 1

Introduction and scope

Several of the oxylipins formed by oxidation of polyunsaturated fatty acids (PUFA), such as arachidonic acid (ARA, 20:4, n6), eicosapentaenoic acid (EPA, 20:5, n3) or docosahexaenoic acid (DHA, 22:6, n3), are potent lipid mediators. They are involved in maintaining physiological homeostasis by regulating e.g., inflammation, platelet aggregation, vasoconstriction or cellular signaling [1]. Oxylipins can be formed by three enzymatic pathways in the ARA cascade or by autoxidation (Fig. 1.1) [2]:

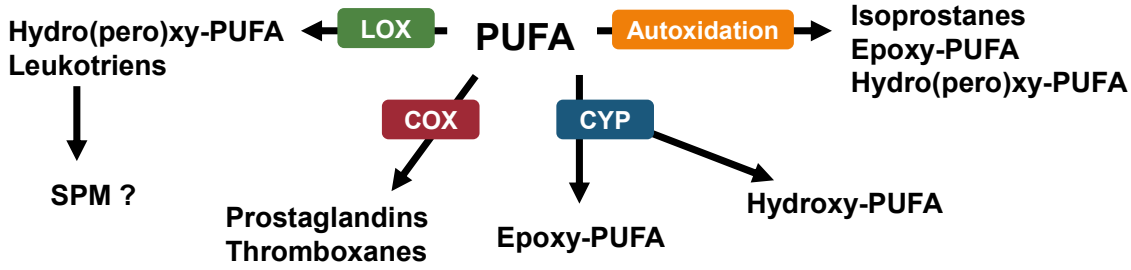

Fig. 1.1: Simplified overview over selected CYP, LOX, COX and autoxidation pathways of PUFA in the ARA cascade.

Cyclooxygenases (COX) catalyze the synthesis *inter alia* of prostaglandins (PG), prostacyclins and thromboxanes (Tx) as major products and hydroxy fatty acids as by-products [1, 3]. There are two isoforms, COX-1 and COX-2, which have similar structural and catalytical characteristics, though they differ in their expression patterns [1]. Whereas the COX-1-coding PTGS1 is constitutively expressed, the COX-2 encoded by PTGS2 expression is dependent on activation by several factors, like cytokines and growth factors. Thus, COX-2 is upregulated in inflammatory sites [1, 4]. The oxygenase active site of COX can be acetylated at a conserved serine residue in the active site by the NSAID aspirin (acetyl-salicylic acid) whereas COX-1 loses all oxygenase activity [5]. Acetylated COX-2 loses the ability to synthesize prostaglandins however maintains to form hydroperoxides giving rise to 15(*R*)-H(p)ETE in case of ARA [3, 5] or 17(*R*)-

H(p)DHA from DHA [5, 6]. Conflicting data exist on the formation of 18-HEPE following aspirin treatment, describing both *S*-isomer [7] and *R*-isomer [8] formation, but the respective concentrations are so low that there is doubt about COX-derived 18-HEPE formation [9]. Lipoxygenases (LOX) catalyze the insertion of molecular oxygen in a bisallylic methylene in the substrate structure [10]. There are six human LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, ALOX5) encoding six different LOX isoforms [11]. The LOX isoforms are classified by the position of the hydroperoxy-group which they insert in ARA, e.g. the products derived from 5-LOX (encoded by ALOX5) are 5-hydroperoxyeicosatetraenoic acid (5-HpETE). Mammalian LOX specifically catalyze the formation of *S*-stereoisomers, only 12(*R*)-LOX (encoded by *ALOX12B*) forms *R*-stereoisomers. 15-LOX (encoded by ALOX15) catalyzes the formation of 12(*S*)- and 15(*S*)-HpETE with ARA as substrate [12-14], whereas 15-LOX-2 (encoded by ALOX15B) exclusively gives rise to 15-HpETE [14, 15]. Both 15-LOX isoforms also oxidize esterified PUFA in e.g. phospholipids or lipoproteins [14, 16-18]. Through a combination of downstream LOX reactions, multihydroxylated metabolites can be synthesized, the so-called ARA-derived lipoxins, EPA- and DHA-derived resolvins or DHA-derived protectins and maresins [9] [19]. Cytochrome P450 monooxygenases (CYP) may act as hydroxylases or epoxygenases when converting PUFA [19]. Besides mid-chain hydroxylation, resulting in the formation of LOX-like hydroxy-PUFA [20], CYP hydroxylate PUFA at the ω-terminus or aliphatic (ω-n)-positions yielding 20-HETE or e.g. 19-HETE from ARA [1]. The ω-hydrolase activity is attributed to the CYP4A und CYP4F subfamilies in human tissue [1]. Moreover, each double bond is susceptible to epoxidation, yielding epoxyeicosatrienoic acids (EpETrEs) in the case of ARA. In humans, the subfamilies CYP2C and CYP2J catalyze this reaction [21].

Oxylipins mediate their biological action via binding to cognate receptors, such as the G-protein coupled receptors [22]. Also the activation of nuclear receptors such as peroxisome proliferator activated receptors (PPAR) [23, 24] or

intervention in intracellular signaling pathways, e.g. TNFα or NF-κB, is known [25-27].

Little is known about the mechanisms underlying an interplay of several PUFA-oxidizing enzymes, such as in the biosynthesis of multihydroxylated PUFA i.e. specialized pro-resolving mediators (SPM), and the proposed formation pathways are under debate based on experimental data [9, 28]. Also, many of the proposed signaling cascades are poorly understood nor studied mechanistically [9, 28]. During homeostasis, the formation of oxylipins is strongly regulated. The increased formation of specific oxylipins due to dysregulation of enzymes and/or receptors is associated with various pathologies [28], leading to a shift of the overall oxylipin profile [29]. Thus, oxylipin 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 [28, 30-32], which increases the need for the implementation of internationally agreed-upon protocols.

In order to establish such methods, several challenges must first be overcome: The endogenous concentration of lipid mediators can span over a range of 4 orders of magnitude and the stability and polarity of oxylipins varies greatly [33, 34]. Therefore, robust analytical methods with high sensitivity, selectivity, accuracy and precision are needed for the analysis of oxylipins. The analysis of oxylipins is predominantly carried out by using reversed phase liquid chromatography coupled with tandem mass spectrometry operated in negative electrospray ionization mode (RP-LC-ESI(-)-MS/MS) to ensure excellent chromatographic separation with high sensitivity over a large dynamic range [28]. Furthermore, authentic standard compounds are required for successful and reliable quantification of oxylipins in biological samples. Currently, standard substances with varying purities can only be purchased from a handful of companies. Thus, in **chapter 2**, a method for the verification of oxylipin standards

is presented that allows reliable and accurate quantification of oxylipin concentrations.

In addition to the quality of the analytical standards, sampling is a source of variability. Factors, like the choice of anticoagulant (EDTA or heparin) or storage conditions during plasma preparation influence the oxylipin composition in the sample greatly [28, 35, 36]. Especially in daily clinical routine, a reproducible short time between blood collection and freezing of plasma can not be guaranteed, so that the biological samples are often stored for different time lengths and temperatures before long-term storage and analysis. The unsuitable handling of blood during plasma generation, storage or sample preparation might bias the results of clinical trials by the artificial formation and/or degradation of oxylipins [37]. However, as several studies investigate the oxylipin modulation following dietary interventions (e.g. supplementation of n3-PUFA) [32, 38, 39] or pharmacological treatment (e.g. modulation of ARA cascade enzymes by drug intervention) [40, 41], robust analytical techniques are required [28, 42]. **Chapter 3** therefore examines the influence of different storage times and temperatures during the transitory stage of plasma generation, long-term storage of plasma at -80 °C and addition of additives during sample preparation on the concentration of total (i.e. sum of free and esterified) oxylipins. So far, only a few studies have investigated the influence of the transitory stage between blood collection and plasma generation on the oxylipin profile. Moreover, the research focused on the non-esterified, i.e. free, oxylipins. However, the comparison of free and total oxylipin levels shows that the major portion of hydroxy- and epoxy-PUFA are found esterified in lipids [32]. The concentration of esterified epoxy-PUFA is 50 to 350 times higher in plasma and serum compared to the levels of free ones. Also, the concentration of esterified hydroxy-PUFA exceeds the concentration of the free ones by 10 to 40-fold in both plasma and serum, whereas dihydroxy-PUFA were detected in the same range while analyzing free and total oxylipins [32, 43, 44]. The study described in **chapter 3** demonstrates the importance of standardized sampling to reduce the variability in oxylipin concentrations and allows a first identification of markers of poor sample handling and storage.

The examination of changes in oxylipin patterns to better understand their role in health and disease is becoming increasingly important in clinical lipidomics. However, in comparison to other established metabolomics methods, there are no standardized procedures or verified standard reference materials for the analysis of oxylipins. In result, so that data from independent laboratories is rarely comparable. In addition to the purity of the standards used for quantification and non-standardized sampling, the use of different sample preparation methods is another reason for the limited comparability of results from different laboratories: The choice of sample preparation approach, like conditions for saponification (of esterified oxylipins), protein precipitation, liquid-liquid extraction, or solid-phase extraction (SPE) [28], has a great influence on the quantified oxylipin concentrations [32, 45]. Harmonization of the sample preparation as well as quantification must be based on robust procedures. Intra- and inter-day as well as inter-operator variability is unavoidable, however it should be kept as low as possible or well characterized for critical analytes. In addition, there should be good recovery (>80%) of internal standards (as this indicates suitable extraction efficancy) and accuracy of external calibration, as well as low matrix effects and high sensitivity with low detection limits [28]. The fulfillment of these factors is of great importance, as oxylipins occur only in low concentrations in biological samples and the detection of shifts in oxylipin profiles has a high potential as complex biomarkers for health and disease. **Chapter 4** describes the laboratory comparison with 5 independent laboratories to investigate the comparability of oxylipin analysis. During the laboratory comparison, critical steps of sample preparation were identified and their influences on certain oxylipin classes were characterized. The study shows the importance of performing laboratory comparisons, as well as harmonizing the steps during sample preparation to achieve reproducible and comparable oxylipin concentrations. This is of crucial importance for adequate potentiation of experimental designs and for improving the identification of reliable and relevant biomarkers for diseases.

Oxylipins produced during inflammation are believed to help fight pathogens and aid in the return to a healthy state. Initially, ARA-derived PGE_2 and LTB_4,

which act as vasodilators and initiate the recruitment of neutrophils, are produced at the inflammation site by perivascular mast cells. The infiltrated neutrophils phagocyte the pathogens and promote infiltration of monocytes. After neutrophils have completed their tasks, they become apoptotic and can be engulfed by macrophages (efferocytosis). This process is believed to initiate the lipid mediator class switch in the macrophages, whereby so-called SPMs are increasingly synthesized with the aid of the upregulated 15-LOX [46-48]. However, the SPM biosynthesis occurs at very low concentrations, challenging their role in inflammatory resolution [9]. Which endogenous factors are involved in the switch from pro- to anti-inflammatory oxylipins has not been fully investigated yet. The lipid mediator switch is believed to be associated with nuclear receptors, therefore a possible role of the nuclear liver X receptor (LXR) in the initiation of this process is presented in **chapter 5**. Stimulation of primary human M0-, M1- and M2-like macrophages with the LXR agonist T09 has no or little effect on the cell differentiation. However, T09 massivly induces ALOX15 in M2-like macrophages in a time- and dose-dependent manner. Cholesterol derivatives were identified as potent endogenous LXR ligands increasing 15-LOX abundance and activity. Thus, the results demonstrate a link between sterols, their activation of LXR and the biosynthesis of anti-inflammatory oxylipins, in M2-like macrophages.

SPMs are thought to reduce neutrophil invasion, stimulate efferocytosis of apoptotic cells, and coordinate the exit of phagocytes into lymphatic vessels to resolve inflammation and return to normal homeostasis [46]. It is assumed that the formation of SPMs occurs through multiple oxidation steps carried out by LOX or alternatively COX-2 or CYP [49, 50] in one cell or in cell-cell interactions [51]. **Chapter 6** investigates the SPM formation capacity by human neutrophils, as the expression of 5-LOX is limited to neutrophil and 5-LOX plays an important role in the biosynthesis of lipoxins and other SPMs [51]. Additionally, 5-LOX is known to require the 5-LOX-activating enzyme (FLAP) for efficient substrate conversion [52, 53]. The studies on 5-LOX-induced SPM formation were performed with three different neutrophil preparations (intact cells, homogenates, supernatant of 100 000 *g* centrifugation) and recombinant 5-LOX supplemented with SPM

precursor lipids 15-HETE, 18-HEPE and 17-HDHA to study FLAP dependency. In intact cells the lipid mediator formation is dominated by dihydroxylated oxylipins, such as 5,15-diHETE and resolvin D5 (7,17-diHDHA), whereas trihydroxylated oxylipins are formed only after addition of the precursor lipids in very low concentration. Upon destruction of cell integrity formation of ARA-derived multi-hydroxylated oxylipins increased, whereas formation of resolvins was attenuated. Additionaly inhibition of FLAP prevented the synthesis of 5-LOX dependent SPMs. The study demonstrates the importance of cell integrity on the formation of SPMs, and furthermore characterizes the preferred 5-LOX substrate among hydro(pero)xylated fatty acids and the primarily formed lipid mediators.

Overall, this thesis demonstrates the importance of standardized and harmonized protocols for sampling and analysis to achieve comparable and reproducible oxylipin concentrations Furthermore, the study of lipid mediator formation in primary human immune cells upon stimulation or supplementation with endogenous lipids contributes to a better understanding of the regulatory pathways and formation of anti-inflammatory oxylipins.

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Chapter 2

A strategy for validating concentrations of oxylipin standards for external calibration

Quantitative analysis of oxylipins by means of chromatography/mass spectrometry is based on (external) calibration with standard compounds. Therefore, the quality of analytical standards is of fundamental importance for accurate results. Recently launched certified standards with an assured concentration within a narrow range are useful tools to verify analytical standards. However, such standards are only available for a few compounds.

Based on the exemplary comparison of certified with none certified standards we suggest a tiered approach to validate and control the concentrations when preparing an external calibration based on non-certified oxylipin standards. Concentrations are evaluated by means of liquid chromatography negative electrospray ionization mass spectrometry (LC-ESI(-)-MS) in selected ion monitoring mode and UV spectroscopy.

Based on the suggested approach, more than 50% of the standards in our calibration mix could be validated. Though most of the non-certified standards are of good quality, several oxylipin concentrations differ considerably demonstrating that a quality control strategy as suggested here is a mandatory prerequisite for quantitative oxylipin metabolomics.

Adapted from Hartung N. M., Mainka M., Kampschulte N., Ostermann A. I., Schebb N. H. (2019). *Prostaglandins Other Lipid Mediat.* 141 pp. 22-24; doi: 10.1016/j.prostaglandins.2019.02.006

2.1 Introduction

Quantitative analysis of oxylipins by means of chromatography/mass spectrometry is based on (external) calibration with standard compounds. Therefore, the quality of analytical standards is of fundamental importance for accurate results. We believe that differences in the used standard material cause the partly massively diverging oxylipin concentrations reported in literature e.g. for human plasma samples by different labs. This hypothesis is supported by our experience that differing results are also obtained from direct laboratory comparison when analyzing the same material.

The correctness of standard concentration needs to be ensured and/or experimentally validated and controlled. Recently launched certified standards with an assured concentration within a narrow range, e.g. MaxSpec standards (Cayman Chemical, Ann Arbor, MI, USA, purity ≥95%, tolerated inter-batch variation within 90–110% and the exact concentration is specified in the certificate of analysis), are useful tools for this purpose. However, such standards are only available for a few compounds at the moment and therefore do not directly serve to validate all of the 100-200 usually non-certified analytes commonly covered by targeted oxylipin metabolomics [1].

In order to overcome this limitation and be able to characterize and validate quality and concentration of a great number of analytes, we here suggest a tiered approach utilizing different analytical techniques, i.e. liquid chromatography-mass spectrometry (LC-MS) and UV spectroscopy.

2.2 Experimental

All non-certified standards in our calibration mix were measured together with the few certified ones (100 nM in methanol) by means of LC-MS on a QTRAP (SCIEX, Darmstadt, Germany) QqQ MS instrument, as established in our lab described in detail elsewhere [2]. The peak areas in selected ion monitoring mode

(SIM) were compared. UV absorption was evaluated in solution of individual compounds (300 µM in ethanol) using a microplate of a Tecan Infinte M Plex Reader (Tecan Austria GmbH, Grädig Austria). All standards were obtained from Cayman Chemical (Ann Arbor, MI, USA) and LC-MS grade acetic acid, acetonitrile and methanol were from Fisher Scientific (Schwerte, Germany).

2.3 Results

The SIM areas of non-certified standards were directly compared to the SIM areas of their corresponding certified standards in case they are also available in higher quality. And if there was no corresponding certified standard available the areas were compared to regiosomers or oxylipins from the same class from different precursor PUFA. UV absorption was also compared in case the compounds contained a structural UV-absorbing moiety and results from both analyses are shown in Fig. 2.1.

2.4 Discussion

For most compounds good comparability between certified and non-certified standards was observed, e.g. 5-HETE, 5(*S*),6(*R*)-DiHETE or $PGF_{2\alpha}$ which were within a range that is acceptable for us of ± 20% of the certified standard's area (Fig. 2.1A). For other oxylipins the differences between both standards were huge (≥ 60%), e.g. 17-HDHA or 6-keto-$PGF_{1\alpha}$ (Fig. 2.1A). UV absorption revealed the same differences between the certified and non-certified material, supporting the results obtained from LC-MS analyses. Moreover, robustness of the approach was investigated by conducting independent LC-MS analyses on different systems in two different labs (Sciex QTrap 6500 and 5500, Fig. 2.2).

A)
% area/abs. of cert. STD
non-cert.
UV Data
5-HETE
12-HETE
17-HDHA
8(9)-EpETrE
11(12)-EpETrE
8,9-DiHETrE
14,15-DiHETrE
19,20-DiHDPE
5(S),6(R)-DiHETE
PGB2
6-keto-PGF1α
PGF2α
B)
rel. area vs. 73%B
62% B
66% B
70% B
73% B
C)
% area/conc of cert. STD
mean (5- and 12-HETE)
5-HETE
8-HETE
9-HETE
11-HETE
c12-HETE
15-HETE
16-HETE
17-HETE
18-HETE
19-HETE
20-HETE
D)
% area of cert. STD mean
(5-HETE, 12-HETE, 17-HDHA)
19-HETE
20-HETE
18-HETE
17-HETE
16-HETE
20-HDHA
15-HETE
16-HDHA
c17-HDHA
13-HDHA
11-HETE
10-HDHA
14-HDHA
8-HETE
c12-HETE
11-HDHA
7-HDHA
9-HETE
8-HDHA
c5-HETE
4-HDHA
E)
% abs. (236 nm) of
cert. 17-HDHA
4-HDHA
7-HDHA
8-HDHA
10-HDHA
11-HDHA
13-HDHA
14-HDHA
16-HDHA
c17-HDHA
20-HDHA
F)
% area of cert. STD mean
(8,9-DiHETrE, 14,15-
DiHETrE, 19,20-DiHDPE)
c14,15-DiHETrE
c19,20-DiHDPE
16,17-DiHDPE
11,12-DiHETrE
13,14-DiHETrE
10,11-DiHDPE
c8,9-DiHETrE
7,8-DiHDPE
5,6-DiHETrE

Fig. 2.1 (left, page 16): Evaluation of purity of oxylipin standards (STD) by means of LC-ESI(-)-MS and UV absorption (Hydroxy-PUFA: 236 nm; 5(*S*),6(R)-DiHETE: 273 nm). **(A)** Comparison of certified (cert.) STD to non-certified STD: Selected ion monitoring (SIM) areas of non-certified STD (100 nM) are shown relatively (%) to the corresponding certified analyte. The accepted range of ± 20% is depicted by dashed lines. **(B)** Influence of mobile phase composition on SIM area of 5-HETE during the retention time window of ARA-derived hydroxy-PUFA, (18.00-21.80 min, 62-72%B). **(C)** Comparison of regioisomers of hydroxy-PUFA: The SIM areas of ARA-derived hydroxy-PUFA (100 nM) are depicted relative to those of certified 5- and 12-HETE STD (cross-hatched). The accepted range of ± 30% is depicted by dashed lines. **(D)** Comparison of hydroxy-FA from different PUFA: The SIM areas of ARA and DHA-derived hydroxy-PUFA (100 nM) are depicted relative to those of certified 5-HETE, 12-HETE and 17-HDHA STD (cross-hatched), analytes are sorted by retention time (RT). The accepted range of ± 30% is indicated by dashed lines. **(E)** UV absorbance (236 nm) of regioisomers of DHA- derived hydroxy-PUFA (300 µM). The chromophore of all analytes (1*Z*, 3*E*-pentadien system) is presented on the right. Shown is the blank corrected absorption relative to the certified 17-HDHA STD. The accepted range of ± 30% is depicted by dashed lines. **(F)** Comparison of dihydroxy-FA from different PUFA: The SIM areas of ARA- and DHA-derived dihydroxy-PUFA (100 nM) are depicted relative to those of certified 8,9-DiHETrE, 14,15-DiHETrE and 19,20-DiHDPE STD (cross-hatched), analytes are sorted by RT. The accepted range of ± 30% is indicated by dashed lines. All results are shown as mean ± SD (n = 3).

For evaluation of standards with no corresponding certified material available we directly compared the MS signal and UV absorption to regioisomeric certified standards. Under isocratic conditions the same response in SIM can be assumed among regioisomers. However, analysis of a large number of structurally different oxylipins requires the use of solvent gradients, influencing the ionization procedure. Though, with a shallow gradient (± 11% B, i.e. organic solvent: 800/150/1 (*v/v/v*) acetonitrile/methanol/acetic acid [2]) the SIM area of ARA-derived hydroxy-fatty acids (HETEs) is not affected as shown exemplarily for 5-HETE (Fig. 2.1B). Thus, we concluded that our standard gradient for oxylipin analysis [2] can be used during validation of standards in SIM-mode. Comparing SIM areas of HETE regioisomers with the mean area of the two certified standards 5- and 12-HETE revealed major differences (> ± 30%) for 15- and 16-HETE (Fig. 2.1C), and precise correction factors based on the certified HETE-standards can be determined and implemented for analysis of samples.

This approach is not only limited to regioisomers derived from the same precursor FA, but also can be used for oxylipins from the same class, e.g. hydroxy-FA (Fig. 2.1D). Similar results for HETEs were obtained when SIM areas of ARA- and DHA-derived hydroxy-FA were compared. Here, the SIM signal of one non-certified standard, i.e. 8-HDHA lied out of the accepted range of ± 30%. In order to substantiate the results of the SIM area approach, ideally, UV absorption (236 nm) can be used for the evaluation of most hydroxy-FA because they contain the same conjugated electron system (1*Z*, 3*E*-pentadien system). Based on this, a comparable molar absorption coefficient can be expected and the absorption can be directly compared (Fig. 2.1E). The approach can be adapted to other classes of oxylipins grouped together based on their chemical structure, such as dihydroxy-FA, exemplarily shown for all ARA- and DHA-derived dihydroxy-FA (Fig. 2.1F).

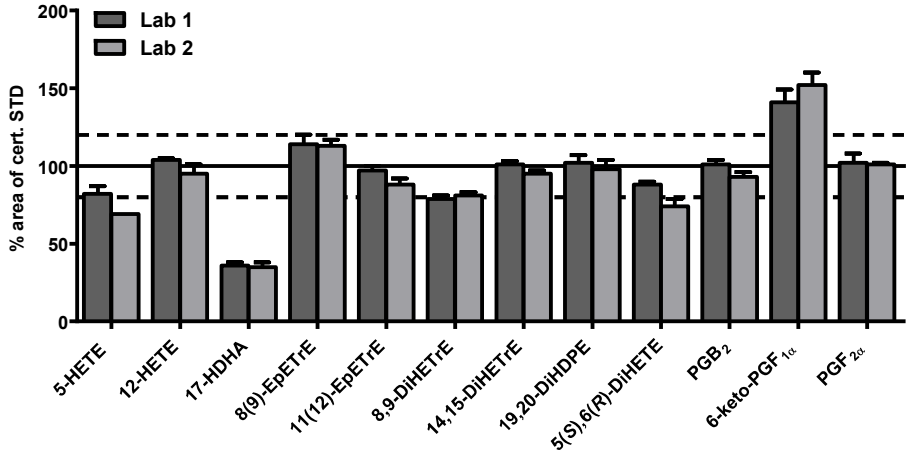

Fig. 2.2: Comparison of certified standards to non-certified standards using LC-ESI(-)-MS in selected ion monitoring (SIM) mode (100 nM) in different labs and instrument types (Lab 1: measured on AB SCIEX 6500 Qtrap, Lab 2: measured on AB SCIEX 5500 Qtrap). Data of non-certified standards is shown relative to specified standard as mean ± SD (n=3). The accepted range ± 20% is depicted by dashed lines.

Conclusively, comparison of SIM areas in LC-ESI(-)-MS has proven to be a helpful tool for evaluating the quality of non-certified standards. Whenever possible, UV absorption should be considered to additionally confirm the results from LC-MS measurements.

Based on these results we suggest the following strategy to validate (multi-analyte) oxylipin standard series since only a limited number of certified materials is currently available:

1.) High quality standards (with tight quality specifications) should be used for direct comparison of the corresponding non-certified standards included in (multianalyte) standard series. SIM areas/UV absorption must be within a ± 20% range of the certified standard's, otherwise factors for correction of the non-certified standard concentration should be implemented in the calibration. The ± 20% range was arbitrarily chosen based on the analytical variation of oxylipin levels for human plasma samples which is generally below 20% (in most cases 5–15%) [3].

2.) Compounds with no corresponding certified standards available should be evaluated by comparing SIM areas/UV absorption to regioisomeric certified standards. Regular gradient methods which are commonly used in targeted oxylipin metabolomics can be applied. If the SIM areas are within a range of ± 30% of the certified standard's, the concentration of the non-certified standard is acceptable. Otherwise, correction factors for these compounds based on the regioisomeric certified standard's SIM area are determined.

3.) For analytes with no corresponding or regioisomeric certified standards at hand, precursor independent comparison of oxylipins from the same class (e.g. hydroxy- or dihydroxy-FA) can be carried out. Here, ratios are calculated relative to the (mean) SIM areas of certified standards from the same class. The same procedure is done for UV data, comparing certified standards with oxylipins containing the same structural absorbing moiety. Again, for compounds whose relative SIM area/UV absorption exceeds a limit of ± 30% of the certified standard's, correction factors based on the certified standard's SIM area are determined via LC-MS.

Not all standards commonly used in targeted oxylipin metabolomics methods can be evaluated by this approach, because no certified standards are available

for all oxylipin classes (e.g. trihydroxy-FA) and some oxylipin classes are composed of structurally diverse compounds with insufficient UV absorption (mainly prostanoids and isoprostanes), and thus, comparison of SIM area and/or UV absorption is hampered. An extended portfolio of certified standards particularly for multiple hydroxylated-FA and isoprostanes is urgently required which would allow validation of the concentration of these oxylipin classes.

2.5 Conclusion

Based on the suggested approach, more than 50% of the standards in our calibration mix could be validated. The results show that most of the non-certified standards are of good quality. Their concentration is indicated correctly and in good accordance with the certified material. However, in some cases concentrations of non-certified standards can differ considerably from the nominal concentration. Therefore, a validation strategy as suggested here and calculation of correction factors in order to adjust concentrations and compensate for the differences is a prerequisite for accurate quantification of oxylipins.

Nevertheless, more certified standards are needed in order to be able to validate the concentration of all oxylipin classes, especially multiple hydroxylated-FA and the structurally diverse isoprostanes.

2.6 References

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Chapter 3

Stability of oxylipins during plasma generation and long-term storage

Oxylipins are lipid mediators involved in the regulation of numerous physiological functions such as inflammation. They have raised strong interest in clinical lipidomics in order to understand their role in health and diseases and their use as biomarkers. However, before the clinical translation, it is crucial to validate the analytical reliability of oxylipins. This notably requires to assess the putative artificial formation or degradation of oxylipins by (unsuitable) blood handling during plasma generation, storage and sample preparation. Using a liquid chromatography-mass spectrometry method covering 133 oxylipins we comprehensively analyzed the total (free + esterified) oxylipin profile in plasma and investigated the influence of i) addition of additives during sample preparation, ii) different storage times and temperatures during the transitory stage of plasma generation and iii) long-term storage of plasma samples at -80 °C. Addition of radical scavenger BHT reduced the apparent concentrations of hydroxy-PUFA and thus should be added to the samples at the beginning of sample preparation. The concentrations of all oxylipin classes remained stable during the transitory stage of plasma generation up to 24 h at 4 °C or 4 h at 20 °C before centrifugation of EDTA-whole blood and up to 5 days at -20 °C after plasma separation. The variations in oxylipin concentrations did not correlate with storage time, storage temperature or stage of plasma generation. A significant increase of potentially lipoxygenase derived hydroxy-PUFA compared to immediate processing was only detected when samples were stored for longer times before centrifugation, plasma separation as well as freezing of plasma revealing residual enzymatic activity. Autoxidative rather than enzymatic processes led to a slightly increased concentration of 9-HETE when plasma samples were stored at -80 °C for 56 months. Overall, we demonstrate that total plasma oxylipins are robust regarding delays during plasma generation and long-term storage at -80 °C supporting the application of oxylipin profiling in clinical research.

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3.1 Introduction

Eicosanoids and other oxylipins are oxygenated metabolites of polyunsaturated fatty acids (PUFA) which are formed via three different enzymatic pathways and autoxidation [1-3]. These pathways result in a diversity of products from different PUFA, e.g. cyclooxygenase (COX) conversion leads to prostanoids such as prostaglandins and thromboxanes [4], lipoxygenase (LOX) conversion leads via hydroperoxy-PUFA to mid-chain hydroxy- or multiple hydroxylated-PUFA [5, 6] and conversion by cytochrome P450 monooxygenases (CYP) leads either to terminal (n) and n-1 hydroxy-PUFA or to epoxy-PUFA [7]. Epoxy-PUFA are further metabolized to dihydroxy-PUFA by soluble epoxide hydrolase (sEH) [8]. Hydro(pero)xy-PUFA and prostaglandin-like structures, called isoprostanes (IsoP), are also formed during autoxidative processes [9, 10]. The biology of free oxylipins has been investigated in numerous studies over the past decades [1-3]. For instance, PGE_2, a prostaglandin formed from arachidonic acid (ARA, C20:4 n6) via COX, is a potent mediator in the regulation of pain, fever and inflammation [4], e.g. by arterial dilation and increase of microvascular permeability leading to increased blood flow in the inflammation site or by interaction with EP1 receptor having an effect on neurons at the inflammation site resulting in hyperalgesia [11, 12]. In contrast, epoxy-PUFA formed via CYP from ARA, eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) have anti-inflammatory properties [13]. Epoxy-PUFA are also involved in several biological processes like vasodilation, angiogenesis and hypertension [14]. However, epoxy-PUFA are rapidly metabolized by sEH to their corresponding dihydroxy-PUFA which are less active [14, 15].

Several studies investigated the modulation of oxylipin synthesis via nutrition or therapeutic treatment. Nutritional intervention by supplementation of n3-PUFA on a Western diet leads to a shift in the relative proportions of n3- and n6-PUFA influencing the oxylipin profile due to variable affinity of the PUFA metabolizing enzymes to the various PUFA and changes in the substrate availability [16-20]. The expression and/or activity of enzymes involved in the ARA cascade can

further be modified by drug intervention leading to altered oxylipin profiles [1]. For instance, COX can be inhibited competitively by non-steroidal anti-inflammatory drugs (NSAIDs) or in case of the NSAID aspirin irreversibly through acetylation of serine at the active enzyme site [12, 21]. Moreover, food derived secondary products like polyphenols can affect the enzyme activity resulting in altered oxylipin patterns [1]. For example, the activity of CYP can be altered *in vitro* by the polyphenol apigenin [22] and the activity and the expression of COX-2 can be influenced by resveratrol, hopeaphenol and apigenin [23].

The influence of these potent lipid mediators on health and disease as well as the interaction with nutrition or drugs have been investigated numerous clinical studies. However, the results can be massively affected by (unsuitable) sample handling, e.g. artificial formation of oxylipins during sample preparation, ion suppression due to insufficient removal of interfering matrix [1, 24-26] or inefficient extraction of oxylipins from biological samples [1].

Besides analytical challenges, the stability of oxylipins is crucial because samples are often stored for different periods of time prior to analysis in daily clinical routine or longitudinal studies. However, only few reports exist on the influence of the transitory stage between blood collection and plasma generation on the oxylipin profile. First studies revealed that the apparent pattern of free oxylipins changes depending on storage procedures of whole blood [23, 27] or plasma [28]. However, the reported results are conflicting, e.g. Dorow et al. found all detectable oxylipins being stable in EDTA-whole blood up to 120 min at 4 °C [27], while Willenberg et al. showed reduced concentrations of hydroxy-PUFA at storage times longer than 60 min at 4 °C [23].

Similarly, little is known about the impact of storage conditions on the profile of total oxylipins. However, the data are urgently needed since the major part of epoxy- and hydroxy-PUFA as well as isoprostanes are found to be esterified in lipids [29, 30]. Total oxylipins might be a good option in the field of biomarker discovery as they represent the plasma oxylipin pattern, might be biologically

active and their levels fluctuate depending on lipoprotein concentration and composition [1].

In the present study we employed a comprehensive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method covering 133 analytes to evaluate the sample preparation/storage induced effects on the pattern of plasma oxylipins. We investigated the efficacy of different additives to prevent artificial formation of oxylipins during sample preparation. Additionally, we monitored the impact of different storage times and temperatures at different stages of plasma generation, which are representative for current practices in clinics, on the apparent total plasma oxylipin concentrations. Finally, we examined the long-term stability of total oxylipins in plasma stored at -80 °C during a period of 56 months.

3.2 Experimental

3.2.1 Chemicals

The analyte standards 10-HODE, 12-HODE, 15-HODE, 13-oxoOTrE, 9,10,11-TriHOME, 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10,11-TriHODE, 9,12,13-TriHODE and 9,10,13-TriHODE were purchased from Larodan (Solna, Sweden). All other oxylipin standards and deuterated internal standards were bought from Cayman Chemical (local distributor Biomol, Hamburg, Germany). LC-MS grade acetic acid, acetonitrile, *iso*-propanol and methanol (MeOH) were obtained from Fisher Scientific (Schwerte, Germany). HPLC grade *n*-hexane was purchased from Carl Roth (Karlsruhe, Germany) and HPLC grade ethyl acetate was purchased from VWR (Darmstadt, Germany). The ultra-pure water (>18 MΩ*cm) was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany).

3.2.2 Generation of the quality standard (QS) plasma

Human EDTA-blood was collected from 4-6 healthy male and female individuals aged between 25-38 years. The blood was centrifuged (1200 x*g*, 15 min, 4 °C), the collected plasma supernatants were pooled, aliquoted and immediately stored at -80 °C as quality standard (QS) plasma.

3.2.3 Calibration

A wide range of oxylipins from different structural classes and derived from different precursor fatty acids (ARA, EPA, DHA, dihomo-γ-linolenic acid (DGLA, C20:3 n6), adrenic acid (AdA, C22:4 n6), n3- and n6-docosapentaenoic acid (DPA, C22:5 n3/n6), γ-linolenic acid (GLA, C18:3 n6), α-linolenic acid (ALA, C18:3 n3), linoleic acid (LA, C18:2 n6) and oleic acid (OA, C18:1 n9)) were selected for calibration. Only alkali stable prostanoids, such as B-ring or F-ring prostaglandins, were included in the calibration. For the quantification of oxylipins the deuterated internal standards $^{2}H_4$-6-keto-$PGF_{1\alpha}$, $^{2}H_4$-8-*iso*-$PGF_{2\alpha}$, $^{2}H_4$-$PGF_{2\alpha}$, $^{2}H_5$-RvD2, $^{2}H_{11}$-8,12-*iso*-$iPF_{2\alpha}$-VI, $^{2}H_5$-LxA_4, $^{2}H_5$-RvD1, $^{2}H_4$-PGB_2, $^{2}H_4$-LTB_4, $^{2}H_4$-9,10-DiHOME, $^{2}H_{11}$-11,12-DiHETrE, $^{2}H_6$-20-HETE, $^{2}H_4$-13-HODE, $^{2}H_4$-9-HODE, $^{2}H_8$-15-HETE, $^{2}H_3$-13-oxo-ODE, $^{2}H_8$-12-HETE, $^{2}H_8$-5-HETE, $^{2}H_4$-12(13)-EpOME, $^{2}H_{11}$-14(15)-EpETrE, $^{2}H_7$-5-oxo-ETE, $^{2}H_{11}$-8(9)-EpETrE were used.

Oxylipins were combined to master mixes according to their molecular weight and sufficient chromatographic separation. Master mixes were prepared in a volumetric flask (5 mL) with a tentative concentration of 10 µM based on the declaration of the manufacturer. Due to limited available standard material the regioisomers of EpODE and DiHODE, 9(10)-EpOME, 7(8)-EpDPE, 8,15-DiHETE, 5(*S*),6(*S*)-DiHETE, 8-HETE, 9-HETE and 11-HETE have been added at lower concentrations.

The IS master was prepared in a volumetric flask (25 mL) with a tentative concentration of 5 µM. The internal standards $^{2}H_5$-RvD1 and $^{2}H_4$-PGB_2 were added at lower concentration because of their contamination with unlabeled

isotopologs. The IS master was diluted with MeOH to a 100 nM “sample preparation IS”-solution in a volumetric flask and aliquoted in amber vials until use. *All oxylipin solutions were prepared avoiding direct light radiation, using only detergent free glassware (no plastic) and stored at -80 °C.*

The purity and the concentration of the analytes in the master mixes was checked before pipetting the standard series (appendix Fig. 8.1). All master mixes were analyzed by means of LC-MS/MS in multiple reaction monitoring (MRM) mode. All transitions of not included analytes were evaluated for contamination/interferences. For detected interferences the standard compound containing the interference was identified. The contamination was quantified by calculating an area ratio between the contamination (at 5 µM) and the analyte standard at 5 µM. If an area ratio was higher than 10%, the contaminated oxylipin was removed from the standard series. The master mixes were analyzed by means of LC-MS in single ion monitoring (SIM) mode and by means of UV spectroscopy according to Hartung et al. [31]. The actual analyte concentration was adjusted by comparison with concentration-verified standard material.

Calibrants with 16 concentrations levels were prepared by a sequential dilution with IS master. The internal standards concentrations were 20 nM, except for $^{2}H_{5}$-RvD1 and $^{2}H_{4}$-PGB_{2} (5 nM and 10 nM in calibrants, respectively).

For calibration the peak area ratio (analyte/IS) was linearly fitted against the analyte concentration using linear least square regression (weighting $1/x^{2}$) (appendix Tab. 8.1). The concentration with a signal to noise ratio of ≥3 was determined as limit of detection (LOD). The lower limit of quantification (LLOQ) was set to the concentration yielding a signal to noise ratio of ≥5 and accuracy of ± 20% within the calibration curve. The accuracy within the calibration curve was ± 15% fulfilling the validation criteria of the European Medicines Agency (EMA) guideline for bioanalyses [6].

3.2.4 Preparation of samples for storage assays

Short-term transitory storage assay: Blood from 4 healthy volunteers aged 25-30 years was collected in EDTA-monovettes (S-Monovette K3E, 02.1066.001, Sarstedt, Nümbrecht, Germany). The subjects did not take NSAIDs. Blood from each individual was pooled, aliquoted and submitted to different treatments before final storage at -80 °C (Tab. 3.1). “Best case” samples were immediately centrifuged (10 min, 4 °C, 1200 x *g*), the plasma was collected, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. “Vortex” samples were strongly shaken (Vortex Genie 2, Scientific Industries (local distributor Carl Roth, Karlsruhe, Germany) max. intensity) for 1 minute directly after blood withdrawal, centrifuged (10 min, 4 °C, 1200 x *g*), the plasma was frozen in liquid nitrogen and stored at -80 °C until further analysis. The influence of storage conditions was investigated in three different ways:

1. Whole blood was stored at room temperature (20 °C) or in the fridge (4 °C) for different time periods, centrifuged plasma was collected and stored at -80 °C until analysis.

2. Whole blood was directly centrifuged, stored at 4 °C for different periods of time, plasma was collected and stored at -80 °C until analysis.

3. Whole blood was directly centrifuged, plasma was collected and temporarily stored at 4 °C or at -20 °C for different time periods and finally stored at -80 °C until analysis.

Additionally, a “worst case” sample was prepared that combines the longest time periods of all three storage conditions described above. Samples from each individual were prepared and analyzed separately together with two QS plasma samples as a quality control.

Long-term storage assay: The human EDTA-plasma used for long-term storage experiments was bought from Etablissement Français du Sang (Saint-Denis, France; pool of 50 donors) aliquoted and stored at -80 °C untill analysis.

The samples were prepared in triplicates together with 3 QS plasma samples as a quality control. In the first 6 months of the long-term storage experiment plasma samples were prepared and analyzed every month and thereafter every three months (month 9, 12 and 15). Finally, the plasma was analyzed again after 4.6 years (month 56).

Tab. 3.1: Overview of the sampling design to investigate the influence of storage at transitory stages on the oxylipin pattern. Blood from 4 individuals was collected into EDTA tubes, aliquoted and submitted to different subsequent treatments before final storage at -80 °C. Best case samples were immediately centrifuged and frozen. Other samples were stored at room temperature or at 4 °C for different time periods before centrifugation. After centrifugation samples were stored at 4 °C without removing the plasma supernatant and at 4 °C as well as room temperature after plasma separation. Additionally, one sample from each individual was vigorously shaked “vortex” for one minute to provoke hemolysis.

	Sample collection	**Vortex**	**Storage before centrifugation**		centrifugation	**Storage after centrifugation**			Storage at – 80°C
			20°C (RT)	4°C (fridge)		Plasma NOT separated 4°C	Plasma separated 4°C	Plasma separated -20°C	
Best case (directly -80°C)		-	-	-		-	-	-	
Storage before centrifugation		-	1 h, 2 h, 3 h	-		-	-	-	
		-	-	1 h, 8 h, 24 h		-	-	-	
Storage after centrifugation		-	-	-		1 h, 2 h	-	-	
		-	-	-		-	1 h, 2 h, 8 h, 24 h	-	
		-	-	-		-	-	8 h, 24 h, 3 d, 5 d	
		-	-	-		-	1 h	3 d	
Shaking of sample		1 min	-	-		-	-	-	
Worst case		-	3 h	24 h		2 h	2 h	5 d	

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

Human plasma samples were extracted using solid phase extraction (SPE) following protein precipitation and alkaline hydrolysis as described [32]. To 100 µL thawed plasma 10 µL of IS solution (100 nM in MeOH) and 10 µL of antioxidant mixture (0.2 mg/mL BHT, 100 µM indomethacin, 100 µM *trans*-4-(-4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*-AUCB) in MeOH) were added. For protein precipitation 400 µL ice-cold iso-propanol was added and the samples were stored at -60 °C for at least 30 min. Following centrifugation (4 °C,

20000 x *g*, 10 min) the supernatant was collected and hydrolyzed at 60 °C for 30 min using 100 µL of 0.6 M potassium hydroxide in MeOH/water (75/25; *v/v*). Afterwards samples were neutralized (pH=6) with acetic acid (HOAc), diluted with 2 mL of 0.1 M disodium hydrogen phosphate buffer (adjusted to pH 6 with HOAc) and loaded onto pre-conditioned SPE cartridges. The extraction of oxylipins with the anion exchange Bond Elut Certify II SPE cartridges (200 mg, 3 mL, Agilent, Waldbronn, Germany) was carried out as described [33, 34]. Oxylipins were eluted with ethyl acetate/n-hexane/acetic acid (75/25/1, *v/v/v*) into a tube containing 6 µL of 30% glycerol in MeOH. After evaporation (vacuum concentrator, 30 °C, 1 mbar; Christ, Osterode, Germany) the residue was reconstituted in 50 µL MeOH containing 40 nM of each, 1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)-phenyl)-urea, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, 12-oxo-phytodienoic acid and aleuritic acid, as IS2 to calculate the extraction efficiency of the deuterated IS. Samples were analyzed by means of LC-ESI(-)-MS/MS (appendix Tab. 8.1) as described [33, 34].

In order to investigate the effects of the compounds included in the antioxidant mixture each compound (butylated hydroxytoluene (BHT) and EDTA, each 0.2 mg/mL and indomethacin and *t*-AUCB, each 100 µM) was added individually and in combination to QS plasma samples.

3.2.6 Data analysis

Inter-individual analyte variance in oxylipin patterns [1, 20] was compensated by normalizing the oxylipin level against the respective oxylipin concentration in the “best case” samples (i.e. directly processed samples) determined in triplicate for each subject. The results are shown as mean (%) ± 95% confidence interval (CI). Normal distribution was shown using the Shapiro-Wilk test. Outliers were identified using the ROUT (Robust regression and OUTlier removal, maximum false discovery rate (Q) = 1%) outlier test and removed. The variability of all conditions was assessed by an unsupervised multivariate analysis, a principal

component analysis (PCA). The differences between best case and storage conditions were determined by one-way ANOVA following Tukey post-test. p values <0.05 were considered significant.

Long-term storage data were evaluated firstly by controlling if the concentrations were comprised within a range of acceptable analytical variance of ±30% relative to month 1 of the monitoring. Then data were evaluated using linear regression model for each oxylipin. The hypothesis of residuals independence, normality and homogeneity was tested to validate the linear models (using Durbin-Watson test, Shapiro-Wilk test and non-constant error variance test, respectively). The significance of the slope of each linear regression model was calculated with a t-test, p-values <0.05 were considered significant. For the oxylipins, which show a significant slope, the trend line was inserted into the respective diagram.

Statistical analyses were performed using GraphPad Prism software (version 6.0, San Diego, CA; USA), R software or SIMCA (version 14, Umetrics). All other calculations were done with Excel Microsoft Office (version 365, Redmond, WA, USA).

3.3 Results

3.3.1 Effects of additives on the oxylipin pattern

The addition of BHT before sample preparation yielded significantly lower apparent concentrations of hydroxy-PUFA (Fig. 3.1A; appendix Tab. 8.2) whereas levels of epoxy-PUFA were mostly unaffected (Fig. 3.1B; appendix Tab. 8.2). For EDTA no effects on the plasma oxylipin pattern were observed (Fig. 3.1). Similarly, the enzyme inhibitors indomethacin (COX-1/-2) and *t*-AUCB (sEH) did not change the apparent oxylipin pattern (Fig. 3.1). The results obtained following addition of an antioxidant mixture containing all four tested additives

(BHT, EDTA, indomethacin and *t*-AUCB) were consistent with the results yielded with BHT addition alone.

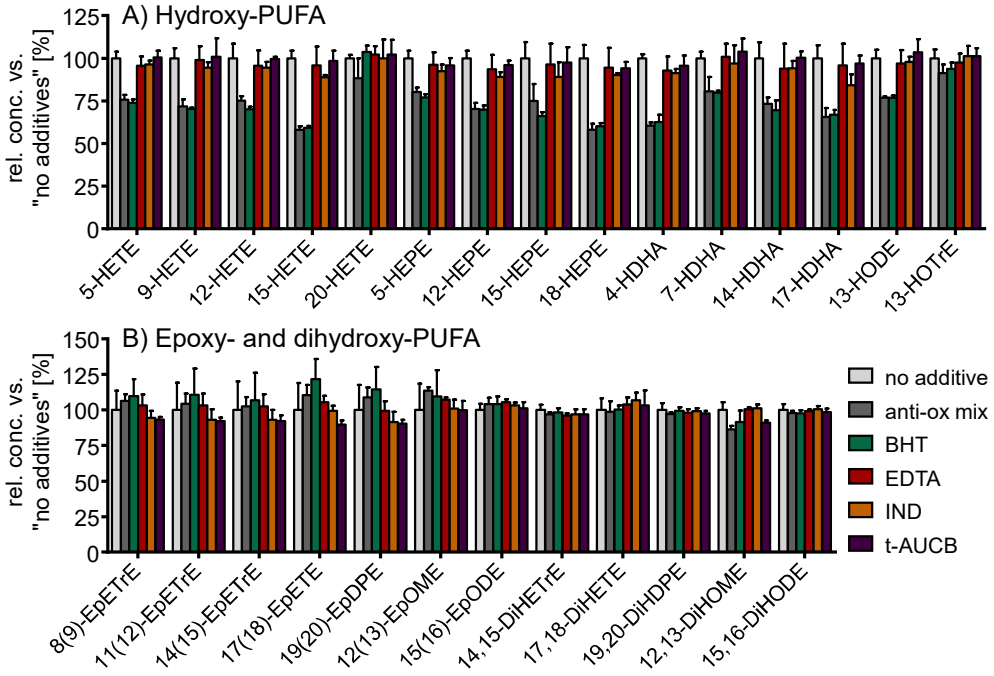


Fig. 3.1: Effects of additives on the apparent oxylipin pattern. Shown are relative oxylipin concentrations of a representative set of analytes using different additives during sample preparation (mean ± SD; n=4). Samples were prepared using no additives, butylated hydroxytoluol (BHT), ethylenediamine-tetraacetic acid (EDTA), indomethacin (IND) and the sEH inhibitor *t*-AUCB, respectively or a mix containing all additives (anti-ox mix). Relative concentrations were calculated against the mean analyte concentration obtained from sample preparation without additives (no additives). Statistical differences between “no additive” and different additives were evaluated by two-way ANOVA followed by Bonferroni post-test (appendix Tab 8.4).

3.3.2 Oxylipin concentrations in plasma

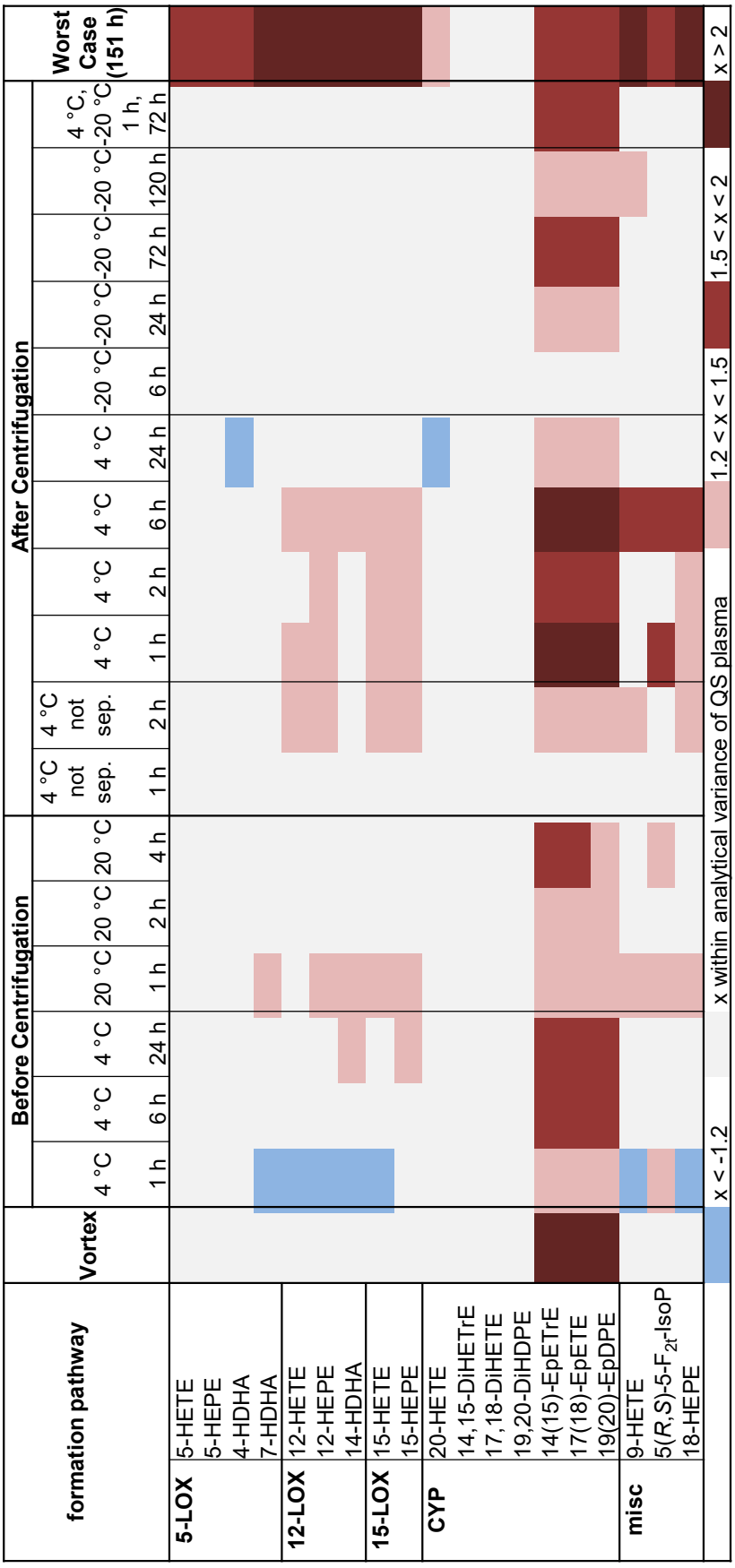
Quality standard (QS) plasma was prepared and analyzed with each data set. The concentrations of total oxylipins in QS plasma are presented in the appendix Tab. 8.3. In this pooled human plasma 78 analytes could be quantified in the range of 150 ± 40 pM (11,12-DiHETE and 14,15-DiHETE) to 186 ± 53 nM (9(10)-Ep-stearic acid). The LLOQ in plasma ranged from 5 pM (9,10-DiHOME) to 500 pM ($PGF_{3\alpha}$ or 8,15-DiHETE) for most of the analytes. Only for 15-F_{3t}-IsoP, 13-γ-HOTrE, 19-HETE, 17-oxo-DPA (n3) and 17-oxo-DHA the method was less sensitive (LLOQ 1.25-5 nM). Regarding intra-day and inter-day variance, for most of the quantified analytes the coefficient of variation (CV) was <20% and thus

slightly higher than for the determination of free oxylipins [35]. For epoxy-PUFA high variations of >30% were observed.

3.3.3 Transitory stage of plasma generation

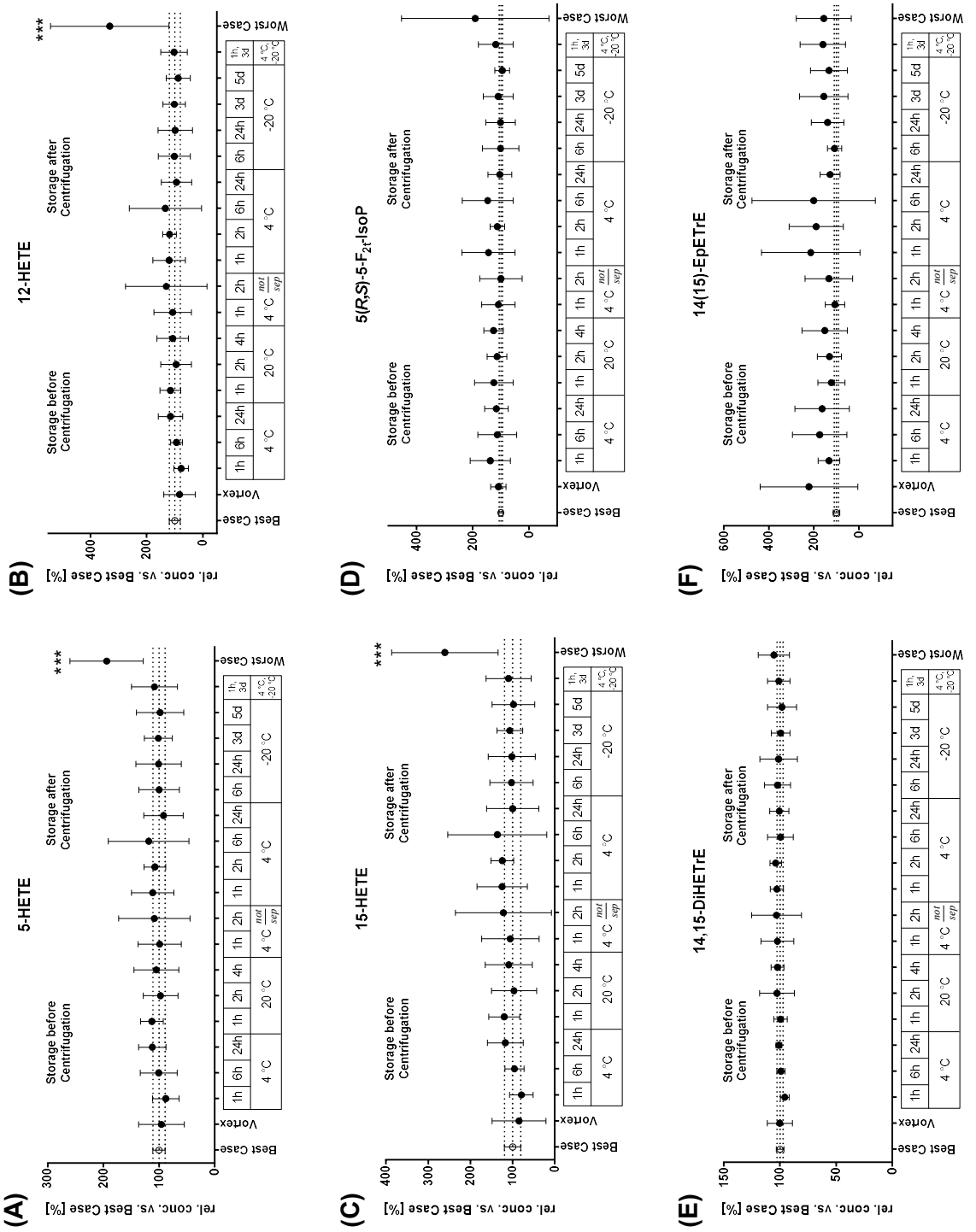
In the best case plasma samples 78 oxylipins could be quantified with concentrations ranging from 149 ± 54 pM (14,15-DiHETE) to 158 ± 65 nM (9(10)-Ep-stearic acid, appendix Tab. 8.4). The concentrations of all detected oxylipins at all time points can be found in the appendix (Tab. 8.4). The PCA, unsupervised multivariate analysis, shows the variability of the dataset (R^2X=0.799 and Q^2=0.506, appendix Fig. 8.2). As shown in the score plot (appendix Fig. 8.2A), the condition “worst case” is the main contributor of the variability of the dataset (i.e. concentrations of the 78 oxylipins) on the first component that brings 48% of the variability. On the second component bringing 19% of the variability, the effect of “vortex” seems to be also significant. The loading plot (appendix Fig. 8.2B) reveals that the condition “worst case” mostly increases the concentration of hydroxy-PUFAs (e.g. 5-HETE, 20-HDHA, …) whereas the “vortex” affects particularly all epoxy-PUFAs of the dataset. Among all these oxylipins, several oxylipins were selected which represent the four branches of the arachidonic acid cascade [1, 2] and can therefore be used to study the effect of different storage conditions on the oxylipin profile. The influence of transitory stage after blood collection until storage of plasma at -80 °C on the oxylipin pattern in plasma is shown exemplarily as fold change relative to immediate processing for a representative set of oxylipins (Fig. 3.2). The concentration of most of the representative analytes did not change markedly at the various storage conditions.

Fig. 3.2 (right, page 35): Effects of different storage conditions on the total oxylipin concentration. The heatmap shows the fold change of the mean concentration for representative analytes relative to mean concentration of best case (processed immediately). Marked in grey are samples within the analytical variance (±20%) of QS plasma. Blue represents a decrease of >1.2-fold against the “best case”. An increase is highlighted in the different shades of red (light red 1.2-1.5-fold; red 1.5-2.0-fold; dark red >2-fold).


formation pathway
Vortex
Before Centrifugation
After Centrifugation
Worst Case (151 h)
4 °C 1 h
4 °C 6 h
4 °C 24 h
20 °C 1 h
20 °C 2 h
20 °C 4 h
4 °C not sep. 1 h
4 °C not sep. 2 h
4 °C 1 h
4 °C 2 h
4 °C 6 h
4 °C 24 h
-20 °C 6 h
-20 °C 24 h
-20 °C 72 h
-20 °C 120 h
4 °C, -20 °C 1 h, 72 h
5-LOX
5-HETE
5-HEPE
4-HDHA
7-HDHA
12-LOX
12-HETE
12-HEPE
14-HDHA
15-LOX
15-HETE
15-HEPE
CYP
20-HETE
14,15-DiHETrE
17,18-DiHETE
19,20-DiHDPE
14(15)-EpETrE
17(18)-EpETE
19(20)-EpDPE
misc
9-HETE
5(R,S)-5-F2t-IsoP
18-HEPE
x < -1.2
x within analytical variance of QS plasma
1.2 < x < 1.5
1.5 < x < 2
x > 2

The levels of the products derived from LOX catalyzed reactions (such as 5-, 12- and 15-HETE) and of the presumably autoxidatively formed products (such as 9-HETE and 5(*R,S*)-F_{2t}-IsoP) were slightly (1.2-1.5 fold) increased when plasma was stored at 4 °C after centrifugation and >1.5 fold elevated for the worst case scenario (Fig. 3.2). The epoxy-PUFA concentrations were generally increased at prolonged storage times at elevated temperatures in comparison to the best case scenario. These trends were also reflected by the mean ± 95% CI of the concentrations for representative oxylipins derived from ARA, EPA, DHA, LA and ALA calculated against concentration in the best case sample (relative concentrations, Fig. 3.3, appendix Fig. 8.3-8.8). 5 HETE, 12 HETE and 15 HETE reached the highest levels in the worst case sample (1.8-3.5-fold increase) which were also significantly different from the best case sample (Fig. 3.3A-C). This accumulative effect towards worst case and the significant difference of the concentrations compared to best case could also be observed for 5-, 12- and 15-LOX products derived from other PUFA (appendix Fig. 8.3-8.6, Tab. 8.4). For 14(15)-EpETrE no significant changes in the concentrations could be detected at any storage condition, however the variance was massively increased, e.g. when plasma was stored for 1 h or 6 h at 4 °C (appendix Fig. 8.9). These strong variations of the concentrations of 14(15)-EpETrE during different storage conditions (Fig. 3.3F) were also detected for other epoxy-PUFA (appendix Fig. 8.7). Levels of the isoprostane 5(*R,S*)-5-F_{2t}-IsoP varied in a similar range (Fig. 3.2, Fig. 3.3D, appendix Fig. 8.8) while dihydroxy-PUFA such as 14,15-DiHETrE were not influenced by the storage (Fig. 3.2, Fig. 3.3E, appendix Fig. 8.7).

Fig. 3.3 (right, page 37): Stability of total oxylipins during plasma generation with different transitory storage times. The relative concentrations of selected ARA derived oxylipins **(A)** 5-HETE, **(B)** 12-HETE, **(C)** 15-HETE, **(D)** 5(*R,S*)-5-F_{2t}-IsoP, **(E)** 14,15-DiHETrE and **(F)** 14(15)-EpETrE were calculated against the concentration of the best case. Shown are mean ± 95% CI (n=4 12). The dotted lines mark the 95% CI of the best case scenario. Statistical differences between best case and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (*** p <0.001). The data for oxylipins derived from other precursor PUFA (ARA, EPA, DHA, ALA, LA) can be found in th e appendix.


(A)
5-HETE
(B)
12-HETE
(C)
15-HETE
(D)
5(R,S)-5-F2t-IsoP
(E)
14,15-DiHETrE
(F)
14(15)-EpETrE
rel. conc. vs. Best Case [%]
Best Case
Vortex
Worst Case
Storage before Centrifugation
Storage after Centrifugation
4 °C
20 °C
not sep
-20 °C
1h; 3d
4 °C; -20 °C
***

3.3.4 Long-term storage of plasma

In plasma samples used to evaluate the effect of long-term storage (at -80 °C, up to 56 months) on the stability of different oxylipin types 74 oxylipins could be quantified (Tab. 3.2, appendix Fig. 8.10). Considering that an analytical variance of 20% up to ±30% is acceptable, 69 oxylipins (93% of the quantified oxylipins) were considered to be stable during the 56 months of monitoring (appendix Tab. 8.5).

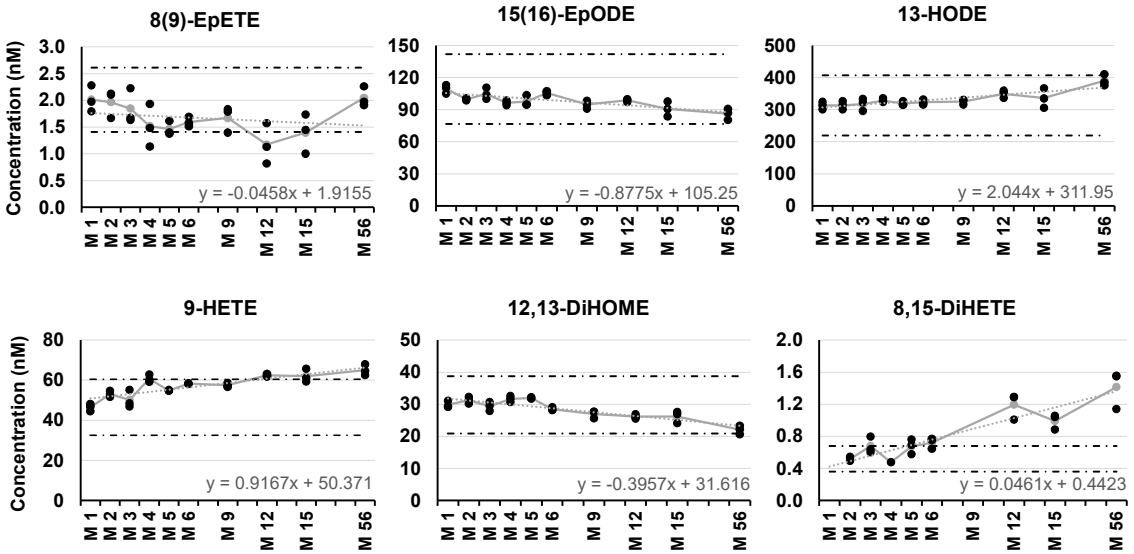


Fig. 3.4: Long-term storage evaluation over 56 months (4.6 years) for oxylipins with significant slopes (positive and negative, n=25). The black dotted lines mark the ±30% of acceptable analytical variance. Black dots represent the samples. Grey dots are the mean of samples by month and a grey line connects these means. The grey dotted lines are the straight linear regression with the mathematical equation shown near the name of oxylipins.

Nevertheless, the concentration of some oxylipins changed over time. On the one side, the concentration of 17 oxylipins (hydroxy-PUFAs) significantly increased (Tab. 3.2, left hand side, highlighted in grey) among which 13-HODE and 9-HETE had the highest coefficients of slope. When considering the difference between month 56 and month 1, the difference of plasma concentration was more pronounced for 9-HETE (+39%) than for 13-HODE (+24%). Importantly, 9-HETE and 8,15-DiHETE were the only two oxylipins for which the change of the concentrations over the investigated period exceeded the threshold of analytical variance (±30%, Fig. 3.4). Of note, the concentration of the isoprostane 5(*R,S*)-F_{2t}-IsoP remained stable over time (non-significant coefficient of slope and

Tab. 3.2: Slopes coefficients (left: positive slope, right: negative slope) and associated p-value from the linear regression models for each oxylipin. Oxylipins with a significant evolution (p-val <0.05) during the long-term storage are highlighted in grey and sorted by the coefficient of slope.

Oxylipins	Coefficient of positive slope	p-value
13-HODE	2.0440	0.0055
9-HETE	0.9167	0.0107
12-HETE	0.6325	0.0136
11-HETE	0.5885	3.00E-04
8-HETE	0.4874	3.00E-04
8-HDHA	0.4538	0.0123
17-HDHA	0.4276	0.0012
15-HETE	0.3777	0.0175
15(S)-HETrE	0.2190	0.0304
14-HDHA	0.2183	0.0248
9-HEPE	0.2018	0.0028
20-HDHA	0.1491	0.0275
13-HDHA	0.1455	0.0011
11-HDHA	0.1325	0.0091
16-HDHA	0.1096	0.0132
8,15-DiHETE	0.0461	0.0097
10-HDHA	0.0450	0.0309
9(10)-Ep-stearic acid	3.6023	0.1841
9-HODE	0.4128	0.6896
5-HETE	0.3128	0.0835
21-HDHA	0.0624	0.2058
4-HDHA	0.0606	0.4082
13-HOTrE	0.0539	0.1937
18-HEPE	0.0530	0.1619
12-HEPE	0.0504	0.3281
11-HEPE	0.0462	0.4821
15,16-DiHODE	0.0362	0.8049
8-HEPE	0.0282	0.2762
5-HEPE	0.0213	0.3121
5(S)-HETrE	0.0169	0.0962
16(17)-EpDPE	0.0135	0.5110
4,5-DiHDPE	0.0117	0.7976
9,10-DiHODE	0.0040	0.4161
17(18)-DiHETE	0.0012	0.7127
13(14)-EpDPE	0.0003	0.9924
14(15)-DiHETE	0.0002	0.7934

Oxylipins	Coefficient of negative slope	p-value
15(16)-EpODE	-0.8775	0.0345
12,13-DiHOME	-0.3957	0.0068
8(9)-EpETE	-0.0458	0.0124
20-HETE	-0.0440	0.0151
20-HEPE	-0.0359	0.0247
19,20-DiHDPE	-0.0236	0.0058
14(15)-DiHETrE	-0.0087	0.0025
11(12)-DiHETE	-0.0021	0.0108
12(13)-EpOME	-1.1686	0.0548
5(6)-EpETrE	-0.5579	0.4088
9(10)-EpOME	-0.3766	0.3078
9,10-DiH-stearic acid	-0.3451	0.2094
5(6)-DiHETrE	-0.1522	0.1221
14(15)-EpETrE	-0.1388	0.4754
7-HDHA	-0.0959	0.0694
11(12)-EpETrE	-0.0953	0.4629
9-HOTrE	-0.0688	0.2634
19(20)-EpDPE	-0.0617	0.1646
9,10-DiHOME	-0.0605	0.1888
8(9)-DiHETrE	-0.0382	0.2585
19-HEPE	-0.0370	0.0864
12-HHTrE	-0.0369	0.4040
15-HEPE	-0.0338	0.1176
9(10)-EpODE	-0.0305	0.0781
12(13)-EpODE	-0.0280	0.1811
10(11)-EpDPE	-0.0268	0.5585
8(9)-EpETrE	-0.0220	0.7249
14(15)-EpETE	-0.0150	0.4343
7,8-DiHDPE	-0.0137	0.5564
17(18)-EpETE	-0.0112	0.4341
11(12)-DiHETrE	-0.0092	0.1581
10,11-DiHDPE	-0.0066	0.1818
8(9)-DiHETE	-0.0057	0.1479
16,17-DiHDPE	-0.0049	0.1034
13,14-DiHDPE	-0.0038	0.2535
5(R,S)-F2t-IsoP	-0.0030	0.5571
22-HDHA	-0.0007	0.9718
12,13-DiHODE	-0.0002	0.9839

concentrations within the range of ±30%). On the other side, the concentration of 8 oxylipins significantly decreased during the 56 months of monitoring (Tab. 3.2, right hand side, highlighted in grey). These oxylipins are all derived from the CYP pathway and 15(16)-EpODE as well as 12,13-DiHOME had the lowest coefficients of slope. When considering the difference between month 56 and month 1, the plasma concentrations of 15(16)-EpODE and 12,13-DiHOME

decreased by 21% and by 26%, respectively. Finally, 8(9)-EpETE was the only oxylipin which exceeded the threshold of analytical variance (±30%, Fig. 3.4).

3.4 Discussion

Oxylipins are formed from polyunsaturated fatty acids which can easily be oxidized. Therefore, in the analysis of oxylipins in biological samples such as blood particular attention should be paid to their stability during plasma generation, storage and sample handling. Only a few studies have been published investigating the influence of the plasma generation procedure and long-term storage on the profile of oxylipins [23, 27, 28]. However, these studies only evaluated free, i.e. non-esterified oxylipins. A major part of oxylipins occurs esterified in biological samples, e.g. bound in lipids [29, 36] and a large number of studies utilizes the total oxylipin pattern to understand the biology of the oxylipins [1, 19, 23].

Our study is the first one investigating the stability of total oxylipins with respect to sample collection, preparation and storage. We assessed the effect of different additives prior to sample preparation, the effect of different duration and temperature during plasma generation as well as the effect of long-term storage on a large pattern of oxylipins using one of the most comprehensive targeted metabolomic platforms (quantitative analysis of >133 oxylipins) available.

Influence of additive addition prior to sample preparation

Additives are commonly added before analytical sample preparation to avoid degradation and artifact formation [23, 33, 37]. Indeed, the addition of BHT at the beginning of sample preparation resulted in a reduction of apparent hydroxy-PUFA concentrations in comparison to samples prepared without additives. The antioxidant BHT reduces peroxyl radical oxidation of PUFA [38] and is therefore commonly used to quench radical catalyzed reactions [39] resulting in a reduction of autoxidative processes. The autoxidation of PUFA is initiated by free radical hydrogen abstraction at a bis-allylic position resulting in a hydroperoxy-PUFA that

is reduced to the corresponding hydroxy-PUFA. In case of ARA 8-, 9-, 11-, 12- and 15-H(p)ETE can be formed [40] whereas for EPA hydrogen is primarily abstracted at positions C7, C10, C13 or C16 [41, 42]. Consistently, after addition of BHT we observed a reduction of almost all hydroxy-PUFA with a slight preference for 15-HETE and 18-HEPE whereas 20-HETE was not affected (Fig. 3.1).

Neither EDTA nor indomethacin nor *t*-AUCB influenced the apparent oxylipin concentrations. It is likely that protein precipitation with iso-propanol which is the first step during sample preparation is sufficient to remove residual enzyme activity in plasma, however inhibition of enzymes involved in oxylipin formation might be relevant for other samples like tissues or other sample preparation strategies. Indomethacin is a non-selective COX-1 and COX-2 inhibitor [43] and is added in order to suppress *ex vivo* formation of prostanoids. Since the majority of COX products is base labile, the effect of indomethacin was hard to deduce in our data set, and inhibition of COX seems to be less relevant for determination of total oxylipins in plasma. However, free oxylipin levels might be influenced by residual COX activity. The metabolism of epoxy-PUFA to vicinal dihydroxy-PUFA by sEH can be prevented by the use of inhibitors such as *t*-AUCB [44]. Particularly, in samples with high sEH activity such as liver or kidney tissue [45] the addition of an inhibitor before homogenization and lipid extraction can be relevant. EDTA can serve as a chelator of metal ions in particular of iron ions which promote lipid peroxidation [46] and of calcium ions which activate phospholipase A2 [47]. However, it is poorly soluble in MeOH and thus may not be included in the additive mixture. Overall, our results show that the different aspects of the sample preparation procedure may have a relevant impact on the apparent oxylipin concentrations. Particularly, the addition of antioxidants like BHT is crucial in order to minimize the artificial formation of oxylipins during sample preparation.

Stability of oxylipins during the transitory stage of plasma generation

Human whole blood contains in addition to the plasma/serum several blood cell types namely erythrocytes, leukocytes and platelets. Most of the enzymes of the ARA cascade are expressed in blood and immune cells. For instance, 5-LOX is expressed in activated polymorphonuclear leukocytes (PMNL) or monocytes [48, 49]. In platelets active 12-LOX and COX-1 can be found [50, 51] whereas 15-LOX can be expressed in macrophages [52]. Additionally, COX-2 is expressed in activated monocytes [53].

Particularly, platelets and thus 12-LOX metabolites are often considered to cause alteration in the apparent plasma oxylipin pattern through unsuitable plasma preparation and storage. Therefore, the required steps during the generation of plasma should be performed immediately in order to minimize ex vivo metabolism. In large study cohorts or daily clinical routine, direct processing of collected whole blood is often not possible resulting in longer periods of time during the transitory stage. Here, we demonstrate that the concentrations of all oxylipin classes remained almost stable during the transitory stage and only drastic intervention in the storage conditions (as it was performed in the worst case) resulted in significantly increased oxylipin levels particularly of hydroxy-PUFA (Fig. 3.3, appendix Fig. 8.3-8.8). The worst case included delays in all steps of plasma generation with a total duration of 151 hours before freezing at -80 °C. It should be noted that apart from the worst case scenario the highest effect of this transitory storage was an increase of epoxy-PUFA by factor 2. Even though a combination of different storage conditions affected the total oxylipin concentration, no clear correlation between storage time, temperature or stage of plasma generation and the total oxylipin concentration was observable. Nevertheless, some trends can be deduced. The concentrations of the 5 /12-/15-LOX metabolites derived from ARA, EPA and DHA were reduced when whole blood was stored at 4 °C for 1 h compared to baseline and increased during 24 h. Additionally, the increased concentrations of products derived from 12- and 15-LOX when storing whole blood for 24 h at 4 °C may be caused by platelet

activation. Although the blood cells are separated from the plasma by centrifugation, residual LOX activity or autoxidation might also lead to increased concentrations of 12- and 15-LOX metabolites when samples were stored at 4 °C after centrifugation before as well as after plasma separation. However, one would assume that this happens more in whole blood than in plasma. It can be assumed that (intact) platelets are efficiently removed by centrifugation at 1200 x *g* for 10 min [54], too harsh centrifugation might also lead to platelet activation [55]. Furthermore, plasma generated from chilled whole blood, as it was performed in our study, has been reported to contain lower concentrations of platelet derived oxylipins due to possibly slowed enzymatic activity and/or cold-induced aggregation of platelets *ex vivo* leading to more efficient removal [56, 57].

Additionally, unsuitable blood handling may cause hemolysis, i.e. the release of hemoglobin upon disruption or damage of erythrocyte membranes [58] which might lead to increased oxylipin concentrations e.g. by Fenton-type catalyzed autoxidation [56]. In case of the vortexed samples which was intended to simulate hemolysis no increase in the concentrations was observed. However, this may also indicate that the sample treatment was not sufficient to cause hemolysis.

The effects of storage temperature and time with regard to the pattern of free oxylipins have been previously described [23, 27, 28, 59]. These studies revealed a clear effect of sample storage at temperatures above -80 °C on the free oxylipin pattern. In a study by Jonasdottir et al. most of the free oxylipins were stable up to 120 min at 4 °C in whole blood and plasma whereas 9-HETE increased after 60 min at 4 °C. Also, the storage of whole blood at room temperature led to higher concentrations of 12-HETE and 12-HHTrE after storing for 30 min [27]. Ramsden et al. also described the increase of 12-LOX and platelet derived oxylipins in whole blood at room temperature in a time dependent manner, whereas storage on ice for up to two hours had no effect on oxylipin concentrations [59]. However, in Willenberg et al. the concentrations of 11-HETE, 15-HETE, $PGF_{2\alpha}$, 11(12)-EpETrE and 14(15)-EpETrE were reduced by half in plasma and whole blood

after 60 min when stored at room temperature or on ice whereas other oxylipins remained stable [23]. In contrast, Jonasdottir et al. reported that several hydroxy-PUFA were significantly increased in plasma after 8 h at room temperature, while no significant differences could be observed at 6 °C up to 24 h. 12-HETE was massively increased after 24 h at -20 °C [28]. Of note, these studies differ regarding their used analytical method which influences the apparent oxylipin concentrations [23, 26].

Although our results for total oxylipins indicate that oxylipins can be considered to be stable during the transitory stage, it cannot be ruled out that oxylipin levels might be affected in other studies. Especially the blood of non-healthy participants might have higher concentrations of released precursor PUFA or ARA cascade enzymes [1]. Thus, this blood samples might be more prone to storage induced changes. The only way to reduce the potential risk of an artificially changed oxylipin pattern is to perform all steps between blood withdrawal and freezing of the plasma samples as soon as possible and as comparable as possible between the study samples. This also contributes to lower variances of oxylipin concentrations close to the analytical variance. Though there was no significant increase in the concentrations during storage higher coefficients of variations were observed for some storage conditions. Storage of centrifuged plasma for 2 h without plasma separation and for 6 h after separation at 4 °C resulted in coefficients of variations (CV) for hydroxy-PUFA being 10% higher than the respective analytical variance determined from the variance of the quality control plasma (appendix Fig. 8.9) and storage for 1 h or 6 h at 4 °C increased the CV of epoxy-PUFA. Of note, the increased variances of the epoxy-PUFA might also be due to possible artificial formation of epoxy-PUFAs during sample preparation using a silica based solid phase extraction material [32]. Additionally, vortexing of samples (simulating hemolysis) increased the CV of many oxylipins. Interestingly, it seems that autoxidative processes do not contribute to increased variances (except for the worst case scenario) since the CV of the isoprostane 5(*R,S*)-F_{2t}-IsoP did not change. The higher variation with no change in the relative concentrations compelled us to conclude that the

blood of the participants is differently affected by storage (appendix Fig. 8.9, Tab. 8.4) [1]. This emphasizes the importance of a standardized method for plasma generation, storage and preparation to ensure a good comparability in clinical studies.

Oxylipin stability during long-term storage at -80 °C

Biological samples such as human plasma are usually stored at -80 °C until analysis to minimize artificial alteration of the oxylipin profile. In longitudinal studies with large cohorts, plasma samples are generated at different time points and thus are often stored for years before analysis. Hence, the storage stability of oxylipins is crucial for the comparability and validity of such studies. Indeed, basically almost all oxylipins were stable and the observed changes in the concentrations were within the analytical variance of ± 20-30% (Fig. 3.4, Tab. 3.2, appendix Fig. 8.10) which is in line with our previous observation on the stability of free oxylipins [1]. Autoxidatively rather than enzymatically formed products seem to be relevantly affected when plasma samples are stored for longer time periods at -80 °C. The concentration of 9-HETE exceeded the analytical variance over the span of 56 months probably due to peroxidation wherein first a hydroperoxide is formed which is subsequently reduced to the corresponding hydroxy-PUFA [60, 61]. Interestingly, oxylipins whose concentrations showed a slight decreasing trend over time are mainly CYP-derived or CYP-derived secondary products like vicinal dihydroxy-PUFA (Fig. 3.4, Tab. 3.2, appendix Tab. 8.5). However, the change was within the analytical variance, thus indicating that epoxy-PUFA, which are of high interest in biological studies due to their pronounced physiological activity, remain stable and were not hydrolyzed (non)enzymatically to their respective dihydroxy-PUFA in stored plasma.

In other long-term studies investigating the influence of storage on the pattern of mainly free oxylipins contradictory results were observed [27, 28]. The storage of plasma at -80 °C for 6 months resulted in decreased levels of most eicosanoids [27] whereas storing of plasma at -80 °C for one year led to increased levels of hydroxy-PUFA and TxB_2 [28]. Barden et al. reported elevated levels of total (i.e.

free and esterified) F2 isoprostanes after 6 months of storage at -80 °C [62]. These findings which are different from our results might be again explained by the generation and the quality, i.e. its initial peroxide content, of the used plasma. Nevertheless, our results indicate that storage of plasma samples at -80 °C is suitable for total oxylipin analysis. This aspect of stability of oxylipins in properly stored plasma samples is another key point regarding their potential use as biomarkers in health and disease.

3.5 Conclusion

In our study we investigated the influence of different additives during sample preparation and the impact of the transitory stage during plasma generation as well as the effects of long-term storage of plasma on the pattern of total oxylipins in human plasma.

Our results demonstrate that the use of the antioxidant BHT reduced the artificial formation of hydroxy-PUFA highlighting the importance of standardized analytical methods for reliable and reproducible quantification of total oxylipins.

The levels of total oxylipins in plasma are stable during the transitory stage of plasma generation. Significant differences in the concentrations result only when delays occur in all steps, namely centrifugation of whole blood, plasma separation as well as freezing of the plasma. Storage of plasma at -80 °C for 56 months slightly increased autoxidatively formed products.

Overall, our results indicate that the total oxylipin pattern is – if antioxidants are used during sample preparation – robust towards minor variations in plasma generation and long-term storage. As a consequence, it is possible to investigate the total oxylipin pattern in clinical samples and in prospective cohorts. Thus, using a targeted metabolomics approach as described here allows to generate biologically meaningful oxylipin pattern which can pave the route towards a mechanistical understanding of oxylipin biology and their role as biomarkers in diseases.

3.6 References

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Chapter 4

Harmonized procedures lead to comparable quantification of total oxylipins across laboratories

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 labs assessed the technical variability and comparability of 133 oxylipins using a harmonized and standardized protocol, common biological materials (i.e. 7 quality control plasmas), standard calibration series and analytical methods. The quantitative analysis was based on a standard calibration series with isotopically labelled 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 labs was examined using consensus value estimates and unsupervised /supervised multivariate analysis (i.e. PCA and PLS-DA). Inter-lab 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. In summary, we show that 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.

Adapted from Mainka M., Dalle C., Petera M., Dalloux-Chioccioli J., Kampschulte N., Ostermann A. I., Rothe M., Bertrand-Michel J., Newman J. W., Gladine C., Schebb N. H. (2020) *J Lipid Res.* 61(11) pp. 1424-1436; doi: 10.1194/jlr.RA120000991.

4.1 Introduction

Eicosanoids and other oxylipins are potent lipid mediators produced via the oxygenation of polyunsaturated fatty acids (PUFA). PUFA can be oxygenated enzymatically by cyclooxygenases to form prostanoids, by lipoxygenases to form hydroperoxy fatty acids which react further to mono- and multihydroxylated fatty acids or by cytochrome P450 monooxygenases giving rise to epoxy and hydroxy fatty acids, or non-enzymatically 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 which 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 in the monitoring of the effects of diet or drugs [1].

Currently, the analysis of oxylipins is mainly carried out by liquid chromatography coupled with mass spectrometry 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 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 pre-treated 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 both 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. 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 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 et al. mainly recommend 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 [24].

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, 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 (intra-laboratory) 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. 7 quality control (QC) plasmas) and standard calibration series.

4.2 Experimental

4.2.1 Chemicals

Acetonitrile, methanol, 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-oxoOTrE, 9,10,11-TriHOME, 9,12,13-TriHOME, 9,10,13-TriHOME, 9,10,11-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 internal standards were purchased from Cayman Chemical (local distributor Biomol, Hamburg, Germany). Calcium ionophore A23187 and all other chemicals were brought from Sigma Aldrich (Schnelldorf, Germany). Pooled human plasma was purchased from BioIVT (West Sussex, United Kingdom).

4.2.2 Biological samples

A total of 7 QC plasmas were prepared in the Schebb Lab for the interlaboratory study. Different types of QC plasma having contrasted oxylipin profiles and biological significance were prepared:

i. QC Plasma 1 – B: Human EDTA-blood was collected from 4-6 healthy male and female individuals aged between 25-38 years, centrifuged (10 min, 4 °C, 1200 x g). Separated plasma was pooled, aliquoted and stored at -80 °C.

ii. QC Plasma 2 – n3: Human EDTA-blood was collected from an individual following n3-rich dietary supplementation, centrifuged (10 min, 4 °C, 1200 x *g*), aliquoted and stored at -80 °C.

iii. QC Plasma 3 – S: Blank plasma pool (QC Plasma 1) was spiked with oxylipin standards PGF2α, 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 at a concentration of 20 nM and LxA_4, 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.

iv. 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, 1200 x *g*), aliquoted and stored at -80 °C.

v. QC Plasma 5 – Ob-H: Human EDTA-blood was collected from 10 obese (BMI ≥ 30 kg/m²) male and female individuals without hypertriglyceridemia and centrifuged. Separated plasma was pooled, aliquoted and stored at -80 °C.

vi. QC Plasma 6 – Ob+H: 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) and centrifuged. Separated plasma was pooled, aliquoted and stored at -80 °C.

vii. QC Plasma 7 – B2: Commercially obtained pooled human plasma (BioIVT, West Sussex, United Kingdom) was aliquoted and stored at -80 °C.

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

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

All laboratories used the same standardized protocol for sample preparation. Human plasma samples were extracted using solid phase extraction (SPE) following protein precipitation and alkaline hydrolysis as described previously (the detailed standard operation procedure which was provided to all labs can be found in the appendix, Tab. 8.6-8.7) [18]. In brief, to 100 µL human plasma 10 µL antioxidant mixture (0.2 mg/mL BHT, 100 µM indomethacin, 100 µM *trans*-4-(-4-(3-adamantan-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*-AUCB) in MeOH) and 10 µL internal standard solution (100 nM of each $^{2}H_4$-8-*iso*-$PGF_{2\alpha}$, $^{2}H_4$-6-keto-$PGF_{1\alpha}$, $^{2}H_4$-$PGF_{2\alpha}$, $^{2}H_{11}$-8,12-*iso*-$iPF_{2\alpha}$-VI, $^{2}H_4$-PGB_2, $^{2}H_5$-LxA_4, $^{2}H_5$-RvD1, $^{2}H_5$-RvD2, $^{2}H_4$-LTB_4, $^{2}H_4$-9,10-DiHOME, $^{2}H_{11}$-11,12-DiHETrE, $^{2}H_4$-13-HODE, $^{2}H_4$-9-HODE, $^{2}H_6$-20-HETE, $^{2}H_8$-15-HETE, $^{2}H_8$-12-HETE, $^{2}H_8$-5-HETE, $^{2}H_4$-12(13)-EpOME, $^{2}H_{11}$-14(15)-EpETrE, $^{2}H_{11}$-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 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-

labelled internal standards [30] allowing the same analyte/internal standard assignment in the laboratories.

4.2.4 Study design

A comprehensive standard operation procedure (appendix SOP, Tab. 8.6-8.7) for sample preparation and MS analysis was standardized and validated in the reference laboratory (laboratory 1). Based on 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.

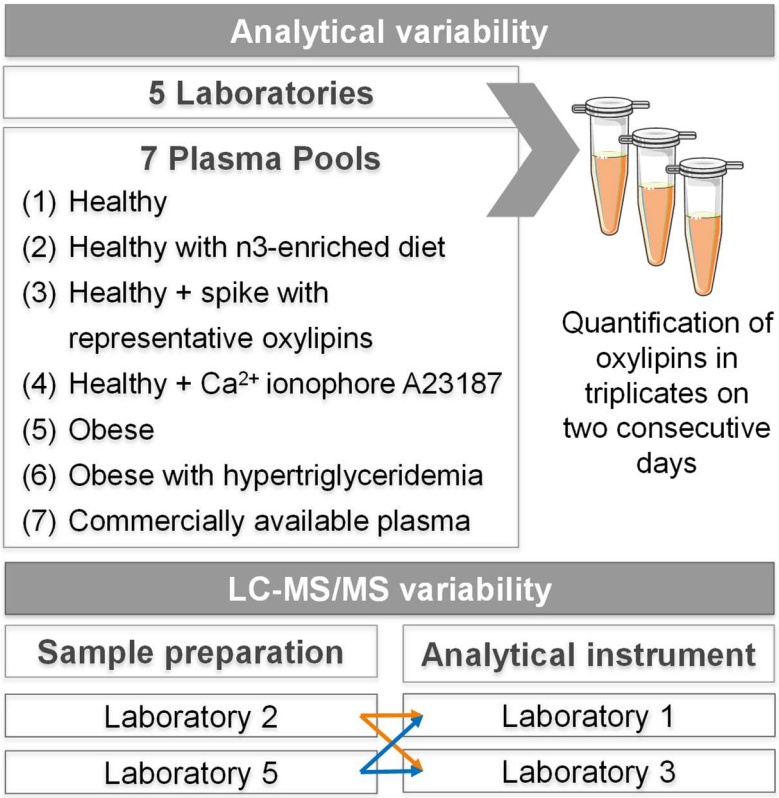


Fig. 4.1: Study design of the interlaboratory comparison. **Top:** For the assessment of the technical variability seven QC plasmas were prepared in triplicates on two consecutive days (in case of plasma 1 on three consecutive days) in the five participating laboratories applying same standardized protocol for sample preparation and LC-MS/MS analysis as well as same standard calibration series for the quantification of total oxylipins. **Bottom:** 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.

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) (Fig. 4.1).

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 nM. For each triplicate determination mean and SD was calculated. If in a triplicate determination the concentration of an analyte in one sample was below 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 LLOQ, the concentration of the analyte was set to LLOQ. Moreover, all participants were asked to fill out a short questionnaire with general remarks on the analysis.

4.2.5 Statistical analysis

Several statistical methodologies were used to assess the intra- and interlaboratory analytical variabilities. Coefficients of variations (CV) 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 6 plasmas on 2 consecutive days + analysis of plasma 1 on 3 consecutive days * 5 laboratories) and 74 oxylipins (> LLOQ for at least 1 QC plasma and at least in 1 laboratory). Firstly, 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 square - 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 its associated uncertainties (u) were calculated by the median of the means (MEDM) method, as in previously described in by Bowden et al. [26]. Briefly, the means and standard deviations (SD) of the triplicates in 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 $u = \sqrt{(\pi/2m)}*1.483*MAD$ 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 plasma and therefore to provide similar biological interpretation, ratios between QC plasma 2, 3, 4 and 7 against 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 was 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).

4.3 Results

4.3.1 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 appendix (appendix Tab. 8.8). Oxylipins were analyzed in seven different QC plasmas in triplicate (i.e. 3 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, Tab. 4.1).

Tab. 4.1: Number of quantified oxylipins. Seven plasma pools were analyzed in five independent laboratories according to the standard operation procedure (SOP; including 133 oxylipins). Shown is the number of oxylipins above lower limit of detection (LLOQ) quantified in each laboratory for each plasma pool.

	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
Lab 1	80	82	90	84	85	92	103
Lab 2	68	71	75	75	69	71	89
Lab 3	71	76	79	80	71	70	97
Lab 4	93	88	96	95	92	95	104
Lab 5	83	79	89	86	84	84	91

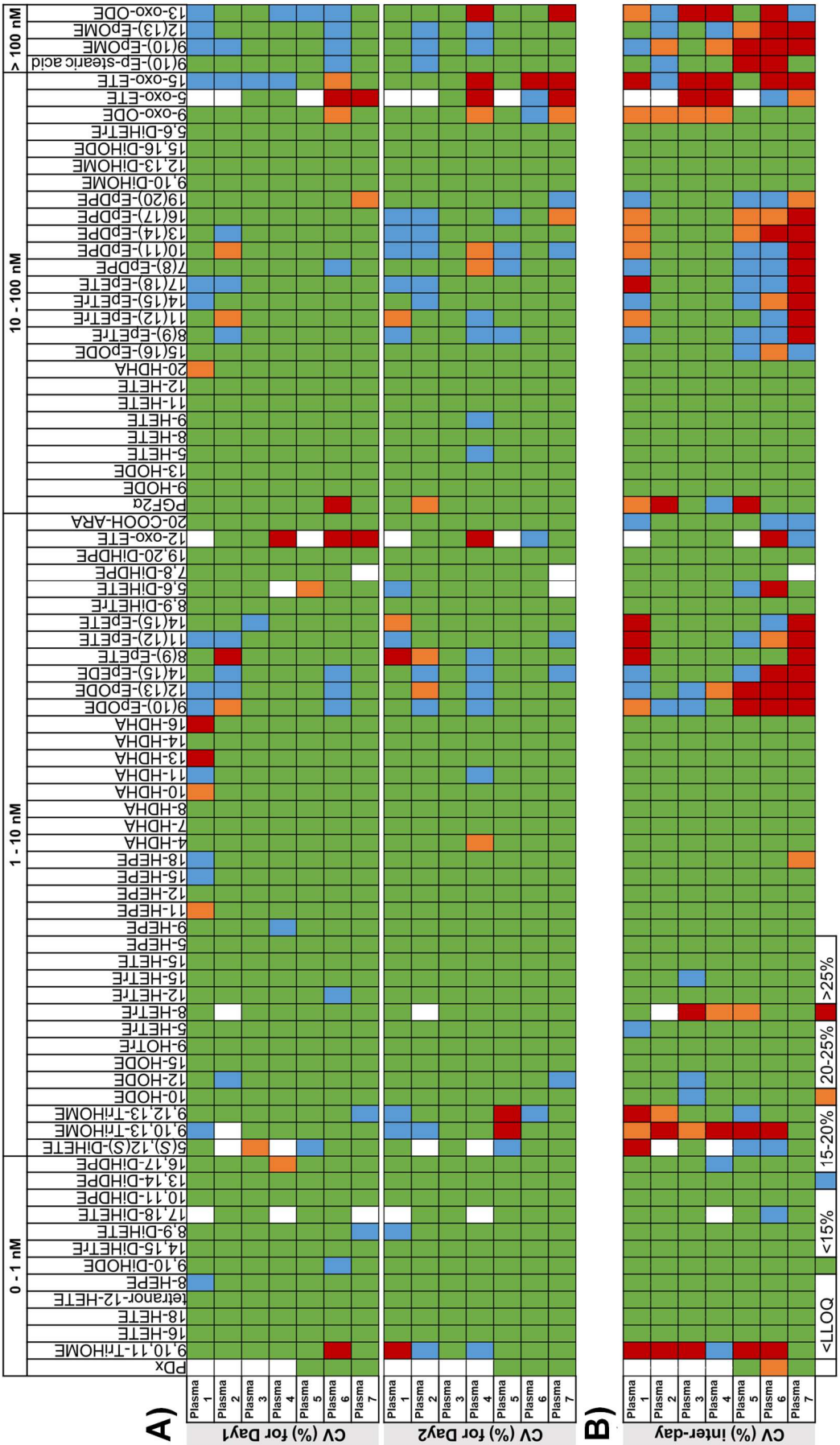
Regarding the oxylipin pattern of QC plasma 1 to 6, the determined oxylipin concentrations were in a similar range (0.16 nM to 547 nM) while in QC plasma 7, mainly for hydroxy-PUFA and multhydroxylated oxylipins, as well as for linoleic acid derived epoxy-PUFA and oxo-PUFA, 10 to 25-fold higher concentrations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

The results of the intra-day and inter-day variability of the oxylipin analysis in the reference laboratory (laboratory 1) are shown in Fig. 4.2. 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-PUFA as well as oxo- and trihydroxy-PUFA 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-PUFA could also be observed in the other participating laboratories (appendix Fig. 8.11-8.14). For laboratories 2, 3, 4 and 5, the variabilities were assessed for 69, 73, 94 and 82 quantified oxylipins, respectively. These laboratories presented overall higher variabilities, except for the laboratory 2, with low intra-day variability (CV < 15%) for 81% of oxylipins for the day 2 and low inter-day variability (CV < 15%) for 72% of oxylipins (appendix Fig. Fig. 8.11) which is consistent to laboratory 1. Of note, for laboratories 2 to 5, the intra-day variability for day 1 was higher than for day 2 which impacted the inter-day variability (appendix Fig. 8.11-8.14). For example, laboratory 3 (appendix Fig. 8.12) 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 to 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.

4.3.2 Laboratory comparison

The quantified oxylipin concentrations in all QC plasmas by all laboratories can be found in the appendix Tab. 8.9.


A)
B)
CV (%) for Day1
CV (%) for Day2
CV (%) inter-day
Plasma 1
Plasma 2
Plasma 3
Plasma 4
Plasma 5
Plasma 6
Plasma 7
0 - 1 nM
1 - 10 nM
10 - 100 nM
> 100 nM
PDx
9,10,11-TriHOME
16-HETE
18-HETE
tetranor-12-HETE
8-HEPE
9,10-DiHODE
14,15-DiHETrE
8,9-DiHETE
17,18-DiHETE
10,11-DiHDPE
13,14-DiHDPE
16,17-DiHDPE
5(S),12(S)-DiHETE
9,10,13-TriHOME
9,12,13-TriHOME
10-HODE
12-HODE
15-HODE
9-HOTrE
5-HETrE
8-HETrE
12-HETrE
15-HETrE
15-HETE
5-HEPE
9-HEPE
11-HEPE
12-HEPE
15-HEPE
18-HEPE
4-HDHA
7-HDHA
8-HDHA
10-HDHA
11-HDHA
13-HDHA
14-HDHA
16-HDHA
9(10)-EpODE
12(13)-EpODE
14(15)-EpEDE
8(9)-EpETE
11(12)-EpETE
14(15)-EpETE
8,9-DiHETrE
5,6-DiHETE
7,8-DiHDPE
19,20-DiHDPE
12-oxo-ETE
20-COOH-ARA
PGF2α
9-HODE
13-HODE
5-HETE
8-HETE
9-HETE
11-HETE
12-HETE
20-HDHA
15(16)-EpODE
8(9)-EpETrE
11(12)-EpETrE
14(15)-EpETrE
17(18)-EpETE
7(8)-EpDPE
10(11)-EpDPE
13(14)-EpDPE
16(17)-EpDPE
19(20)-EpDPE
9,10-DiHOME
12,13-DiHOME
15,16-DiHODE
5,6-DiHETrE
9-oxo-ODE
5-oxo-ETE
15-oxo-ETE
9(10)-EpStearic acid
9(10)-EpOME
12(13)-EpOME
13-oxo-ODE
<LLOQ
<15%
15-20%
20-25%
>25%

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 1st component) is related to the type of QC plasma with QC plasma 7 – B2 being different from the others. The variability on the 2nd component is much lower (i.e. 18.6%), and mainly driven by laboratory 4 (appendix Fig. 8.15A). The loading plot shows that the discrimination of laboratory 4 is based mainly on epoxy-PUFA (appendix Fig. 8.15B). Epoxy-PUFA 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 appendix Tab. 8.8) the latter of which results in increased concentrations of epoxy-PUFA (appendix Fig. 8.16). Therefore, we speculated that including the epoxy-PUFA in the PCA model might have artificially driven the discrimination of laboratory 4. To exclude this possibility and precisely determine the influence of the laboratory on the variability of the oxylipin profiles, the 12 epoxy-PUFA were excluded from further biostatistical analysis.

Fig. 4.2 (right, page 64): 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 triplicates on two consecutive days in the reference laboratory (Lab 1) 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 ($\boldsymbol{CV} = \frac{\boldsymbol{SD}}{\boldsymbol{mean}} * \boldsymbol{100}$). Shown are the CVs of 81 quantified oxylipins in the seven plasmas in laboratory 1 displayed in four different colors.

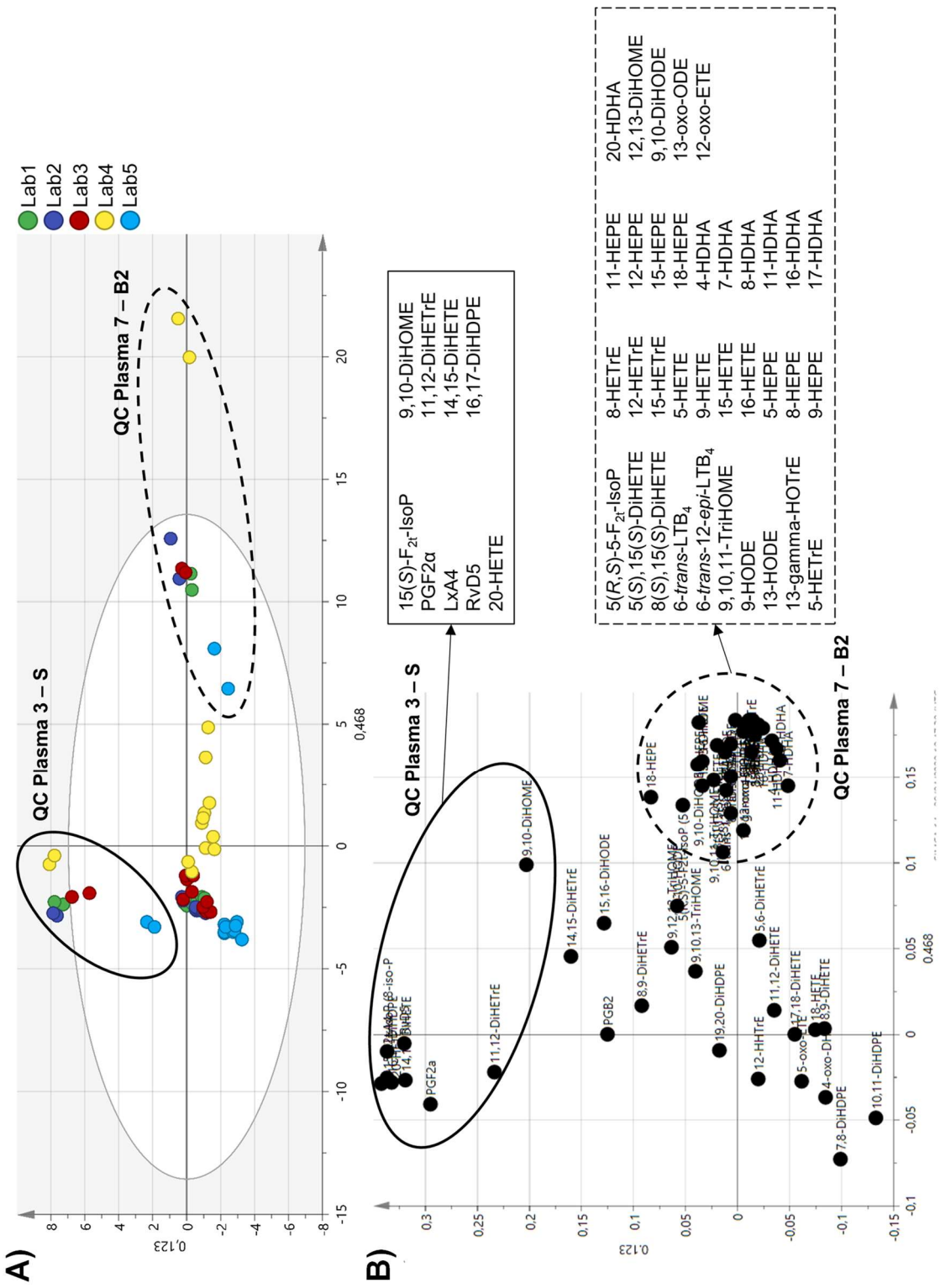

A)
Lab1
Lab2
Lab3
Lab4
Lab5
QC Plasma 3 – S
QC Plasma 7 – B2
0,123
0,468
B)
QC Plasma 3 – S
QC Plasma 7 – B2
15(S)-F2t-IsoP
PGF2α
LxA4
RvD5
20-HETE
9,10-DiHOME
11,12-DiHETrE
14,15-DiHETE
16,17-DiHDPE
5(R,S)-5-F2t-IsoP
5(S),15(S)-DiHETE
8(S),15(S)-DiHETE
6-trans-LTB4
6-trans-12-epi-LTB4
9,10,11-TriHOME
9-HODE
13-HODE
13-gamma-HOTrE
5-HETrE
8-HETrE
12-HETrE
15-HETrE
5-HETE
9-HETE
15-HETE
16-HETE
5-HEPE
8-HEPE
9-HEPE
11-HEPE
12-HEPE
15-HEPE
18-HEPE
4-HDHA
7-HDHA
8-HDHA
11-HDHA
16-HDHA
17-HDHA
20-HDHA
12,13-DiHOME
9,10-DiHODE
13-oxo-ODE
12-oxo-ETE
9,10-DiHOME
14,15-DiHETrE
15,16-DiHODE
11,12-DiHETrE
PGF2a
PGB2
8,9-DiHETrE
9,10,13-TriHOME
19,20-DiHDPE
12-HHTrE
11,12-DiHETE
5,6-DiHETrE
17,18-DiHETE
18-HEPE
10,11-DiHDPE
0,123
0,468

Fig. 4.3 (right, page 66): Principal components analysis (PCA) model without epoxy-PUFAs. The model 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 is depicted in respective boxes.

A second PCA model was built without the epoxy-PUFA (matrix with 75 samples and 62 oxylipins). The score plot (Fig. 4.3A) confirms that the variability on the 1st component (46.8%) is related to the type of plasma with QC plasma 7 (commercial plasma rich in hydroxy-PUFA) being clearly different from the others. In this second model, the variability on the 2nd 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, LxA_4, 20-HETE, 17(18)-EpETE, 19(20)-EpDPE, 16,17-DiHDPE and 13-HODE) being different from the others. The loading plot (Fig. 4.3B) shows that the oxylipins which contribute the most to the discrimination of QC plasma 7 and 3 are consistent with the highly concentrated oxylipins in the profiles of the plasma i.e. enriched with hydroxy-PUFA 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 excluded again the 12 epoxy-PUFA from the PCA model as well as samples from the QC plasma7 (i.e. plasma with 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 (R2Y=0.78 and Q2=0.729). On the 1st component of the cross-validated score plot (CV score plot) bringing 42.7% of variability (Fig. 4.4A), laboratory 4 clearly distinguishes itself. The loading plot (Fig. 4.4B) shows that this difference is driven by the hydroxy-PUFA. On the 2nd component, the variability is much lower (13.1%). However, it shows that laboratory 5 is different

from the others. Concerning the other 3 laboratories, no separation could be achieved with the model, suggesting very similar oxylipin profiles.

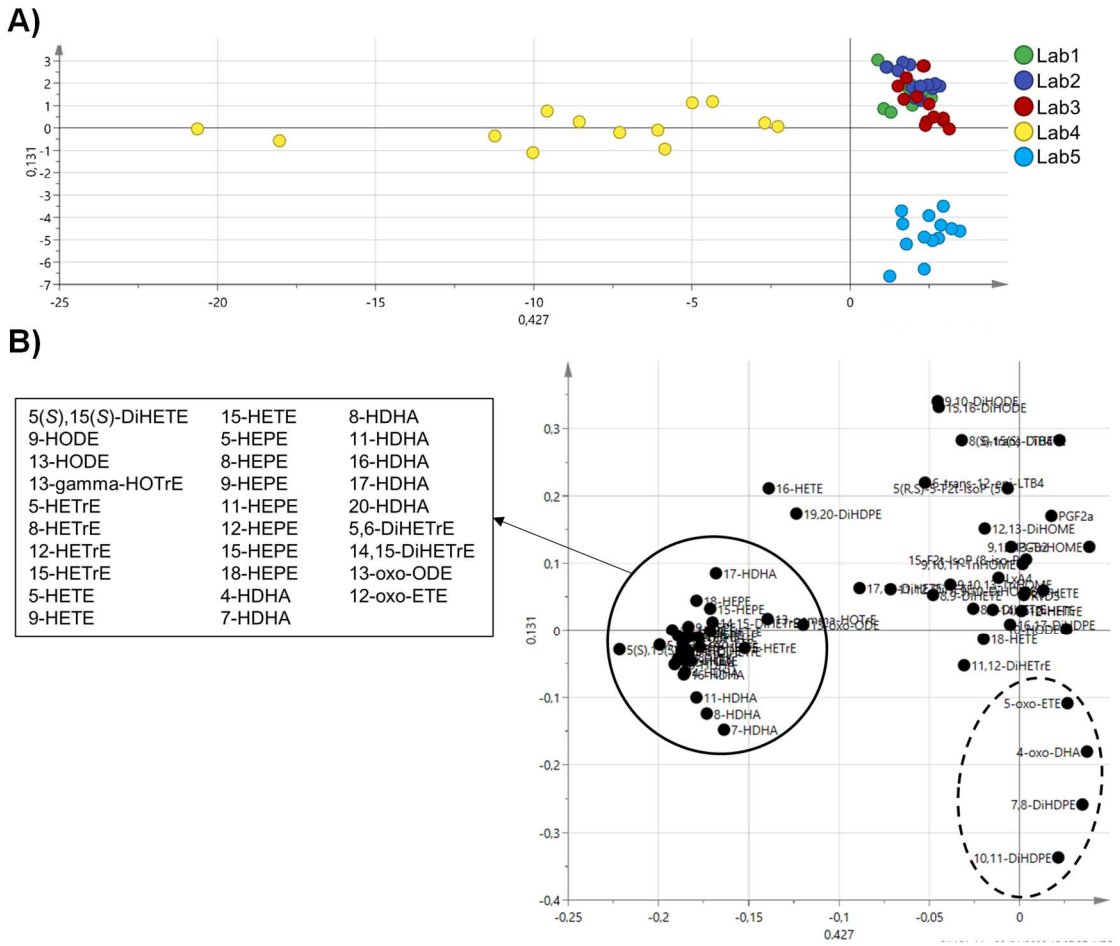


Fig. 4.4: Partial least square discriminant analysis (PLS-DA). The model was built with 65 samples and 62 oxylipins (R^2Y=0.78 and Q^2=0.729). **A)** The cross-validated score plot shows that laboratories 4 and 5 distinguish themselves from the others. **B)** The loading plot shows the oxylipins contributing to the discrimination of laboratory 4 (solid circle), the list of these oxylipins indicates that this discrimination is driven by hydroxy-PUFA. With less variability, 4 oxylipins (dotted circle) contribute to the discrimination of laboratory 5.

The consensus values were evaluated using the median of means (MEDM) approach to assess the interlaboratory comparability. The consensus values were deemed acceptable when the coefficient of dispersion (COD = 100**u*/MEDM) was less than 40% as described in Bowden et al [26]. Means ± 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 appendix Tab. 8.10-8.11 and Fig. 8.18-8.23.

In total 78 oxylipins were reported for QC plasma 1 in all laboratories, with an acceptable consensus value for 17 oxylipins (22%) (Fig. 4.5). Of note, 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) (appendix Fig. 8.17). In QC plasma 2, 3, 4, 5, 6 and 7 the consensus values were acceptable for 19, 29, 29, 21, 23 and 60 oxylipins, respectively.

4.3.3 Identification of differences between plasma pools

To assess the ability of the labs to identify similar differences (in magnitude and direction) between two plasmas, we compared the ratios calculated between different QC plasmas by each lab. 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-PUFA and linoleic acid metabolites) vs 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 vs QC plasma 1 was similar for the five laboratories. The only noticeable difference in concentration ratios of compares QC plasmas occurred between laboratory 2 and laboratory 3 for 13-oxo-ODE (ratio 5-15 and < 0.067, respectively), whereas the laboratories 1, 4 and 5 obtained very similar ratios between compared plasmas (ratio 1.33-5; Fig. 4.6A).

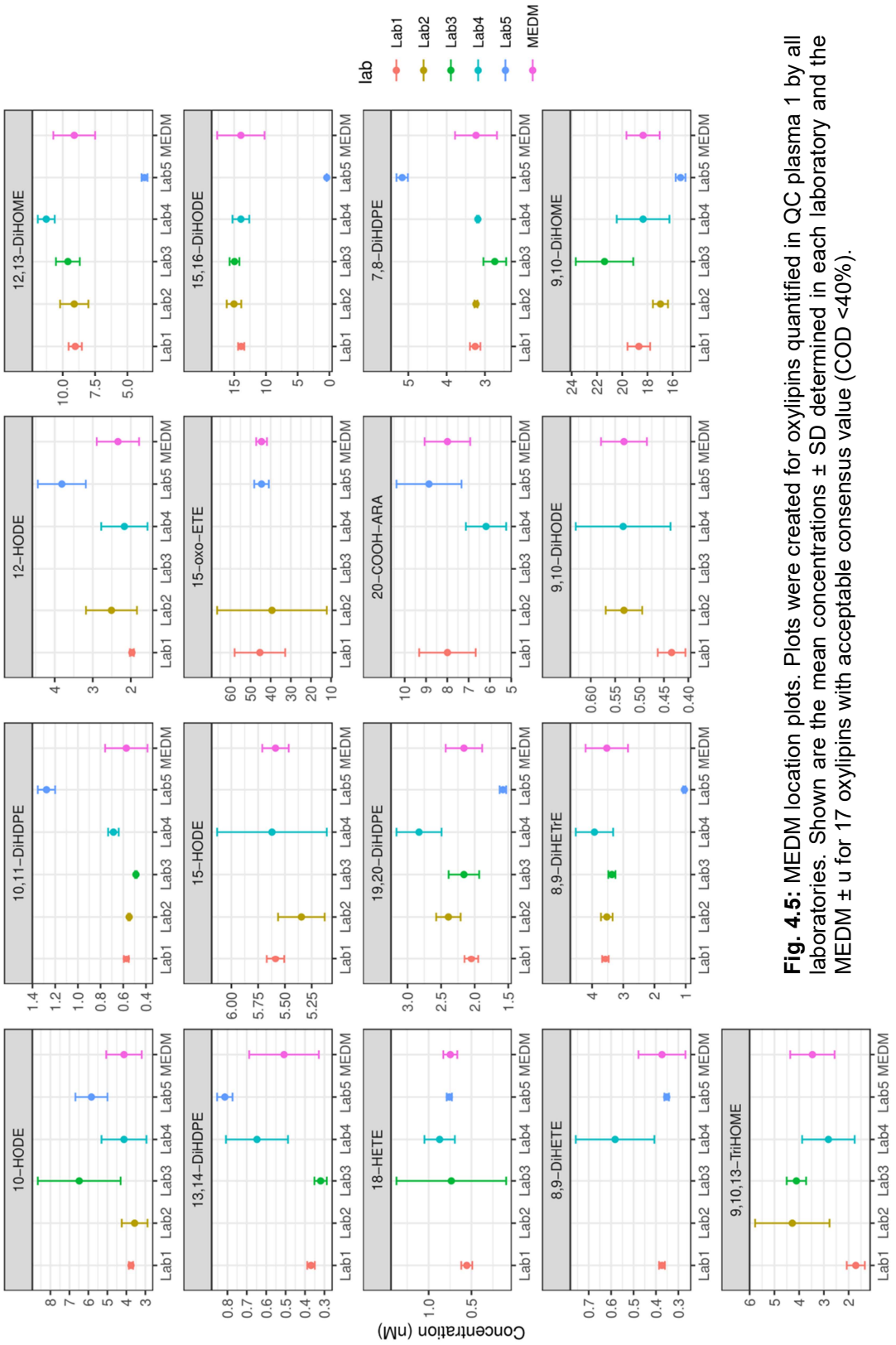
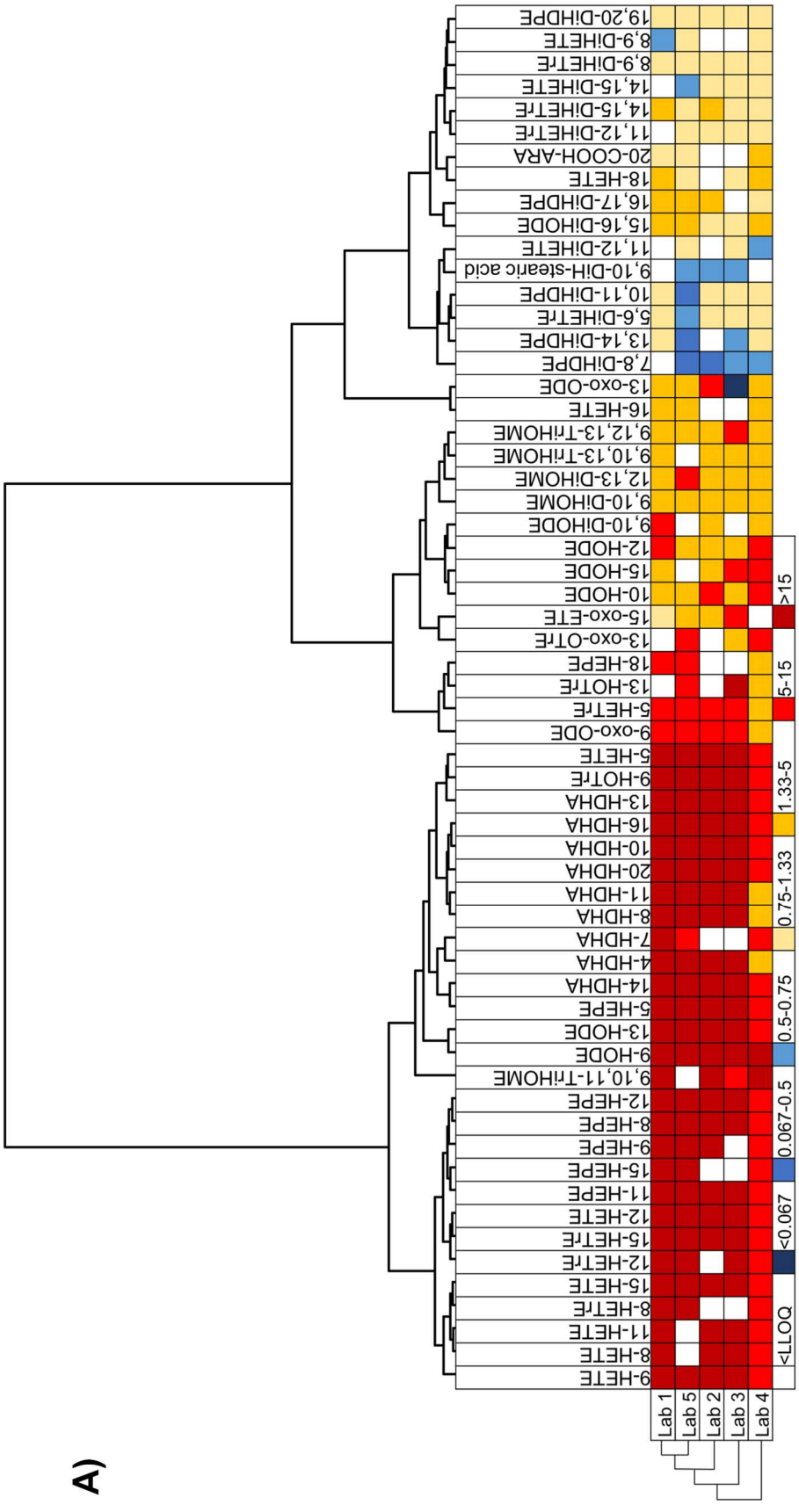


Fig. 4.5: MEDM location plots. Plots were created for oxylipins quantified in QC plasma 1 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and the MEDM ± u for 17 oxylipins with acceptable consensus value (COD <40%).

A second laboratory comparison was made with the ratios calculated between the QC plasmas obtained in obese individuals with or without hypertriglyceridemia (plasma 5 vs plasma 6). For 92% of the oxylipins (matrix consisting of 61 oxylipins) the ratios were very similar between the 5 laboratories. However, ratios in opposite directions were obtained for 9,10,11-TriHOME, 11,12-DiHETE, 9-HODE, 13-HODE and 15-oxoETE (Fig. 4.6B).

4.3.4 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 which were detected at a concentration above LLOQ in all laboratories). The oxylipin profiles were generated from samples prepared in two different laboratories (Lab 2 and Lab 5) and the prepared samples were either analyzed on 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 Lab 2, seven oxylipins (i.e. 9,10,13-TriHOME, 9,12,13-TriHOME, 10-HODE, 12-HETrE, 12-HEPE, 7-HDHA, 11(12)-EpETrE) had different concentration depending on where the LC-MS analysis was performed (MS1 or MS3) whereas in samples prepared in Lab 5, only one oxylipin (i.e. 12-HETrE) had different concentration depending on the MS platform (appendix Tab. 8.12, Fig. 8.27). Of note, the intra-MS variabilities (i.e. variability within each independent triplicate) were generally higher for the samples prepared in Lab 5 which impairs the detection of valid differences between the two sets of triplicate analyses


A)
Lab 1
Lab 5
Lab 2
Lab 3
Lab 4
9-HETE
8-HETE
11-HETE
8-HETrE
15-HETE
12-HETrE
15-HETrE
12-HETE
11-HEPE
15-HEPE
9-HEPE
8-HEPE
12-HEPE
9,10,11-TriHOME
9-HODE
13-HODE
5-HEPE
14-HDHA
4-HDHA
7-HDHA
8-HDHA
11-HDHA
20-HDHA
10-HDHA
16-HDHA
13-HDHA
9-HOTrE
5-HETE
9-oxo-ODE
5-HETrE
13-HOTrE
18-HEPE
13-oxo-OTrE
15-oxo-ETE
10-HODE
15-HODE
12-HODE
9,10-DiHOME
12,13-DiHOME
9,10,13-TriHOME
9,12,13-TriHOME
16-HETE
13-oxo-ODE
7,8-DiHDPE
13,14-DiHDPE
5,6-DiHETrE
10,11-DiHDPE
9,10-DiH-stearic acid
11,12-DiHETE
15,16-DiHODE
16,17-DiHDPE
18-HETE
20-COOH-ARA
11,12-DiHETrE
14,15-DiHETrE
14,15-DiHETE
8,9-DiHETrE
8,9-DiHETE
19,20-DiHDPE
>15
5-15
1.33-5
0.75-1.33
0.5-0.75
0.067-0.5
<0.067
<LLOQ

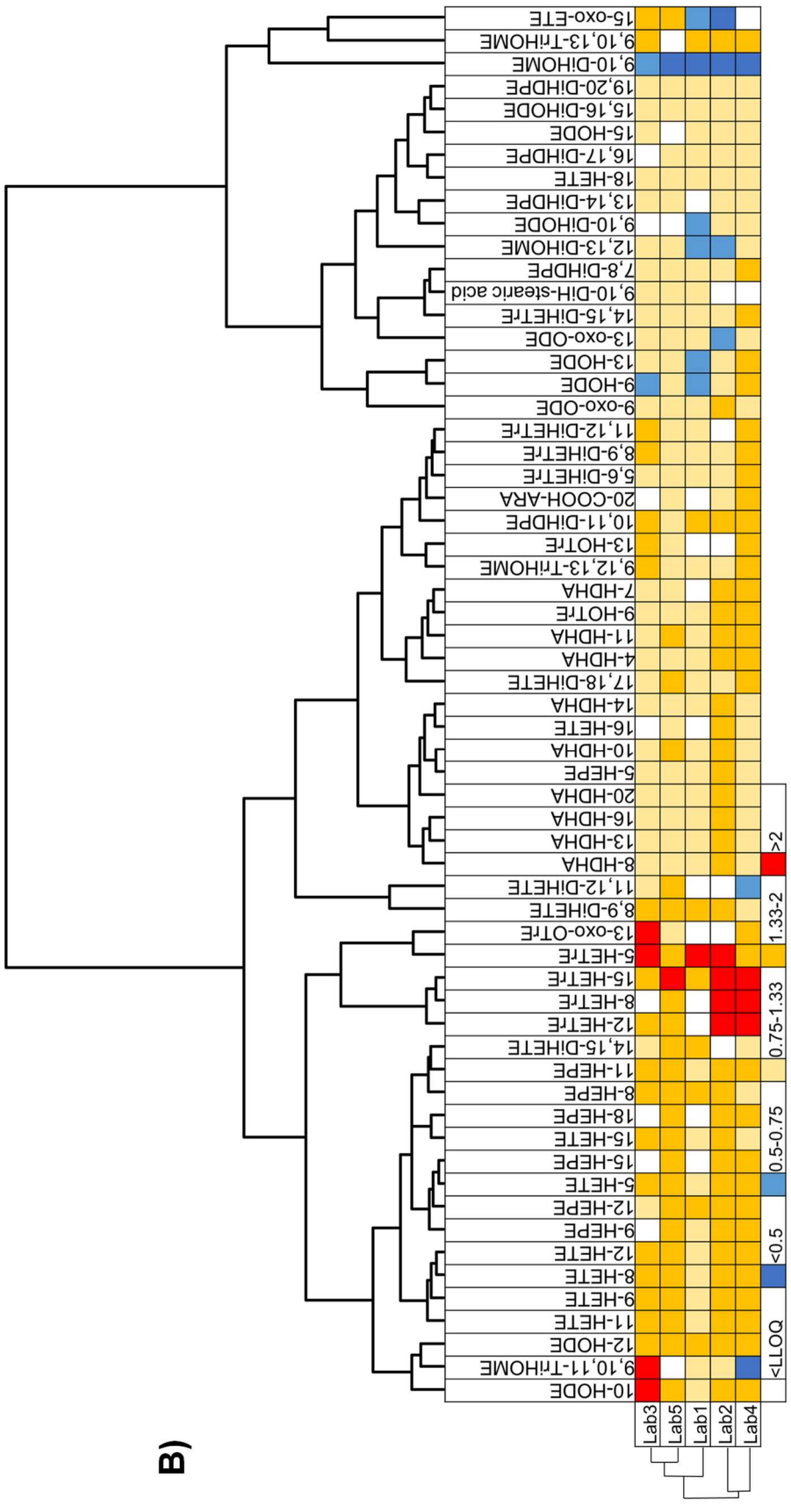


Fig. 4.6: The ability of laboratories to identify similar differences in oxylipin profiles. Shown are heatmaps with hierarchical clustering (Euclidean distances and Ward aggregation method) for laboratories and oxylipins. **A)** Heatmap of ratio between QC plasma 7 – B2 (rich in hydroxy-PUFAs) and QC 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.

4.4 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 standard operation procedure (appendix), calibration series and internal standard solutions to all the laboratories. This harmonization allows to assess the extent and sources of technical variability and to investigate which oxylipins can be robustly quantified as reliable potential biomarkers.

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. 4.2). 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 LLOQ should have a precision of < 15% [32, 33]. Low inter-day variances for 72% of oxylipins (< 15%, appendix Fig. 8.11) were also observed in laboratory 2, whose personnel was trained in the reference laboratory. In laboratories where the analysis was carried out only based on the SOP slightly higher variances (> 20%, appendix Fig. 8.12-8.14) were obtained for 38-56% of oxylipins. Although all participating laboratories are experienced in the field of oxylipin analysis they have established methods [1] which 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 two of analysis than at day one. It is quite possible that 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 the use of the same protocol is 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 the reference laboratory 1 were comparable to those described by Ostermann et al. (CV < 20% using same sample preparation protocol) [18]. However, when comparing with an independent method for the quantitation of total oxylipins described by Quehenberger et al (CV 5-20%), the observed CV are slightly higher [17], indicating further potential of optimizing the protocol.

Notably, in all laboratories, higher CVs were observed for epoxy- and oxo-PUFA as well as for TriHOMEs (up to ≥ 25%). We believe 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-PUFA can be artificially formed during sample preparation. Ostermann et al. described the drying of samples on the SPE cartridges as a particularly critical step during sample preparation [18]. During SPE, the polyunsaturated fatty acids released in large quantities during hydrolysis can adsorb on the phase material (mainly on non-endcapped silica groups) and be oxidized by atmospheric oxygen, mainly to epoxy-PUFA [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-PUFA. 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. for ARA derived eicosanoids.

Reinicke et al. examined the within-run (reinjection of same sample ten times within one analytical run) and between-run (reinjection of same sample on ten consecutive days) variability of oxylipins, whereby the variability was in a range of 1-24% in each case [37].

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 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 labelled 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 deals 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 is only a very small number of interlaboratory comparisons that investigate the comparability of lipidomic approaches [25-28] and even less 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 high 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 non-comparable results [23]. In order to solve this issue Hartung et al. recently described a strategy to verify the concentration of commercially available standards [22]. 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 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 evaluating of relative results no difference between laboratories are identified (Fig. 4.6, appendix Fig. 8.24-8.26).

The major field of application of targeted oxylipin metabolomics are 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. 4.6B). 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 desired. 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. and Quehenberger et al. where solely the NIST plasma was analyzed [26, 43]. When comparing the results of Bowden et al. [26] with the results of Quehenberger et al. [43], clear differences in the absolute oxylipin concentrations become apparent. Bowden et al. quantified 10, 6.8 and 2.4 nM for 5-HETE, 12-HETE and 15-HETE, respectively. While Quehenberger et al. 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. mol/amount per volume or gram sample. In order to compare molar concentration determined in independent laboratories often the consensus value is evaluated. There are different methods whose advantages and disadvantages should be weighed beforehand [38]. In the laboratory comparison by Bowden et al. the median of means (MEDM) approach was used to compare the results and the coefficient of dispersion 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. participating laboratories quantified all possible lipid classes, however, in only one laboratory a large number of oxylipins (143 oxylipins) were quantified. 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 is 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 LOD and LLOQ using a provided protocol, which takes into account the validation criteria of the European Medicines Agency (EMA) guideline on bioanalytical method validation (LOD 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 3 of 5 laboratories no differences between the results can be observed (Fig. 4.3). 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. 4.4). If laboratory 4 results are excluded the consensus values estimates were acceptable for 73% of the oxylipins in QC plasma 1. In general, excluding laboratory 4 increased the number of acceptable consensus values (appendix Tab. 8.10-8.11). It is particularly enlightening that the long post extraction acquisition delays and repeated injections led to higher concentrations of epoxy-PUFA in these samples (appendix Fig. 8.15-8.16). Furthermore, excluding the epoxy-PUFA from the analysis, higher levels and variance in hydroxy-PUFA were also observed in laboratory 4 data (Fig. 4.4). 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 not only allows an interlaboratory comparison in terms of relative results, but also the absolute concentrations obtained are comparable. In the lipidomics community there is an increasing demand for standardized methods [1, 10]. Our study could be used as first step for the development of an 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.

4.5 Conclusion

Our study is the first to investigate the technical variability and interlaboratory comparability, of the targeted metabolomics analysis of total oxylipins. Epoxy- and oxo-PUFA appear particularly sensitive to analytical sample handling, and delayed post-processing analyses are to be avoided. In addition, when analyzing total oxylipins special care should be taken during the drying steps when using non-end capped 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.

4.6 Limitations

The small number of participating laboratories in this exercise 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.

4.7 References

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Chapter 5

Sterol derivatives specifically increase anti-inflammatory oxylipin formation in M2-like macrophages by LXR mediated induction of 15-LOX

The understanding of the role of LXR in regulation of macrophages during inflammation is increasingly emerging. Here, we show that LXR agonist T09 specifically increases 15-LOX abundance in primary human M2 macrophages. In time- and dose-dependent incubations with T09 an increase of 3-fold for ALOX15 and up to 15-fold for 15-LOX derived oxylipins was observed. Besides that, LXR activation has no or moderate effects on the polarization of macrophages based on abundance of phenotype specific proteins (TLR2, TLR4, PPARγ and IL-1RII) and surface markers (CD14, CD86, CD163). Stimulation of M2 macrophages with FXR and RXR agonists leads to moderate ALOX15 induction, probably due to side activity on LXR. Finally, desmosterol, 24(S),25-Ep cholesterol and 22(R)-OH cholesterol were identified as potent endogenous LXR ligands leading to a strong ALOX15 induction.

LXR mediated ALOX15 regulation is a new link between the two lipid mediator classes sterols and oxylipins, possibly being an important tool in inflammatory regulation through anti-inflammatory oxylipins.

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5.1 Introduction

Inflammation is a protective mechanism of the organism against infection or tissue injury. During the course of inflammation immune cells, such as neutrophils and macrophages, are recruited in several phases and stimulate the formation and release of different cytokines, chemokines, growth factors and lipid mediators [1-3]. In the inflammatory process, macrophages have three main tasks: production of immunomodulators, phagocytosis and antigen presentation [4, 5].

The phagocytosis of cell debris and apoptotic neutrophils serves to restore tissue homeostasis [5, 6]. The produced and released immunomodulators, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-α/β, IL-10, IL-12, or IL-18 regulate immune responses, such as stimulation of proliferation of activated natural killer cells or regulation of leukocyte migration from blood to tissue [5, 7, 8]. In addition, T- and B-cells are attracted by antigen presentation and release of chemoattractants [5, 6].

Primary cell culture of monocyte derived macrophages allows to investigate signaling and regulatory pathways of the innate immune response. Under cell culture conditions, macrophage polarization in vivo is mimicked using different stimuli. The (simplified) classification of macrophages is characterized by the expression pattern of characteristic markers, including surface marker as well as pro/anti-inflammatory proteins [9]. Stimulation with granulocyte/macrophage colony-stimulating factor (GM-CSF) leads to the development of a pro-inflammatory state with increased TNF expression and release. After priming with macrophage colony-stimulating factor (M-CSF) the cells exert an anti-inflammatory character with increased IL-10 expression and release. In addition, the polarization of macrophages is induced by stimuli such as cytokines or ligands of the innate immune response such as bacterial lipopolysaccharide (LPS) [10]. Commonly, stimulation of macrophages with IFNγ and LPS is used to generate classically activated macrophages, i.e. M1-like macrophages [9, 11]. The polarization to alternatively activated macrophages is induced by IL-4 or IL-13, i.e. M2-like macrophages [9, 12].

During the inflammatory process, the polarization of macrophages can switch from pro- to anti-inflammatory depending on the inflammatory environment [13-15]. This process can be initiated by the efferocytosis, i.e. uptake, of apoptotic cells [14]. The macrophage switch associated with efferocytosis is initiated by nuclear receptors, such as retinoid X receptors (RXR), peroxisome proliferator activated receptors (PPAR) or liver X receptors (LXR) [16]. While inactivation of PPAR or RXR impairs efferocytosis of apoptotic cells, LXR deficient macrophages are unable to clear apoptotic cells altogether [17, 18]. In addition, LXR also drives efferocytosis indirectly by increasing its own expression via an autoregulatory manner [19].

LXR belongs to the family of nuclear receptors involved in the regulation of metabolic homeostasis and inflammation [20]. There are two LXR isoforms that are expressed differently in tissues despite their high structural similarity (77%). While LXRα is mainly expressed in liver, kidney, intestine, adipose tissue, or macrophages, LXRβ can be found in all human cells [21, 22]. LXR target genes include sterol response element binding protein 1c (SREBP1c), apolipoprotein (apo) E, ATP-binding cassette transporters (ABC)A1, ABCG1, ABCG5, cytochrome P-450 7A1 (CYP7A1) and fatty acid synthase (FAS) [23, 24].

The resolution-phase macrophages produce a variety of anti-inflammatory cytokines and chemokines. In addition, the formation of lipid mediators – oxylipins – plays a decisive role in the course of inflammation. While high levels of cyclooxygenase (COX)- and 5-lipoxygenase (LOX)-derived pro-inflammatory oxylipins, such as prostaglandin (PG) E_2 and D_2 or leukotriene (LT) B_4, are predominantly formed and released at the beginning of the inflammation, multihydroxylated metabolites of arachidonic acid (ARA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) are formed during the resolution phase [25, 26]. The formation of multihydroxylated oxylipins predominantly involves different lipoxygenases (LOX) interacting with each other (Fig. 5.1), 15-LOX being a key enzyme [27]. Although there is doubt about the formation and detectability of multihydroxylated LOX products, so-called specialized pro-

resolving mediators (SPM), the anti-inflammatory effect of 15-LOX is undisputed [28].

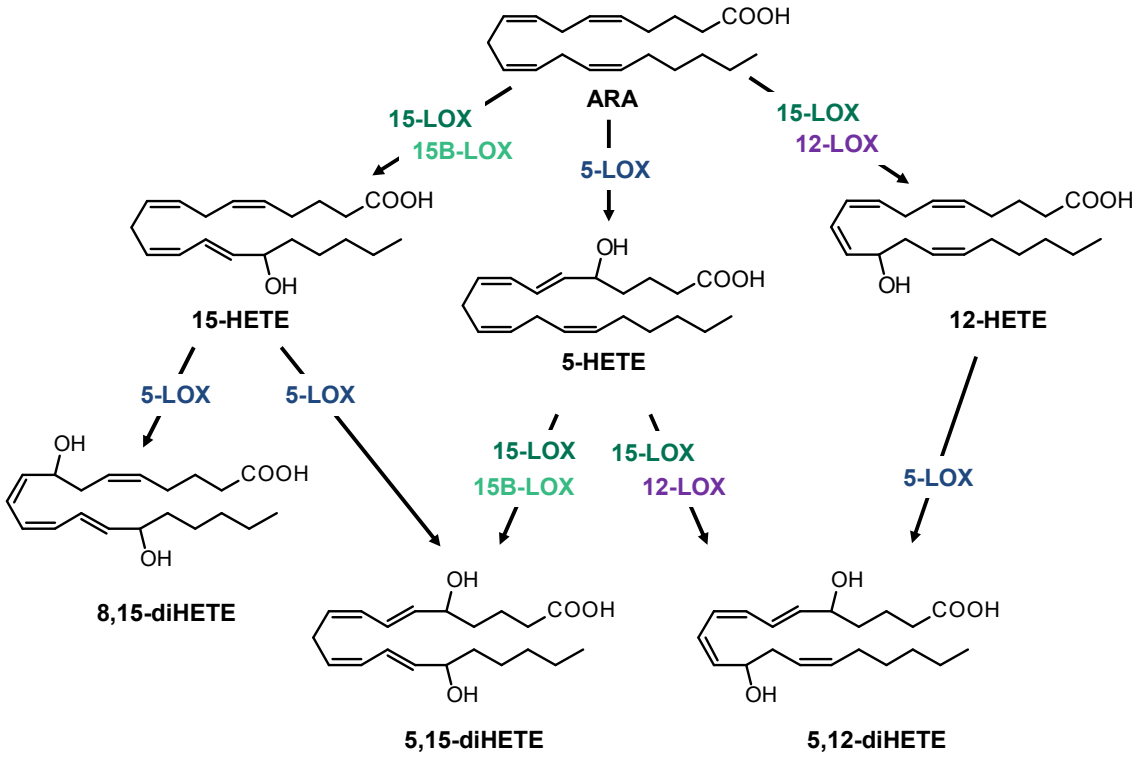


Fig. 5.1: Simplified overview of structures and formation routes of (multiple) hydroxylated ARA metabolites by the human 5-, 12- and 15-lipoxygenases.

Recently we have shown that stimulation of macrophages with synthetic LXR agonists T0901317 (T09) increases the expression of anti-inflammatory genes, such as ALOX15 (15-LOX gene) [29]. Here, we aim to investigate the LXR-induced influence on macrophage differentiation and polarization as well as the formation of oxylipins. For this, human peripheral blood mononuclear cells (PBMC) differentiated to M0-, M1- and M2-like macrophages were stimulated with T09 and the formation of oxylipins, protein levels and expression of surface markers was compared to non-stimulated cells. The specificity of the nuclear receptor activation was investigated using FXR agonists, RXR agonists. Finally, sterols were identified as endogenous LXR ligands elevating 15-LOX abundance and upregulating oxylipin formation. In summary, with the sterol mediated induction of oxylipins we demonstrated a new link between these lipid mediator classes in the regulation of M2-like macrophages.

5.2 Experimental

5.2.1 Chemicals

Human AB plasma was obtained from the blood donor service of the University Hospital of Düsseldorf (Düsseldorf, Germany). Lymphocyte separation medium 1077 was from PromoCell (Heidelberg, Germany). Recombinant human colony stimulating factors M-CSF and GM-CSF, IFNγ and IL-4 produced in *E.coli* were purchased from PeproTech Germany (Hamburg, Germany). RPMI 1640 cell culture medium, L-glutamine and penicillin/streptomycin (5.000 units penicillin and 5 mg streptomycin/mL), lipopolysaccharide (LPS) from *E.coli* (0111:B4, product number: L2630), dextran from *Leuconostoc* spp. (molecular weight 450.000-650.000), copper sulfate pentahydrate, iodoacetamide, dimethylsulfoxide (DMSO) and desmosterol were obtained from Sigma (Schnellendorf, Germany). Trypsin (>6000 U/g, from porcine pancreas), protease-inhibitor mix M (AEBSF, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A) and resazurin were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Ammonium hydrogen carbonate, sodium deoxycholate, urea and formaldehyde were from Carl Roth (Karlsruhe, Germany).

Acetonitrile, methanol (LC-MS grade), acetone (HPLC grade), acetic acid (Optima LC-MS grade) and BCA reagent A were purchased from Fisher Scientific (Schwerte, Germany). Ethyl acetate (HPLC grade) and n-hexane (HPLC grade) were bought from VWR (Darmstadt, Germany). The ultra-pure water with a conductivity of >18 MΩ*cm was generated by the Barnstead Genpure Pro system from Thermo Fisher Scientific (Langenselbold, Germany). Oxylipin standards and deuterated oxylipin standards used as internal standards and tested compounds (T0901317 (T09), 22(*R*)-OH cholesterol, 24(*S*)-OH cholesterol, 25-OH cholesterol, 24(*S*),25-Ep cholesterol, 7-keto cholesterol, GSK2033, fexaramine, hyodeoxycholic acid, bexarotene, 9-*cis* retinoic acid) were purchased from Cayman Chemical (local distributor Biomol, Hamburg, Germany). Unlabeled and

heavy labeled (lys: uniformly labeled (U)-$^{13}C_6$; U-$^{15}N_2$; arg: U-$^{13}C_6$; U-$^{15}N_4$) peptide standards were purchased from JPT Peptides (Berlin, Germany).

5.2.2 Cultivation of macrophages from human PBMC

Primary human macrophages were purified and differentiated as described [30]. Buffy coats were obtained from the generation of erythrocyte concentrates from the blood donor services at the University Hospital of Düsseldorf, Germany, drawn with the informed consent from healthy human subjects. The study was approved by the Ethical Committee of the University of Wuppertal. In brief, primary human blood monocytic cells (PBMC) were isolated from fresh buffy coats by dextran (5%) sedimentation for 45 min. The supernatant was layered on lymphocyte separation medium and centrifuged for 10 min at 1000 *g* without deceleration. PBMC sedimented on top of the lymphocyte separation medium were collected. Following washing with PBS twice, cell pellets were resuspended in RPMI 1640 medium supplemented with 1% L-glutamine and 1% penicillin/streptomycin (P/S). Cell suspensions were transferred to petri dishes and incubated for 1 h (37 °C; 5% CO_2; humidified atmosphere). Afterwards, dishes were washed twice with RPMI 1640 medium supplemented with 1% L-glutamine and 1% P/S to remove non-adherent cells and layered with RPMI 1640 growth medium supplemented with 1% L-glutamine, 1% P/S and 5% heat inactivated human AB plasma. The monocytes were differentiated into different macrophage phenotypes for 8 days. For the M1-like phenotype the growth medium was supplemented with 10 ng/mL GM-CSF and for the M2-like phenotype with 10 ng/mL with M-CSF. The growth medium was refreshed every other day. In addition, for the last 48 h the cells were incubated with 10 ng/mL IFNγ (M1) or 10 ng/mL IL-4 (M2). Six hours before the harvest the M1-like cells were additionally stimulated with 100 ng/mL LPS. For the M0-like phenotype cells were cultivated in growth medium without addition of cytokines.

The differentiated macrophages were treated with test compounds at different time-points and concentrations.

The cytotoxic effect of the test substances was investigated by means of the resazurin (alamar blue) assay [31] and only non-toxic concentrations were used (appendix Fig. 8.30).

The cells were harvested using cold shock method by washing with PBS and incubating in ice cold PBS/EDTA (20 min/4 °C). The cells were collected by scraping, centrifugation and pelleted following washing with PBS containing protease inhibitor. The harvested primary macrophages pellets were frozen at -80 °C until use.

5.2.3 Analysis of oxylipins and protein levels by LC-MS/MS

The analysis of oxylipins and protein levels was carried out from the same cell pellet. Cell pellets were resuspended in PBS containing antioxidant/inhibitor mixture (0.2 mg/mL BHT, 100 µM indomethacin, 100 µM *trans*-4-(-4-(3-adamantane-1-yl-ureido)-cyclohexyloxy)-benzoic acid (*t*-AUCB) in MeOH) and sonicated [32, 33] [34]. The protein content was determined by the bicinchoninic acid (BCA) assay. Following addition of 10 µL of the oxylipin internal standards ($^2H_4$-13,14-dihydro-15-keto PGE_2, $^2H_4$-15-deoxy-Δ12,14-PGJ_2, $^2H_4$-6-keto-$PGF_{1\alpha}$, $^2H_4$-8-*iso*-$PGF_{2\alpha}$, $^2H_4$-PGE_2, $^2H_4$-PGD_2, $^2H_4$-TxB_2, $^2H_4$-$PGF_{2\alpha}$, $^2H5$-RvD_2, $^2H_{11}$-8,12-*iso*-iP$F_{2\alpha}$-VI, $^2H_5$-LxA_4, $^2H_5$-RvD1, $^2H_4$-PGB_2, $^2H_4$-LTB_4, $^2H_4$-9,10-DiHOME, $^2H_{11}$-11,12-DiHETrE, $^2H_6$-20-HETE, $^2H_4$-13-HODE, $^2H_4$-9-HODE, $^2H_8$-15-HETE, $^2H_3$-13-oxoODE, $^2H_8$-12-HETE, $^2H_8$-5-HETE, $^2H_4$-12(13)-EpOME, $^2H_{11}$-14(15)-EpETrE, $^2H_7$-5-oxoETE, $^2H_{11}$-8(9)-EpETrE, each 100 nM in MeOH) and protein precipitation in methanol (-80 °C; 30 min), the supernatant was used for the oxylipin analysis whereas the protein pellet was frozen at -80 °C for a later protein level analysis [34].

The oxylipin analysis was carried out as described [32, 33]. In brief, the oxylipins were extracted from the supernatants using Bond Elut Certify II SPE cartridges (200 mg, 3 mL, Agilent, Waldbronn, Germany). Following elution of the oxylipins using ethyl acetate/n hexane/acetic acid (75/25/1, *v/v/v*) samples were

evaporated (vacuum concentrator, 30 °C, 1 mbar; Christ, Osterode, Germany), and the residue was reconstituted in 50 µL internal standard 2 (1-(1-(ethylsulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)¬phenyl)¬urea, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid, 12-oxo-phytodienoic acid and aleuritic acid) [32, 33]. Oxylipins were analyzed using an 1290 Infinity II System (Agilent) coupled to a 5500 QTRAP instrument (Sciex, Darmstadt, Germany) in ESI(-)-mode operated in scheduled selected reaction monitoring. [35].

The analysis of the protein levels was carried out as described [34, 36]. In brief, the protein pellet was dissolved in 5% (*w/v*) sodium deoxycholate with protease inhibitor (100/1; *v/v*), precipitated with ice-cold acetone (4 volumes) and centrifugated (4 °C, 15 000 *g*, 20 min). Following subsequent incubations with 200 mM dithiothreitol (in 50 mM NH_4HCO_3), 200 mM iodoacetamide (in 50 mM NH_4HCO_3) and again 200 mM dithiothreitol the proteins were digested overnight (15 h) using 100 µg/mL trypsin in 50 mM acetic acid (trypsin-to-protein ratio of 1:50). The reaction was stopped by acidification with concentrated acetic acid (pH 3-4). Following addition of internal standards (heavy labeled peptides with sequences corresponding to each analyte peptide) the samples were extracted using Strata-X SPE 33 µm Polymeric Reversed Phase cartridges (100 mg / 3mL, Phenomenex LTD, Aschaffenburg, Germany). The peptides were eluted with 70% ACN/0.1% acetic acid, evaporated and finally reconstituted in 15% acetonitrile/0.1% acetic acid. The peptides were analyzed on an 1290 Infinity II System (Agilent) coupled to a 6500+ QTRAP instrument (Sciex) in ESI(+)-mode operated in scheduled selected reaction monitoring mode [34, 36].

MS data analysis was done with the software MultiQuantTM 3.0.2 (Sciex) using a Gaussian smooth width of 1 and the MQ4 integration algorithm. Concentrations were calculated via external calibration using internal standards based on the ratio of analyte and internal standard areas. The quantified peptide/protein and oxylipin concentrations were normalized to the absolute protein content determined via BCA assay.

5.2.4 Immunofluorescence labelling

PBMC were seeded in 24-well plates on coverslips (Sarstedt, Nümbrecht, Germany) and cultivated as described above. For labelling, polarized macrophages were fixed in 4% formaldehyde in PBS for 15 min at 37 °C. Following washing with TBS the cells were permeabilized and blocked with TBS containing 0.3% Tween 20 and 1% bovine serum albumin (BSA) for 30 min at 30 °C. The primary macrophages were then incubated overnight with the primary antibodies 1:100 diluted in TBS/0.3% Tween 20/1% BSA against CD14, CD86 and CD163 (all mouse anti-human; Bio-Rad Laboratories, Feldkirchen, Germany). The coverslips were washed extensively with TBS/0.3% Tween 20 and then incubated with FITC-conjugated secondary antibody (1:300 in TBS/0.3% Tween 20/1% BSA; goat anti-mouse IgG Alexa Fluor 488, Thermo Scientific) for 45 min at 30 °C and again extensively washed with TBS/0.3% Tween 20. The nuclei were stained with 10 µg/mL Hoechst 33258 (Sigma) for 45 sec and finally the coverslips were observed using a fluorescence microscope (Leica DM6 B, Wetzlar, Germany) [37].

5.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc. version 6.01, San Diego, CA, USA). Data are presented as mean ± standard error of mean (SEM). Statistical analyses were performed by two-tailed unpaired student's t-test. Differences were considered significant at p-values <0.05.

5.3 Results

In the present study, we investigated the effect of LXR activation by different nuclear receptor agonists and endogenous lipids on the induction of ARA cascade enzymes and oxylipin formation as well as overall effects on the polarization of human primary macrophages.

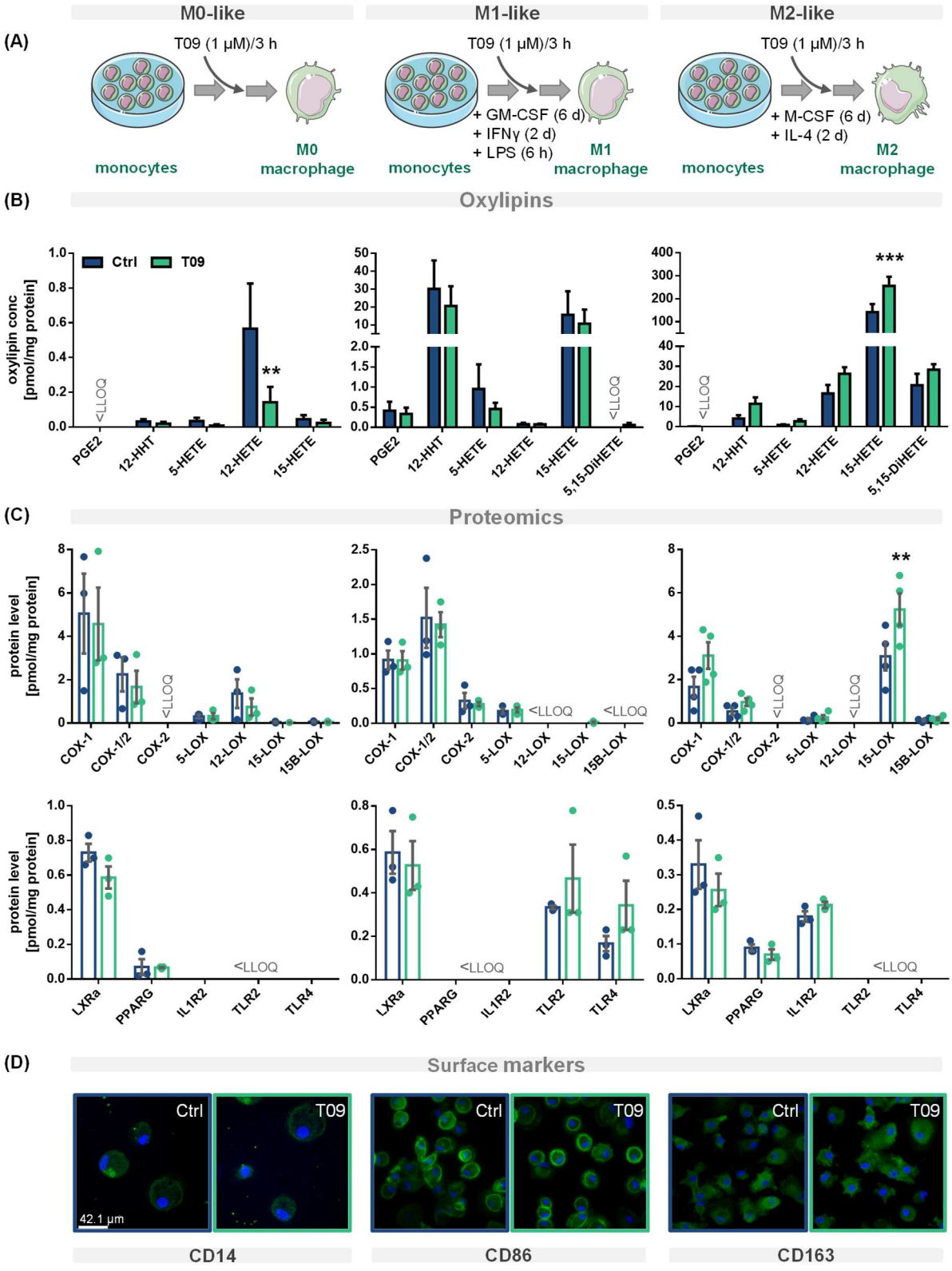

(A)
M0-like
M1-like
M2-like
T09 (1 µM)/3 h
T09 (1 µM)/3 h
T09 (1 µM)/3 h
+ GM-CSF (6 d)
+ IFNγ (2 d)
+ LPS (6 h)
+ M-CSF (6 d)
+ IL-4 (2 d)
monocytes
M0 macrophage
monocytes
M1 macrophage
monocytes
M2 macrophage
(B)
Oxylipins
oxylipin conc [pmol/mg protein]
Ctrl
T09
<LLOQ
PGE2
12-HHT
5-HETE
12-HETE
15-HETE
5,15-DIHETE
(C)
Proteomics
protein level [pmol/mg protein]
COX-1
COX-1/2
COX-2
5-LOX
12-LOX
15-LOX
15B-LOX
LXRa
PPARG
IL1R2
TLR2
TLR4
(D)
Surface markers
Ctrl
T09
42.1 µm
CD14
CD86
CD163

Fig. 5.2 (right, page 96): Effect of the LXR agonist T09 on the ARA cascade and differentiation of primary macrophages. **(A)** Primary human blood monocytic cells were treated with 10 ng/mL GM-CSF (M1 type) for 8 days or with M-CSF (M2 type) for 8 days as well as 10 ng/mL IFNγ (M1 type) or IL-4 (M2 type) for the final 2 days. Additionally, M1 cells were challenged with 100 ng/mL LPS for 6 h. For M0 type, the adhered monocytes were left untreated for 8 days. The three different macrophage phenotypes were incubated w/o (blue) or w/ the synthetic LXR agonist T09 (1 µM; green) for the final three hours. Shown are **(B)** oxylipin concentrations and **(C)** protein levels (mean ± SEM; cells from 3-5 donors). **(D)** The three macrophage phenotypes w/ or w/o T09 (1 µM) were immunostained for specific macrophage markers CD14 (M0), CD86 (M1) or CD163 (M2) using specific mouse anti-human antibodies (green). Nuclei were counterstained with Hoechst (blue). The immuno-positivity of the antibodies towards the respective macrophage types is shown in appendix Fig. 8.28.

PBMC were differentiated to M0-, M1- or M2-like macrophages and stimulated with the synthetic LXR agonist T09 (1 µM) for 3 h (Fig. 5.2A). Oxylipins were analyzed using an LC-MS/MS targeted oxylipin metabolomics method. Fig. 5.2B shows representative oxylipins derived from ARA. The analysis of the protein levels was carried out by LC-MS/MS based targeted proteomics (appendix “Selection of peptides” and Tab. 8.13-8.14). The effect of T09-mediated LXR activation on ARA cascade enzymes, receptors and other protein levels specific for macrophages is depicted in Fig. 5.2C. The expression of macrophage surface markers reflecting their polarization was analyzed by immunofluorescence staining (Fig. 5.2D). The T09-induced 15-LOX abundance and activity was further evaluated in a time- and dose-dependent manner (Fig. 5.3 and appendix Tab. 8.16). Following the evaluation of the optimal incubation conditions, the induction of 15-LOX by other LXR, FXR and RXR specific compounds as well as cholesterol derivatives (Fig. 5.4) was investigated. The results are shown in Fig. 5.5 and appendix Tab. 8.17.

5.4 Discussion

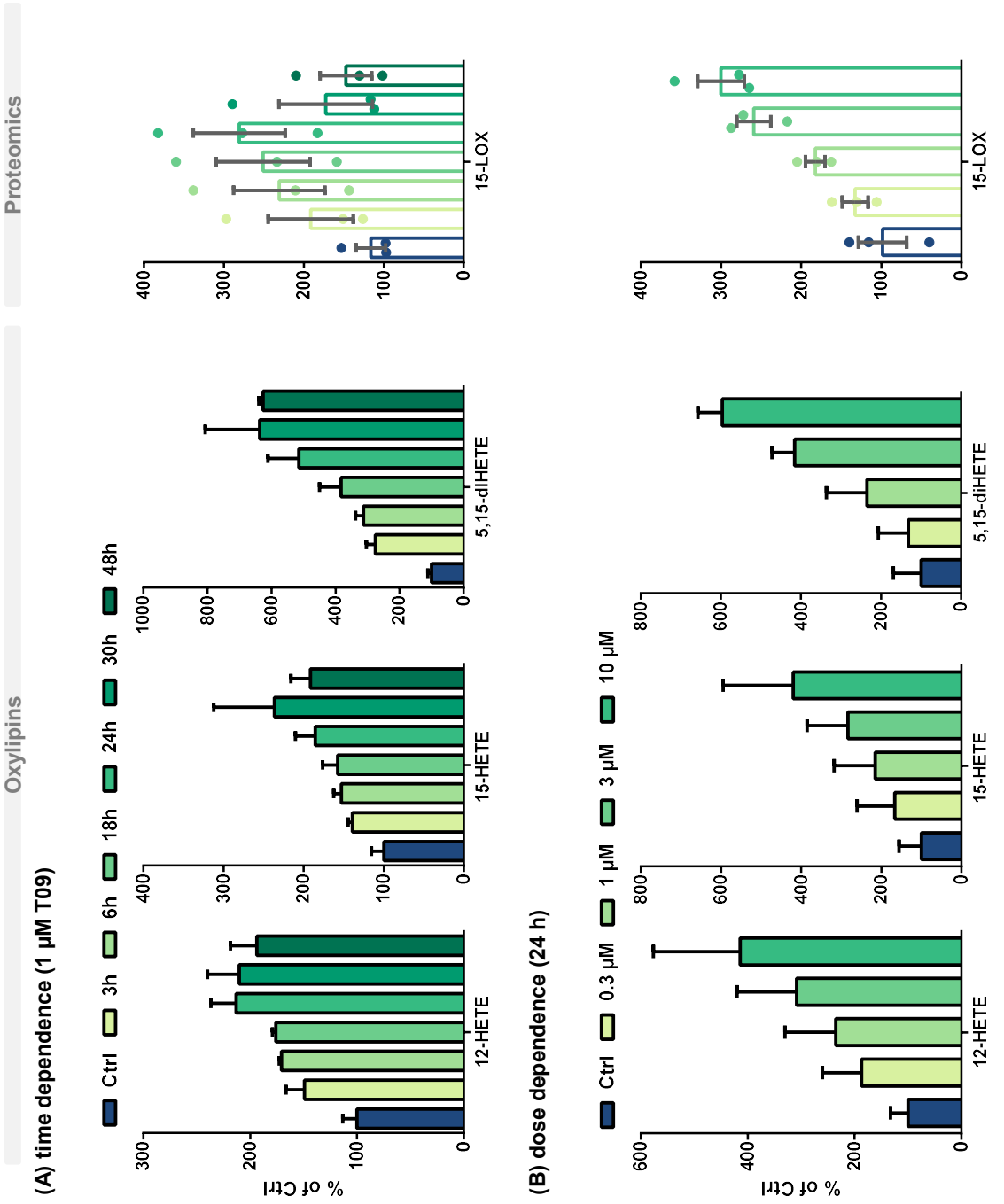
The nuclear receptor LXR is associated with different immune cell functions [38]. We have recently shown that stimulation of M2-like macrophages with LXR agonist T09 leads to overexpression of ALOX15, as well as increased formation of 15-LOX derived oxylipins [29]. 15-LOX as well as its products, such as 15-HETE or 15-HEPE, are discussed to have anti-inflammatory properties [39-41] [28]

Here, we investigated the effect of LXR activation on the enzymes of the ARA cascade and oxylipin formation as well as the effect on the polarization of human primary macrophages in more detail (Fig. 5.2A). Our experiments demonstrate that in IL-4-stimulated M2-like macrophages, ALOX15 induction is strongly increased by T09 (Fig. 5.2B-C). The LXR agonist T09 has a specific effect on 15-LOX abundance and its metabolites (15-HETE, 5,15-diHETE). It has no or low effect on PPARγ, IL-1RII (Fig. 5.2C) and the surface marker CD163 (Fig. 5.2D), which are highly expressed in M2-like macrophages [42, 43], and thus polarization of M2 macrophages was not influenced.

In M1-like macrophages, T09 also has a neglectable effect on the polarization (Fig. 5.2C-D). The surface marker CD86, which is highly expressed in M1-like macrophages after IFNγ stimulation [44] remained unchanged. Stimulation with T09 had no effect on the 15-LOX-derived 15-HETE and did not lead to an ALOX15 expression in these cells. Stimulation with T09 hardly modulated M1 specific proteins, with a trend towards higher TLR2 and TLR4 amounts and a decrease of COX-2 and its products PGE_2 and 12-HHT (Fig. 5.2B-C).

Of note, inducible nitric oxide synthase (iNOS) is often described as an M1-like macrophage marker and has therefore been included in the proteomics method (appendix “Selection of peptides” and Tab. 8.13-8.15). However, there are conflicting opinions about iNOS expression in human macrophages. Many studies have described that iNOS is not expressed in human macrophages, in contrast to murine macrophages, and that the nitric oxide found are formed by other mechanisms [45, 46]. While other studies have shown expression of iNOS under certain disease conditions [47]. In our experiments, we were not able to detect iNOS in human M1-like macrophages using our method.

Fig. 5.3 (right, page 99): Time- and dose-dependent T09 induced 15-LOX abundance and activity in primary M2-like macrophages. Shown is the increase of ARA derived 15-LOX metabolites 12-HETE, 15-HETE and 5,15-diHETE **(left)** and 15-LOX abundance **(right)**. Results are shown as % of Ctrl (mean ± SEM, cells from 3-5 donors).


Proteomics
Oxylipins
(A) time dependence (1 µM T09)
Ctrl
3h
6h
18h
24h
30h
48h
% of Ctrl
12-HETE
15-HETE
5,15-diHETE
15-LOX
(B) dose dependence (24 h)
Ctrl
0.3 µM
1 µM
3 µM
10 µM

The M0-like macrophages express low levels of PPARγ and the surface marker CD14, which remain unchanged after stimulation with T09. Interestingly, incubations with T09 reduce 12-HETE formation (Fig. 5.2B) and 12-LOX protein levels (Fig. 5.2C) present in cell preparations due to unavoidable platelet contamination [34]. Again, neither elevated 15-HETE, nor ALOX15 expression were observed with T09 treatment in M0-like macrophages. While all macrophage phenotypes express comparable levels of LXRα (Fig. 5.2C) only incubations of M2-like macrophages with the LXR agonist T09 specifically increase 15-LOX abundance and its oxylipins. No other major effect on macrophage polarization, as shown by specific expression pattern of macrophage proteins, namely IL-1RII and PPARγ in M2-like and TLR2 and TLR4 in M1-like macrophages, (Fig. 5.2C) and the immunostaining of the surface markers CD14, CD86 and CD163 (Fig. 5.2D) was observed.

This effect on 15-LOX in M2-like macrophages was studied in more detail in terms of the time- and dose-dependent influence of T09-mediated LXR activation (Fig. 5.3). The highest 15-LOX concentration (3-fold) was after a 24-hour incubation (Fig. 5.3A; appendix Tab. 8.16). An increase in oxylipin concentrations was detected up to 30 h (Fig. 5.3A; appendix Tab. 8.16). Overall, LXR induced relative increase in oxylipin formation is more pronounced for multihydroxylated oxylipins (4 to 6-fold increase), such as ARA derived 5,15-diHETE (Fig. 5.3A), 5,12-diHETE and 8,15-diHETE, EPA derived 5,15-diHEPE or DHA derived 7,17-diHDHA (appendix Fig. 8.29A) than for the monohydroxylated oxylipins (2.5-fold increase) (Fig. 5.3A, appendix Tab. 8.16). However, when considering the absolute amount of formed oxylipins, the levels of monohydroxylated oxylipins are several times higher: 180 pmol/mg protein for 15-HETE vs. 0.17 pmol/mg protein for 5,15-diHETE (appendix Tab. 8.16).

A strong LXR-induced dose dependent ALOX15 expression in M2-like macrophages was observed after 24 h of incubation (Fig. 5.3B). At 10 µM T09, 15-LOX abundance can be maximally increased 3-4-fold (Fig. 5.3B). The monohydroxylated oxylipins show a 4-fold higher concentration (Fig. 5.3B),

whereas the concentration of the multihydroxylated metabolites is increased up to 15-fold (Fig. 5.3B, appendix Fig. 8.29B). No saturation could be achieved in the dose-dependent stimulation of M2-like cells, as higher T09 concentrations were found to be cytotoxic (appendix Fig. 8.30). Nevertheless, incubations with 10 µM T09 reached maximum levels of 15-LOX and oxylipins, higher than all other reports about this enzyme and its products even compared to IL-4 induced macrophages [30, 40].

So far, stimulation of M2-like macrophages with the T-helper type 2 cytokines IL-4 and IL-13 has been the only way to induce ALOX15 at the mRNA and protein level in human macrophages [40, 48]. Even with prolonged incubations with LPS for more than 16 h, the 15-LOX abundance at the mRNA as well as protein level remain unchanged, whereas the 15-LOX-derived oxylipins increase by factor 1.5-2 [30]. Also, the concentrations of multihydroxylated oxylipins, such as 5,15-diHETE and 7,17-diHDHA, were at most increased by factor 2 [30]. The LXR-mediated effect is stronger than the previously described results on 15-LOX activity in macrophages, and thus could be of high biological relevance in the regulation of inflammatory pathways in macrophages.

15-LOX is involved in the regulation of inflammation and is thought to play a protective role in arthritis, to promote wound healing and host defense, and counteract fibrosis [49-51]. This is supported on the one hand by experiments of ALOX15 silencing in various experimental models, which was associated with inflammation and tissue damage [49]. On the other hand, overexpression of human reticulocyte 15-LOX in experimental models protected transgenic animals against atherosclerosis [52].

Anti-inflammatory properties have been described for several of the 15-LOX products. ARA derived 15-HETE can activate PPARγ and inhibit neutrophil migration, degranulation, and superoxide formation [52-57]. 15-HETE can also reduce inflammatory signaling by regulating TNFα mRNA half-life [58, 59]. EPA-

derived 15-HEPE is also thought to have anti-inflammatory properties. In transgenic fat1 mice, expressing an n3-PUFA generating desaturase, a protective effect was observed after DSS-induced colitis, which was attributed to the high concentrations of 15-HEPE, the major 15-LOX metabolite of EPA in mice. The results were corroborated by silencing of ALOX15 in fat1 transgenic mice, where 15-HEPE concentration was strongly reduced. Moreover, wild-type mice were protected against the development of DSS-induced colitis after intraperitoneal injections with 15-HEPE [41].

Upon interaction of several LOX enzymes, 5,15-diHETE or 8,15-diHETE can be formed, which are associated with neutrophil and eosinophil chemotactic activity [53, 60, 61]. Moreover, 15-HETE is the precursor in the biosynthesis of lipoxins, [58, 62, 63] which are described to play a role in inflammatory resolution associated clearance of apoptotic neutrophils, migration stop of neutrophils or regulation of cytokine and chemokine gene expression and formation [58, 64-66]. EPA-derived 5,15-diHEPE, also known as resolvin E4, has been described to induce human macrophages to efferocytosis of apoptotic neutrophils and senescent red blood cells and thus, leading to a resolution of inflammation [67]. Also, the ALOX15 DHA-derived multihydroxylated metabolite 7,17-diHDHA, also called resolvin D5, increases phagocytosis activity in neutrophils and macrophages and decreases the formation of pro-inflammatory mediators, such as TNFα and NF-κB [68, 69]. However, these multihydroxylated oxylipins are synthesized in cells and tissues only at low concentrations and many of the proposed effects and signaling pathways are a matter of discussion [28].

Here we show that LXR agonist T09 specifically elevates ALOX15 expression while the effect on polarization and proteins involved in the regulation of inflammation (PPARγ, TLR2 and TLR4 or COX) were minor. 15-LOX activation in macrophages is clearly associated with an anti-inflammatory biology. Thus, with the correlation of LXR activation and formation of oxylipins we conclude that part of anti-inflammatory effects of LXR [16, 19, 29] are mediated by 15-LOX and its products. Given that the effects of 15-LOX modulation are more pronounced

compared to TLR4 ligand LPS, argues that LXR seems to be a major regulatory pathway in macrophages.

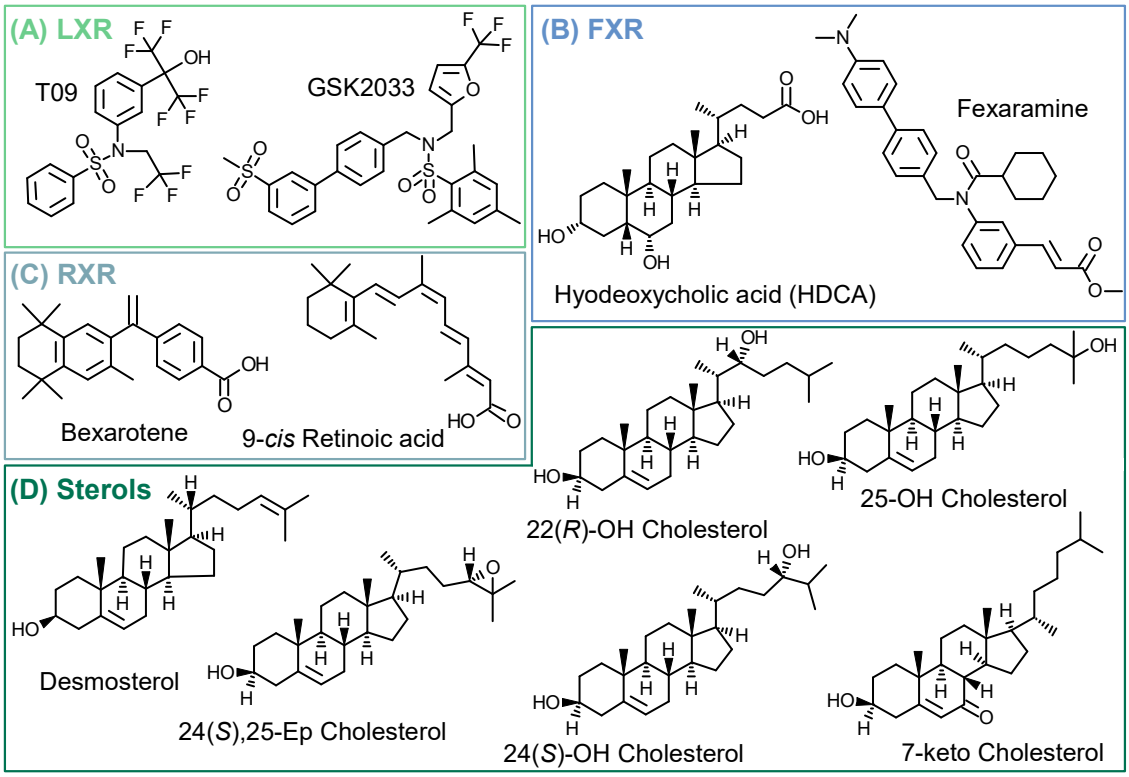


Fig. 5.4: Structures of investigated compounds. **(A)** LXR agonist (T09) and antagonist (GSK2033), **(B)** FXR agonists (fexaramine and hyodeoxycholic acid), **(C)** RXR agonists (bexarotene and 9-*cis* retinoic acid) and **(D)** tested cholesterol precursor (desmosterol) and oxidized metabolites.

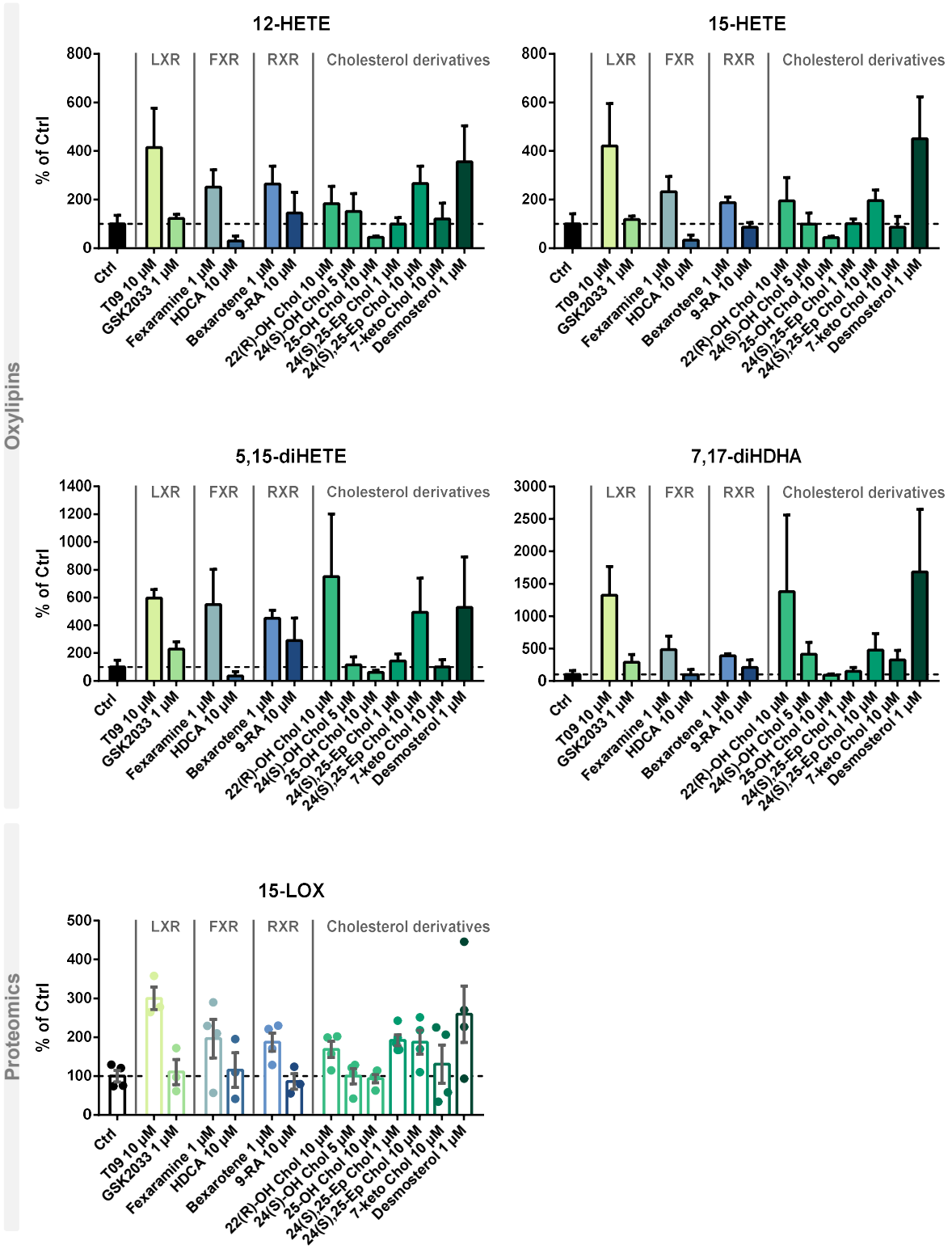
Next, we examined the specificity of the ALOX15 regulation by LXR activation. We analyzed the effects of the related nuclear receptors FXR and RXR (Fig. 5.4) with regard to the 15-LOX modulation in M2-like macrophages using specific agonists and antagonists. Of note, all investigated compounds share a sterol scaffold or other similar structural properties as well as side chains with polar groups. The binding to LXR, as described for T09 [70], occurs via a hydrophobic cavity to which the ligand can bind. In addition, a hydrogen bond is formed between a histamine residue at the end of the binding pocket and the ligand [70]. Thus, the selected compounds could also be accepted by LXR as ligands and modulate 15-LOX expression and activity since they all share a hydrophobic backbone and a side chain with polar groups to form hydrogen bonds.

Incubations with the LXR antagonist GSK2033 did not lead to changes in 15-LOX abundance and formation of its metabolites (Fig. 5.5).

Following treatment of M2-like cells with the FXR agonist hyodeoxycholic acid (HDCA) [71] the 15-LOX abundance remains unchanged. The FXR agonist fexaramine leads to a 2-fold increase in 15-LOX expression (Fig. 5.5 Proteomics, appendix Tab. 8.17) and the corresponding oxylipin concentrations are also increased 3 to 5-fold (Fig. 5.5 Oxylipins, appendix Tab. 8.17). However, due to the rather nonspecific binding pocket [70, 72] of the nuclear receptors, the binding of ligands overlaps. Thus, the effect of fexaramine could be caused by a side activity on LXR, though, it was previously described that fexaramine cannot activate LXR [73]. Consistently, it has been shown that the RXR agonists bexarotene and 9-*cis* retinoic acid (9-RA) have agonistic activity toward LXR [74] and thus, bexarotene increased 15-LOX expression 2-fold (Fig. 5.5 Proteomics, appendix Tab. 8.17) and its products 3 to 5-fold (Fig. 5.5 Oxylipins). 9-RA showed lower effects on 15-LOX: The 15-LOX concentrations apparently remained unchanged while 12-HETE and 5,15-diHETE formation increased (Fig. 5.5, appendix Tab. 8.17).

Finally, we searched for endogenous ligands eliciting the LXR mediated effects on the ARA cascade in M2-like macrophages (Fig. 5.4D). Oxysterols and metabolites from cholesterol biosynthesis have been previously described as LXR activators [21, 75-77].

Fig. 5.5 (right, page 105): Investigation of 15-LOX induction by LXR, FXR and RXR agonists and identification of cholesterol precursors and metabolites. M2-like macrophages were incubated with test compounds for 24 h. Shown is the increase of 15-LOX derived monohydroxylated metabolites 12-HETE and 15-HETE **(top)** and multihydroxylated metabolites 5,15-diHETE and 7,17-diHDHA **(middle)** as well as 15-LOX abundance **(bottom)**. Results are shown as % of Ctrl (mean ± SEM, cells from 3-5 donors).


Oxylipins
12-HETE
15-HETE
5,15-diHETE
7,17-diHDHA
% of Ctrl
LXR
FXR
RXR
Cholesterol derivatives
Ctrl
T09 10 µM
GSK2033 1 µM
Fexaramine 1 µM
HDCA 10 µM
Bexarotene 1 µM
9-RA 10 µM
22(R)-OH Chol 10 µM
24(S)-OH Chol 5 µM
25-OH Chol 10 µM
24(S),25-Ep Chol 1 µM
24(S),25-Ep Chol 10 µM
7-keto Chol 10 µM
Desmosterol 1 µM
Proteomics
15-LOX

The treatment with the autoxidatively formed 7-keto cholesterol leads to a moderate increase in 15-LOX abundance and activity (Fig. 5.5). The 24(*S*),25-Ep cholesterol elevates 15-LOX products in a concentration-independent manner (2-fold) (Fig. 5.5 Oxylipins, appendix Tab. 8.17), whereas only incubations with 10 µM 24(*S*),25-Ep cholesterol increase monohydroxylated oxylipins 3-fold and multihydroxylated oxylipins 5-fold (Fig. 5.5 Oxylipins, appendix Tab. 8.17). For 24(*S*)-OH cholesterol and 25-OH cholesterol a weaker effect was observed as they moderately increased LOX products, while no difference in 15-LOX abundance was observed (Fig. 5.5 Proteomics, appendix Tab. 8.17). For 24(*S*)-OH cholesterol a trend towards increased 15-LOX-derived oxylipin concentrations (up to 3-fold) is observed (Fig. 5.5 Oxylipins, appendix Tab. 8.17). In incubations with 25-OH cholesterol the oxylipin concentrations were reduced by half (Fig. 5.5 Oxylipins, appendix Tab. 8.17). 22(*R*)-OH cholesterol has the strongest effect on 15-LOX activity of all the oxysterols studied. While the monohydroxylated oxylipins are increased 2-fold, the increase of the multihydroxylated oxylipins is up to 15-fold (Fig. 5.5 Oxylipins, appendix Tab. 8.17) together with a 2-fold increase in 15-LOX abundance.

The activity of oxysterols on ALOX15 in M2-like macrophages is consistent with ligand binding affinity to the LXR [20, 76]. In structure/activity studies it was shown that the position of the functional group is of great importance, the essential positions being C-22 and C-24 [76, 78]. In our experiments, we were also able to correlate the effect strength with the position of the functional group. Thus, when incubated with 22(*R*)-OH cholesterol and 24(*S*),25-Ep cholesterol we observed the strongest effect on 15-LOX abundance and activity, whereas with 25-OH cholesterol hardly any changes were observed. Our results are consistent with the effects described, where a significant LXR activation is observed for 22(*R*)-OH cholesterol > 24(*S*)-OH cholesterol > 25-OH cholesterol, whereas 25-OH cholesterol has little to no effect [20, 76, 77, 79]. Other significantly important ligands are described to be 24(*S*),25-Ep cholesterol, 20(*S*)-OH cholesterol and 27-OH cholesterol [76, 77, 80]. However, the stereochemistry of the functional groups also appears to be important, with the synthetic enantiomers, namely

22(S)-OH cholesterol and 24(R)-OH cholesterol, having no effect on LXR activation [76, 79]. Of note, 24(*S*),25-Ep cholesterol is not formed from cholesterol like other oxysterols but is a by-product of cholesterol biosynthesis (mevalonate pathway) [76, 80, 81]. Also, the central intermediate from cholesterol biosynthesis, desmosterol, caused strong effects on 15-LOX. Following stimulation with desmosterol 15-LOX is overexpressed almost 3-fold, which is comparable to the effect of T09 on 15-LOX expression (Fig. 5.5 Proteomics, appendix Tab. 8.17). 15-LOX activity in incubations with desmosterol and T09 is also comparable, with the monohydroxylated oxylipins also being increased 4-fold and the multihydroxylated oxylipins up to 16-fold (Fig. 5.5 Oxylipins, appendix Tab. 8.17). Thus, we have found active endogenous LXR ligands, namely desmosterol, 22(*R*)-OH cholesterol and 24(*S*),25-Ep cholesterol that are able to massively increase 15-LOX abundance and activity through LXR.

Oxysterols occur in mammalian tissues in micromolar concentrations and circulating in only nanomolar concentrations [76, 80, 82]. Elevated (circulating) oxysterol levels are associated with pathological structures, such as foam cells or atherosclerotic lesions [38, 80, 83, 84]. High 24(*S*)-OH cholesterol plasma levels are associated with Alzheimer's disease [80, 85, 86]. Desmosterol can be found accumulated mainly in macrophage foam cells and atherosclerotic plaques, where it regulates via LXR the activation of genes involved in cholesterol efflux [87]. Due to the induction of ALOX15 expression and elevated formation of anti-inflammatory oxylipins, desmosterol might also be involved in the reduction of atherosclerotic lesions via LXR activation. 22(*R*)-OH cholesterol, 24(*S*)-OH cholesterol and 24(*S*),25-Ep cholesterol has been shown to inhibit inflammatory signaling in cell culture models [88]. As many oxysterols and metabolites of cholesterol biosynthesis are involved in the regulation of inflammatory and immune response [16, 89-91], this study of the effect of cholesterol derivatives on macrophages is of great importance to better understand the interactions between sterol metabolism and immune regulation: The induction of ALOX15 in macrophages is associated with anti-inflammatory properties [52], our results show that the anti-inflammatory roles of LXR may be related to the modulation of

15-LOX and the formation of anti-inflammatory oxylipins. Thus, the observed relationship between sterols, LXR, and oxylipin formation may represent an important feedback regulation in macrophages, where sterols indirectly modulate anti-inflammatory 15-LOX through their binding to LXR initiating inflammatory resolution.

5.5 Conclusion

We demonstrate that LXR activation by T09 exerts dramatic effects on ARA cascade specifically in M2-like macrophages by overexpression of 15-LOX and increased formation of its products. No or only a moderate effect of T09 on M0-like and M1-like macrophages as well as polarization and PPARγ, IL-1RII, TLR2 and TLR4 abundance was observed.

The investigated agonists of FXR and RXR only had a moderate effect compared to T09 on 15-LOX abundance and activity, presumably mediated by LXR through side activity.

We found that sterols are important endogenous LXR agonists regulating ALOX15. We could show that particularly the oxysterols 24(*S*),25-Ep cholesterol and 22(*R*)-OH cholesterol and the cholesterols precursor desmosterol modulate the ARA cascade in macrophages through LXR. Thus, our results show a new cross-link between two lipid mediator classes: sterols/oxysterols and oxylipins. Sterols affect the LXR-mediated formation of 15-LOX-derived oxylipins. This regulatory mechanism should be further investigated as the 15-LOX derived oxylipins are suggested to play an important role in the inflammatory resolution. A detailed characterization of this regulatory pathway could provide insights into the lipid mediator switch in macrophages leading to the formation of anti-inflammatory oxylipins.

5.6 References

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Chapter 6

On the biosynthesis of specialized pro-resolving mediators in human neutrophils and the influence of cell integrity

Neutrophils are key players in inflammation initiation and resolution. Little attention has been paid to the detailed biosynthesis of specialized pro-resolving mediators (SPM) in these cells. We investigated SPM formation in human polymorphonuclear leukocytes (PMNL), in broken PMNL preparations and recombinant human 5-lipoxygenase (5-LO) supplemented with the SPM precursor lipids 15-Hydroxyeicosatetraenoic acid (15-HETE), 18-Hydroxyeicosapentaenoic acid (18-HEPE) or 17-Hydroxydocosahexaenoic acid (17-HDHA). In addition, the influence of 5-LO activating protein (FLAP) inhibition on SPM formation in PMNL was assessed.

Intact human PMNL preferred ARA over DHA for lipid mediator formation. In contrast, in incubations supplemented with the SPM precursor lipids DHA-derived 17-HDHA was preferred over 15-HETE and 18-HEPE. SPM formation in the cells was dominated by 5(S),15(S)-diHETE (800 pmol / 20 mio cells) and Resolvin D5 (2300 pmol / 20 mio cells). Formation of lipoxins (< 10 pmol / 20 mio cells), E-series (< 70 pmol / 20 mio cells) and other D-series resolvins (< 20 pmol / 20 mio cells) was low and only detected after addition of the precursor lipids. Upon destruction of cell integrity, formation of lipoxins and 5(S),15(S)-diHETE increased while formation of 17-HDHA- and 18-HEPE-derived SPMs was attenuated. Recombinant 5-LO did not accept the precursors for SPM formation and FLAP inhibition prevented the formation of the 5-LO-dependent SPMs. Together with the data on FLAP inhibition our results point to unknown factors that control SPM formation in human neutrophils and also render lipoxin and 5(S),15(S)-diHETE formation independent of membrane association and FLAP when cellular integrity is destroyed.

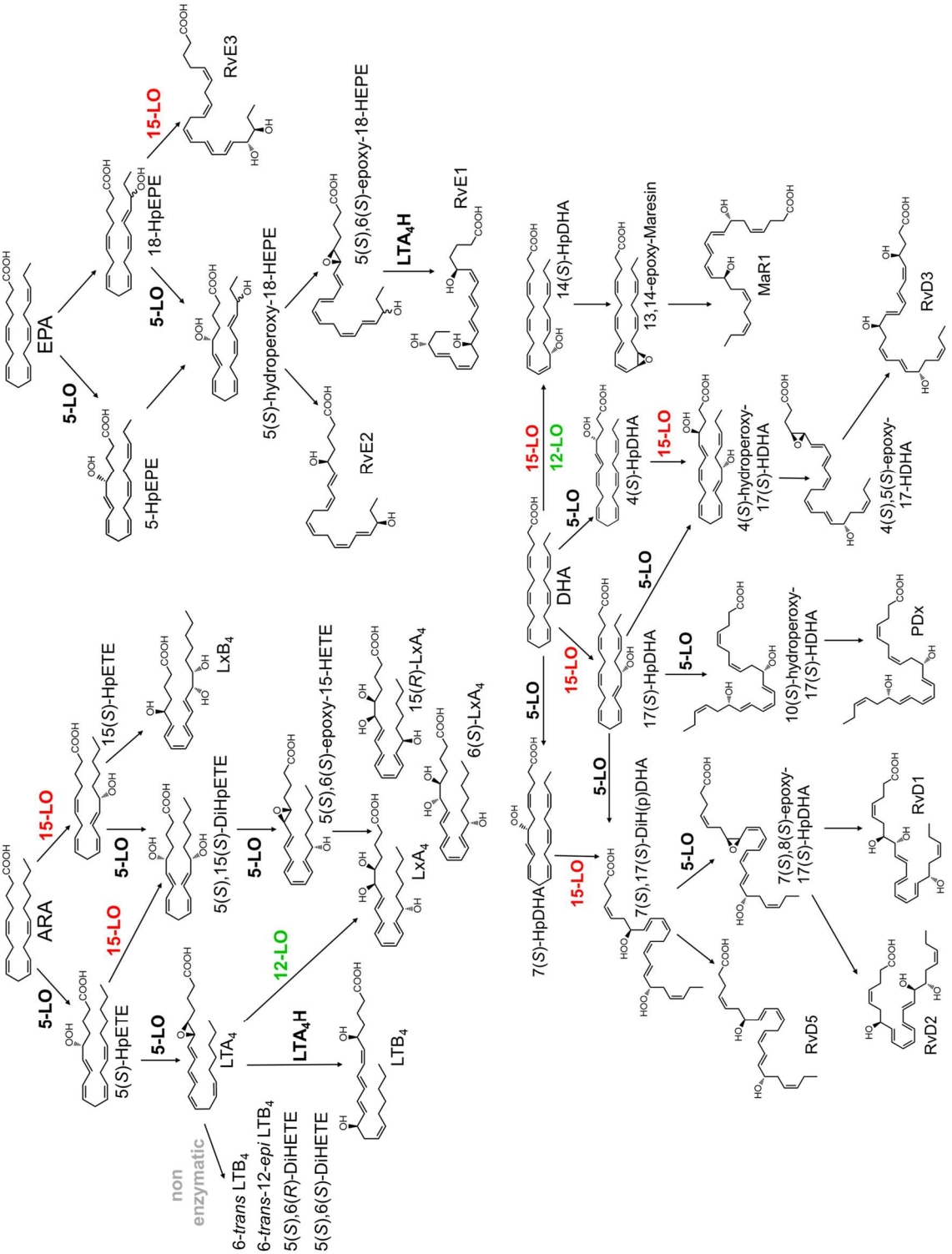
Adapted from Mainka M., George S., Angioni C., Ebert R., Goebel T., Kampschulte N., Krommes A., Weigert A., Thomas D., Schebb N. H., Steinhilber D., Kahnt A. S. (2021) *Biochim Biophys Acta Mol Cell Biol Lipids.* 1867(3) pp. 159093; doi: 10.1016/j.bbalip.2021.159093.

6.1 Introduction

Specialized pro-resolving mediators (SPM) are a group of polyhydroxylated polyunsaturated fatty acid (PUFA) derivatives which are thought to play an integral part in the resolution of inflammation. Together with pro-inflammatory lipid mediators such as leukotrienes and prostaglandins, SPM are released from immune cells at specific time points during the time-course of zymosan-induced peritonitis in mice - a model system for inflammation resolution. Here, SPMs repress the invasion of neutrophils into the inflamed tissue at the same time promoting the influx of monocytes, stimulate efferocytosis of dying neutrophils by macrophages and coordinate the egress of the phagocytes into the lymphatics thus restoring tissue function and homeostasis resulting in resolution of inflammation [1]. A large body of evidence from animal models shows that exogenous application of individual SPMs such as D-series resolvins (RvD), protectin D1 and lipoxins can positively influence resolution and ameliorate disease severity [2]. Even though these data are promising, little attention has been paid to the details of SPM biosynthesis in humans in the past. The in vivo half live and distribution of SPMs is rather short which rules out direct therapeutic application [3-5]. Therefore, for the successful development of resolution supportive therapies, the in-depth knowledge of the participating cells and the molecular details of SPM biosynthesis in humans is important.

SPMs have been detected in various fluids and tissues of the human body such as blood, breast milk, adipose and muscle tissues, spleen and lymph nodes and have been inversely correlated with disease severity in some studies, for review see [6]. Furthermore, SPMs were found in human blister exudates during resolution in an experimental model of skin inflammation [7, 8]. However, reported SPM concentrations in humans are often very low which renders SPM research an analytically challenging issue.

Fig. 6.1 (right, page 119): Suggested formation routes for LO-derived leukotrienes and SPMs. Note: The enzymes are only indicative, because several oxylipins can be formed by different pathways as well as by autoxidation.


ARA
5-LO
15-LO
5(S)-HpETE
15(S)-HpETE
5-LO
5(S),15(S)-DiHpETE
LxB4
LTA4
12-LO
LTA4H
LTB4
5(S),6(S)-epoxy-15-HETE
LxA4
15(R)-LxA4
6(S)-LxA4
non enzymatic
6-trans LTB4
6-trans-12-epi LTB4
5(S),6(R)-DiHETE
5(S),6(S)-DiHETE
EPA
5-HpEPE
18-HpEPE
RvE3
5(S)-hydroperoxy-18-HEPE
RvE2
5(S),6(S)-epoxy-18-HEPE
RvE1
DHA
7(S)-HpDHA
7(S),17(S)-DiH(p)DHA
RvD5
7(S),8(S)-epoxy-17(S)-HpDHA
RvD1
RvD2
17(S)-HpDHA
10(S)-hydroperoxy-17(S)-HDHA
PDx
4(S)-HpDHA
4(S)-hydroperoxy-17(S)-HDHA
4(S),5(S)-epoxy-17-HDHA
RvD3
14(S)-HpDHA
13,14-epoxy-Maresin
MaR1

SPMs are either formed from the n6-PUFA arachidonic acid (ARA) or the n3-PUFAs eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) by sequential oxidation steps. These steps are carried out by 5-lipoxygenase (LO) together with 12-LO or 15-LO. Alternatively, formation routes of the intermediates can also involve cytochrome P450 enzymes, (acetylated) cyclooxygenase-2 or autoxidation [9, 10]. Fig. 6.1 shows the proposed biosynthetic pathways for SPMs in detail.

5-LO also plays an integral part in the biosynthesis of pro-inflammatory leukotrienes from ARA [11]. What controls the switch from pro-inflammatory to pro-resolving lipid mediator formation by 5-LO remains elusive so far. It has been discussed that 5-LO operates in the cytosol of macrophages during SPM formation instead of translocation to the nucleus which is crucial for leukotriene formation [12]. This would be a way to separate pro-inflammatory from pro-resolving lipid mediator formation in leukocytes. However, we could recently show that the 5-LO activating protein (FLAP) is essential for LxA_4 and RvD1 biosynthesis in intact neutrophils and monocytes [13, 14]. Since FLAP is an integral membrane protein, cytosolic SPM formation does not really fit. If FLAP dependency also applies to other 5-LO-dependent SPMs such as D- and E-series resolvins and maresins is not known.

Lipoxygenases are expressed in different leukocyte subtypes in humans: Active 5-LO can be found in myeloid cells such as monocytes, macrophages, eosinophils and neutrophils while platelets express 12-LO [11]. 15-LO-1 is present in alternatively activated (M2) macrophages following IL-4 or IL-13 stimulation as well as liver X receptor activation and in eosinophils [15, 16]. 15-LO-2 is expressed in M2 macrophages as well and its expression can be further elevated by persistent stimulation of TLR-2 and -4 [17]. Although alternatively activated macrophages co-express 5- and 15-LO-1/-2, SPM formation is low in these cells compared to pro-inflammatory leukotrienes, prostaglandins and 15-LO-derived monohydroxylated PUFAs (15-HETE, 17-HDHA) released alongside [17, 18]. These data show that SPM release from a single cell is not efficient and

clearly argues for transcellular SPM biosynthesis where alternatively activated macrophages are probably the source of 15-LO-dependent SPM precursors while a second 5-LO expressing leukocyte subpopulation takes over the final oxidation steps. Or vice versa, neutrophil-derived 5-H(p)ETE might be taken up by 15-LO-expressing macrophages. Indeed, interaction of macrophages with neutrophils plays an important role in inflammation resolution and neutrophils supplemented with 15-HETE or 17-HDHA have been shown to release lipoxins and RvD1, respectively upon stimulation [13, 19-21].

Since expression of active 5-LO is limited to leukocytes, leukocytic SPM formation is an important hallmark to estimate human SPM formation capacity. In the present study, we took a closer look on SPM formation in human neutrophils. For this, polymorphonuclear leukocytes (PMNL) supplemented with ARA, DHA or the monohydroxylated SPM precursor PUFAs (15-HETE, 15-HpETE, 18-HEPE, 17-HDHA, 17-HpDHA) were stimulated with Ca^{2+} ionophore and lipid mediator formation was monitored and compared to cells stimulated in absence of these fatty acid precursors. Furthermore, SPM formation in broken cell preparations as well as from recombinant 5-LO was investigated and FLAP dependency was tested. This should answer the question whether 5-LO is able to accept monohydroxylated PUFAs for SPM formation on its own, whether FLAP dependency in PMNL applies to 5-LO-dependent SPM formation in general and whether other factors apart from 5- and 15-LOs play a role in PMNL-dependent SPM biosynthesis in humans.

6.2 Experimental

6.2.1 Drugs, chemical reagents and other materials

Dextran, $CaCl_2$ and calcium ionophore (A23187) were obtained from Sigma-Aldrich (Steinheim, Germany). PBS was purchased from Gibco Life Technologies (Paisley, UK). EDTA and SDS were purchased from Merck KGaA (Darmstadt,

Germany). Peroxide free ARA, 15(*S*)-HETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*R*)-HDHA, 18(*R/S*)-HEPE, the oxylipin standards as well as the deuterated standards were bought from Cayman Chemical (Ann Arbor, MI, USA). UPLC grade methanol, D-(+)-glucose, HPLC grade n-hexane and ATP (disodium salt) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Ultrapure tris(hydroxymethyl)aminomethane (TRIS), acrylamide 4K solution 30%, dimethyl sulfoxide (DMSO) were purchased from AppliChem GmbH (Darmstadt, Germany). LC-MS grade methanol, acetonitrile and acetic acid were bought from Fisher Scientific (Schwerte, Germany). HPLC grade ethyl acetate was from VWR (Darmstadt, Germany).

6.2.2 Purification of polymorphonuclear leukocytes (PMNL) from human leukocyte concentrates

Human PMNL were freshly isolated from leukocyte concentrates (Institut für Transfusionsmedizin und Immunhämatologie, DRK-Bluspendedienst, Frankfurt, Germany). Blood was drawn with the informed consent of the patients. For PMNL purification, total leukocytes were separated from erythrocytes by dextran sedimentation for 30 min. After this, leukocyte subsets were isolated on NycoPrep cushions (PAA Laboratory, Pasching, Austria) by centrifugation (10 min without deceleration) at 800 *g*. After this, the supernatant as well as the separation medium were removed resulting in the crude PMNL pellet. To get rid of remaining erythrocyte contaminations in the PMNL fraction, the cell pellet was then subjected to hypotonic lysis with ice-cold water for 45 sec. After this, the lysis was stopped by addition of PBS followed by centrifugation. The resulting erythrocyte free PMNL pellet was then resuspended in PGC buffer (PBS + 1 mg/mL glucose), counted and kept on ice until further processing.

6.2.3 PMNL homogenates and 100,000 g supernatants (S100)

PMNL homogenates were prepared by sonicating (Sonoplus HD 200, sonotrode MS72, BANDELIN electronic GmbH & Co, Berlin, Germany) a

suspension of freshly isolated PMNL in ice-cold PBS/EDTA (PBS + 1 mM EDTA) 3 times at 90% intensity for 10 s at 4 °C. Cell concentration was 107 human PMNL per 500 µL PBS/EDTA. To prepare PMNL 100,000 *g* supernatants (S100), PMNL homogenates were additionally subjected to an ultracentrifugation step at 100,000 *g* (70 min, 4 °C). The resulting supernatants were then further used for activity assays.

6.2.4 Recombinant 5-LO

Recombinant human 5-LO was expressed in Escherichia coli BL21 (DE3). For this the cells were transformed with the plasmid pT3-5-LOX (kind gift of Prof. Olof Rådmark, Karolinska Institutet, Stockholm, Sweden). The protein was expressed and purified as previously described using an ÄKTA Xpress system (GE Healthcare, Uppsala, Sweden) [22].

6.2.5 Lipid mediator formation assays

Activity assays with intact cells were carried out with 10^7 freshly isolated human PMNL resuspended in 500 µL PGC buffer (PBS + 1mg/mL glucose) supplemented with $CaCl_2$ (final concentration 1 mM). Lipid mediator formation was triggered by addition of 10 µM SPM precursor oxylipins (15(*S*)-HETE, 15(*S*)-HpETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*S*)-HpDHA, 17(*R*)-HDHA or 18(*R/S*)-HEPE) or ARA plus 5 µM Ca^{2+} ionophore A23187 for 15 min at 37 °C in a water bath. The reaction was terminated by addition of 500 µL ice-cold methanol. After sonication, the cell supernatants were then frozen at -80 °C until further analysis by LC-MS/MS. In incubations where the FLAP inhibitor Mk-886 was employed, PMNL were pre-incubated with the inhibitor or its vehicle control (DMSO) for 15 min (37 °C, water bath) prior to the addition of Ca^{2+} ionophore A23187.

For activity assays with broken PMNL preparations, homogenates or S100 corresponding to 10^7 PMNL were diluted in 500 µL PGC buffer supplemented with $CaCl_2$ (final concentration 2 mM) per sample. For assays employing purified

human 5-LO, 1.5 µg of the recombinant protein were diluted in 500 µL PGC buffer supplemented with $CaCl_2$ (1 mM). After addition of ATP (final concentration 1 mM), the samples were pre-heated for 30 seconds in a water bath. After this, the reaction was started by addition of 10 µM SPM precursor oxylipins (15(*S*)-HETE, 15(*S*)-HpETE, 15(*R*)-HETE, 17(*S*)-HDHA, 17(*S*)-HpDHA, 17(*R*)-HDHA or 18(*R/S*)-HEPE), ARA or DHA for 10 min at 37 °C. Finally, the reaction was terminated by addition of 500 µL ice-cold methanol. For further processing, all samples were sonicated after assay termination and immediately frozen at -80 °C until further analysis by LC-MS/MS. Tab. 6.1 shows the impurities and autoxidation products of the different SPM precursor oxylipins and their effective concentrations in the activity assays.

6.2.6 Extraction and analysis of lipid mediators

Eicosanoids and other oxylipins were analyzed as described [23-25]. In brief, antioxidants and inhibitors as well as and internal standards were added to the sonicated cell suspension. Cell suspensions from incubations with hydroperoxy PUFAs were additionally treated with 25 µL $SnCl_2$ (10 mg/mL in methanol) to reduce the hydroperoxy PUFAs to their corresponding hydroxyl PUFAs. Following protein precipitation, the lipid mediators were extracted by solid phase extraction on a mixed mode anion exchange/reversed phase column (Bond Elute Certify II, Agilent). Separation was carried out on a Zorbax Eclipse Plus C18 reversed phase column (2.1 × 150 mm, particle size 1.8 µm, pore size 9.5 nm). Detection was carried out following electrospray ionization in negative mode by a triple quadrupole mass spectrometer (Sciex QTRAP 5500) operated in scheduled selected reaction monitoring mode.

Tab. 6.1: Hydroxy-PUFA impurities, autoxidation products and effective concentrations used in the activity assays. Hydroxy-PUFAs were incubated in PGC buffer without addition of cells, cell preparations or recombinant 5-LO during the activity assays (background controls). HydroxyPUFA concentrations are shown in nM. Effective concentrations of the monohydroxy-PUFA SPM precursors are marked in bold red. Oxylipins in concentrations below 0.01 % of the total monohydroxyPUFAs (15-HETE, 18-HEPE, 17-HDHA) added were considered unspecific and are depicted as < 0.01% in light grey. nd, not determined.

[nM]	15-HETE		17-HDHA		18-HEPE
	(*S*)	(*R*)	(*S*)	(*R*)	(*R/S*)
5(*R/S*)-HETE	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
12(*R/S*)-HETE	1	15	15	1	nd
15(*R/S*)-HETE	**12689**	**12292**	4	3	20
5(*S*),15(*S*)-DiHETE	17	10	< 0.01 %	< 0.01 %	< 0.01 %
5(*S*),12(*S*)-DiHETE	8	13	< 0.01 %	< 0.01 %	nd
8(*S*),15(*S*)-DiHETE	2	2	< 0.01 %	< 0.01 %	< 0.01 %
5(*S*),6(*R*)-DiHETE	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
5(*S*),6(*S*)-DiHETE	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
LTB_4	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
6-trans-LTB_4	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
6-trans-12-epi-LTB_4	< 0.01 %	< 0.01 %	< 0.01 %	1	nd
LxA_4	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
15(*R*)-LxA_4	< 0.01 %	< 0.01%	< 0.01 %	< 0.01 %	< 0.01 %
6(*S*)-LxA_4	< 0.01%	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
LxB_4	< 0.01%	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
5(*R/S*)-HEPE	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
12(*R/S*)-HEPE	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
15(*R/S*)-HEPE	28	< 0.01 %	10	< 0.01 %	nd
18(*R/S*)-HEPE	< 0.01%	5	26	5	**11011**
RvE1	< 0.01 %	< 0.01 %	1	1	< 0.01 %
18(*R*)-RvE2	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	7
18(*S*)-RvE3	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	5
18(*R*)-RvE3	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	1
4(*R/S*)-HDHA	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
7(*R/S*)-HDHA	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	nd
14(*R/S*-)HDHA	< 0.01 %	< 0.01 %	214	1	nd
17(*R/S*)-HDHA	< 0.01%	< 0.01%	**7708**	**5664**	< 0.01 %
RvD1	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
17(*R*)-RvD1	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
RvD2	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
RvD3	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
RvD5	< 0.01 %	< 0.01 %	< 0.01 %	2	< 0.01 %
MaR1	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
7epi-MaR1	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %	< 0.01 %
PDx	< 0.01 %	< 0.01 %	< 0.01 %	2	< 0.01 %

6.2.7 SDS-PAGE/Western Blotting

Western Blotting samples were obtained by lysis of freshly isolated PMNL or the pellet resulting after 100,000 *g* centrifugation of the PMNL homogenates (P100) with SDS buffer (2.2% SDS, 11% glycerol, 56 mM TrisHCl). For this, the boiling buffer was added to the cell pellets followed by further incubation of the samples at 96 °C for 5 minutes to assure complete protein denaturation. S100 protein lysates were obtained by addition of ice-cold methanol to the S100 fraction followed by overnight precipitation of the proteins at -20 °C. The resulting protein pellet was also lysed in hot SDS buffer as described above. Protein concentrations of the lysates were determined using the Pierce bicinchoninic acid method according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA) employing a microplate reader (infinite M200, Tecan Group Ltd., Crailsheim, Germany). Equal quantities of the cell lysates were separated on 10% or 16% polyacrylamide gels via electrophoresis (SDS-PAGE) and proteins were electrophoretically blotted onto nitrocellulose membranes (Odyssey, LI-COR Biosciences, Bad Homburg, Germany). Membranes were then incubated in Odyssey blocking reagent (LI-COR Biosciences, Bad Homburg, Germany) followed by treatment with the respective primary antibodies directed against either 5-LO (sc-515821, Santa Cruz Biotechnology, Heidelberg, Germany), 15-LO-1 (ab119774, abcam, Cambridge, UK), 15-LO-2 (sc271290, Santa Cruz Biotechnology, Hamburg, Germany), FLAP (ab53536, abcam, Cambridge, UK) or β-actin (sc-47778 or sc-1616, Santa Cruz Biotechnology, Heidelberg, Germany). After several washing steps and incubation with the corresponding IRDye680- or IRDye800-conjugated secondary antibodies (LI-COR Biosciences, Bad Homburg, Germany), immunoreactive bands were visualized on the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

6.3 Results

6.3.1 Formation of lipoxygenase-derived lipid mediators in human PMNL stimulated with Ca2+ ionophore (A23187)

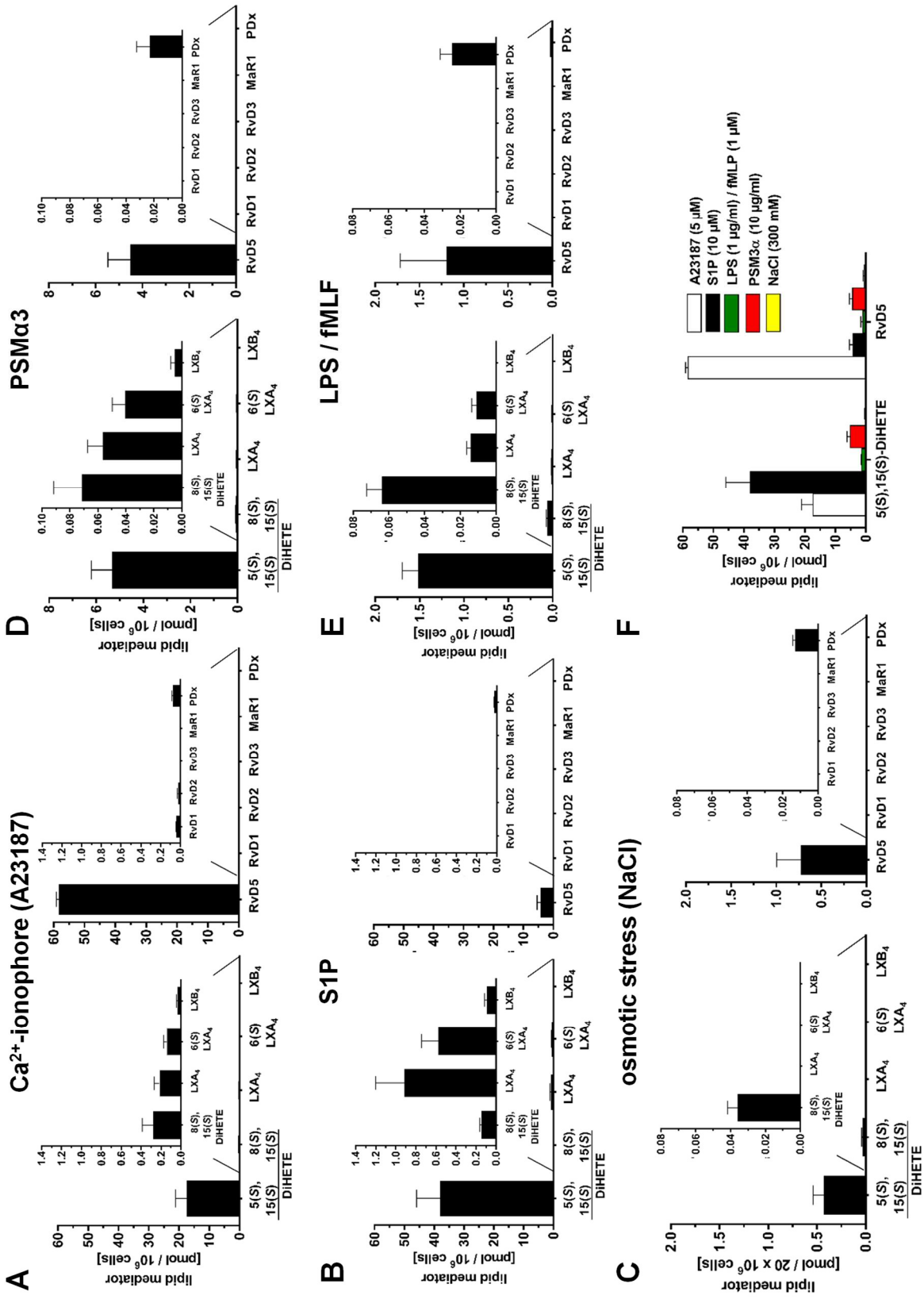
In order to investigate the formation of SPM from human neutrophils, the PMN fraction was isolated from leukocyte concentrates. In addition to neutrophils, PMNL may contain small numbers of eosinophils which express 15-LO-1 in addition to 5-LO. To ascertain proper cell isolation and processing as well as to enable correct data interpretation, the expression patterns of 5-LO as well as 15-LO-1, 15-LO-2 and FLAP were assessed in intact PMNL as well as PMNL 100,000 *g* supernatants (S100) and pellets (P100) (Fig. 6.3E). As expected, intact human PMNL expressed high amounts of 5-LO and FLAP.

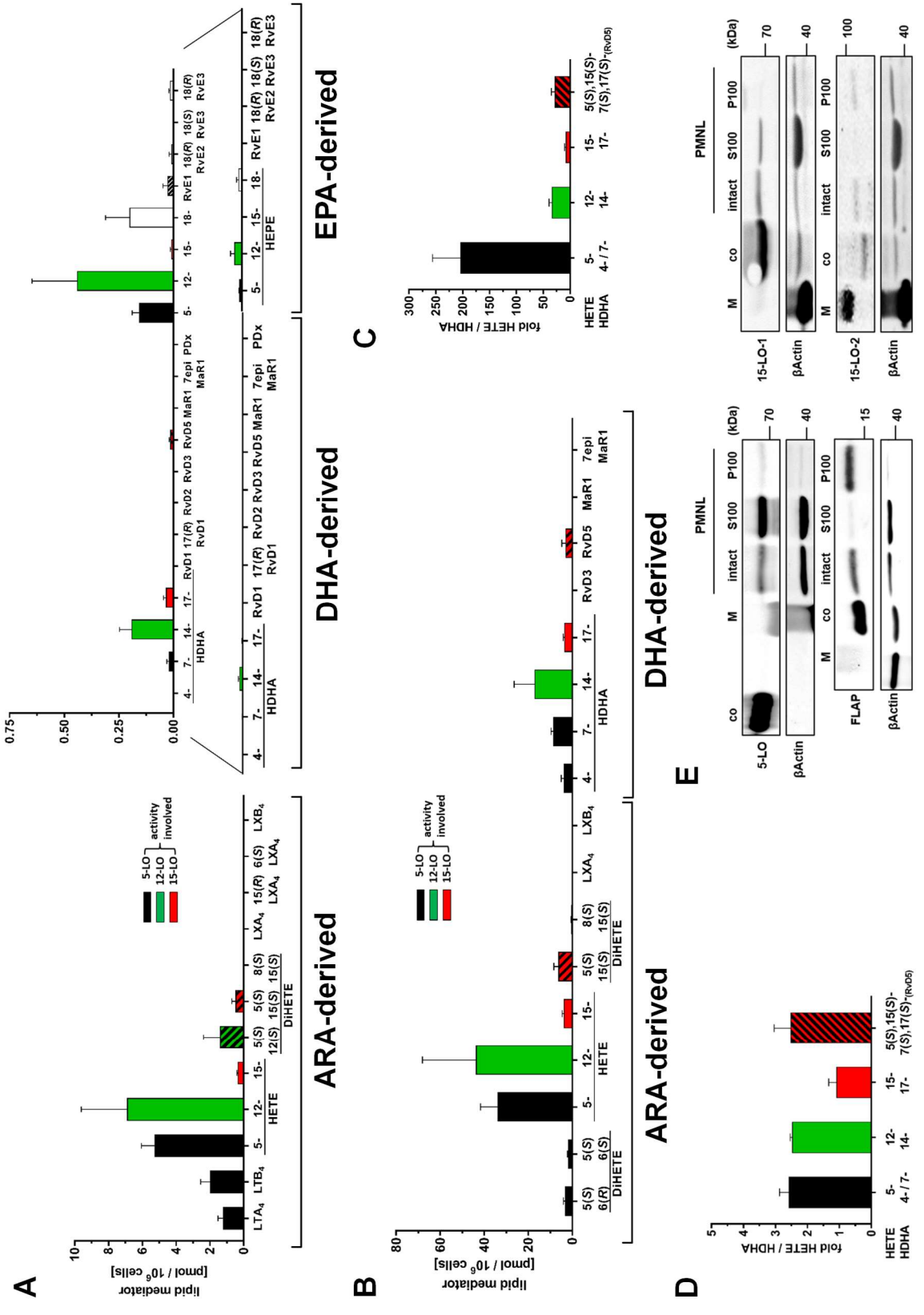
When the cell integrity was destroyed and the corresponding homogenates were subjected to 100,000 *g* centrifugation, 5-LO was found in the soluble fraction (S100) whereas the integral membrane protein FLAP was found in the pellet (P100). All donors displayed low 15-LO-1 expression confirming the presence of small numbers of eosinophils in our preparations. Comparable to 5-LO, 15-LO-1 was found in the soluble fraction after 100,000 *g* centrifugation. 15-LO-2 was not detected in any donor.

Before conducting the experiments, different stimuli were tested for their SPM inducing capacity in human PMNL (Fig. 6.2A-E). From this, we chose Ca^{2+} ionophore as most potent inducer of lipoxin and resolvin biosynthesis (Fig. 6.2F). First, we investigated the lipid mediator formation from endogenous lipid stores in human PMNL. For this, PMNL were stimulated with 5 µM Ca^{2+} ionophore A23187 for 15 minutes at 37 °C (Fig. 6.3A). While PMNL formed substantial amounts of ARA-derived lipid mediators, formation of EPA- and DHA-based hydroxylated PUFAs was considerably lower. The monohydroxylated ARA metabolites 5- and 12-HETE showed the highest formation of all lipid mediators measured (5.3 ± 0.8 pmol/10^6 cells and 6.9 ± 2.7 pmol/10^6 cells) followed by LTB_4

(2 ± 0.5 pmol/ 10^6 cells). The 5-LO-derived non-enzymatic hydrolysis products of LTA_4 [5(*S*),6(*S*)-diHETE, 5(*S*),6(*R*)-diHETE, 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4 - summed up in the graph as LTA_4] and the 5-LO/12-LO double oxygenation product 5(*S*),12(*S*)-diHETE were found in low amounts of 1.25 ± 0.3 pmol/10^6 cells and 1.4 ± 1 pmol/106 cells, respectively.

Fig. 6.2 (right, page 129): Comparison of lipid mediator formation from different stimuli. Cells were stimulated with either (A) 5 µM Ca^{2+} ionophore, (B) 10 µM sphingosine-1-phosphate (S1P), (C) osmotic stress (0.3 M NaCl), (D) 10 µg/ml phenol-soluble modulin α3 (PSMα3) or (E) 1 µM N-formyl-leucyl-phenylalanine (fMLF) at 37 °C in presence of 10 µM 15(*S*)-HETE or 17(*S*)-HDHA. fMLF incubations were additionally primed with 1 µg/ml bacterial lipopolysaccharides (LPS) for 20 min at RT prior stimulation. After 15 minutes of stimulation, the reactions were terminated by addition of ice-cold methanol and lipid mediators in the samples were quantified by LC-MS/MS. (F) Comparison of 5(*S*),15(*S*)-diHETE and RvD5 formation efficiency of the different stimuli. All data are depicted as mean + SEM from 3 independent experiments.


A
Ca2+-ionophore (A23187)
B
S1P
C
osmotic stress (NaCl)
D
PSMα3
E
LPS / fMLF
F
A23187 (5 µM)
S1P (10 µM)
LPS (1 µg/ml) / fMLP (1 µM)
PSM3α (10 µg/ml)
NaCl (300 mM)
lipid mediator
[pmol / 10^6 cells]
[pmol / 20 x 10^6 cells]
RvD5 RvD1 RvD2 RvD3 MaR1 PDx
5(S),15(S)-DiHETE
8(S),15(S) DiHETE
LXA4
LXB4
6(S) LXA4


A
B
C
D
E
ARA-derived
DHA-derived
EPA-derived
lipid mediator [pmol / 10^6 cells]
5-LO
12-LO
15-LO
activity involved
LTA4 LTB4 5-HETE 12-HETE 15-HETE 5(S),12(S)-DiHETE 5(S),15(S)-DiHETE LXA4 15(R)-LXA4 8(S)-LXA4 LXB4 6(S)-LXA4
5(S),6(R)-DiHETE 5(S),6(S)-DiHETE 5(S),15(S)-DiHETE 8(S),15(S)-DiHETE
4-HDHA 7-HDHA 14-HDHA 17-HDHA 17-HETE RvD1 17(R)-RvD1 RvD2 RvD3 RvD5 MaR1 7epi-MaR1 PDx
5-HEPE 12-HEPE 15-HEPE 18-HEPE RvE1 18(R)-RvE2 18(S)-RvE3 18(R)-RvE3
fold HETE / HDHA
HETE
HDHA
5(S),15(S)-7(S),17(S)-(RvD5)
PMNL
M co intact S100 P100 (kDa)
5-LO
15-LO-1
15-LO-2
FLAP
βActin

Formation of 15-LO-dependent 15-HETE and 5(*S*),15(*S*)-diHETE was even lower (0.4 ± 0.04 pmol/10^6 cells and 0.5 ± 0.2 pmol/10^6 ± cells, respectively). No lipoxins were detected in the incubations. Compared to the ARA metabolites, EPA-derived lipid mediator formation was much lower. Only small amounts of 5- and 12-HEPE (0.2 ± 0.03 pmol/10^6 cells and 0.4 ± 0.2 pmol/10^6 cells, respectively) as well as 18-HEPE (0.2 ± 0.1 pmol/10^6 cells) were detected. In addition, even lower amounts of 15-HEPE, RvE1 and 18(*R*)-RvE3 were found. Comparable to EPA, formation of DHA-derived lipid mediators was very low. Again, the 12-LO product 14-HDHA showed the highest formation of all DHA products (0.2 + 0.06 pmol/10^6 cells) followed by 17-HDHA (0.03 ± 0.01 pmol/10^6 cells), 7-HDHA, 4-HDHA as well as RvD5. RvD1, RvD2, RvD3, PDx and maresins were below the detection limit in these incubations. Comparison of the corresponding ARA and DHA mono- and dihydroxylated PUFAs for each lipoxygenase action showed that the ARA metabolites were always more abundant in PMNL (Fig. 6.3C). 5-LO-derived 5-HETE formation was 205-fold higher compared to the sum of both corresponding DHA metabolites 4- and 7-HDHA. 12-HETE was about 34-fold higher compared to 14-HDHA and 15-HETE was 8-fold higher than 17-HDHA. Also, 5(*S*),15(*S*)-diHETE exceeded RvD5 formation by 29-fold.

Fig. 6.3 (left, page 130): Enzyme expression and lipoxygenase activities in stimulated human PMNL. Lipid mediator formation in PMNL was stimulated with 5 µM Ca^{2+} ionophore **(A)** or with 2.5 µM Ca^{2+} ionophore in presence of an ARA/DHA mix (10 µM each) **(B)** for 15 **(A)** or 10 **(B)** minutes at 37 °C. After this, the reaction was terminated by addition of ice-cold methanol and lipid mediators in the samples were quantified by LC-MS/MS. Data are depicted as mean ± SEM from 6 **(A)** and 4 **(B)** independent experiments. Ratio of corresponding ARA and DHA LO metabolites (HETE:HDHA) from A **(C)** and B **(D)**. **(E)** Western Blots from PMNL lysates. After isolation the cells were either directly lysed (intact) or homogenized by sonication followed by 100,000 *g* centrifugation resulting in a soluble fraction (S100) and the pelleted insoluble membrane fraction (P100). Proteins in the S100 were precipitated with methanol. S100 proteins and P100 pellets were then solubilized in boiling SDS buffer and equal quantities of the lysates were electrophoretically separated followed by Western Blotting. Recombinant human 5-LO or FLAP, 15-LO-1 and 15-LO-2 overexpressing 239T cells were used as positive controls. One representative experiment out of 4 is shown. Co, control; M, molecular weight marker.

This dominance of ARA over DHA products might be due to a net enzymatic preference of ARA in PMNL. To investigate this further, we supplemented PMNL with a mixture of ARA and DHA (10 µM each) during stimulation with Ca^{2+} ionophore (Fig. 6.3B). This exogenous PUFA supplementation resulted in an overall increase in hydroxylated PUFAs with the DHA metabolites being more heavily affected. Nevertheless, ARA-derived lipid mediators still dominated the 5- and 12-lipoxygenation products although the gap between DHA and ARA metabolites was much smaller (3-fold for 5-HETE and 2-fold for 12-HETE and 5(*S*),15(*S*)-diHETE) compared to PMNL stimulated in absence of exogenous PUFAs (Fig. 6.3D). Interestingly, 15-LO-derived 15-HETE and 17-HDHA were formed in equal amounts under these conditions. Although simultaneous supplementation with ARA and DHA resulted in an upregulation of 5(*S*),15(*S*)-diHETE and its DHA-derived analogue RvD5, other SPMs were not detected in our incubations.

6.3.2 Lipid mediator formation in human PMNL supplemented with 15-HETE, 18-HEPE or 17-HDHA

PMNL alone did not form substantial amounts of SPM upon stimulation with Ca^{2+} ionophore even though 5-LO and 15-LO-1 expression was found in the incubations. Apparently, endogenous formation of the hydroxylated PUFAs that serve as 5-LO substrates for the formation of SPMs (15-H(p)ETE, 17-H(p)DHA, 18-H(p)EPE) was too low.

In order to mimic possible transcellular biosynthetic pathways where SPM precursors are released from cells or tissues expressing 15-LO and taken up by PMNL, we treated PMNL with the precursors for lipoxins (15(*S*)- and 15(*R*)-HETE), E-series resolvins (18(*R/S*)-HEPE), D-series resolvins and protectins (17(*S*)- and 17(*R*)-HDHA). For this, PMNL were stimulated with Ca^{2+} ionophore in the presence of each precursor (10 µM).

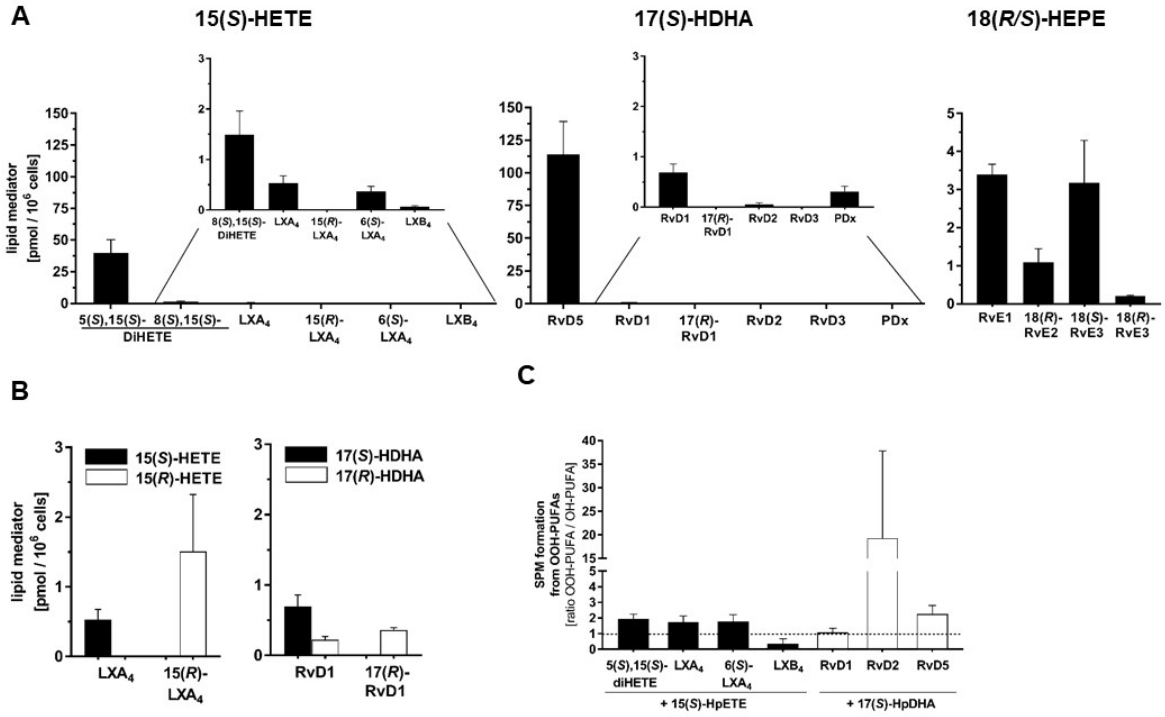


Fig. 6.4: Lipid mediator formation in intact human PMNL supplemented with 15-HETE, 18-HEPE or 17-HDHA. **(A)** Freshly isolated human PMNL were resuspended in PGC buffer (2×10^7 cells/mL) followed by stimulation with 5 µM Ca^{2+} ionophore at 37 °C. In addition, 10 µM of the respective monohydroxylated PUFA were added. The reaction was terminated after 15 minutes by addition of ice-cold methanol. The lipid mediators formed in these incubations were then quantified by LC-MS/MS. Data are depicted as mean ± SEM from 4-5 independent experiments. **(B)** Comparison of LxA_4 and RvD1 formation upon stimulation with the 15-HETE or 17-HDHA enantiomers. **(C)** Comparison of SPM formation from 10 µM hydroxyl PUFA and hydroperoxy PUFA precursors in intact human PMNL. Data are depicted as mean ± SEM from 3 independent experiments.

15(*S*)-HETE supplementation led to a substantial increase in the 5-LO-dependent 15-HETE metabolite 5(*S*),15(*S*)-diHETE from 0.5 ± 0.2 pmol/10^6 cells treated with ionophore only to 40.1 ± 10.2 pmol/10^6 cells in precursor supplemented cells (Fig. 6.4A). In contrast to the ionophore only treated cells, LxA4, 6(*S*)-LxA_4 and LxB_4 formation was detectable in the precursor-supplemented preparations but lipoxin yields were about 75, 110 and 570 times lower (LxA4 0.5 ± 0.1 pmol/10^6 cells; 6(*S*)-LxA_4 0.4 ± 0.1 pmol/10^6 cells; LXB4 0.07 ± 0.02 pmol/10^6 cells) compared to 5(*S*),15(*S*)-diHETE. When 17(*S*)-HDHA was used instead of 15(*S*)-HETE, RvD5 levels were substantially elevated from

0.02 ± 0.005 pmol/10^6 cells to 114.2 ± 25.1 pmol/106 cells. Interestingly, utilization of 17(*S*)-HDHA was about 4 times more efficient compared to 15(*S*)-HETE and even about 30 times higher than 18(*R/S*)-HEPE (Fig. 6.6C). In addition to RvD5, only small amounts of RvD1 (0.7 ± 0.2 pmol/10^6 cells) and PDx (0.3 ± 0.1 pmol/10^6 cells) were detected. 18(*R/S*)-HEPE supplementation resulted in small amounts of RvE1 (3.4 ± 0.3 pmol/10^6 cells), 18(*R*)-RvE2 (1.1 ± 0.4 pmol/10^6cells), 18(*S*)-RvE3 (3.2 ± 1.1 pmol/10^6cells) and 18(*R*)-RvE3 (0.2 ± 0.03 pmol/10^6cells). Furthermore, we compared both 15-HETE and 17-HDHA enantiomers and found that formation of 15(*R*)-LxA_4 from 15(*R*)-HETE was twofold higher compared to LxA_4 while formation of 17(*R*)-RvD1 from 17(*R*)-HDHA was about half compared to RvD1 derived from 17(*S*)-HDHA (Fig. 6.4B).

In order to investigate if the capacity for SPM biosynthesis substantially differs between the hydroxy-PUFAs (15-HETE, 17-HDHA, 18-HEPE) and their corresponding hydroperoxy precursors (15-HpETE, 17-HpDHA, 18-HpEPE), we compared SPM formation from 10 µM 15(*S*)-HETE and 15(*S*)-HpETE as well as from 17(*S*)-HDHA and 17(S)-HpHDA in intact PMNL (Fig. 6.4C). Since 18-HpEPE is not commercially available, E-series resolvins could not be tested in this assay. Formation of 5(*S*),15(*S*)-diHETE, LxA4 and 6(*S*)-LxA_4 was increased by a factor of 2 in 15(*S*)-HpETE compared to 15(*S*)-HETE treated cells, while LXB_4 formation was lower from the hydroperoxy precursor. D-resolvin formation from 17(*S*)-HpDHA was more efficient for RvD5 (2-fold) and RvD2 (20-fold) but had no influence on RvD1 formation.

6.3.3 Influence of cell integrity on SPM formation in human PMNL

In the following experiments, PMNL cell integrity was destroyed by sonication to investigate if 5-LO-dependent SPM formation is based on an intact cellular environment or if 5-LO can form SPM independently of other cellular factors in neutrophils. Cell homogenates corresponding to 2 × 10^7 cells/mL were supplemented with 10 µM of the SPM precursor lipids (15-HETE, 18-HEPE or 17-HDHA) and the SPM synthesis capacity was determined in presence of 1 mM

ATP. In addition, the homogenates were further subjected to 100,000 *g* centrifugation in order to remove the membrane fraction. Then, the resulting soluble fraction (S100) containing 5-LO and 15-LO-1 as well as other soluble PMNL proteins was tested for SPM formation in presence of 10 µM SPM precursors and 1 mM ATP. Intact PMNL stimulated with Ca^{2+} ionophore and supplemented with the SPM precursors served as controls.

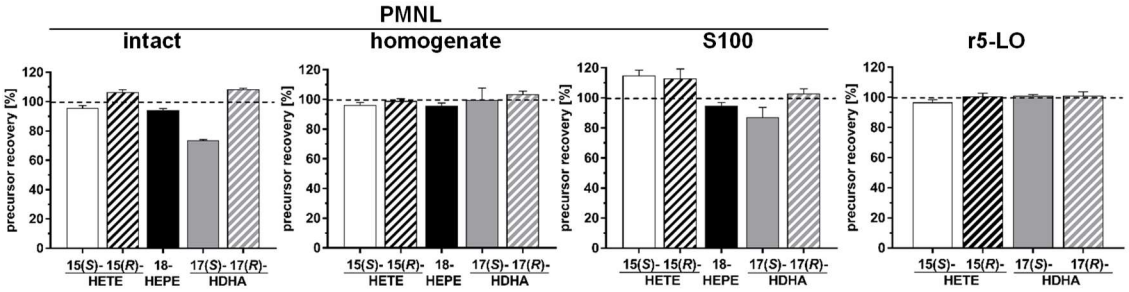


Fig. 6.5: Total precursor recovery. Percent recovery for each monohydroxylated PUFA in intact PMNL, PMNL homogenates, PMNL 100,000 *g* preparations (S100) as well as with recombinant 5-LO. Values were calculated as the sum of the non-metabolized monohydroxylated PUFA plus the metabolites in relation to the total monohydroxylated PUFA added to each assay.

First, we had a look on the total precursor recovery (proportion of the sum of unchanged monohydroxy-PUFAs plus corresponding metabolites to the total monohydroxy-PUFAs added) in the samples to assure that our method captured all relevant metabolites formed from the SPM precursors in the PMNL incubations (Fig. 6.5). Total precursor recovery of 15(*S*)- and 15(*R*)-HETE, 18(*R/S*)-HEPE and 17(*R*)-HDHA added up to almost 100% or more in intact PMNL. Interestingly, 17(*S*)-HDHA total precursor recovery was only 74% in intact cells suggesting that not all metabolites were captured in our LC-MS/MS method. Total precursor recovery in PMNL homogenates was about 100% for all hydroxylated PUFAs. A loss of lipids due to adherence to plastic ware or cellular debris that might hamper lipid analysis could therefore be ruled out. With the exception of 17(*S*)-HDHA (87%), recovery in S100 preparations was again 100%.

Next, we investigated the formation of lipid mediators from PMNL homogenates and S100 preparations compared to intact cells (Fig. 6.6A). Unexpectedly, destruction of PMNL cell integrity led to a 5-fold increase in 5(*S*),15(*S*)-diHETE (241.9 ± 12.5 pmol/10^6 cells) and a 9-fold increase in LxA_4

formation (4.8 ± 0.4 pmol/10^6 cells) in 15(*S*)-HETE treated homogenates compared to intact cells. 8(*S*),15(*S*)-diHETE, 6(*S*)-LxA_4 and LXB_4 levels also increased. Removal of insoluble cell components such as membrane fragments and organelles by 100,000 *g* centrifugation (S100) resulted in even higher 5(*S*),15(*S*)- and 8(*S*),15(*S*)-diHETE levels and had only a low inhibitory effect on the elevated LxA_4 and 6(*S*)-LxA_4 formation compared to the homogenates. Interestingly, PMNL homogenates supplemented with 17(*S*)-HDHA or 18(*R/S*)-HEPE showed the opposite effect. Here, biosynthesis of RvD1, RvD5, RvE1 and 18(*R*)-RvE2 that was detected in the intact cells was inhibited in the homogenates about 2- to 3-fold upon destruction of cell integrity. In contrast, formation of PDx was not impaired. When we used PMNL S100 preparations instead of the homogenates in the activity assay, resolvin and PDx formation was completely abrogated (Fig. 6.6A).

For comparison, leukotriene formation from ARA in intact PMNL, homogenates and S100 was investigated (Fig. 6.6B). For this, intact cells and cell preparations were supplemented with 10 µM ARA instead of the monohydroxy-PUFAs. Interestingly, destruction of cell integrity impaired 5-HETE formation while 15-HETE and LTB_4 levels were 2-fold elevated. 12-HETE formation was not influenced.

In contrast to intact PMNL where 17(*S*)-HDHA is preferentially metabolized, destruction of cell integrity and 100,000 *g* centrifugation reversed this effect: In homogenates utilization of 15(*S*)-HETE exceeded that of 17(*S*)-HDHA by 24-fold. Even more, in S100 preparations 15(*S*)-HETE was preferred 126-fold over 17(*S*)-HDHA (Fig. 6.6C, D).

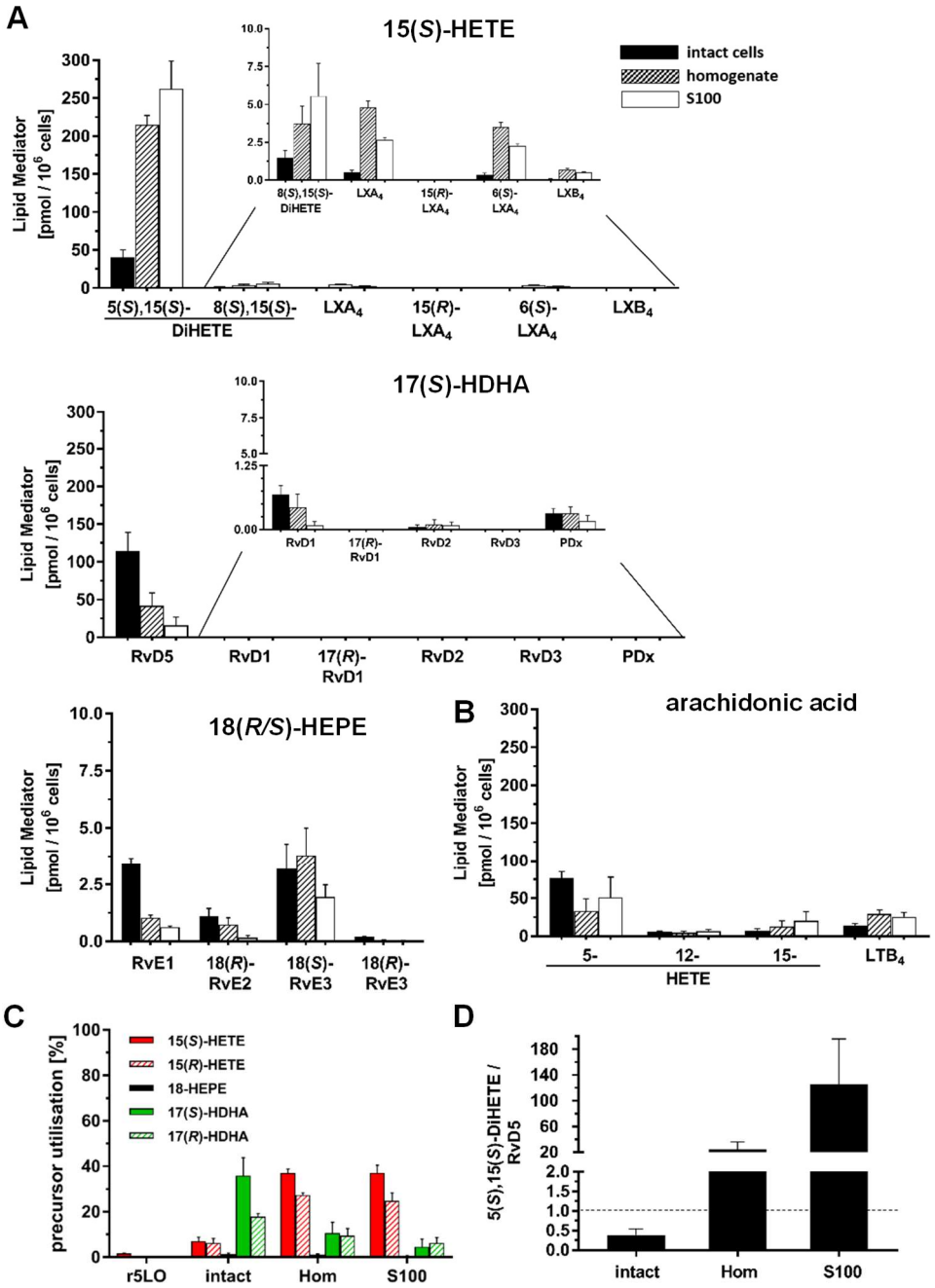


Fig. 6.6: Influence of cell integrity on SPM formation in human PMNL supplemented with 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA. **(A)** Freshly isolated PMNL were homogenized by sonication followed by 100,000 *g* centrifugation. Resulting homogenates and 100,000 *g* supernatants (S100) corresponding to (2×10^7 cells/mL) were supplemented with 10 µM 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA in presence of 1 mM ATP and Ca^{2+}. Intact PMNL (2×10^7 cells/mL) stimulated with 5 µM Ca^{2+} ionophore and supplemented with 10 µM of the respective monohydroxylated PUFA served as controls. The reaction was terminated after 15 minutes by addition of ice-cold methanol. Lipid mediators formed in these incubations were then quantified by LC-MS/MS. **(B)** Cells were treated with 10 µM ARA instead of the monohydroxylated PUFAs and lipid mediator formation was quantified via LC-MS/MS [44]. Data are depicted as mean ± SEM from 4-8 independent experiments. **(C)** Precursor utilisation calculated as sum of all metabolites detected from a respective monohydroxylated PUFA in relation to the total monohydroxylated PUFA added. **(D)** Ratio of 5(*S*),15(*S*)-diHETE to the corresponding DHA metabolite RvD5.

6.3.4 Influence of FLAP inhibition on SPM formation in human PMNL

FLAP dependency has been shown for lipoxin, 5(*S*),15(*S*)-diHETE and RvD1 formation [13, 26]. To investigate this further, intact PMNL were pre-treated with 3 µM of the FLAP inhibitor Mk-886 followed by stimulation with 5 µM Ca^{2+} ionophore in presence or absence of 15(*S*)-HETE, 17(*S*)-HDHA or 18(*R/S*)-HEPE (10 µM). Cells treated with 10 µM ARA instead of the monohydroxy-PUFAs were used as control. Inhibition of FLAP resulted in an overall shift from 5-LO to 12-/15-LO products in PMNL treated with ARA (Fig. 6.7B): 5-LO-derived 5-HETE and LTB4 were strongly inhibited (residual activity below 10%) while 15-HETE levels were elevated about 3.2-fold. 12-HETE formation was also augmented by Mk-886 treatment although to a lower extent (~ 1.6-fold). When cell integrity was disrupted in ARA supplemented PMNL, the inhibitory effect of MK-886 on 5-LO activity was completely abolished and no shunting into other LO pathways was detectable. Intact PMNL stimulated with Ca^{2+}-ionophore in the absence of exogenously added fatty acids displayed a lower formation of 5-HETE and LTB_4 as well as an increase in 15-HETE but no effect on 12-HETE was detectable (Fig. 6.7A). This effect was also found for the DHA- and EPA-derived monohydroxylated PUFAs.

In intact PMNL supplemented with 15(*S*)-HETE, 17(*S*)-HDHA or 18(*R/S*)-HEPE instead of ARA, formation of 5(*S*),15(*S*)-diHETE, LxA_4, 6(*S*)-LxA4, RvD1, RvD5 and RvE1 was potently inhibited by Mk-886 supporting the FLAP dependency of their biosynthesis (Fig. 6.7B).

Destruction of cell integrity led to a loss of inhibition of 5(*S*),15(*S*)-diHETE, RvD1 and RvD5 formation in homogenates (residual activities being 91%, 110% and 89%, respectively). Interestingly, inhibition of LxA_4 and 6(*S*)-LxA_4 formation was not completely rescued (residual activity 66%) in the cell homogenates. Further 100,000 *g* centrifugation completely abolished the inhibitory effect of Mk-886 seen in the intact cells and also elevated the formation of RvD5 and PDx. As expected, formation of 8(*S*),15(*S*)-diHETE and PDx was barely affected by MK-886 treatment since 5-LO is not involved in their biosynthesis.

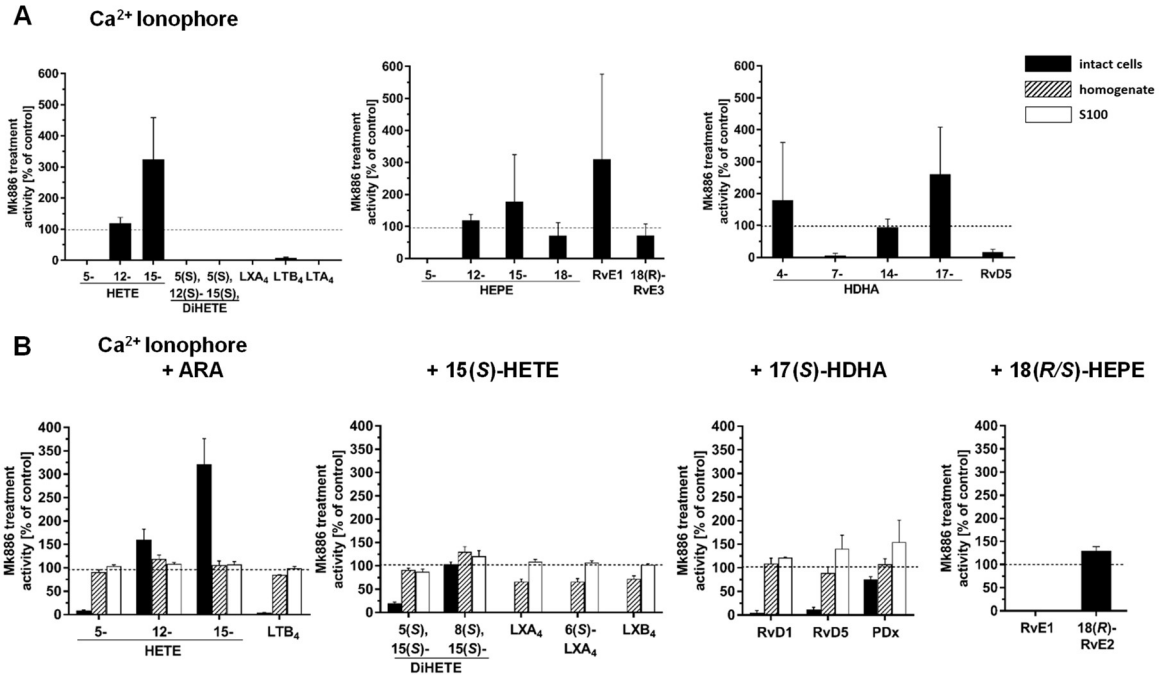


Fig. 6.7: Influence of FLAP inhibition on SPM formation in human PMNL supplemented with 15(*S*)-HETE, 18(*R/S*)-HEPE or 17(*S*)-HDHA. **(A)** Intact PMNL were resuspended in PGC-buffer and preincubated with Mk-886 (3 µM) for 15 minutes followed by stimulation with 5 µM Ca^{2+} ionophore for 15 minutes at 37 °C. **(B)** Either 2 × 10^7/mL intact or homogenized PMNL or PMNL homogenates centrifuged at 100,000 *g* (S100) were preincubated with the FLAP inhibitor Mk-886 (3 µM) for 15 minutes. The reaction was started by addition of 10 µM of 15(*S*)-HETE, 18(*R/S*)-HEPE, 17(*S*)-HDHA or ARA together with 5 µM Ca^{2+} ionophore (intact cells) or 1 mM ATP (homogenates, S100). Reactions were terminated after 15 minutes with ice-cold methanol and the lipid mediators formed were quantified using LC-MS/MS. Data on lipid mediator formation in ARA treated PMNL were measured according to [44]. Data are depicted as mean + SEM from 3-4 independent experiments.

6.3.5 Recombinant human 5-LO alone is not able to form SPM

Due to the unexpected upregulation of lipoxin and 5(*S*),15(*S*)-diHETE formation in PMNL homogenates and S100 preparations, recombinant purified human 5-LO (r5-LO) was assayed in presence of 15(*S*)-HETE or 17(*S*)-HDHA and compared to ARA.

Upon ARA supplementation, r5-LO released a number of hydroxylated products (Fig. 6.8A). The non-enzymatic hydrolysis products of LTA4, 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4 (855 ± 51 pmol/3µg protein and 1406 ± 88 pmol/3µg protein) were the most abundant products found in the incubations followed by 5-HETE (370 ± 38 pmol/3 µg protein). In addition, small amounts of LTA_4-derived 5(*S*),6(*R*)- and 5(*S*),6(*S*)-diHETE were formed. When r5-LO was supplemented with 15(*S*)-HETE the enzyme released minute amounts of 5(*S*),15(*S*)-diHETE (86 ± 32 pmol/ 3 µg protein) and even lower amounts of lipoxins. After addition of 17(*S*)-HDHA no oxidation products were detected. Total precursor recovery for 15(*S*)-HETE and 17(*S*)-HDHA in these incubations was almost 100% with negligible precursor utilization (Fig. 6.5).

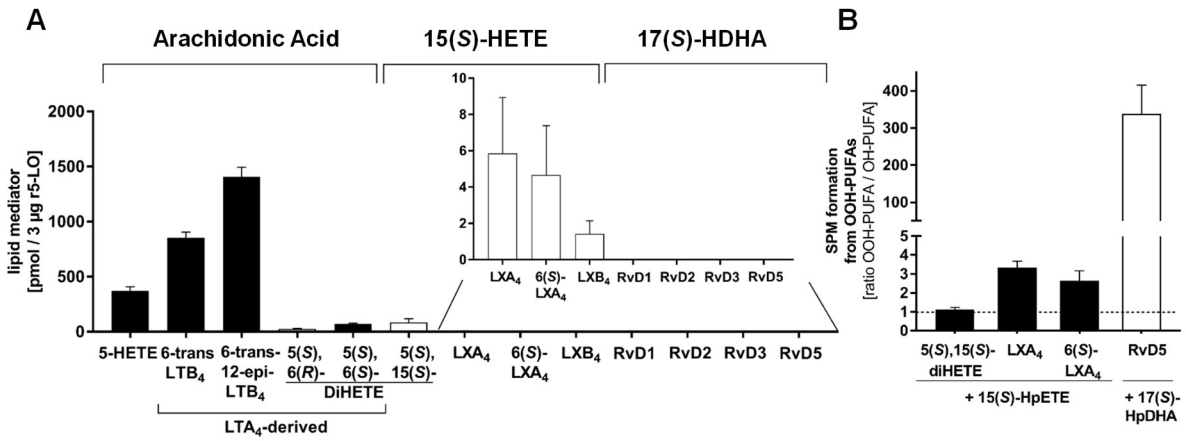


Fig. 6.8: Lipid mediator formation by human recombinant 5-LO supplemented with 15(*S*)-HETE, 17(*S*)-HDHA or ARA. Human recombinant 5-LO was isolated from E. coli by gel filtration. For each sample 1.5 µg of the purified enzyme were diluted in 500 µl PGC buffer. **(A)** The reaction was started by addition of 10 µM of 15(*S*)-HETE, 17(*S*)-HDHA or ARA together with 1 mM ATP in presence of Ca^{2+}. Reactions were terminated after 15 minutes with ice-cold methanol and the lipid mediators formed were quantified by LC-MS/MS. Data are depicted as mean ± SEM from 4 independent experiments. **(B)** Comparison of SPM formation from 10 µM hydroxy-PUFA and hydroperoxy-PUFA precursors by recombinant human 5-LO. Data are depicted as mean ± SEM from 3 independent experiments.

In addition to the monohydroxy precursors, SPM formation from their corresponding hydroperoxy precursors by isolated human 5-LO was studied (Fig. 6.8B). We found that formation of 5(*S*),15(*S*)-diHETE did not differ between 15(*S*)-HETE and 15(*S*)-HpETE while LxA_4 and 6(S)-LxA_4 formation was 2-3 times more efficient from the hydroperoxy-PUFA. LxB_4 was below the LLOQ in these

incubations. Furthermore, neither 17(*S*)-HDHA nor 17(*S*)-HpDHA were converted into the trihydroxylated D-series resolvins RvD1, RvD2 and RvD3. In contrast, isolated 5-LO readily accepted 17(*S*)-HpHDA for the formation of RvD5 while 17(*S*)-HDHA was hardly converted by the enzyme.

6.4 Discussion

In the present study we investigated the formation of SPM in human neutrophil preparations (PMNL). As expected, the cells displayed abundant 5-LO and FLAP expression (Fig. 6.3E). Expression of 15-LO-1 was detected as well, probably due to low numbers of eosinophils present in the PMNL fraction, while 15-LO-2 was absent in the preparations. In contrast to this, it was recently found that neutrophils from human peripheral blood isolated by CD16 positive selection express 15-LO-2 while eosinophils exclusively express 15-LO-1 [27]. In our hands, PMNL showed a close band running above 15-LO-2 in the Western Blots. We can therefore not rule out that a post-transcriptionally modified 15-LO-2 was expressed in our neutrophil preparations.

We found that PMNL primarily form ARA-derived lipid mediators such as leukotrienes and 5-HETE from endogenous sources whereas 15-HETE, 5(*S*),15(*S*)-diHETE and the formation of monohydroxylated EPA and DHA derivatives as well as RvD5 was very low (Fig. 6.3A). Lipoxins, E-series resolvins, maresins, protectins as well as other D-series resolvins were not detected in these incubations. The high levels of 12-lipoxygenation products such as 12-HETE and 14-HDHA were probably due to inevitable platelet contaminations in our preparations.

The formation of low amounts of DHA- and EPA-derived metabolites in Ca^{2+} ionophore stimulated PMNL (Fig. 6.3A) seems to be due to a low abundance of EPA and DHA in PMNL membranes since supplementation of the cells with these fatty acids strongly increased the formation of EPA- and DHA-derived oxylipins (Fig. 6.3B). This co-incubation levelled the difference between 15-HETE and 17-

HDHA formation suggesting that 15-LO has no substrate preference in PMNL. Nevertheless, PMNL still preferred ARA over DHA for 5- and 12-lipoxygenation. This is in line with our previous finding of a preference for ARA over DHA in the formation of LO-derived lipid mediators in human monocyte-derived M1 and M2 macrophages [17].

With the exception of RvD5, SPMs were not formed by PMNL even if the cells were supplemented with ARA and DHA. RvD5 is a 5-LO/15-LO dioxygenation product that can be considered as the DHA-derived analogue to 5(*S*),15(*S*)-diHETE. This lack of SPM formation is surprising since PMNL release substantial amounts of 5-HETE, 15-HETE and 5,15-diHETE under these conditions. Apparently, neither the abundant amounts of 5-H(p)ETE/7-H(p)DHA formed in PMNL were converted into SPMs by 15-LO-1 present in the preparations nor was 15-H(p)ETE/17-H(p)DHA efficiently metabolized by 5-LO. Therefore, our data suggest that PMNL alone are not able to synthesize significant amounts of trihydroxylated SPMs such as lipoxins, RvD1, RvD2 and RvD3 from endogenous sources although 5-LO-derived precursors are available.

In order to mimic the transcellular 15-LO/5-LO route of SPM formation, we added the SPM precursors 15-HETE, 17-HDHA or 18-HEPE to PMNL and stimulated the cells with Ca^{2+} ionophore (Fig. 6.4A). Exogenous 15-HETE has been shown to switch the substrate utilization of 5-LO from ARA to 15-HETE products in neutrophils and eosinophils when both lipids are present at the same time thereby reducing the formation of leukotrienes and 5-HETE [28-33]. This would argue for a lipid mediator switch in PMNL upon accumulation of extracellular 15-LO products. In addition to this preference for 15-HETE, we show here that PMNL also change their lipid 5-lipoxygenation preference. Upon addition of 15-HETE, 17-HDHA or 18-HEPE, SPM formation from DHA-derived 17(*S*)-HDHA was 4-fold more efficient compared to 15(*S*)-HETE. Again, 5(*S*),15(*S*)-diHETE and its DHA analogue RvD5 clearly dominated the SPMs released in PMNL supplemented with 15(*S*)-HETE or 17(*S*)-HDHA, respectively. Although formation of lipoxins, RvD1, RvE1, 18(R)-RvE2 and PDx was detectable

upon supplementation with the monohydroxy precursors, the levels formed were very low. Our data show that even upon exogenous addition of substantial quantities, the SPM-precursors 15-HETE, 17-HDHA and 18-HEPE are scarcely used for the formation of SPM. Thus, the capacity to form pro-inflammatory lipid mediators such as leukotrienes or 5-HETE exceeds that for SPM formation in PMNL by far. Exceptions from this are the dihydroxylated 5-/15-lipoxygenation products 5(*S*),15(*S)*-diHETE and RvD5. In line with these data, we recently published that TLR-4-stimulated human M2 macrophages which co-express 15-LO-1 and -2 release substantial amounts of 5(*S*),15(*S*)-diHETE and RvD5 but only low concentrations of other SPMs such as lipoxins, E-series resolvins, RvD1 and RvD2 upon supplementation with a mix of ARA, EPA and DHA [17]. Apparently, oxylipins formed in human neutrophils, eosinophils and macrophages are dominated by the dihydroxylated ARA and DHA derivatives 5(*S*),15(*S*)-diHETE and RvD5 (7(*S*),17(*S*)-diHDHA) derived from the sequential dioxygenation by 5-LO and 15-LO. 5-LO's downstream epoxidase activity on 5(*S*)-hydroperoxy,15(*S*)-HETE and 7(*S*)-hydroperoxy-17(*S*)-HDHA seems to be poor. Judged by the amounts of lipid mediators formed in intact PMNL, precursor turnover can therefore be classified as follows: 17-HDHA > ARA = 15-HETE > DHA > 18-HEPE for 5-LO products formed by dioxygenation and 18-HEPE > 15-HETE = 17-HDHA > ARA = DHA for the trihydroxylated products mainly formed by 5-LO's epoxidation reaction.

Regarding ARA, 5-LO catalyses LTA_4 formation via two reactions: First 5(*S*)-HpETE is formed by insertion of molecular oxygen (deoxygenation reaction). If 5(*S*)-HpETE stays in the active center, epoxidation of the hydroperoxide follows resulting in the formation of LTA_4 [11]. Due to a superior substrate capture rate (kcat/kM) 5-LO prefers ARA over 5(*S*)-HpETE. Therefore, LTA_4 accumulation can be observed when ARA availability is limited [33-35]. It seems as if 5-LO's preference for substrates carrying an unoxidized C atom in position 5 also applies to 15-HETE/17-HDHA in comparison to 5(*S*)-hydroperoxy-15(*S*)-HETE/7(*S*)-hydroperoxy-15(*S*)-HDHA in our incubations favoring the formation of dihydroxylated products instead of trihydroxylated ones. In accordance with this,

it was recently shown that recombinant 5-LO does not accept 5(*S*),15(*S*)-diHpETE for the formation of LxA_4 due to a much slower substrate capture rate of 5(*S*),15(*S*)-diHpETE compared to 15(*S*)-HETE [36].

There is a lack of comprehensive studies comparing SPM formation capacity from monohydroxy-PUFAs (15-HETE, 17-HDHA, 18-HEPE) with their hydroperoxy precursors (15-HpETE, 17-HpDHA, 18-HpEPE) in cellular systems. It was recently shown that the turnover of 15(*S*)-HETE and 15(*S*)-HpETE by isolated 5-LO is comparable and differs only by a factor of 1.5 [37]. In line with this, earlier studies compared lipoxin formation from 15-HETE or 15-HpETE in PMNL and found little difference in the product quantities [19, 21, 38]. Since this issue has never been addressed systematically for a larger number of SPM, we compared formation of 5(*S*),15(*S*)-diHETE, lipoxins and D-series resolvins in intact human PMNL und with isolated recombinant human 5-LO. In line with recent studies on isolated 5-LO [37], 5(*S*),15(*S*)-diHETE, lipoxins and D-resolvin formation was only moderately elevated upon treatment with the hydroperoxy precursors in intact PMNL. The same was true for lipoxin and 5(*S*),15(*S*)-diHETE formation from isolated recombinant 5-LO. Of note, the use of 17(*S*)-HpDHA instead of 17(*S*)-HDHA strongly elevated the formation of RvD5 by the recombinant enzyme. This shows again that 5-LO only accepted the monohydroxy / monohydroperoxy-PUFAs while the epoxidation reaction at position 5/6 or 7/8 is not efficiently executed.

Two alternative conclusions can be drawn from these data: A) Formation of SPMs other than 5(*S*),15(*S*)-diHETE and RvD5 is highly inefficient in humans due to a decidedly slow substrate capture rate of the dihydroperoxylated PUFAs by 5-LO epoxidase activity. B) Unknown factors might exist which are triggered by stimuli other than Ca^{2+} ionophore that allosterically control the substrate acceptance of 5-LO to facilitate a higher turn-over of bulkier, more hydrophilic substrates.

Since cellular 5-LO activity is regulated in a complex manner by factors such as the intracellular calcium level [39], phosphorylation [40] and the cellular redox

tone [41], we also investigated the activity of the PMNL LOs in broken cell preparations. This better reflects the biosynthetic capacity and usually correlates with LO protein expression [11]. Furthermore, disruption of PMNL cell integrity can mimic cell death and lysis during inflammatory processes which is known to contribute to the switch of inflammation to resolution. Interestingly, destruction of PMNL integrity followed by 100,000 *g* centrifugation led to the gradual inhibition of 17(*S*)-HDHA- and 18(*R/S*)-HEPE-derived SPMs while 15(*S*)-HETE-derived 5(*S*),15(*S*)-diHETE and lipoxin formation strongly increased (Fig. 6.6). On the other hand, when recombinant human 5-LO was treated with 17(*S*)-HDHA or 15(*S*)-HETE the enzyme did not accept these substrates for further oxidation.

These data clearly show that the utilization of the SPM precursors 18-HEPE and 17-HDHA needs an intact cellular environment. In contrast to resolvins, formation of lipoxins and 5(*S*),15(*S*)-diHETE was dramatically enhanced in homogenates and S100 preparations treated with 15(*S*)-HETE (Fig. 6.6A). However, recombinant human 5-LO alone only poorly accepted 15(*S*)-HETE as substrate whereas ARA was readily converted by the recombinant enzyme. We have recently shown that isolated human 5-LO accepts 18-HEPE for further conversion into RvE2, RvE3 and RvE1 isomers in the presence of phosphatidylcholine (PC) [42]. Since PC was not added to incubations of recombinant 5-LO in this study, it can be speculated that presence of PC might trigger acceptance of 15(*S*)-HETE as well suggesting that binding to phospholipid bilayers in the presence of Ca^{2+} and ATP might be sufficient to induce changes in 5-LO that allow the acceptance of bulkier, more hydrophilic lipids such as 15-HETE, 17-HDHA and 18-HEPE. Indeed, it is known that the activity of purified recombinant 5-LO is upregulated in presence of PC vesicles [34]. Furthermore, membrane association of 5-LO has been shown to change the substrate specificity of the enzyme allowing the utilization of 12(*S*)-HETE and 15(*S*)-HETE as substrates [43]. This also fits our recent findings that a 5-LO triple tryptophan mutant deficient in membrane binding and translocation shows attenuated lipoxin formation in intact HEK293 cells [13].

Taken together, these data strongly argue for a dependency of SPM biosynthesis on 5-LO translocation in intact cells but does neither explain the elevated lipoxin nor the attenuated resolvin levels in broken PMNL preparations. Since elevated lipoxin levels were also observed in PMNL S100 supernatants which do not contain membrane fragments, it seems that an additional cytosolic factor in PMNL is involved in lipoxin formation.

Total precursor recovery encompassing the LO-derived metabolites plus the unchanged remains of the respective monohydroxylated PUFA averaged almost 100% for both 15-HETE enantiomers, 17(*R*)-HDHA and 18(*R/S*)-HEPE in intact PMNL as well as in the broken cell preparations (Fig. 6.5). This suggests that the detection method we used captured the vast majority of oxidation products formed from the respective precursor in PMNL and also ruled out a substantial loss of precursor lipids due to adherence to plastic ware or cell debris. In contrast, recovery of 17(*S*)-HDHA was only around 70% in intact cells and 80% in S100 preparations suggesting that one or more unknown oxidation products exist which were not covered by our method. Since S100 preparations which are devoid of cell membrane debris also showed reduced recovery of 17(*S*)-HDHA, elevated membrane incorporation can be ruled out as main cause for the reduced recovery in our assays. The nature of the unknown 17(*S*)-HDHA metabolite(s) has to be elucidated in the future.

In addition to 5-LO translocation and membrane association, we have recently shown that FLAP is essential for LxA4 and RvD1 formation in neutrophils which are exogenously supplied with 15-HETE and 17-HDHA [13]. Formation of 5(*S*),15(*S*)-diHETE has been shown to be FLAP-dependent as well [26]. FLAP is an integral membrane protein of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) family. It is thought to transfer substrate to 5-LO thereby being essential for the enzyme's activity in intact cells. In addition, FLAP serves as a membrane anchor for 5-LO [11]. In this study we could support the FLAP dependency of RvD1, LxA_4 and 5(*S*),15(*S*)-diHETE biosynthesis.

Furthermore, we show here that formation of LXB_4, RvE1 and RvD5 are also sensitive to inhibition by Mk-886.

6.5 Conclusion

Biosynthesis of pro-resolving lipid mediators in humans is far from understood. Here, we could show that SPMs formed in human neutrophils are dominated by the dihydroxylated ARA and DHA derivatives 5(*S*),15(*S*)-diHETE and RvD5 derived from the dioxygenation by 5-LO and 15-LO. In contrast, 5-LO's downstream epoxidase activity, a prerequisite for lipoxin and resolvin biosynthesis, is poor. Furthermore, we show that FLAP is important for biosynthesis of all 5-LO-dependent SPMs measured in intact PMNL and that membrane association is a prerequisite for the acceptance of monohydroxy-PUFAs by 5-LO. In addition, we show that neutrophils prefer ARA over DHA for SPM biosynthesis while 17-HDHA is preferred over 15-HETE and 18-HEPE for the generation of the dioxygenation products. Of note, formation of E- and D-series resolvins was restricted to intact cells, whereas biosynthesis of lipoxins and 5(*S*),15(*S*)-diHETE was strongly upregulated upon destruction of cell integrity. Together with the data on FLAP inhibition our results point to unknown factors that control SPM formation and also render lipoxin and 5(*S*),15(*S*)-diHETE formation independent of membrane association and FLAP when cellular integrity is destroyed.

6.6 References

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Chapter 7

Concluding remarks and future perspectives

This thesis aimed to establish pre-analytical and analytical methods for a reliable and comprehensive oxylipin analysis as well as investigation of formation of lipid mediators with particular emphasis on biosynthesis of mono- and multihydroxylated oxylipins in immune cells.

Eicosanoids and other oxylipins are involved in the regulation of numerous physiological processes, such as inflammation, blood coagulation, vascular tone and endothelial permeability [1]. In recent years, oxylipins have attracted great interest in clinical lipidomics as potential biomarkers to better understand the mechanisms of diseases [2-12]. The most common method to analyze oxylipins is liquid chromatography coupled with mass spectrometry [1]. Nevertheless, it is striking that oxylipin concentrations reported from different laboratories are rarely comparable [13] showing that the analysis of oxylipins faces many challenges. This thesis describes the importance of harmonized pre-analytical, analytical and post-analytical methodologies for the generation of biologically meaningful oxylipin patterns.

In clinical studies, it is difficult to store biological samples adequately and immediately after collection. The samples often remain for a long-time on the laboratory bench or in the refrigerator before they are frozen. In addition, the generated samples are sometimes stored in the freezer for several years before they are analyzed, especially in long-term studies. How such delays in sample generation and storage affect the analyzed metabolites is rarely examined. However, such studies are of great importance in the case of oxylipins because they can be formed autoxidatively and by residual enzymatic activity or degraded by improper sample handling. Some studies on the pre-analytical stability of oxylipins have already been carried out [14-16], however these studies are limited to changes in free oxylipin concentrations. The majority of circulating oxylipins

are found esterified in lipids [13]. Therefore, it is important to investigate the storage stability of the total oxylipins (free and esterified in lipids) in plasma to assess their biomarker suitability (**chapter 3**). We found that most of the analyzed oxylipins are stable during the transitory stage of plasma generation. Only if all steps of plasma generation are delayed, i.e. whole blood centrifugation, plasma separation and freezing, significant differences in concentrations are quantified for all oxylipins (increase up to 3.5-fold). During long-term storage of plasma, oxylipins are robust and only autoxidative metabolites increase slightly after 56 months (4.6 years) of storage. Furthermore, to reduce the formation or degradation of oxylipins during sample preparation, the addition of antioxidants and enzyme inhibitors is recommended. PUFA are susceptible to autoxidation initiated by free radicals, whereby hydro(pero)xy-PUFA or prostaglandin-like isoprostanes can be formed [17-19]. The artificial formation of mainly hydroxy-PUFA was prevented by the addition of the radical scavenger butylated hydroxytoluene (BHT).

In biological samples, oxylipins occur in both free and esterified form. Depending on the biological matrix, oxylipins are present in a very wide range of concentrations. In addition, oxylipins differ in structure and stereochemistry, resulting in different polarities [1]. Therefore, the choice of analytical approach is particularly important to avoid discrimination. Typical sample preparation steps are protein precipitation, liquid-liquid extraction, saponification (release of esterified oxylipins from lipids) or solid phase extraction (SPE). However, the choice of methods or conditions used have a major influence on the quantified oxylipin concentrations [1]. Furthermore, efficient matrix removal is of particular importance in order to minimize ion suppression and to obtain good oxylipin recoveries [20]. To ensure comparability of results from different studies and laboratories, the performance of comprehensive laboratory comparisons as described in **chapter 4** is essential. Using identical biological samples, standardized sample preparation protocols, external standard calibration series and analytical methods, as well as trained personnel, a technical variance of ±15% can be achieved. The use of standardized protocols shows that

comparable and reproducible oxylipin concentrations can be quantified between different laboratories. Furthermore, epoxy- and oxo-PUFA are characterized as critical analytes showing high variances in case of insufficient sample handling, such as delayed processing or sample drying on SPE cartridge.

Post-analytics, e.g. raw data processing, internal standard selection or quantification [21], can bias accurate quantification of oxylipins. The quantification of oxylipins in biological samples is performed by external calibration using authentic standard substances. Accordingly, it is important to use standards with a high degree of purity to generate reliable results. However, only a limited number of standard substances can be purchased with a certified concentration (certified standards). Using these standards to validate the concentration of commercial standards we found that the most purchased oxylipin standards have the concertation declared by the manufacturer. Nevertheless, a comparison with certified standard substances is important, since for several oxylipins deviating target concentrations from the purchased non-certified standards were found. If deviations exceed 20%, correction factors are calculated to adjust the concentrations (**chapter 2**). Substances with no corresponding certified standard were compared with regioisomeric certified standards, also precursor fatty acid independent, and in case of a deviation of ≥30% a factor for concentration adjustment was calculated.

Conclusively, the direct comparison of absolute oxylipin concentrations generated in different clinical studies and laboratories is possible following appropriate standardization of pre-analytical, analytical and post-analytical methods. Based on these findings, the method standardization described in this thesis was applied to identify oxylipins associated with cardiometabolic syndrome in a large case-control study (Polish branch of the Prospective Urban and Rural Epidemiological (PURE) cohort) and to validate the detected oxylipin signature using the French NutriNet-Santé cohort (EU JPI-HDHL OXYGENATE project (https://www6.inrae.fr/jpi-hdhl-biomarkers-oxygenate/)). The OXYGENATE project focuses on the identification and validation of candidate oxylipins and how

they differ at different stages of the cardiometabolic syndrome, as well as the influence of dietary intervention on cardiometabolic endpoints. Using the methodologies described in this thesis helps to ensure that the identified oxylipins can be reproducibly determined in participating laboratories leading to high quality oxylipin profiling.

In the second part of the thesis, the focus was set on the biosynthesis of oxylipins. Oxylipins are formed e.g. during inflammation and play different functions during the course of inflammatory progression and resolution. In order to better understand oxylipin biosynthesis, the study of the immune cells involved in the progression of inflammation is very important. Special attention should be focused on the enzymes expressed in these cells, but also the interaction of different immune cells or the response to exogenous stimuli influences the formation and release of these lipid mediators from immune cells. 5-LOX is expressed in neutrophils and to a lesser extent in monocytes, macrophages or eosinophils [22, 23]. Activated platelets, on the other hand, express 12-LOX and COX-1 [24, 25]. 15-LOX coding ALOX15 is expressed in M2-like macrophages or eosinophils [26, 27], while COX-2 is mainly expressed in LPS-stimulated M1-like macrophages [28].

While pro-inflammatory prostaglandins and leukotrienes are predominantly released at the beginning of the inflammatory response, macrophages adopt anti-inflammatory and pro-resolving functions later in the process producing many anti-inflammatory oxylipins, which are believed to initiate inflammatory resolution and tissue repair. In general, efferocytosis, i.e. recognition, engulfment and digestion of apoptotic cells, initiates the lipid mediator class switch in macrophages [29]. Changes in gene transcription induced by efferocytosis are supported by nuclear receptors, especially liver X receptors (LXR). LXR are regulated by oxysterols and sterols from cholesterol biosynthesis [30, 31]. Moreover, high concentrations of cholesterol precursors, such as desmosterol, lanosterol or lathosterol, and oxysterols are present in apoptotic cells [32]. Through efferocytosis, these sterols are accumulated in macrophages, where

they can activate LXR and consequently upregulate LXR-dependent genes. Upon LXR activation, e.g. with the synthetic agonist T09, Th2 cytokine-dependent genes ALOX15, IL1RN, CIITA, XXYLT1 and LILRB1 are amplified [27]. Moreover, 15-LOX is involved in clearance of apoptotic cells through the oxidation of esterified PUFA and thus production of oxidized phospholipids [32]. Therefore, the link between LXR activation and 15-LOX overexpression may represent an important route of anti-inflammatory oxylipin formation (**chapter 5**). In general, LXR activation (by the potent synthetic agonist T09) induces ALOX15 in M2- but not in M1- and M0-like macrophages, although all three cell types have LXR. Overall, LXR activation has no or only moderate effect on cell polarization based on the abundance of TLR2, TLR4, PPARγ and IL-1RII as well as expression of surface markers (CD14, CD86, CD163). In M2-like macrophages upon time- and dose-dependent stimulation with T09 15-LOX abundance and activity is massively increased. The 15-LOX derived oxylipins such as 15-HETE, 5,15-diHETE or 7,17-diHDHA, are associated with anti-inflammatory properties. 15-HETE can activate PPARy [33-38] or regulate the half-life of TNFa mRNA [39, 40]. 5,15-diHETE is associated with the chemotactic activity of neutrophils [34, 41, 42], while 7,17-diHDHA is thought to increase the phagocytosis activity of neutrophils and macrophages [43, 44]. In particular, the oxysterols 22(*R*)-OH cholesterol and 24(*S*),25-Ep cholesterol and the cholesterol precursor desmosterol are identified as strong modulators by overexpressing and upregulating of 15-LOX and its metabolites, which are comparable to the results following T09 stimulation. Thus, these results demonstrate a link between two lipid mediator classes, namely sterols and oxylipins, and indicate a possible influence of sterols on the formation of anti-inflammatory oxylipins and thus resolution of inflammatory processes.

In a collaborative project we investigated the role of human polymorphonuclear leukocytes (PMNL) in the formation of specialized pro-resolving mediators (SPM) (**chapter 6**). SPMs represent a group of multihydroxylated PUFA that are thought to be associated with resolution of inflammation [45, 46]. They are formed by several oxidation steps modulated by

5-LOX together with 15-LOX or 12-LOX from ARA, EPA or DHA in one cell or after an interaction of several cell types [47, 48]. As a relevant amount of active 5-LOX is expressed in leukocytes, leukocyte SPM formation was investigated after supplementation with SPM precursors, namely 15-HETE, 18-HEPE and 17-HDHA, to mimic the transcellular route. PMNLs convert precursor oxylipins predominantly to dihydroxylated metabolites, namely 5,15-diHETE (ARA) and 7,17-diHDHA (resolvin D5; DHA). The formation of trihydroxylated metabolites was very low. After destruction of cell integrity, i.e. in PMNL homogenates or supernatants after ultracentrifugation, increased formation of ARA derived SPMs was quantified, whereas the formation of EPA and DHA derived SPMs was attenuated pointing toward still unknown factors that control SPM formation.

Conclusively, immune cells have the biosynthetic capacity to form multihydroxylated oxylipins by means of 5-LOX and 15-LOX. In this process, mainly dihydroxylated products, such as 5,15-diHETE or 7,17-diHDHA, are formed to a considerable extent. However, the levels are many times lower than those of hydroxy-PUFA or pro-inflammatory oxylipins (e.g. prostaglandins). In contrast, the biosynthesis of trihydroxylated oxylipins occurs at a much lower rate and can be detected only following supplementation with precursor oxylipins. Importantly, when considering *in vitro* or cell culture experiments, it must be kept in mind that these often are performed under extreme conditions (e.g. stimulation with non-physiological Ca^{2+} ionophore) and do not reflect *in vivo* situations. However, the use of other stimuli such as LPS or S1P leads to only moderate increases in multihydroxylated oxylipins [49]. Thus, these findings raise doubts about the biological relevance and effect of these multihydroxylated metabolites [50]. Although it is unclear whether or to what extent the multihydroxylated oxylipins exert anti-inflammatory and pro-resolving properties [50], the anti-inflammatory nature of 15-LOX is undisputed [33, 50, 51]. The experiments and results presented in the second part of the thesis reveal a new regulatory pathway of 15-LOX in macrophages, however detailed characterization of the presented regulatory pathway *in vivo* could provide important insights into the biosynthesis

of anti-inflammatory oxylipins and their involvement in the lipid mediator switch in macrophages.

Overall, this thesis highlights the importance of i) carefully optimized sample preparation, analytical and quantification procedures as well as ii) using harmonized and standardized pre-analytical, analytical and post-analytical methods to generate biologically meaningful oxylipin patterns to better understand the role of oxylipin in health and diseases by investigating the formation of anti-inflammatory oxylipins in immune cells.

7.1 References

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Summary

Eicosanoids and other oxylipins are a class of potent lipid mediators formed by oxidation of polyunsaturated fatty acids (PUFA). Oxylipins possess important homeostatic properties that include the regulation of immunity, blood pressure, blood coagulation, endothelium permeability or renal function. Due to the regulatory crosstalk and complex interactions of different oxylipin classes during physiological processes, the modulation of oxylipin patterns in response to e.g. pharmaceutical intervention is increasingly being investigated. Therefore, in the present thesis, the pre-analytical, analytical and post-analytical methodologies were optimized to ensure a reliable and comparable quantification of oxylipins. In the second part of the thesis the formation of anti-inflammatory oxylipins in immune cells was investigated using the optimized methods.

In the first part of the thesis, the pre-analytical, analytical and post-analytical prerequisites for an optimized quantification of oxylipins were created. For this purpose, an oxylipin standard series was first established. Assuming that structurally similar oxylipins of equimolar concentration should show comparable areas in LC-MS measurements in selected ion monitoring (SIM) mode, the oxylipin standards were compared with certified standards and the concentrations verified (**chapter 2**). For oxylipins with a conjugated electron system, e.g. hydroxy-PUFA, additional UV absorptions were recorded. For more than 50% of the commercially obtained oxylipins, good comparability to certified standards was obtained. A concentration factor was calculated for oxylipins with SIM areas outside a range of ±20% of the corresponding certified standard. If a corresponding certified standard was not available, oxylipins of the same class were compared independently of precursor PUFA and a factor was determined in case of a deviation of ±30%.

As the pre-analytical handling of biological samples can artificialy alter the oxylipin levels, the stability of endogenous oxylipin concentrations during

transitory stage of plasma preparation and long-term storage was investigated (**chapter 3**). To mimic common procedures in clinical laboratories, the effects of time and temperature on storage at different stages of plasma generation were studied in detail after blood collection. Analysis of the sum of free and esterified oxylipins showed that the concentrations of most oxylipin classes remain stable during transitory storage. Only a delay in all steps of plasma generation over a total time of 151 hours led to an increase in oxylipin levels of up to 3.5-fold. If the plasma was generated without delays and frozen directly at -80 °C, most oxylipins were unchanged for up to 56 months. Autoxidatively formed oxylipins, such as 9-HETE or 13-HODE, were increased while the concentrations of some cytochome P450 monooxygenase metabolites were slightly degraded.

When investigating the addition of additives at the beginning of sample preparation, significantly lower hydroxy-PUFA concentrations were quantified with the radical scavenger BHT. The other additives, namely EDTA and the enzyme inhibitors indomethacin and *t*-AUCB, had no effect on the artificial formation of oxylipins in plasma. However, with the presented sample preparation procedure, the residual enzyme activity in plasma is negligible, yet the use of enzyme inhibitors might be relevant for other biological samples such as tissue or with other sample preparation procedures to inhibit enzymes involved in oxylipin formation.

For a comprehensive LC-MS based oxylipin analysis that obtains reliable and comparable concentrations, the performance of laboratory comparisons is of great importance (**chapter 4**). Using the same sample material and external standard calibration, as well as optimized methods for sample preparation and analysis, a laboratory comparison was performed with five independent laboratories. The intra-day and inter-day variability of the method used in all laboratories was <15%. Only for epoxy-PUFA and oxo-PUFA an increased variance of up to >25% was achieved, so that these could be identified as critical oxylipins, which are preferentially artificially formed during sample preparation. Using unsupervised and supervised statistical models, the overall variability of

the recorded oxylipin profiles was investigated. Two plasma samples with particularly high oxylipin concentrations contributed to the variability. When considering the supervised multivariate analysis, laboratory 4 distinguished itself due to deviating hydroxy-PUFA concentrations, while the results of the other laboratories were comparable. Furthermore, all participating laboratories were able to identify similar differences (in direction and magnitude) between plasmas when comparing ratios between two plasmas.

In the second part of the thesis, the biosynthesis of oxylipins in immune cells was investigated using the optimized methods. The nuclear liver X receptor (LXR) is discussed to support the polarization switch in macrophages, so that the influence of LXR activation on the formation of oxylipins was investigated (**chapter 5**). M0-, M1- and M2-like human primary macrophages were incubated with the potent synthetic LXR agonist T09 and oxylipin concentrations and protein levels were analyzed as well as phenotypic surface markers. After treatment with T09, only moderate effects on macrophage cell polarization were observed. However, LXR activation (by the potent synthetic agonist T09) induced ALOX15 in M2- but not in M1- and M0-like macrophages. In M2-like macrophages 15-lipoxygenase (LOX) was upregulated up to 3-fold in a time- and dose-dependent manner, and 15-LOX metabolites were quantified at up to 15-fold higher concentrations. Treatment with agonists of other nuclear receptors had a moderate effect on 15-LOX modulation, presumably mediated by LXR through side activity. Sterols had the greatest effect on 15-LOX modulation. Here, significant changes were determined mainly with 24(*S*),25-Ep cholesterol (15-LOX 2-fold; monohydroxylated oxylipins 3-fold; multihydroxylated oxylipins 5-fold increased) and 22(*R*)-OH cholesterol (15-LOX 2-fold; monohydroxylated oxylipins 2-fold; multihydroxylated oxylipins 15-fold increased). However, the greatest influence on 15-LOX modulation was exerted by the cholesterol precursor desmosterol (15-LOX 3-fold; monohydroxylated oxylipins 4-fold; multi-hydroxylated oxylipins 16-fold increased). Thus, cholesterol derivatives were identified as potent ALOX15 modulators by upregulating 15-LOX abundance and activity, which were comparable to the results following T09 threatment.

Neutrophils represent other important key players in the inflammatory process, so their role in the formation of inflammation-solving oxylipins, the so-called specialized pro-resolving mediators (SPM), was investigated (**chapter 6**). Polymorphonuclear leukocytes (PMNL), broken PMNL, and recombinant human 5-LOX were incubated with SPM precursor oxylipins, 15-HETE, 18-HEPE, and 17-HDHA. In intact cells, the direct metabolites were predominantly formed from the precursor oxylipins, namely 5,15-diHETE from 15-HETE and 7,17-diHDHA (RvD5) from 17-HDHA, with 17-HDHA being the preferred substrate. After destruction of cell integrity, higher 5,15-diHETE and lipoxin concentrations were quantified, whereas the formation of 18-HEPE- and 17-HDHA-derived SPMs decreased.

Overall, the combination of harmonized protocols for sampling and analysis allows the reliable investigation of oxylipin formation in biological systems. The use of immune cells has provided new insights into the regulatory pathways of lipid mediator formation and a better understanding of the complex interaction of cellular enzymes was obtained.

Appendix

Chapter 3

Tab. 8.1 (pages 168-173): Parameters of the LC-ESI(-)-MS/MS method. Shown are transitions for each analyte for quantification in scheduled single reaction monitoring (SRM) mode, MS potentials (declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP)), assigned internal standards (IS), retention time (t_R), full width at half maximum (FWHM), limit of detection (LOD), calibration range with lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), slope and correlation coefficient of the calibration curve (r^2).

[1] full peak width at half maximum (FWHM) determined as mean width of standards (0.25 - 5 nM)
[2] limit of detection (LOD) set to lowest concentration with a signal to noise ratio ≥3
[3] lower limit of quantification (LLOQ) set to lowest calibration standards with a signal to noise ratio ≥5 and accuracy ± 20%
[4] upper limit of quantification (ULOQ) set to calibration of the highest injected standard
[5] calibration was performed as weighted regression using 1/x2 weighting
[6] other isoprostanes, isofuranes and phytoprostanes can be included as described in Rund et al., 2017 (Anal Chim Acta 1037, 63-74)

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP		[min]	[min]	LOD [2] [nM]	LLOQ [3] [nM]	ULOQ [4] [nM]		
			[V]											
18-COOH-dinor-LTB_4	337.2	195.2	-85	-10	-24	-9	$^2H_4$-8-iso-$PGF_{2\alpha}$	4.26	0.066	0.75	1.0	500	0.9981	0.014
Δ^{17}-6-keto-PGF_{1a}	367.2	163.2	-60	-10	-35	-10	$^2H_4$-6-keto-$PGF_{1\alpha}$	4.75	0.302	0.25	0.5	500	0.9994	0.069
2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP [6]	325.2	237.0	-70	-10	-18	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	5.10	0.062	0.100	0.25	500	0.9993	0.087
$^2H_4$-6-keto-$PGF_{1\alpha}$	373.0	167.0	-100	-10	-35	-8	*internal standard*	5.63	0.379					
6-keto-$PGF_{1\alpha}$	369.3	163.2	-90	-10	-35	-6	$^2H_4$-6-keto-$PGF_{1\alpha}$	5.65	0.373	0.64	0.96	640	0.9985	0.077
20-COOH-LTB_4	365.2	347.2	-100	-10	-24	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	5.80	0.059	0.066	0.17	330	0.9987	0.108
15-F_{3t}-IsoP (8-iso-$PGF_{3\alpha}$) [6]	351.1	193.0	-95	-10	-27	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	5.94	0.060	1	2.5	500	0.9968	0.024
20-OH-LTB_4	351.2	195.2	-100	-10	-24	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	6.02	0.058	0.025	0.05	500	0.9984	0.215
9,12,13-TriHODE	327.2	211.0	-80	-10	-29	-10	$^2H_5$-LxA_4	6.76	0.059	0.05	0.1	250	0.9986	0.255
9,10,13-TriHODE	327.2	201.0	-80	-10	-28	-8	$^2H_5$-LxA_4	6.76	0.066	0.75	1	500	0.9968	0.027
$PGF_{3\alpha}$	351.2	193.2	-95	-10	-30	-7	$^2H_4$-$PGF_{2\alpha}$	6.76	0.061	0.75	1	250	0.9991	0.043
15-F_{1t}-IsoP (8-iso-$PGF_{1\alpha}$) [6]	355.2	211.0	-100	-10	-35	-8	$^2H_4$-$PGF_{2\alpha}$	6.93	0.063	0.75	1	500	0.9976	0.111
$^2H_4$-8-iso-$PGF_{2\alpha}$	357.2	196.8	-75	-10	-34	-8	*internal standard*	6.95	0.061					
15-F_{2t}-IsoP (8-iso-PGF_{2a}) [6]	353.1	193.1	-95	-10	-34	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	6.97	0.058	0.10	0.25	500	0.9980	0.139
5(*R,S*)-5-F_{2t}-IsoP (5-i$PF_{2\alpha}$-VI) [6]	353.2	114.8	-85	-10	-27	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	7.45	0.077	0.1	0.25	500	0.9976	0.124
15-oxo-15-F_{2t}-IsoP [6]	351.2	219.0	-75	-10	-23	-8	$^2H_4$-8-iso-$PGF_{2\alpha}$	7.59	0.063	0.3	0.5	500	0.9979	0.121
9,10,11-TriHODE	327.0	171.0	-80	-10	-25	-8	$^2H_5$-LxA_4	7.71	0.066	0.025	0.05	250	0.9986	0.483
9,12,13-TriHOME	329.2	211.1	-100	-10	-31	-10	$^2H_5$-LxA_4	7.76	0.065	0.025	0.05	250	0.9978	0.460
9,10,13-TriHOME	329.2	171.1	-100	-10	-31	-8	$^2H_5$-LxA_4	7.91	0.063	0.05	0.1	250	0.9961	0.538
$^2H_4$-$PGF_{2\alpha}$	357.2	197.0	-80	-10	-33	-7	*internal standard*	7.95	0.065					
$PGF_{2\alpha}$	353.2	193.0	-80	-10	-33	-7	$^2H_4$-$PGF_{2\alpha}$	7.97	0.062	0.25	0.5	500	0.9986	0.083
$PGF_{1\alpha}$	355.4	293.2	-110	-10	-35	-6	$^2H_4$-$PGF_{2\alpha}$	8.02	0.063	0.025	0.05	250	0.9980	0.120
13,14-dihydro-15-oxo-15-F_{2t}-IsoP [6]	353.2	291.1	-110	-10	-30	-8	$^2H_4$-$PGF_{2\alpha}$	8.23	0.062	0.25	0.50	500	0.9976	0.025
$^2H_9$-PGE_2	360.2	280.1	-85	-10	-24	-6	*internal standard*	8.28	0.065					
$^2H_9$-PGD_2	360.2	280.2	-60	-10	-24	-6	*internal standard*	8.75	0.066					
$^2H_5$-RvD2	380.2	175.0	-80	-10	-31	-10	*internal standard*	8.89	0.066					

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP				LOD [2]	LLOQ [3]	ULOQ [4]		
			[V]					[min]	[min]	[nM]	[nM]			
9,10,11-TriHOME	329.1	201.1	-95	-10	-30	-8	$^2H_5$-LxA_4	9.17	0.069	0.05	0.1	250	0.9985	0.347
$^2H_{11}$-8,12-*iso*-$iPF_{2\alpha}$-VI	364.2	320.1	-90	-10	-30	-8	*internal standard*	9.48	0.162					
5(*R,S*)-5-F_{2c}-IsoP (8,12-iso-$iPF_{2\alpha}$-VI) [6]	353.2	219.2	-90	-10	-30	-8	$^2H_{11}$-8,12-*iso*-$iPF_{2\alpha}$-VI	9.55	0.069	0.25	0.5	500	0.9988	0.018
$^2H_5$-LxA_4	356.3	222.2	-80	-10	-24	-13	*internal standard*	9.57	0.068					
LxA_4	351.2	235.1	-70	-10	-18	-15	$^2H_5$-LxA_4	9.63	0.063	0.1	0.25	500	0.9987	0.047
$^2H_5$-RvD1	380.3	141.0	-80	-10	-18	-8	*internal standard*	9.71	0.066					
PGB_3	331.2	223.0	-75	-10	-24	-8	$^2H_4$-PGB_2	9.93	0.071	0.5	0.75	500	0.9975	0.065
1a,1b-dihomo-$PGF_{2\alpha}$ [6]	381.4	221.1	-80	-10	-37	-10	$^2H_4$-PGB_2	10.49	0.065	0.5	0.75	500	0.9967	0.037
17(*R,S*)-RvD4	375.2	131.0	-80	-10	-19	-10	$^2H_5$-RvD1	11.04	0.061	0.1	0.25	500	0.9980	0.037
PGJ_2	333.3	189.2	-80	-10	-24	-8	$^2H_4$-PGB_2	11.48	0.069	0.014	0.027	135	0.9970	0.141
$^2H_4$-PGB_2	337.2	179.0	-80	-10	-27	-8	*internal standard*	11.52	0.070					
PGB_2	333.3	175.1	-80	-10	-27	-8	$^2H_4$-PGB_2	11.62	0.069	0.025	0.05	500	0.9980	0.168
15,16-DiHODE	311.2	223.2	-105	-10	-28	-10	$^2H_4$-9,10-DiHOME	12.51	0.074	0.18	0.45	181	0.9991	0.338
9,10-DiHODE	311.2	201.2	-90	-10	-26	-10	$^2H_4$-9,10-DiHOME	12.57	0.071	0.01	0.025	100	0.9991	0.271
12,13-DiHODE	311.2	183.1	-105	-10	-29	-8	$^2H_4$-9,10-DiHOME	12.67	0.075	0.1	0.25	250	0.9983	0.166
8(*S*),15(*S*)-DiHETE	335.2	235.2	-90	-10	-21	-4	$^2H_4$-LTB_4	12.72	0.074	0.51	1.26	253	0.9995	0.012
6-*trans*-LTB_4	335.2	195.1	-80	-10	-22	-9	$^2H_4$-LTB_4	13.12	0.072	0.1	0.25	250	0.9993	0.079
5(*S*),15(*S*)-DiHETE	335.3	173.2	-80	-10	-20	-8	$^2H_4$-LTB_4	13.15	0.067	0.05	0.1	500	0.9988	0.045
17,18-DiHETE	335.3	247.2	-90	-10	-23	-8	$^2H_4$-LTB_4	13.24	0.073	0.083	0.11	55	0.9985	0.013
6-*trans*-12-*epi*-LTB_4	335.2	195.1	-85	-10	-20	-9	$^2H_4$-LTB_4	13.28	0.074	0.1	0.25	500	0.9986	0.044
PDx	359.1	153.1	-80	-10	-21	-10	$^2H_4$-LTB_4	13.32	0.075	0.16	0.39	393	0.9985	0.079
RvD5	359.1	199.1	-70	-10	-19	-10	$^2H_4$-LTB_4	13.40	0.074	0.1	0.25	500	0.9986	0.030
$^2H_4$-LTB_4	339.2	197.2	-80	-10	-22	-9	*internal standard*	13.54	0.076					
LTB_4	335.2	195.1	-80	-10	-22	-9	$^2H_4$-LTB_4	13.61	0.078	0.05	0.1	500	0.9996	0.094
14,15-DiHETE	335.3	207.2	-90	-10	-24	-10	$^2H_4$-LTB_4	13.86	0.074	0.025	0.05	250	0.9992	0.184
7(*S*),17(*S*)-DiH-n3DPA	361.5	263.3	-80	-10	-19	-4	$^2H_4$-LTB_4	13.86	0.077	0.5	0.75	500	0.9975	0.007

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP		[min]	[min]	LOD [2] [nM]	LLOQ [3] [nM]	ULOQ [4] [nM]		
			[V]											
11,12-DiHETE	335.2	167.1	-90	-10	-25	-5	$^2H_4$-LTB$_4$	14.09	0.075	0.025	0.05	250	0.9994	0.162
5(*S*),12(*S*)-DiHETE	335.2	195.1	-80	-10	-21	-8	$^2H_4$-LTB$_4$	14.19	0.074	0.025	0.05	500	0.9987	0.060
12,13-DiHOME	313.2	183.2	-105	-10	-29	-8	$^2H_4$-9,10-DiHOME	14.27	0.076	0.015	0.029	145	0.9980	0.164
8,9-DiHETE	335.2	127.1	-90	-10	-25	-5	$^2H_4$-LTB$_4$	14.47	0.076	0.05	0.100	500	0.9981	0.052
$^2H_4$-9,10-DiHOME	317.2	203.4	-105	-10	-28	-8	*internal standard*	14.63	0.082					
9,10-DiHOME	313.2	201.2	-105	-10	-28	-8	$^2H_4$-9,10-DiHOME	14.73	0.077	0.0075	0.01	250	0.9982	0.183
12-oxo-LTB$_4$	333.2	178.9	-80	-10	-20	-9	$^2H_4$-LTB$_4$	14.77	0.103	0.1	0.25	500	0.9986	0.103
tetranor-12-HETE	265.2	109.1	-70	-10	-15	-8	$^2H_4$-9,10-DiHOME	14.91	0.080	0.025	0.05	250	0.9988	0.104
MaR2	359.1	221.0	-70	-10	-16	-14	$^2H_4$-9,10-DiHOME	14.95	0.078	0.1	0.25	500	0.9974	0.033
5,6-DiHETE	335.2	115.1	-85	-10	-20	-8	$^2H_4$-LTB$_4$	15.27	0.067	0.23	0.3	15	0.9989	0.000
12-HHTrE	279.1	179.0	-70	-10	-15	-8	$^2H_{11}$-11,12-DiHETrE	15.45	0.083	0.25	0.5	500	0.9982	0.021
14,15-DiHETrE	337.2	207.1	-90	-10	-24	-10	$^2H_{11}$-11,12-DiHETrE	15.49	0.080	0.01	0.025	100	0.9994	0.131
19,20-DiHDPE	361.2	273.2	-90	-10	-23	-6	$^2H_{11}$-11,12-DiHETrE	15.56	0.084	0.25	0.5	500	0.9985	0.029
LTB$_3$	337.2	195.2	-80	-10	-21	-8	$^2H_{11}$-11,12-DiHETrE	15.77	0.085	0.1	0.25	500	0.9984	0.045
$^2H_{11}$-11,12-DiHETrE	348.2	167.2	-85	-10	-26	-8	*internal standard*	16.13	0.082					
16,17-DiHDPE	361.2	233.2	-90	-10	-23	-6	$^2H_{11}$-11,12-DiHETrE	16.20	0.080	0.05	0.1	500	0.9982	0.045
11,12-DiHETrE	337.2	167.1	-90	-10	-25	-8	$^2H_{11}$-11,12-DiHETrE	16.30	0.083	0.032	0.064	160	0.9999	0.080
13,14-DiHDPE	361.2	193.2	-90	-10	-23	-6	$^2H_{11}$-11,12-DiHETrE	16.48	0.081	0.05	0.1	500	0.9986	0.051
9-HOTrE	293.2	171.2	-85	-10	-20	-8	$^2H_4$-13-HODE	16.71	0.080	0.1	0.25	500	0.9993	0.091
10,11-DiHDPE	361.2	153.2	-90	-10	-23	-6	$^2H_{11}$-11,12-DiHETrE	16.86	0.081	0.05	0.1	250	0.9985	0.050
8,9-DiHETrE	337.2	127.1	-95	-10	-29	-8	$^2H_{11}$-11,12-DiHETrE	16.94	0.082	0.034	0.068	170	0.9990	0.035
20-COOH-ARA	333.2	271.0	-95	-10	-23	-6	$^2H_6$-20-HETE	16.94	0.080	0.1	0.25	500	0.9982	0.260
5(*S*),6(*R*)-DiHETE (ARA)	335.2	115.1	-70	-10	-20	-8	$^2H_{11}$-11,12-DiHETrE	16.91	0.082	0.020	0.039	390	0.9992	0.041
13-HOTrE	293.2	195.1	-90	-10	-22	-8	$^2H_4$-13-HODE	17.08	0.078	0.25	0.5	250	0.9971	0.040
18-HEPE	317.2	259.2	-75	-10	-15	-7	$^2H_8$-15-HETE	17.16	0.084	0.05	0.1	500	0.9979	0.041
13-γ-HOTrE	293.0	193.0	-90	-10	-23	-8	$^2H_4$-LTB$_4$	17.44	0.086	1	2.5	500	0.9989	0.059

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP		[min]	[min]	LOD [2] [nM]	LLOQ [3] [nM]	ULOQ [4] [nM]		
5(*S*),6(*S*)-DiHETE (ARA)	335.2	115.1	-70	-10	-20	-8	$^2H_{11}$-11,12-DiHETrE	17.51	0.084	0.022	0.045	223	0.9994	0.048
19-HETE	319.3	230.9	-70	-10	-19	-6	$^2H_8$-15-HETE	17.63	0.079	1	2.5	500	0.9980	0.006
7,8-DiHDPE	361.2	113.1	-90	-10	-23	-6	$^2H_{11}$-11,12-DiHETrE	17.68	0.086	0.25	0.5	500	0.9978	0.014
$^2H_6$-20-HETE	325.2	295.2	-90	-10	-22	-6	*internal standard*	17.80	0.083					
5,6-DiHETrE	337.2	145.1	-95	-10	-25	-10	$^2H_{11}$-11,12-DiHETrE	17.87	0.087	0.05	0.1	500	0.9963	0.043
20-HETE	319.2	289.1	-100	-10	-22	-6	$^2H_6$-20-HETE	17.89	0.084	0.25	0.5	500	0.9987	0.097
15-HEPE	317.2	219.2	-80	-10	-18	-10	$^2H_8$-15-HETE	17.91	0.078	0.05	0.1	500	0.9979	0.063
13-oxo-OTrE	291.1	195.0	-100	-10	-27	-8	$^2H_3$-13-oxo-ODE	17.95	0.084	0.05	0.1	100	0.9974	1.537
15-HODE	295.2	223.0	-95	-10	-24	-9	$^2H_4$-13-HODE	18.01	0.085	0.089	0.18	177	0.9986	1.028
11-HEPE	317.2	167.0	-70	-10	-19	-8	$^2H_8$-15-HETE	18.02	0.081	0.031	0.062	310	0.9988	0.083
9-oxo-OTrE	291.0	185.0	-100	-10	-24	-8	$^2H_3$-13-oxo-ODE	18.16	0.088	0.1	0.25	500	0.9973	0.213
8-HEPE	317.2	155.2	-80	-10	-18	-8	$^2H_8$-15-HETE	18.24	0.081	0.03	0.06	300	0.9991	0.062
18-HETE	319.2	261.0	-85	-10	-20	-8	$^2H_8$-15-HETE	18.34	0.082	0.1	0.25	500	0.9982	0.068
12-HEPE	317.2	179.2	-85	-10	-18	-8	$^2H_8$-15-HETE	18.44	0.081	0.05	0.1	500	0.9982	0.061
17-HETE	319.2	247.0	-85	-10	-19	-8	$^2H_8$-15-HETE	18.56	0.077	0.1	0.25	500	0.9987	0.081
9-HEPE	317.2	167.0	-70	-10	-17	-8	$^2H_8$-15-HETE	18.60	0.080	0.1	0.25	500	0.9982	0.037
16-HETE	319.2	233.1	-85	-10	-18	-8	$^2H_8$-15-HETE	18.72	0.086	0.1	0.25	500	0.9983	0.089
12-HODE	295.1	183.0	-80	-10	-24	-9	$^2H_4$-13-HODE	18.82	0.084	0.025	0.05	100	0.9984	0.455
5-HEPE	317.2	115.1	-80	-10	-18	-6	$^2H_8$-15-HETE	18.94	0.084	0.03	0.06	295	0.9979	0.077
$^2H_4$-13-HODE	299.2	198.1	-80	-10	-24	-9	*internal standard*	19.03	0.087					
13-HODE	295.2	195.2	-100	-10	-24	-9	$^2H_4$-9-HODE	19.15	0.089	0.1	0.25	500	0.9984	0.060
$^2H_4$-9-HODE	299.2	172.3	-100	-10	-24	-6	*internal standard*	19.14	0.086					
10-HODE	295.1	183.0	-90	-10	-25	-7	$^2H_4$-9-HODE	19.18	0.088	0.038	0.076	380	0.9986	0.202
9-HODE	295.2	171.1	-100	-10	-24	-7	$^2H_4$-9-HODE	19.26	0.084	0.14	0.35	348	0.9995	0.130
20-HDHA	343.2	241.2	-75	-10	-17	-7	$^2H_8$-15-HETE	19.56	0.083	0.1	0.25	500	0.9981	0.025
$^2H_8$-15-HETE	327.2	226.0	-90	-10	-18	-8	*internal standard*	19.70	0.091					

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP		[min]	[min]	LOD [2] [nM]	LLOQ [3] [nM]	ULOQ [4] [nM]		
15(16)-EpODE	293.3	235.2	-90	-10	-16	-4	$^2H_4$-12(13)-EpOME	19.75	0.085	0.092	0.185	924	0.9987	0.108
15-HETE	319.2	219.2	-80	-10	-18	-8	$^2H_8$-15-HETE	19.90	0.086	0.11	0.22	220	0.9985	0.030
9(10)-EpODE	293.2	171.2	-90	-10	-16	-8	$^2H_4$-12(13)-EpOME	19.92	0.090	0.058	0.116	582	0.9985	0.090
$^2H_3$-13-oxo-ODE	296.2	198.2	-85	-10	-28	-8	*internal standard*	19.97	0.088					
17(18)-EpETE	317.2	215.2	-90	-10	-16	-6	$^2H_{11}$-14(15)-EpETrE	20.02	0.086	0.5	0.75	500	0.9985	0.029
13-oxo-ODE	293.2	113.0	-100	-10	-27	-8	$^2H_3$-13-oxo-ODE	20.06	0.090	0.25	0.5	500	0.9982	0.250
16-HDHA	343.2	233.2	-75	-10	-17	-7	$^2H_8$-15-HETE	20.10	0.078	0.1	0.25	500	0.9995	0.087
17-HDHA	343.2	201.2	-80	-10	-18	-6	$^2H_8$-15-HETE	20.23	0.085	0.34	0.9	170	0.9996	0.010
12(13)-EpODE	293.2	183.1	-90	-10	-20	-8	$^2H_4$-12(13)-EpOME	20.38	0.084	0.17	0.33	331	0.9980	0.062
13-HDHA	343.2	193.2	-75	-10	-17	-7	$^2H_8$-12-HETE	20.42	0.087	0.05	0.1	500	0.9984	0.074
11-HETE	319.2	167.2	-80	-10	-21	-7	$^2H_8$-12-HETE	20.47	0.085	0.022	0.044	219	0.9980	0.105
9-oxo-ODE	293.2	185.1	-115	-10	-26	-8	$^2H_3$-13-oxo-ODE	20.67	0.082	0.25	0.5	500	0.9972	0.167
15-oxo-ETE	317.2	113.1	-90	-10	-23	-8	$^2H_7$-5-oxo-ETE	20.70	0.084	0.05	0.1	250	0.9964	2.365
10-HDHA	343.2	153.2	-70	-10	-19	-7	$^2H_8$-12-HETE	20.69	0.080	0.025	0.05	500	0.9987	0.090
14-HDHA	343.2	205.2	-70	-10	-17	-7	$^2H_8$-12-HETE	20.69	0.080	0.055	0.14	275	0.9966	0.029
$^2H_8$-12-HETE	327.2	184.2	-85	-10	-20	-8	*internal standard*	20.74	0.088					
14(15)-EpETE	317.2	207.2	-90	-10	-16	-6	$^2H_{11}$-14(15)-EpETrE	20.79	0.081	0.1	0.25	500	0.9988	0.071
8-HETE	319.2	155.2	-80	-10	-20	-6	$^2H_8$-12-HETE	20.89	0.081	0.094	0.23	468	0.9983	0.099
12-HETE	319.2	179.2	-80	-10	-19	-8	$^2H_8$-12-HETE	20.93	0.079	0.1	0.25	500	0.9990	0.100
11(12)-EpETE	317.2	167.0	-90	-10	-16	-6	$^2H_{11}$-14(15)-EpETrE	20.98	0.079	0.1	0.25	500	0.9992	0.081
11-HDHA	343.2	121.1	-70	-10	-18	-7	$^2H_8$-5-HETE	21.04	0.073	0.1	0.25	500	0.9987	0.054
8(9)-EpETE	317.2	127.2	-90	-10	-16	-6	$^2H_{11}$-8(9)-EpETrE	21.17	0.094	0.5	0.75	500	0.9965	0.048
7-HDHA	343.2	141.2	-75	-10	-17	-7	$^2H_8$-5-HETE	21.18	0.079	0.05	0.1	500	0.9989	0.050
9-HETE	319.2	167.2	-80	-10	-21	-7	$^2H_8$-5-HETE	21.29	0.079	0.27	0.4	265	0.9993	0.022
15-HETrE	321.2	221.2	-90	-10	-21	-10	$^2H_8$-5-HETE	21.36	0.084	0.05	0.1	500	0.9986	0.170
$^2H_8$-5-HETE	327.2	116.1	-80	-10	-19	-8	*internal standard*	21.43	0.086					

Tab. 8.1: Continued.

analyte	mass transition		mass parameters				internal standard	t_R	FWHM [1]		calibration range		r^2 [5]	slope
	Q1	Q3	DP	EP	CE	CXP		[min]	[min]	LOD [2] [nM]	LLOQ [3] [nM]	ULOQ [4] [nM]		
			[V]											
8-HDHA	343.2	189.2	-70	-10	-17	-7	$^2H_8$-5-HETE	21.42	0.081	0.038	0.1	190	0.9984	0.036
12-oxo-ETE	317.2	153.1	-90	-10	-23	-8	$^2H_7$-5-oxo-ETE	21.54	0.082	0.41	1.0	103	0.9959	0.420
5-HETE	319.2	115.2	-80	-10	-19	-7	$^2H_8$-5-HETE	21.59	0.079	0.018	0.035	350	0.9989	0.096
8-HETrE	321.2	157.1	-85	-10	-22	-9	$^2H_8$-5-HETE	21.76	0.076	0.25	0.5	500	0.9986	0.095
12-HETrE	321.0	181.0	-85	-10	-24	-10	$^2H_8$-5-HETE	22.00	0.073	0.1	0.25	500	0.9959	0.074
$^2H_4$-12(13)-EpOME	299.2	198.1	-100	-10	-21	-8	*internal standard*	22.16	0.081					
17-oxo-DHA	341.3	111.0	-85	-10	-21	-8	$^2H_7$-5-oxo-ETE	22.15	0.069	7.5	10	500	0.9908	0.010
4-HDHA	343.2	101.1	-75	-10	-17	-7	$^2H_8$-5-HETE	22.17	0.077	0.05	0.1	500	0.9988	0.074
17-oxo-n3DPA	343.1	247.0	-85	-10	-24	-8	$^2H_7$-5-oxo-ETE	22.29	0.077	2.5	5	100		
12(13)-EpOME	295.2	195.2	-105	-10	-19	-8	$^2H_4$-12(13)-EpOME	22.28	0.077	0.019	0.037	185	0.9988	0.033
19(20)-EpDPE	343.2	241.2	-90	-10	-16	-7	$^2H_{11}$-14(15)-EpETrE	22.27	0.078	0.25	0.5	500	0.9981	0.033
$^2H_{11}$-14(15)-EpETrE	330.2	219.3	-90	-10	-16	-4	*internal standard*	22.35	0.073					
9(10)-EpOME	295.2	171.1	-105	-10	-19	-8	$^2H_4$-12(13)-EpOME	22.50	0.076	0.081	0.2	405	0.9990	0.076
14(15)-EpETrE	319.2	219.2	-90	-10	-15	-4	$^2H_{11}$-14(15)-EpETrE	22.50	0.071	0.1	0.25	500	0.9993	0.066
16(17)-EpDPE	343.2	233.2	-90	-10	-16	-7	$^2H_{11}$-14(15)-EpETrE	22.79	0.065	0.1	0.25	500	0.9982	0.043
$^2H_7$-5-oxo-ETE	324.2	210.2	-90	-10	-24	-8	*internal standard*	22.80	0.074					
13(14)-EpDPE	343.2	193.2	-90	-10	-16	-7	$^2H_{11}$-14(15)-EpETrE	22.89	0.063	0.05	0.1	250	0.9986	0.054
5-oxo-ETE	317.2	273.0	-90	-10	-20	-6	$^2H_7$-5-oxo-ETE	22.92	0.068	0.5	0.75	500	0.9932	0.297
10(11)-EpDPE	343.2	153.2	-90	-10	-16	-7	$^2H_{11}$-8(9)-EpETrE	23.00	0.063	0.01	0.025	500	0.9987	0.293
11(12)-EpETrE	319.2	167.2	-85	-10	-16	-7	$^2H_{11}$-8(9)-EpETrE	23.10	0.067	0.05	0.1	500	0.9982	0.212
$^2H_{11}$-8(9)-EpETrE	330.2	155.0	-80	-10	-16	-7	*internal standard*	23.17	0.066					
7(8)-EpDPE	343.2	141.2	-75	-10	-16	-7	$^2H_{11}$-8(9)-EpETrE	23.22	0.060	0.44	0.65	436	0.9991	0.041
8(9)-EpETrE	319.2	155.2	-90	-10	-16	-6	$^2H_{11}$-8(9)-EpETrE	23.39	0.058	0.25	0.5	500	0.9981	0.082
4-oxo-DHA	341.0	115.1	-100	-10	-20	-6	$^2H_7$-5-oxo-ETE	23.36	0.061	0.1	0.25	500	0.9964	0.175
14(15)-EpEDE	321.2	221.2	-85	-10	-19	-4	$^2H_{11}$-14(15)-EpETrE	23.65	0.062	0.025	0.05	100	0.9977	0.121
5-HETrE	321.2	115.1	-90	-10	-17	-9	$^2H_8$-5-HETE	23.69	0.063	0.01	0.025	250	0.9982	0.087
9(10)-Ep-stearic acid	297.0	170.8	-120	-10	-24	-11	$^2H_4$-12(13)-EpOME	24.10	0.068	0.25	0.5	500	0.9988	0.013

Tab. 8.2 Statistical analysis of the effects of additives on the apparent oxylipin pattern. Statistical differences between “no additive” and different additives were evaluated by two-way ANOVA followed by Bonferroni post-test.

	anti-ox mix	BHT	EDTA	IND	*t*-AUCB
	p-val				
5-HETE	p <0.05	p <0.05	ns	ns	ns
9-HETE	p <0.01	p <0.01	ns	ns	ns
12-HETE	p <0.05	p <0.01	ns	ns	ns
15-HETE	p <0.001	p <0.001	ns	ns	ns
20-HETE	ns	ns	ns	ns	ns
5-HEPE	ns	p <0.05	ns	ns	ns
12-HEPE	p <0.01	p <0.01	ns	ns	ns
15-HEPE	p <0.05	p <0.001	ns	ns	ns
18-HEPE	p <0.001	p <0.001	ns	ns	ns
4-HDHA	p <0.001	p <0.001	ns	ns	ns
7-HDHA	ns	ns	ns	ns	ns
14-HDHA	p <0.01	p <0.01	ns	ns	ns
17-HDHA	p <0.001	p <0.001	ns	ns	ns
13-HODE	p <0.05	p <0.05	ns	ns	ns
13-HOTrE	ns	ns	ns	ns	ns
5(6)-EpETrE	ns	ns	ns	ns	ns
8(9)-EpETrE	ns	ns	ns	ns	ns
11(12)-EpETrE	ns	ns	ns	ns	ns
14(15)-EpETrE	ns	ns	ns	ns	ns
17(18)-EpETE	ns	p <0.05	ns	ns	ns
19(20)-EpDPE	ns	ns	ns	ns	ns
12(13)-EpOME	ns	ns	ns	ns	ns
15(16)-EpODE	ns	ns	ns	ns	ns
14,15-DiHETrE	ns	ns	ns	ns	ns
17,18-DiHETE	ns	ns	ns	ns	ns
19,20-DiHDPE	ns	ns	ns	ns	ns
12,13-DiHOME	ns	ns	ns	ns	ns
15,16-DiHODE	ns	ns	ns	ns	ns

Tab. 8.3 (pages 175-177): Oxylipin concentrations in quality standard (QS) plasma. Shown are analyzed oxylipins sorted by their retention time (tR) with their mass transitions used for quantification in scheduled SRM mode, limit of detection (LOD) and calibration range with lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) in vial. For each analyte mean ± SD (n=8) concentration in plasma and ratio of determined concentration to LLOQ (fold LLOQ) was calculated. Plasma samples are concentrated during sample preparation by factor 2 yielding lower LLOQ in human plasma. The LLOQ is provided in grey when concentration of analyte was <LLOQ in more than 50% of the samples.

analyte	mass transition		t_R		calibration range		QS plasma (total oxylipins)				
				LOD	LLOQ	ULOQ	Mean	SD	RSD	Fold
	Q1	Q3	[min]	[nM]	[nM]		[nM]	[nM]	%	LLOQ
18-COOH-dinor-LTB_4	337.2	195.2	4.26	0.75	1.0	500	0.50 ± 0.00			
Δ^{17}-6-keto-PGF_{1a}	367.2	163.2	4.75	0.25	0.5	500	0.25 ± 0.00			
2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	325.2	237.0	5.10	0.100	0.25	500	0.13 ± 0.00			
6-keto-$PGF_{1\alpha}$	369.3	163.2	5.65	0.64	0.96	640	0.48 ± 0.00			
20-COOH-LTB_4	365.2	347.2	5.80	0.066	0.17	330	0.08 ± 0.00			
15-F_{3t}-IsoP (8-iso-$PGF_{3\alpha}$)	351.1	193.0	5.94	1	2.5	500	1.25 ± 0.00			
20-OH-LTB_4	351.2	195.2	6.02	0.025	0.05	500	0.03 ± 0.00			
9,12,13-TriHODE	327.2	211.0	6.76	0.05	0.1	250	0.05 ± 0.00			
9,10,13-TriHODE	327.2	201.0	6.76	0.75	1	500	0.50 ± 0.00			
$PGF_{3\alpha}$	351.2	193.2	6.76	0.75	1	250	0.50 ± 0.00			
15-F_{1t}-IsoP (8-iso-$PGF_{1\alpha}$)	355.2	211.0	6.93	0.75	1	500	0.50 ± 0.00			
15-F_{2t}-IsoP (8-iso-PGF_{2a})	353.1	193.1	6.97	0.10	0.25	500	0.13 ± 0.00			
5(*R,S*)-5-F_{2t}-IsoP (5-i$PF_{2\alpha}$-VI)	353.2	114.8	7.45	0.1	0.25	500	0.44 ± 0.18		42	4
15-oxo-15-F_{2t}-IsoP	351.2	219.0	7.59	0.3	0.5	500	0.25 ± 0.00			
9,10,11-TriHODE	327.0	171.0	7.71	0.025	0.05	250	0.03 ± 0.00			
9,12,13-TriHOME	329.2	211.1	7.76	0.025	0.05	250	0.03 ± 0.00			
9,10,13-TriHOME	329.2	171.1	7.91	0.05	0.1	250	0.05 ± 0.00			
$PGF_{2\alpha}$	353.2	193.0	7.97	0.25	0.5	500	0.25 ± 0.00			
$PGF_{1\alpha}$	355.4	293.2	8.02	0.025	0.05	250	0.03 ± 0.00			
13,14-dihydro-15-oxo-15-F_{2t}-IsoP	353.2	291.1	8.23	0.25	0.50	500	0.25 ± 0.00			
9,10,11-TriHOME	329.1	201.1	9.17	0.05	0.1	250	1.22 ± 0.60		49	24
5(*R,S*)-5-F_{2c}-IsoP (8,12-iso-i$PF_{2\alpha}$-VI)	353.2	219.2	9.55	0.25	0.5	500	0.25 ± 0.00			
LxA_4	351.2	235.1	9.63	0.1	0.25	500	0.13 ± 0.00			
PGB_3	331.2	223.0	9.93	0.5	0.75	500	0.38 ± 0.00			
1a,1b-dihomo-$PGF_{2\alpha}$	381.4	221.1	10.49	0.5	0.75	500	0.38 ± 0.00			
17(*R,S*)-RvD4	375.2	131.0	11.04	0.1	0.25	500	0.13 ± 0.00			
PGJ_2	333.3	189.2	11.48	0.014	0.027	135	0.01 ± 0.00			
PGB_2	333.3	175.1	11.62	0.025	0.05	500	0.03 ± 0.00			
15,16-DiHODE	311.2	223.2	12.51	0.18	0.45	181	15.08 ± 0.56		4	67
9,10-DiHODE	311.2	201.2	12.57	0.01	0.025	100	0.44 ± 0.02		5	35
12,13-DiHODE	311.2	183.1	12.67	0.1	0.25	250	0.13 ± 0.00			
8(*S*),15(*S*)-DiHETE	335.2	235.2	12.72	0.51	1.26	253	0.63 ± 0.00			
6-*trans*-LTB_4	335.2	195.1	13.12	0.1	0.25	250	0.13 ± 0.00			
5(*S*),15(*S*)-DiHETE	335.3	173.2	13.15	0.05	0.1	500	0.05 ± 0.00			
17,18-DiHETE	335.3	247.2	13.24	0.083	0.11	55	0.63 ± 0.07		11	11
6-*trans*-12-*epi*-LTB_4	335.2	195.1	13.28	0.1	0.25	500	0.13 ± 0.00			
PDx	359.1	153.1	13.32	0.16	0.39	393	0.20 ± 0.00			
RvD5	359.1	199.1	13.40	0.1	0.25	500	0.13 ± 0.00			
LTB_4	335.2	195.1	13.61	0.05	0.1	500	0.05 ± 0.00			
14,15-DiHETE	335.3	207.2	13.86	0.025	0.05	250	0.15 ± 0.04		27	6
7(*S*),17(*S*)-DiH-n3DPA	361.5	263.3	13.86	0.5	0.75	500	0.38 ± 0.00			
11,12-DiHETE	335.2	167.1	14.09	0.025	0.05	250	0.15 ± 0.02		11	6
5(*S*),12(*S*)-DiHETE	335.2	195.1	14.19	0.025	0.05	500	0.03 ± 0.00			
12,13-DiHOME	313.2	183.2	14.27	0.015	0.029	145	5.24 ± 0.49		9	361
8,9-DiHETE	335.2	127.1	14.47	0.05	0.100	500	0.37 ± 0.05		14	7
9,10-DiHOME	313.2	201.2	14.73	0.008	0.01	250	18.40 ± 1.53		8	3680
12-oxo-LTB_4	333.2	178.9	14.77	0.1	0.25	500	0.13 ± 0.00			
tetranor-12-HETE	265.2	109.1	14.91	0.025	0.05	250	0.25 ± 0.02		8	10
MaR2	359.1	221.0	14.95	0.1	0.25	500	0.13 ± 0.00			
5,6-DiHETE	335.2	115.1	15.27	0.23	0.3	15	2.05 ± 0.17		9	14

Tab. 8.3: Continued.

analyte	mass transition		t_R		calibration range		QS plasma (total oxylipins)				
				LOD	LLOQ	ULOQ	Mean		SD	RSD	Fold
	Q1	Q3	[min]	[nM]	[nM]		[nM]		[nM]	%	LLOQ
12-HHTrE	279.1	179.0	15.45	0.25	0.5	500	0.25	±	0.00		
14,15-DiHETrE	337.2	207.1	15.49	0.01	0.025	100	0.75	±	0.05	7	60
19,20-DiHDPE	361.2	273.2	15.56	0.25	0.5	500	2.15	±	0.15	7	9
LTB_3	337.2	195.2	15.77	0.1	0.25	500	0.13	±	0.00		
16,17-DiHDPE	361.2	233.2	16.20	0.05	0.1	500	0.42	±	0.05	11	8
11,12-DiHETrE	337.2	167.1	16.30	0.032	0.064	160	1.10	±	0.09	8	34
13,14-DiHDPE	361.2	193.2	16.48	0.05	0.1	500	0.35	±	0.03	9	7
9-HOTrE	293.2	171.2	16.71	0.1	0.25	500	2.73	±	0.28	10	22
10,11-DiHDPE	361.2	153.2	16.86	0.05	0.1	250	0.51	±	0.03	6	10
8,9-DiHETrE	337.2	127.1	16.94	0.034	0.068	170	3.17	±	0.25	8	93
20-COOH-ARA	333.2	271.0	16.94	0.1	0.25	500	7.90	±	0.96	12	63
5(*S*),6(*R*)-DiHETE (ARA)	335.2	115.1	16.91	0.020	0.039	390	0.02	±	0.00		
13-HOTrE	293.2	195.1	17.08	0.25	0.5	250	2.62	±	0.38	15	10
18-HEPE	317.2	259.2	17.16	0.05	0.1	500	2.78	±	0.35	13	56
13-γ-HOTrE	293.0	193.0	17.44	1	2.5	500	1.25	±	0.00		
5(*S*),6(*S*)-DiHETE (ARA)	335.2	115.1	17.51	0.022	0.045	223	0.02	±	0.00		
19-HETE	319.3	230.9	17.63	1	2.5	500	1.25	±	0.00		
7,8-DiHDPE	361.2	113.1	17.68	0.25	0.5	500	2.62	±	0.30	11	10
5,6-DiHETrE	337.2	145.1	17.87	0.05	0.1	500	14.71	±	1.26	9	294
20-HETE	319.2	289.1	17.89	0.25	0.5	500	1.56	±	0.15	10	6
15-HEPE	317.2	219.2	17.91	0.05	0.1	500	1.79	±	0.30	17	36
13-oxo-OTrE	291.1	195.0	17.95	0.05	0.1	100	0.05	±	0.00		
15-HODE	295.2	223.0	18.01	0.089	0.18	177	5.64	±	0.28	5	64
11-HEPE	317.2	167.0	18.02	0.031	0.062	310	1.15	±	0.21	18	37
9-oxo-OTrE	291.0	185.0	18.16	0.1	0.25	500	0.13	±	0.00		
8-HEPE	317.2	155.2	18.24	0.03	0.06	300	1.09	±	0.21	19	36
18-HETE	319.2	261.0	18.34	0.1	0.25	500	0.65	±	0.07	11	5
12-HEPE	317.2	179.2	18.44	0.05	0.1	500	2.03	±	0.31	15	41
17-HETE	319.2	247.0	18.56	0.1	0.25	500	0.30	±	0.02	7	2
9-HEPE	317.2	167.0	18.60	0.1	0.25	500	2.06	±	0.33	16	17
16-HETE	319.2	233.1	18.72	0.1	0.25	500	0.73	±	0.06	8	6
12-HODE	295.1	183.0	18.82	0.025	0.05	100	2.25	±	0.72	32	90
5-HEPE	317.2	115.1	18.94	0.03	0.06	295	2.67	±	0.31	12	91
13-HODE	295.2	195.2	19.15	0.1	0.25	500	78.33	±	18.40	23	627
10-HODE	295.1	183.0	19.18	0.038	0.076	380	4.10	±	1.13	27	108
9-HODE	295.2	171.1	19.26	0.14	0.35	348	60.38	±	14.30	24	347
20-HDHA	343.2	241.2	19.56	0.1	0.25	500	6.30	±	1.11	18	50
15(16)-EpODE	293.3	235.2	19.75	0.092	0.185	924	19.38	±	3.39	17	210
15-HETE	319.2	219.2	19.90	0.11	0.22	220	14.25	±	1.90	13	130
9(10)-EpODE	293.2	171.2	19.92	0.058	0.116	582	5.38	±	2.29	43	93
17(18)-EpETE	317.2	215.2	20.02	0.5	0.75	500	8.93	±	3.48	39	24
13-oxo-ODE	293.2	113.0	20.06	0.25	0.5	500	0.25	±	0.00		
16-HDHA	343.2	233.2	20.10	0.1	0.25	500	3.63	±	0.71	20	29
17-HDHA	343.2	201.2	20.23	0.34	0.9	170	0.43	±	0.00		
12(13)-EpODE	293.2	183.1	20.38	0.17	0.33	331	2.60	±	0.94	36	16
13-HDHA	343.2	193.2	20.42	0.05	0.1	500	4.41	±	0.92	21	88
11-HETE	319.2	167.2	20.47	0.022	0.044	219	15.04	±	2.80	19	688
9-oxo-ODE	293.2	185.1	20.67	0.25	0.5	500	0.25	±	0.00		
15-oxo-ETE	317.2	113.1	20.70	0.05	0.1	250	0.05	±	0.00		
10-HDHA	343.2	153.2	20.69	0.025	0.05	500	4.00	±	0.77	19	160

Tab. 8.3: Continued.

analyte	mass transition		t_R		calibration range		QS plasma (total oxylipins)				
				LOD	LLOQ	ULOQ	Mean		SD	RSD	Fold
	Q1	Q3	[min]	[nM]	[nM]		[nM]		[nM]	%	LLOQ
14-HDHA	343.2	205.2	20.69	0.055	0.14	275	3.67	±	0.83	23	53
14(15)-EpETE	317.2	207.2	20.79	0.1	0.25	500	5.34	±	1.75	33	43
8-HETE	319.2	155.2	20.89	0.094	0.23	468	10.91	±	2.22	20	93
12-HETE	319.2	179.2	20.93	0.1	0.25	500	11.44	±	2.30	20	92
11(12)-EpETE	317.2	167.0	20.98	0.1	0.25	500	4.66	±	1.87	40	37
11-HDHA	343.2	121.1	21.04	0.1	0.25	500	4.90	±	1.07	22	39
8(9)-EpETE	317.2	127.2	21.17	0.5	0.75	500	4.38	±	1.62	37	12
7-HDHA	343.2	141.2	21.18	0.05	0.1	500	3.78	±	0.64	17	76
9-HETE	319.2	167.2	21.29	0.27	0.4	265	12.09	±	2.23	18	61
15-HETrE	321.2	221.2	21.36	0.05	0.1	500	3.17	±	0.44	14	63
8-HDHA	343.2	189.2	21.42	0.038	0.1	190	3.38	±	0.66	19	71
12-oxo-ETE	317.2	153.1	21.54	0.41	1.0	103	0.51	±	0.00		
5-HETE	319.2	115.2	21.59	0.018	0.035	350	19.14	±	2.05	11	1094
8-HETrE	321.2	157.1	21.76	0.25	0.5	500	2.32	±	0.64	28	9
12-HETrE	321.0	181.0	22.00	0.1	0.25	500	2.93	±	0.71	24	23
17-oxo-DHA	341.3	111.0	22.15	7.5	10	500	5.00	±	0.00		
4-HDHA	343.2	101.1	22.17	0.05	0.1	500	6.52	±	1.41	22	130
17-oxo-n3DPA	343.1	247.0	22.29	2.5	5	100	2.50	±	0.00		
12(13)-EpOME	295.2	195.2	22.28	0.019	0.037	185	126.92	±	50.52	40	6860
19(20)-EpDPE	343.2	241.2	22.27	0.25	0.5	500	14.11	±	5.39	38	56
9(10)-EpOME	295.2	171.1	22.50	0.081	0.2	405	156.78	±	63.03	40	1550
14(15)-EpETrE	319.2	219.2	22.50	0.1	0.25	500	47.40	±	17.50	37	379
16(17)-EpDPE	343.2	233.2	22.79	0.1	0.25	500	7.51	±	2.53	34	60
13(14)-EpDPE	343.2	193.2	22.89	0.05	0.1	250	7.85	±	2.74	35	157
5-oxo-ETE	317.2	273.0	22.92	0.5	0.75	500	0.38	±	0.00		
10(11)-EpDPE	343.2	153.2	23.00	0.01	0.025	500	8.06	±	3.18	39	645
11(12)-EpETrE	319.2	167.2	23.10	0.05	0.1	500	30.44	±	10.37	34	609
7(8)-EpDPE	343.2	141.2	23.22	0.44	0.65	436	8.81	±	3.15	36	27
8(9)-EpETrE	319.2	155.2	23.39	0.25	0.5	500	25.62	±	8.70	34	102
4-oxo-DHA	341.0	115.1	23.36	0.1	0.25	500	0.13	±	0.00		
14(15)-EpEDE	321.2	221.2	23.65	0.025	0.05	100	6.20	±	2.69	43	248
5-HETrE	321.2	115.1	23.69	0.01	0.025	250	1.57	±	0.25	16	126
9(10)-Ep-stearic acid	297.0	170.8	24.10	0.25	0.5	500	186.37	±	53.06	28	745

Tab. 8.4 (pages 179-186): Analyte concentrations of total oxylipins in human EDTA plasma during the transitory stage. Shown are mean ± SD concentrations for all storage conditions and times as well as for the QS plasma. Additionally, for each analyte lower limit of quantification (LLOQ) is displayed. When analyte concentration was <LLOQ in more than 50% of the samples the LLOQ (highlighted in grey) is shown instead of mean.

Tab. 8.4: Continued (best case, vortex, before centrifugation, worst case)

	best case		vortex		before centrifugation												worst case	
					4°C; 1h		4°C; 6h		4°C; 24h		20°C; 1h		20°C; 2h		20°C; 4h			
concentration [nM]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
18-COOH-dinor-LTB_4	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
Δ^{17}-6-keto-PGF_{1a}	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
6-keto-$PGF_{1\alpha}$	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
20-COOH-LTB_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
15-F_{3t}-IsoP (8-iso-$PGF_{3\alpha}$)	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0
20-OH-LTB_4	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
9,12,13-TriHODE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
9,10,13-TriHODE	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
$PGF_{3\alpha}$	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
15-F_{1t}-IsoP (8-iso-$PGF_{1\alpha}$)	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
15-F_{2t}-IsoP (8-iso-PGF_{2a})	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
5(*R,S*)-5-F_{2t}-IsoP (5-i$PF_{2\alpha}$-VI)	0.5 ±	0.1	0.5 ±	0.2	0.6 ±	0.2	0.5 ±	0.2	0.5 ±	0.2	0.6 ±	0.2	0.5 ±	0.0	0.6 ±	0.2	0.8 ±	0.5
15-oxo-15-F_{2t}-IsoP	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
9,10,11-TriHODE	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
9,12,13-TriHOME	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
9,10,13-TriHOME	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
$PGF_{2\alpha}$	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
$PGF_{1\alpha}$	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
13,14-dihydro-15-oxo-15-F_{2t}-IsoP	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
9,10,11-TriHOME	1.2 ±	0.7	1.2 ±	0.7	1.1 ±	0.8	1.0 ±	0.8	1.1 ±	0.7	1.3 ±	0.8	1.3 ±	0.7	1.1 ±	0.7	1.4 ±	1.0
5(*R,S*)-5-F_{2c}-IsoP (8,12-iso-i$PF_{2\alpha}$-VI)	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
LxA_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
PGB_3	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
1a,1b-dihomo-$PGF_{2\alpha}$	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
17(*R,S*)-RvD4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
PGJ_2	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
PGB_2	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
15,16-DiHODE	17.6 ±	2.1	18.5 ±	2.2	16.7 ±	1.6	17.4 ±	1.5	16.9 ±	2.4	17.7 ±	2.2	18.0 ±	2.0	17.5 ±	2.5	19.6 ±	3.6
9,10-DiHODE	0.4 ±	0.0	0.5 ±	0.1	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.5 ±	0.0	0.5 ±	0.1	0.5 ±	0.0	0.5 ±	0.0
12,13-DiHODE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
8(*S*),15(*S*)-DiHETE	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0	0.6 ±	0.0
6-*trans*-LTB_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
5(*S*),15(*S*)-DiHETE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
17,18-DiHETE	0.8 ±	0.6	0.8 ±	0.7	0.8 ±	0.7	0.7 ±	0.7	0.7 ±	0.6	0.8 ±	0.8	0.9 ±	0.9	0.7 ±	0.6	0.9 ±	0.7
6-*trans*-12-*epi*-LTB_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
PDx	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0	0.2 ±	0.0

Tab. 8.4: Continued (best case, vortex, before centrifugation, worst case)

	best case		vortex		before centrifugation										worst case	
					4°C; 1h		4°C; 6h		4°C; 24h		20°C; 1h		20°C; 2h		20°C; 4h			
concentration [nM]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
RvD5	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
LTB_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
14,15-DiHETE	0.1 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.1 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1
7(*S*),17(*S*)-DiH-n3DPA	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
11,12-DiHETE	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.1	0.2 ±	0.2	0.2 ±	0.1	0.2 ±	0.1
5(*S*),12(*S*)-DiHETE	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
12,13-DiHOME	5.7 ±	1.1	5.8 ±	1.1	5.2 ±	1.4	5.7 ±	1.6	5.5 ±	1.4	5.8 ±	1.5	6.0 ±	1.4	5.7 ±	1.4	6.3 ±	1.9
8,9-DiHETE	0.5 ±	0.3	0.4 ±	0.2	0.4 ±	0.3	0.4 ±	0.2	0.4 ±	0.3	0.5 ±	0.4	0.5 ±	0.4	0.5 ±	0.4	0.5 ±	0.3
9,10-DiHOME	18.3 ±	5.1	18.0 ±	4.8	17.2 ±	5.6	17.9 ±	5.2	17.8 ±	5.4	18.0 ±	5.1	18.7 ±	6.4	18.2 ±	5.3	18.9 ±	5.8
12-oxo-LTB_4	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
tetranor-12-HETE	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.4 ±	0.1
MaR2	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
5,6-DiHETE	2.6 ±	2.2	2.6 ±	2.6	2.6 ±	2.3	2.6 ±	2.6	2.5 ±	2.2	2.7 ±	2.7	2.9 ±	3.0	2.5 ±	2.5	2.9 ±	2.5
12-HHTrE	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
14,15-DiHETrE	0.8 ±	0.2	0.8 ±	0.1	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2
19,20-DiHDPE	2.5 ±	0.4	2.5 ±	0.5	2.4 ±	0.4	2.5 ±	0.5	2.4 ±	0.4	2.5 ±	0.5	2.5 ±	0.5	2.5 ±	0.5	2.7 ±	0.7
LTB_3	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
16,17-DiHDPE	0.5 ±	0.1	0.5 ±	0.0	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.1	0.5 ±	0.1
11,12-DiHETrE	1.2 ±	0.2	1.1 ±	0.1	1.2 ±	0.2	1.2 ±	0.2	1.2 ±	0.2	1.2 ±	0.2	1.2 ±	0.2	1.2 ±	0.1	1.2 ±	0.2
13,14-DiHDPE	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.1	0.4 ±	0.1	0.4 ±	0.0	0.4 ±	0.1
9-HOTrE	2.8 ±	1.2	2.7 ±	1.4	2.6 ±	1.4	2.8 ±	1.5	3.1 ±	1.6	2.8 ±	1.0	2.7 ±	1.2	3.1 ±	1.6	6.6 ±	6.3
10,11-DiHDPE	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1	0.6 ±	0.1
8,9-DiHETrE	3.5 ±	0.6	3.3 ±	0.5	3.4 ±	0.7	3.4 ±	0.5	3.4 ±	0.7	3.4 ±	0.6	3.6 ±	0.8	3.4 ±	0.7	3.5 ±	0.6
20-COOH-ARA	7.7 ±	2.4	7.3 ±	2.4	6.8 ±	2.5	6.9 ±	2.4	7.7 ±	2.9	6.8 ±	2.9	7.3 ±	2.8	7.4 ±	3.2	8.4 ±	3.0
5(*S*),6(*R*)-DiHETE (ARA)	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
13-HOTrE	3.1 ±	1.9	2.6 ±	1.2	2.6 ±	1.2	2.7 ±	1.6	3.0 ±	1.9	2.9 ±	1.2	2.6 ±	1.3	3.0 ±	1.9	11.5 ±	12.3
18-HEPE	3.4 ±	2.5	2.6 ±	1.2	2.6 ±	1.4	3.3 ±	2.3	4.5 ±	4.4	4.4 ±	3.8	3.8 ±	3.8	4.4 ±	4.4	9.2 ±	6.9
13-γ-HOTrE	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0
5(*S*),6(*S*)-DiHETE (ARA)	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0	0.0 ±	0.0
19-HETE	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0	1.3 ±	0.0
7,8-DiHDPE	3.0 ±	0.5	2.9 ±	0.6	3.0 ±	0.6	3.0 ±	0.6	3.1 ±	0.6	3.1 ±	0.6	3.1 ±	0.8	3.1 ±	0.8	3.0 ±	0.6
5,6-DiHETrE	16.7 ±	5.2	15.4 ±	5.6	16.1 ±	5.8	16.0 ±	5.4	16.2 ±	6.1	16.5 ±	6.0	17.0 ±	6.2	16.5 ±	6.3	16.6 ±	6.6
20-HETE	1.5 ±	0.4	1.5 ±	0.3	1.5 ±	0.3	1.6 ±	0.4	1.5 ±	0.5	1.7 ±	0.4	1.9 ±	0.6	1.9 ±	0.5	2.0 ±	0.7
15-HEPE	2.3 ±	1.7	1.5 ±	0.6	1.7 ±	0.9	2.2 ±	1.6	3.1 ±	2.8	3.0 ±	2.6	2.6 ±	2.6	2.8 ±	2.8	7.6 ±	6.1
13-oxo-OTrE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
15-HODE	5.9 ±	2.6	5.8 ±	3.5	5.6 ±	2.9	5.8 ±	3.0	5.5 ±	2.7	5.8 ±	2.8	5.9 ±	3.1	5.7 ±	2.6	6.2 ±	3.2
11-HEPE	1.4 ±	1.1	1.0 ±	0.4	1.1 ±	0.6	1.3 ±	0.9	2.0 ±	1.9	2.0 ±	1.8	1.6 ±	1.7	1.9 ±	1.9	4.5 ±	3.8

Tab. 8.4: Continued (best case, vortex, before centrifugation, worst case)

	best case		vortex		before centrifugation												worst case	
					4°C; 1h		4°C; 6h		4°C; 24h		20°C; 1h		20°C; 2h		20°C; 4h			
concentration [nM]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
9-oxo-OTrE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
8-HEPE	1.4 ±	1.1	1.0 ±	0.4	1.0 ±	0.6	1.2 ±	0.9	1.8 ±	1.8	1.8 ±	1.7	1.6 ±	1.7	1.7 ±	1.8	3.9 ±	3.3
18-HETE	0.7 ±	0.2	0.7 ±	0.3	0.6 ±	0.2	0.7 ±	0.3	0.7 ±	0.4	0.7 ±	0.3	0.7 ±	0.5	0.6 ±	0.3	0.7 ±	0.3
12-HEPE	2.4 ±	1.8	1.7 ±	0.7	1.8 ±	0.9	2.3 ±	1.6	3.2 ±	3.1	3.3 ±	3.0	2.8 ±	2.9	3.1 ±	3.1	9.1 ±	7.4
17-HETE	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1	0.3 ±	0.1
9-HEPE	2.5 ±	1.9	1.7 ±	0.7	1.9 ±	0.9	2.4 ±	1.7	3.3 ±	3.3	3.3 ±	3.0	2.8 ±	2.7	3.1 ±	3.5	9.0 ±	7.3
16-HETE	0.8 ±	0.3	0.8 ±	0.4	0.7 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.2	0.8 ±	0.3	1.0 ±	0.4
12-HODE	2.3 ±	0.8	2.0 ±	1.2	2.0 ±	0.6	2.2 ±	0.7	2.4 ±	0.7	2.4 ±	0.8	2.0 ±	0.4	2.4 ±	1.0	2.6 ±	0.8
5-HEPE	3.2 ±	1.8	2.7 ±	1.2	2.7 ±	1.3	3.1 ±	1.9	3.8 ±	3.0	3.6 ±	2.5	3.3 ±	2.6	3.6 ±	2.8	5.8 ±	4.2
13-HODE	101.7 ±	83.1	75.9 ±	31.9	68.5 ±	18.3	76.4 ±	20.5	88.5 ±	15.0	102.0 ±	45.8	88.1 ±	30.0	90.1 ±	19.5	342.8 ±	287.8
10-HODE	4.4 ±	1.4	3.7 ±	2.0	3.7 ±	1.1	4.1 ±	1.5	4.3 ±	1.1	4.4 ±	1.4	3.7 ±	0.8	4.4 ±	1.6	4.6 ±	1.4
9-HODE	73.9 ±	54.2	57.3 ±	23.4	50.5 ±	14.4	57.2 ±	15.1	65.5 ±	10.0	74.2 ±	34.0	65.3 ±	25.5	67.4 ±	11.9	220.5 ±	187.0
20-HDHA	7.0 ±	2.0	6.4 ±	3.5	5.8 ±	1.8	6.9 ±	1.8	8.7 ±	2.5	8.4 ±	1.3	6.6 ±	2.4	7.9 ±	2.7	17.1 ±	5.2
15(16)-EpODE	18.2 ±	10.0	29.9 ±	24.5	20.4 ±	14.0	25.4 ±	19.4	20.4 ±	9.4	18.9 ±	11.4	19.4 ±	10.5	19.5 ±	8.4	22.8 ±	15.2
15-HETE	15.6 ±	5.2	13.7 ±	8.2	12.3 ±	3.1	14.8 ±	2.5	18.1 ±	3.8	18.6 ±	4.2	14.6 ±	3.9	16.6 ±	3.9	39.5 ±	10.4
9(10)-EpODE	4.7 ±	3.1	12.1 ±	11.3	6.5 ±	5.4	9.6 ±	9.1	6.8 ±	4.6	5.5 ±	3.9	5.5 ±	2.8	6.3 ±	3.2	7.5 ±	6.3
17(18)-EpETE	9.8 ±	7.0	17.5 ±	8.9	10.9 ±	5.1	13.4 ±	5.2	16.9 ±	16.4	12.0 ±	9.2	14.6 ±	15.3	18.4 ±	22.2	14.0 ±	9.9
13-oxo-ODE	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
16-HDHA	3.9 ±	1.3	3.6 ±	2.0	3.4 ±	1.1	4.0 ±	1.1	5.0 ±	1.5	4.9 ±	0.9	3.8 ±	1.4	4.4 ±	1.6	11.1 ±	4.6
17-HDHA	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
12(13)-EpODE	2.2 ±	1.5	5.3 ±	4.8	3.0 ±	2.5	4.2 ±	3.7	3.1 ±	1.9	2.5 ±	1.7	2.6 ±	1.4	2.9 ±	1.5	3.5 ±	2.9
13-HDHA	4.9 ±	1.7	4.1 ±	2.1	4.1 ±	1.9	5.0 ±	1.8	6.1 ±	2.1	5.9 ±	1.3	4.8 ±	2.0	5.4 ±	2.3	12.7 ±	5.2
11-HETE	16.4 ±	6.5	13.6 ±	8.7	12.3 ±	3.6	14.8 ±	2.6	18.1 ±	3.4	18.8 ±	4.0	15.0 ±	4.5	16.5 ±	4.1	45.5 ±	14.2
9-oxo-ODE	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0	0.3 ±	0.0
15-oxo-ETE	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
10-HDHA	4.6 ±	1.4	3.9 ±	1.8	3.9 ±	1.7	4.5 ±	1.4	5.5 ±	1.9	5.6 ±	1.2	4.4 ±	1.8	5.0 ±	1.9	11.2 ±	4.8
14-HDHA	3.5 ±	1.3	3.0 ±	1.6	3.0 ±	1.4	3.6 ±	1.1	4.2 ±	2.0	4.2 ±	1.3	3.1 ±	1.7	4.1 ±	1.5	10.7 ±	4.6
14(15)-EpETE	5.6 ±	4.0	10.1 ±	5.1	6.2 ±	3.1	7.6 ±	2.9	9.4 ±	8.9	6.6 ±	5.0	7.9 ±	8.1	10.0 ±	12.0	7.7 ±	5.4
8-HETE	12.5 ±	4.5	10.7 ±	6.4	9.5 ±	2.5	11.0 ±	1.9	13.3 ±	2.5	13.8 ±	2.7	11.2 ±	2.6	12.1 ±	2.5	28.7 ±	6.6
12-HETE	12.0 ±	4.4	10.3 ±	5.9	9.2 ±	2.6	11.1 ±	1.8	13.4 ±	1.9	13.8 ±	3.6	11.0 ±	2.9	12.2 ±	1.9	37.6 ±	12.0
11(12)-EpETE	4.9 ±	3.4	9.1 ±	4.7	5.5 ±	2.4	6.6 ±	2.5	8.4 ±	7.8	6.0 ±	4.5	7.3 ±	7.7	9.2 ±	11.1	7.2 ±	5.2
11-HDHA	5.9 ±	2.0	5.2 ±	2.9	5.3 ±	2.8	6.2 ±	2.5	7.5 ±	2.6	7.2 ±	1.8	5.6 ±	2.2	6.4 ±	2.8	18.2 ±	8.9
8(9)-EpETE	4.9 ±	3.5	7.7 ±	4.0	4.6 ±	3.0	6.0 ±	2.9	7.7 ±	7.2	6.5 ±	5.2	7.7 ±	8.4	8.2 ±	9.4	7.5 ±	5.8
7-HDHA	4.7 ±	1.3	4.1 ±	1.5	3.5 ±	0.7	4.2 ±	0.8	5.1 ±	1.1	5.6 ±	1.2	4.9 ±	1.5	4.9 ±	1.5	10.7 ±	3.6
9-HETE	14.4 ±	6.6	12.7 ±	8.7	10.5 ±	2.9	13.1 ±	3.8	15.9 ±	2.9	16.9 ±	3.3	12.5 ±	2.7	13.3 ±	2.3	47.2 ±	16.8
15-HETrE	3.4 ±	1.5	3.4 ±	2.8	2.8 ±	1.3	3.3 ±	1.5	3.8 ±	1.3	3.8 ±	0.9	2.9 ±	0.3	3.4 ±	1.1	8.2 ±	1.8
8-HDHA	4.0 ±	1.1	3.4 ±	1.1	3.4 ±	1.0	3.9 ±	0.8	4.8 ±	1.4	4.7 ±	0.9	3.9 ±	1.3	4.3 ±	1.6	11.6 ±	4.9

Tab. 8.4: Continued (best case, vortex, before centrifugation, worst case)

	best case		vortex		before centrifugation												worst case	
					4°C; 1h		4°C; 6h		4°C; 24h		20°C; 1h		20°C; 2h		20°C; 4h			
concentration [nM]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
12-oxo-ETE	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
5-HETE	22.0 ±	6.9	21.7 ±	11.6	19.1 ±	5.4	21.6 ±	5.4	24.2 ±	5.7	24.5 ±	5.5	20.7 ±	3.7	22.2 ±	4.4	40.8 ±	5.1
8-HETrE	2.5 ±	1.5	2.1 ±	1.4	1.3 ±	0.5	2.1 ±	1.3	2.6 ±	1.0	3.1 ±	1.3	2.3 ±	0.4	2.5 ±	1.0	5.7 ±	1.8
12-HETrE	3.6 ±	2.2	3.0 ±	2.8	2.6 ±	1.5	3.2 ±	1.6	3.7 ±	1.5	3.7 ±	0.9	2.8 ±	0.3	3.1 ±	0.8	10.0 ±	5.0
17-oxo-DHA	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0
4-HDHA	8.0 ±	2.2	7.2 ±	3.9	7.4 ±	3.4	8.2 ±	3.1	9.1 ±	2.2	9.0 ±	1.2	7.0 ±	1.4	8.5 ±	2.5	13.6 ±	2.7
17-oxo-n3DPA	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0
12(13)-EpOME	109.4 ±	29.7	250.6 ±	209.7	151.6 ±	60.9	206.4 ±	139.8	187.4 ±	126.1	124.5 ±	54.8	133.6 ±	37.8	156.3 ±	69.8	162.1 ±	116.7
19(20)-EpDPE	14.8 ±	4.7	35.9 ±	35.0	19.9 ±	7.2	26.3 ±	18.0	25.9 ±	18.8	19.2 ±	11.6	19.6 ±	10.2	23.1 ±	13.4	26.6 ±	23.0
9(10)-EpOME	126.0 ±	35.1	304.8 ±	242.0	172.4 ±	67.9	242.6 ±	167.7	217.3 ±	148.4	147.2 ±	63.9	157.4 ±	41.3	185.0 ±	77.8	193.5 ±	130.2
14(15)-EpETrE	43.3 ±	16.7	107.5 ±	109.3	57.7 ±	28.8	79.4 ±	62.9	80.0 ±	64.6	55.5 ±	36.8	58.1 ±	29.7	67.7 ±	38.9	74.2 ±	65.4
16(17)-EpDPE	7.5 ±	2.5	17.9 ±	16.1	10.2 ±	3.7	13.2 ±	8.8	13.4 ±	9.5	10.0 ±	5.8	10.3 ±	5.1	11.8 ±	6.1	13.2 ±	10.8
13(14)-EpDPE	8.0 ±	2.6	19.4 ±	17.9	10.2 ±	3.8	14.4 ±	10.0	13.9 ±	10.3	10.2 ±	6.4	10.6 ±	5.5	12.5 ±	6.7	13.8 ±	11.6
5-oxo-ETE	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
10(11)-EpDPE	7.9 ±	3.0	19.2 ±	18.3	10.6 ±	4.5	14.9 ±	11.8	14.1 ±	11.5	10.5 ±	7.5	10.6 ±	6.0	12.8 ±	7.7	14.1 ±	12.5
11(12)-EpETrE	29.3 ±	11.1	68.9 ±	71.4	38.7 ±	18.8	53.9 ±	45.7	53.8 ±	44.9	36.8 ±	26.0	37.4 ±	20.3	44.8 ±	27.5	47.8 ±	44.6
7(8)-EpDPE	8.8 ±	2.9	19.6 ±	16.9	11.9 ±	4.4	16.1 ±	11.8	15.4 ±	12.0	11.5 ±	7.4	11.7 ±	6.3	13.8 ±	8.1	15.2 ±	12.5
8(9)-EpETrE	24.5 ±	9.5	52.4 ±	50.6	31.5 ±	15.5	44.0 ±	38.0	44.2 ±	37.8	31.0 ±	22.5	31.2 ±	17.4	37.6 ±	23.4	38.7 ±	33.9
4-oxo-DHA	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
14(15)-EpEDE	5.7 ±	3.8	18.6 ±	25.1	8.4 ±	6.6	12.6 ±	14.3	11.9 ±	14.4	8.3 ±	8.6	8.0 ±	7.1	8.9 ±	7.0	12.2 ±	15.5
5-HETrE	2.1 ±	1.4	2.0 ±	1.8	1.9 ±	1.4	2.0 ±	1.4	2.0 ±	1.4	1.9 ±	1.0	1.8 ±	1.0	1.8 ±	1.1	2.7 ±	1.3
9(10)-Ep-stearic acid	158.3 ±	65.1	381.6 ±	358.2	209.1 ±	100.7	288.2 ±	240.7	265.0 ±	221.7	190.5 ±	122.7	195.7 ±	98.1	221.0 ±	101.0	273.0 ±	242.5

Tab. 8.4: Continued (after centrifugation)

concentration [nM]	4°C not sep.; 1h mean	SD	4°C not sep.; 2h mean	SD	4°C; 1h mean	SD	4°C; 2h mean	SD	4°C; 6h mean	SD	4°C; 6h mean	SD	-20°C; 6h mean	SD	-20°C; 24h mean	SD	-20°C; 3d mean	SD	-20°C; 5d mean	SD	4°C; 1h; -20°C; 3d mean	SD
18-COOH-dinor-LTB_4	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
Δ^{17}-6-keto-PGF_{1a}	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0
2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
6-keto-$PGF_{1\alpha}$	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
20-COOH-LTB_4	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
15-F_{3t}-IsoP	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0	1.3	0.0
20-OH-LTB_4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,12,13-TriHODE	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
9,10,13-TriHODE	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
$PGF_{3\alpha}$	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
15-F_{1t}-IsoP	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0	0.5	0.0
15-F_{2t}-IsoP	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
5(*R,S*)-5-F_{2t}-IsoP	0.5	0.1	0.5	0.2	0.6	0.2	0.5	0.1	0.6	0.1	0.5	0.2	0.5	0.2	0.4	0.1	0.5	0.1	0.4	0.2	0.5	0.1
15-oxo-15-F_{2t}-IsoP	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0
9,10,11-TriHODE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,12,13-TriHOME	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9,10,13-TriHOME	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
$PGF_{2\alpha}$	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0
$PGF_{1\alpha}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13,14-dihydro-15-oxo-15-F_{2t}-IsoP	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0
9,10,11-TriHOME	1.3	0.9	1.2	0.6	1.2	0.8	1.8	1.7	1.2	0.6	1.2	0.8	1.3	0.7	1.1	0.7	1.1	0.8	1.0	0.4	1.2	0.7
5(*R,S*)-5-F_{2c}-IsoP	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0	0.3	0.0
LxA_4	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
PGB_3	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0
1a,1b-dihomo-$PGF_{2\alpha}$	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0	0.4	0.0
17(*R,S*)-RvD4	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
PGJ_2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PGB_2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15,16-DiHODE	17.5	2.5	18.1	1.6	17.6	2.2	18.5	3.2	18.0	1.5	17.9	2.5	17.5	1.8	18.2	1.6	18.1	2.0	18.0	2.4	18.8	3.2
9,10-DiHODE	0.4	0.1	0.5	0.1	0.5	0.0	0.5	0.0	0.4	0.1	0.4	0.0	0.5	0.1	0.5	0.1	0.4	0.1	0.4	0.0	0.4	0.1
12,13-DiHODE	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
8(*S*),15(*S*)-DiHETE	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0	0.6	0.0
6-*trans*-LTB_4	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
5(*S*),15(*S*)-DiHETE	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
17,18-DiHETE	0.7	0.6	0.8	0.7	0.8	0.7	0.8	0.6	0.8	0.7	0.8	0.6	0.8	0.8	0.8	0.8	0.8	0.7	0.8	0.6	0.8	0.7
6-*trans*-12-*epi*-LTB_4	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
PDx	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0	0.2	0.0

Tab. 8.4: Continued (after centrifugation)

	after centrifugation																																	
	4°C not sep.; 1h			4°C not sep.; 2h			4°C; 1h			4°C; 2h			4°C; 6h			4°C; 6h			-20°C; 6h			-20°C; 24h			-20°C; 3d			-20°C; 5d			4°C; 1h; -20°C; 3d		
concentration [nM]	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**	**mean**		**SD**
RvD5	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
LTB_4	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
14,15-DiHETE	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1
7(*S*),17(*S*)-DiH-n3DPA	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0
11,12-DiHETE	0.2	±	0.1	0.2	±	0.2	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1
5(*S*),12(*S*)-DiHETE	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
12,13-DiHOME	5.5	±	1.1	5.7	±	1.2	5.7	±	1.3	5.9	±	1.0	5.7	±	1.2	5.7	±	1.3	5.7	±	1.3	5.8	±	1.3	5.7	±	0.9	5.6	±	1.1	5.8	±	1.4
8,9-DiHETE	0.5	±	0.4	0.5	±	0.5	0.5	±	0.4	0.5	±	0.3	0.5	±	0.3	0.4	±	0.3	0.5	±	0.4	0.5	±	0.4	0.5	±	0.3	0.4	±	0.2	0.4	±	0.3
9,10-DiHOME	17.8	±	5.9	18.7	±	5.8	18.9	±	5.0	18.0	±	6.6	17.9	±	6.0	16.9	±	5.4	18.1	±	5.5	18.1	±	5.4	17.4	±	6.3	17.4	±	5.8	17.8	±	5.8
12-oxo-LTB_4	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
tetranor-12-HETE	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1
MaR2	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
5,6-DiHETE	2.7	±	2.6	3.0	±	3.4	2.9	±	2.4	2.7	±	2.5	2.7	±	2.5	2.8	±	2.3	2.8	±	2.8	3.0	±	3.0	2.7	±	2.4	2.5	±	2.3	2.7	±	2.5
12-HHTrE	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0
14,15-DiHETrE	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2	0.8	±	0.2
19,20-DiHDPE	2.5	±	0.5	2.5	±	0.5	2.5	±	0.5	2.4	±	0.4	2.5	±	0.5	2.5	±	0.5	2.5	±	0.6	2.5	±	0.6	2.5	±	0.4	2.5	±	0.4	2.5	±	0.4
LTB_3	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
16,17-DiHDPE	0.5	±	0.1	0.5	±	0.1	0.5	±	0.1	0.5	±	0.1	0.5	±	0.1	0.5	±	0.0	0.5	±	0.1	0.5	±	0.1	0.5	±	0.0	0.5	±	0.0	0.5	±	0.1
11,12-DiHETrE	1.2	±	0.2	1.2	±	0.2	1.2	±	0.1	1.3	±	0.2	1.2	±	0.2	1.1	±	0.2	1.2	±	0.2	1.2	±	0.2	1.1	±	0.2	1.1	±	0.2	1.2	±	0.2
13,14-DiHDPE	0.4	±	0.0	0.4	±	0.1	0.4	±	0.1	0.4	±	0.1	0.4	±	0.0	0.4	±	0.0	0.4	±	0.1	0.4	±	0.1	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0
9-HOTrE	2.8	±	1.3	3.1	±	1.6	3.3	±	1.5	3.3	±	1.3	4.1	±	2.5	3.3	±	2.5	2.8	±	0.9	2.6	±	0.8	3.0	±	1.5	3.3	±	2.3	3.3	±	2.1
10,11-DiHDPE	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1	0.6	±	0.0	0.6	±	0.1	0.6	±	0.1	0.6	±	0.1
8,9-DiHETrE	3.5	±	0.8	3.6	±	0.9	3.6	±	0.7	3.6	±	0.8	3.3	±	0.5	3.2	±	0.5	3.4	±	0.7	3.4	±	0.8	3.2	±	0.6	3.2	±	0.5	3.3	±	0.6
20-COOH-ARA	7.4	±	3.2	8.0	±	3.6	7.5	±	2.7	6.9	±	3.6	7.2	±	2.8	6.6	±	2.3	7.4	±	2.7	7.4	±	2.7	6.9	±	2.6	7.1	±	2.4	7.3	±	2.6
5(*S*),6(*R*)-DiHETE	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
13-HOTrE	2.7	±	1.3	3.2	±	1.8	3.0	±	1.5	3.5	±	1.5	6.5	±	5.7	3.3	±	3.0	2.6	±	1.1	2.6	±	0.9	2.9	±	1.6	2.9	±	2.0	3.5	±	2.0
18-HEPE	4.5	±	4.9	5.5	±	6.7	5.0	±	4.8	4.4	±	3.2	4.4	±	1.8	3.3	±	1.6	4.0	±	4.0	3.9	±	3.8	3.9	±	3.0	3.1	±	1.4	3.3	±	1.5
13-γ-HOTrE	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0
5(*S*),6(*S*)-DiHETE	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
19-HETE	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0	1.3	±	0.0
7,8-DiHDPE	3.2	±	0.8	2.8	±	1.0	3.2	±	0.7	3.3	±	0.8	2.9	±	0.5	2.8	±	0.5	3.1	±	0.7	3.0	±	0.8	2.8	±	0.6	2.8	±	0.5	2.9	±	0.7
5,6-DiHETrE	16.7	±	6.0	17.0	±	6.4	16.9	±	6.0	16.6	±	6.3	16.8	±	5.9	15.6	±	5.3	16.2	±	6.0	16.8	±	6.3	15.6	±	5.5	15.4	±	5.4	16.1	±	6.2
20-HETE	1.6	±	0.4	1.6	±	0.5	1.6	±	0.3	1.6	±	0.4	1.5	±	0.2	1.4	±	0.3	1.5	±	0.4	1.5	±	0.3	1.5	±	0.2	1.3	±	0.2	1.6	±	0.3
15-HEPE	2.8	±	3.1	3.8	±	4.7	3.2	±	3.0	2.9	±	2.1	2.7	±	1.2	2.0	±	1.2	2.7	±	2.8	2.6	±	2.6	2.4	±	1.7	1.7	±	0.7	2.0	±	0.9
13-oxo-OTrE	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
15-HODE	5.9	±	3.0	6.0	±	3.2	6.1	±	3.2	5.9	±	3.1	6.3	±	3.8	5.8	±	3.5	5.9	±	2.9	5.9	±	2.8	5.9	±	3.1	5.8	±	3.2	6.0	±	3.2
11-HEPE	1.8	±	2.0	2.4	±	3.0	2.1	±	2.1	1.9	±	1.5	1.7	±	0.7	1.2	±	0.6	1.8	±	2.0	1.7	±	1.8	1.5	±	1.2	1.1	±	0.4	1.2	±	0.7

Tab. 8.4: Continued (after centrifugation)

concentration [nM]	4°C not sep.; 1h			4°C not sep.; 2h			4°C; 1h			4°C; 2h			4°C; 6h			4°C; 6h			-20°C; 6h			-20°C; 24h			-20°C; 3d			-20°C; 5d			4°C; 1h; -20°C; 3d		
	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD
9-oxo-OTrE	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
8-HEPE	1.7	±	2.0	2.1	±	2.8	1.9	±	1.9	1.7	±	1.5	1.5	±	0.6	1.1	±	0.6	1.6	±	1.8	1.6	±	1.8	1.4	±	1.2	1.0	±	0.5	1.2	±	0.6
18-HETE	0.7	±	0.4	0.7	±	0.4	0.7	±	0.4	0.7	±	0.3	0.7	±	0.3	0.7	±	0.2	0.7	±	0.4	0.7	±	0.4	0.7	±	0.4	0.7	±	0.3	0.7	±	0.2
12-HEPE	3.1	±	3.4	4.0	±	5.2	3.3	±	3.1	3.2	±	2.5	3.0	±	1.4	2.1	±	1.2	2.9	±	3.2	2.7	±	2.9	2.7	±	2.0	1.8	±	0.9	2.2	±	1.0
17-HETE	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.4	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1
9-HEPE	3.2	±	3.8	4.1	±	5.5	3.5	±	3.4	3.3	±	2.6	3.1	±	1.7	2.1	±	1.2	3.1	±	3.5	2.8	±	2.9	2.6	±	1.9	1.9	±	0.9	2.2	±	1.2
16-HETE	0.9	±	0.2	0.9	±	0.2	0.9	±	0.3	0.9	±	0.3	0.9	±	0.2	0.9	±	0.3	0.8	±	0.2	0.9	±	0.2	0.8	±	0.2	0.9	±	0.2	0.9	±	0.3
12-HODE	2.7	±	0.9	2.6	±	0.3	2.5	±	0.6	2.7	±	0.5	3.1	±	1.0	2.3	±	1.0	2.5	±	0.8	2.4	±	0.9	2.3	±	0.7	2.5	±	0.9	2.5	±	1.1
5-HEPE	3.5	±	3.0	3.9	±	3.7	3.9	±	3.1	3.4	±	2.1	3.4	±	1.3	2.9	±	1.3	3.4	±	2.8	3.5	±	2.7	3.4	±	2.4	2.8	±	1.1	3.0	±	1.3
13-HODE	84.2	±	17.9	96.4	±	30.0	96.7	±	12.3	106	±	32.4	262	±	355	88.6	±	39.5	80.9	±	11.8	80.9	±	21.3	84.3	±	24.5	86.4	±	34.3	163	±	166
10-HODE	4.9	±	1.5	4.9	±	0.8	4.7	±	1.2	4.9	±	0.9	5.6	±	1.8	4.1	±	1.6	4.5	±	1.3	4.4	±	1.5	4.1	±	1.2	4.3	±	1.4	4.5	±	1.9
9-HODE	63.3	±	11.8	70.5	±	18.7	71.8	±	6.2	78.4	±	26.4	176	±	241	65.4	±	27.4	62.5	±	11.0	62.0	±	19.7	64.4	±	21.5	66.2	±	28.5	118	±	119
20-HDHA	7.3	±	2.9	8.2	±	4.3	8.9	±	2.9	8.4	±	1.9	8.9	±	3.4	7.4	±	3.3	7.3	±	2.0	7.1	±	2.0	7.3	±	0.8	6.9	±	2.6	7.5	±	1.4
15(16)-EpODE	17.4	±	10.5	19.8	±	11.1	33.1	±	35.2	25.1	±	15.8	29.4	±	32.7	20.4	±	15.5	18.6	±	9.3	22.8	±	16.9	23.1	±	19.1	22.7	±	19.7	24.6	±	19.8
15-HETE	15.8	±	4.9	18.1	±	9.1	19.2	±	5.7	19.4	±	4.2	21.6	±	13.5	15.3	±	5.5	15.7	±	4.2	15.7	±	5.6	16.4	±	3.1	15.2	±	4.7	17.1	±	6.2
9(10)-EpODE	3.7	±	2.1	5.4	±	3.3	15.5	±	21.5	9.0	±	7.1	12.8	±	19.1	6.2	±	5.7	4.5	±	2.4	7.5	±	6.7	8.1	±	8.8	7.6	±	8.4	8.8	±	8.5
17(18)-EpETE	10.7	±	11.0	16.5	±	21.1	20.4	±	14.5	16.7	±	9.3	21.2	±	19.1	11.2	±	5.9	11.3	±	10.9	15.4	±	15.1	17.2	±	18.3	11.7	±	6.9	13.6	±	7.5
13-oxo-ODE	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0
16-HDHA	4.1	±	1.8	4.8	±	2.8	5.1	±	1.7	5.0	±	1.0	5.4	±	2.7	4.0	±	2.2	4.2	±	1.5	4.0	±	1.4	4.2	±	0.9	3.6	±	1.6	4.2	±	1.2
17-HDHA	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0	0.4	±	0.0
12(13)-EpODE	1.9	±	1.1	2.5	±	1.5	6.9	±	9.4	4.1	±	3.1	6.1	±	8.8	3.0	±	2.7	2.2	±	1.1	3.5	±	3.0	3.7	±	3.9	3.6	±	3.9	3.9	±	3.7
13-HDHA	4.8	±	2.3	5.8	±	3.5	6.1	±	2.1	5.8	±	1.0	5.8	±	2.6	4.6	±	2.1	5.0	±	1.8	4.8	±	1.9	4.9	±	1.1	4.3	±	2.0	4.9	±	1.4
11-HETE	15.6	±	5.4	19.1	±	11.0	19.6	±	6.5	19.9	±	4.6	25.6	±	23.1	14.8	±	5.2	16.0	±	5.1	15.8	±	6.0	15.8	±	3.9	13.5	±	4.6	16.9	±	7.3
9-oxo-ODE	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0
15-oxo-ETE	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
10-HDHA	4.5	±	2.2	5.3	±	3.2	5.5	±	1.9	5.3	±	1.1	5.0	±	2.0	4.1	±	1.7	4.6	±	1.8	4.4	±	1.7	4.4	±	0.9	3.9	±	1.7	4.5	±	1.2
14-HDHA	3.5	±	1.6	4.4	±	2.6	4.4	±	1.5	4.3	±	0.7	4.4	±	2.1	3.4	±	1.6	3.7	±	1.3	3.5	±	1.4	3.6	±	0.7	2.9	±	1.6	3.8	±	1.2
14(15)-EpETE	6.8	±	7.4	9.4	±	12.0	11.1	±	7.6	9.8	±	5.9	10.8	±	9.5	6.5	±	3.8	6.7	±	6.4	8.5	±	8.2	9.6	±	9.9	6.7	±	4.2	7.8	±	4.5
8-HETE	12.0	±	3.8	13.9	±	7.0	14.4	±	5.1	14.6	±	3.8	15.4	±	9.7	10.8	±	3.4	12.1	±	3.5	12.0	±	3.9	12.1	±	2.7	10.6	±	3.4	12.3	±	3.9
12-HETE	12.3	±	3.1	14.4	±	8.0	14.1	±	3.9	14.2	±	3.1	16.8	±	12.9	11.1	±	3.4	11.9	±	3.9	11.7	±	4.8	12.2	±	4.4	10.5	±	3.7	12.5	±	5.4
11(12)-EpETE	5.8	±	6.1	8.5	±	11.1	10.4	±	7.3	8.8	±	5.0	10.2	±	9.2	5.7	±	3.2	5.6	±	5.5	8.0	±	8.0	9.2	±	9.8	6.0	±	3.6	7.2	±	4.0
11-HDHA	5.7	±	2.6	6.8	±	4.1	7.2	±	2.5	7.1	±	1.6	7.7	±	3.7	5.5	±	2.7	5.9	±	2.2	5.5	±	2.1	5.8	±	1.2	5.1	±	2.2	5.8	±	1.7
8(9)-EpETE	5.6	±	6.0	8.4	±	10.5	8.6	±	5.7	7.9	±	5.4	7.1	±	6.0	5.6	±	3.7	6.2	±	6.0	8.3	±	8.6	9.0	±	9.9	5.3	±	3.2	7.1	±	4.2
7-HDHA	4.9	±	1.9	5.3	±	2.4	5.2	±	2.3	5.1	±	1.5	4.9	±	1.3	4.1	±	1.0	5.0	±	1.7	4.6	±	1.8	4.7	±	0.8	4.1	±	0.8	4.7	±	0.2
9-HETE	13.5	±	4.0	15.7	±	8.3	15.6	±	4.9	16.4	±	3.8	23.9	±	25.3	12.2	±	3.7	13.5	±	4.4	13.5	±	5.7	13.7	±	4.4	11.7	±	4.4	15.5	±	7.9
15-HETrE	3.2	±	0.9	3.4	±	1.1	3.8	±	1.7	3.9	±	1.7	5.4	±	4.0	3.4	±	1.9	3.1	±	0.5	3.0	±	0.3	3.4	±	0.8	3.3	±	1.6	4.0	±	1.6
8-HDHA	3.9	±	1.6	4.5	±	2.9	4.4	±	1.5	4.5	±	0.8	5.0	±	2.3	3.6	±	1.1	3.9	±	1.4	3.8	±	1.6	3.9	±	0.8	3.4	±	1.0	4.0	±	0.9

Tab. 8.4: Continued (after centrifugation)

	after centrifugation																							
	4°C not sep.; 1h		4°C not sep.; 2h		4°C; 1h		4°C; 2h		4°C; 6h		4°C; 6h		-20°C; 6h		-20°C; 24h		-20°C; 3d		-20°C; 5d		4°C; 1h; -20°C; 3d	
concentration [nM]	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
12-oxo-ETE	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0	0.5 ±	0.0
5-HETE	20.9 ±	4.1	22.6 ±	6.5	23.9 ±	6.0	23.2 ±	4.8	25.0 ±	8.8	19.9 ±	6.0	21.2 ±	4.2	21.4 ±	5.1	21.6 ±	3.2	21.0 ±	6.2	23.2 ±	6.3
8-HETrE	2.7 ±	0.7	2.8 ±	0.8	2.8 ±	1.9	2.9 ±	1.2	3.8 ±	3.9	2.5 ±	1.7	2.7 ±	0.4	2.5 ±	0.6	2.6 ±	1.1	2.6 ±	1.5	3.0 ±	1.8
12-HETrE	2.8 ±	0.6	3.2 ±	1.0	3.5 ±	1.5	3.9 ±	1.6	8.0 ±	9.4	3.0 ±	1.5	2.7 ±	0.3	2.7 ±	0.4	2.7 ±	0.8	2.8 ±	1.4	4.5 ±	3.7
17-oxo-DHA	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0	5.0 ±	0.0
4-HDHA	7.1 ±	2.3	7.4 ±	2.9	8.9 ±	2.8	8.0 ±	1.9	8.2 ±	2.7	6.5 ±	2.1	7.0 ±	2.0	7.0 ±	2.2	7.3 ±	1.2	7.0 ±	2.1	7.7 ±	1.0
17-oxo-n3DPA	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0	2.5 ±	0.0
12(13)-EpOME	92.2 ±	13.4	134 ±	69.3	253 ±	211	196 ±	135	201 ±	182	129 ±	42.9	109 ±	30.3	153 ±	58.9	148 ±	62.0	138 ±	76.1	163 ±	92.5
19(20)-EpDPE	13.1 ±	2.8	17.9 ±	9.9	31.9 ±	22.6	27.1 ±	21.8	29.8 ±	25.1	17.7 ±	5.3	15.2 ±	7.7	21.4 ±	10.4	22.5 ±	10.5	20.0 ±	10.5	24.6 ±	17.1
9(10)-EpOME	111 ±	19.3	162 ±	79.7	288 ±	227	244 ±	157	234 ±	206	158 ±	58.0	130 ±	35.0	185 ±	70.1	182 ±	88.1	173 ±	105	204 ±	119
14(15)-EpETrE	44.2 ±	13.9	56.5 ±	31.8	84.7 ±	42.1	88.8 ±	71.1	69.7 ±	34.6	53.2 ±	17.6	48.5 ±	23.9	58.8 ±	25.5	61.8 ±	20.8	55.2 ±	24.8	70.8 ±	47.7
16(17)-EpDPE	7.2 ±	1.8	9.7 ±	5.3	16.5 ±	11.3	15.0 ±	11.4	15.3 ±	12.8	9.6 ±	2.9	8.3 ±	3.9	10.9 ±	5.0	11.5 ±	5.4	10.0 ±	4.8	12.9 ±	8.3
13(14)-EpDPE	7.5 ±	1.8	10.1 ±	5.9	18.0 ±	13.5	15.5 ±	11.6	16.5 ±	14.8	9.9 ±	3.1	8.5 ±	4.2	11.4 ±	5.4	12.3 ±	6.0	10.7 ±	5.3	13.4 ±	8.7
5-oxo-ETE	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0	0.4 ±	0.0
10(11)-EpDPE	6.9 ±	1.6	10.2 ±	6.2	18.0 ±	13.4	16.0 ±	14.0	15.4 ±	13.0	9.8 ±	3.6	8.5 ±	4.9	11.6 ±	5.9	11.7 ±	5.5	10.4 ±	5.6	13.7 ±	9.6
11(12)-EpETrE	27.5 ±	9.1	37.9 ±	23.2	57.7 ±	30.1	58.6 ±	50.7	45.1 ±	23.0	35.1 ±	13.4	31.8 ±	16.7	39.7 ±	18.9	37.9 ±	13.2	34.6 ±	16.2	46.2 ±	33.5
7(8)-EpDPE	7.8 ±	1.7	11.0 ±	6.4	18.6 ±	12.6	16.9 ±	13.8	15.9 ±	12.2	10.9 ±	4.0	9.6 ±	5.2	13.2 ±	6.3	12.8 ±	5.6	11.4 ±	5.5	15.0 ±	9.9
8(9)-EpETrE	22.7 ±	7.2	30.9 ±	18.2	47.6 ±	26.5	47.7 ±	42.4	36.9 ±	17.8	28.4 ±	11.0	26.1 ±	13.5	32.5 ±	15.3	30.9 ±	10.1	27.8 ±	11.9	37.8 ±	26.5
4-oxo-DHA	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0	0.1 ±	0.0
14(15)-EpEDE	4.7 ±	1.9	6.3 ±	3.2	13.7 ±	11.6	14.0 ±	17.5	10.6 ±	9.4	7.3 ±	5.1	6.1 ±	5.9	8.3 ±	6.4	7.9 ±	3.5	8.1 ±	6.8	11.0 ±	11.6
5-HETrE	1.7 ±	1.0	1.8 ±	0.9	2.0 ±	1.2	1.9 ±	1.3	2.0 ±	1.0	1.7 ±	1.2	1.7 ±	0.9	1.7 ±	0.9	1.7 ±	1.0	1.8 ±	1.2	1.8 ±	1.2
9(10)-Ep-stearic acid	135 ±	25.7	183.1 ±	49.5	356 ±	303	304 ±	264	273 ±	226	197 ±	96.0	168 ±	95.4	229 ±	116.3	222 ±	105.1	219 ±	139	266 ±	196

Tab. 8.5 (page 188): Analyte concentrations of total oxylipins during long-term storage (15 months) of human EDTA plasma. Shown are mean concentrations of oxylipins >LLOQ for all storage times. In addition, a concentration range of ±30% (corresponds to the analytical variance) was determined for each oxylipin based on the concentration quantified in month 1.

Mean [nM]	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 9	Month 12	Month 15	Range of ±30%
5(*R,S*)-F_{2t}-IsoP	0.73	0.80	0.85	0.92	0.80	0.84	0.83	0.82	0.72	0.507 - 0.942
5(6)-EpETrE	87.9	90.7	97.0	76.1	69.9	76.2	78.9	80.4	82.5	61.5 - 114.2
8(9)-EpETrE	15.1	16.1	16.4	14.9	14.5	14.7	15.6	14.2	15.9	10.5 - 19.5
11(12)-EpETrE	16.3	17.6	18.0	17.2	13.7	13.4	15.3	15.6	16.1	11.4 - 21.2
14(15)-EpETrE	24.8	27.4	28.8	31.3	25.0	23.4	26.7	26.2	25.0	17.3 - 32.2
8(9)-EpETE	2.01	1.97	1.84	1.52	1.46	1.59	1.67	1.17	1.39	1.40 - 2.61
17(18)-EpETE	2.71	2.74	2.96	2.86	2.63	2.78	2.37	2.62	2.16	1.89 - 3.52
14(15)-EpETE	2.43	2.44	2.73	2.74	2.25	2.09	2.42	2.55	2.83	1.70 - 3.16
10(11)-EpDPE	5.57	5.79	6.24	5.04	4.51	4.66	5.32	4.88	5.57	3.90 - 7.24
13(14)-EpDPE	3.93	4.37	4.93	4.38	3.89	3.92	4.47	4.29	4.25	2.74 - 5.10
16(17)-EpDPE	4.38	4.51	4.88	4.51	4.10	4.14	4.64	4.60	4.68	3.06 - 5.69
19(20)-EpDPE	6.92	6.98	7.94	6.57	6.71	5.90	6.54	6.17	6.50	4.84 - 8.99
9(10)-EpODE	4.86	4.57	4.77	4.26	4.23	4.62	4.36	4.35	4.26	3.40 - 6.32
12(13)-EpODE	5.24	4.84	4.82	4.48	4.48	4.81	4.76	4.88	4.34	3.66 - 6.80
15(16)-EpODE	109	99.6	105	96.3	97.6	106	94.7	98.7	90.5	76.5 - 142
9(10)-EpOME	114	113	107	107	100	107	113	106	104	80.0 - 148
12(13)-EpOME	161	149	157	146	139	141	149	145	135	112 - 209
9(10)-Ep-stearic acid	344	332	352	306	264	311	355	375	368	240 - 446
5-HETE	63.0	65.4	59.6	61.7	62.8	64.7	64.7	68.0	65.2	44.0 - 81.8
8-HETE	45.7	48.2	45.7	47.3	46.9	47.4	50.4	51.2	52.7	31.9 - 59.4
9-HETE	46.5	53.1	50.2	60.7	54.8	58.2	57.6	62.4	62.0	32.5 - 60.4
11-HETE	45.5	47.7	44.6	47.1	49.0	46.9	50.0	52.1	53.8	31.8 - 59.1
12-HETE	55.2	61.8	55.0	59.7	57.5	56.6	63.2	63.6	65.3	38.6 - 71.8
15-HETE	67.8	70.2	67.1	67.7	70.2	68.1	68.3	71.3	74.5	47.4 - 88.1
20-HETE	2.79	2.74	2.80	2.50	2.47	2.35	2.76	2.10	2.17	1.94 - 3.62
12-HHTrE	6.60	6.13	6.61	6.16	5.36	5.15	5.31	5.76	6.18	4.61 - 8.57
5-HEPE	8.27	7.66	8.27	7.87	8.24	8.07	7.88	8.08	8.53	5.79 - 10.7
8-HEPE	7.14	6.27	7.01	6.48	6.76	6.64	6.58	6.97	7.27	4.99 - 9.27
9-HEPE	8.97	8.22	9.17	8.55	8.86	9.21	8.87	11.2	11.3	6.27 - 11.6
11-HEPE	8.89	7.51	8.74	7.02	7.14	7.39	7.38	8.48	9.01	6.22 - 11.5
12-HEPE	9.38	8.54	9.22	7.99	8.10	7.98	8.56	9.34	9.59	6.56 - 12.1
15-HEPE	7.39	7.15	7.26	6.91	6.71	6.58	6.66	7.00	6.74	5.17 - 9.60
18-HEPE	9.33	9.21	9.21	8.70	8.67	8.56	8.74	9.62	10.0	6.53 - 12.1
19-HEPE	1.63	2.03	1.85	1.25	1.46	1.43	1.13	1.31	1.38	1.14 - 2.12
20-HEPE	3.23	3.35	3.02	2.91	2.96	2.96	2.59	2.74	2.84	2.25 - 4.19
4-HDHA	22.0	22.2	21.3	19.6	20.2	21.4	21.6	21.9	22.3	15.3 - 28.5
7-HDHA	12.0	12.7	12.4	11.2	10.7	12.2	11.3	11.3	10.8	8.39 - 15.5
8-HDHA	56.4	58.6	57.4	60.2	58.9	62.7	61.5	65.0	61.8	39.4 - 73.3
10-HDHA	11.7	12.5	11.9	11.9	12.2	12.1	12.3	12.5	12.6	8.19 - 15.2
11-HDHA	15.9	16.1	16.1	16.5	15.6	16.9	16.3	18.1	17.4	11.1- 20.6
13-HDHA	17.0	17.4	16.9	17.5	18.2	17.6	17.9	19.1	18.9	11.8 - 22.0
14-HDHA	24.6	27.3	24.6	25.4	26.3	25.2	27.9	27.5	28.0	17.4 - 31.9
16-HDHA	16.1	16.7	16.2	16.5	17.1	16.4	16.3	18.0	17.7	11.5 - 20.8
17-HDHA	47.6	49.2	48.3	50.1	52.1	50.7	50.4	53.8	54.1	33.3 - 61.8
20-HDHA	23.1	23.6	23.1	23.2	25.1	23.7	23.2	24.9	25.6	16.2 - 30.0
21-HDHA	6.62	6.59	7.46	6.76	7.24	7.20	5.95	7.23	8.18	4.63 - 8.60
22-HDHA	5.17	5.39	5.14	4.91	5.48	5.26	4.72	5.12	5.41	3.69 - 6.72
9-HOTrE	22.6	23.9	23.3	24.1	22.4	23.7	22.2	23.4	22.1	15.9 - 29.3
13-HOTrE	12.8	14.0	13.3	13.7	13.0	13.9	13.0	14.4	13.9	8.9 - 16.7
9-HODE	419	424	426	451	431	452	434	445	423	293 - 544
13-HODE	313	314	317	329	319	323	325	349	335	218 - 406
5(*S*)-HETrE	1.92	2.07	1.86	1.89	1.80	1.85	1.86	2.11	2.19	1.34 - 2.49
15(*S*)-HETrE	14.1	15.4	14.3	18.1	16.4	16.4	16.6	17.6	17.8	9.84 - 18.2
5(6)-DiHETrE	15.6	12.6	15.8	12.9	13.8	13.6	14.3	12.2	12.6	10.9 - 20.2
8(9)-DiHETrE	6.14	5.33	6.16	5.04	5.03	5.35	5.61	5.23	5.21	4.29 - 7.98
11(12)-DiHETrE	1.93	1.82	1.85	1.69	1.68	1.70	1.75	1.76	1.71	1.35 - 2.51
14(15)-DiHETrE	1.27	1.28	1.29	1.22	1.22	1.20	1.19	1.21	1.15	0.892 - 1.65
8(9)-DiHETE	0.55	0.44	0.47	0.51	0.47	0.43	0.37	0.42	0.46	0.387 - 0.718
11(12)-DiHETE	0.19	0.18	0.18	0.17	0.18	0.17	0.16	0.16	0.16	0.136 - 0.252
14(15)-DiHETE	0.17	0.18	0.19	0.17	0.16	0.17	0.17	0.19	0.17	0.116 - 0.216
17(18)-DiHETE	0.76	0.73	0.75	0.68	0.66	0.72	0.72	0.80	0.72	0.529 - 0.982
4,5-DiHDPE	10.00	8.63	9.81	8.40	9.86	9.37	9.41	9.73	9.36	6.99 - 12.9
7,8-DiHDPE	3.63	2.86	3.76	3.02	3.28	3.08	3.27	3.24	3.14	2.54 - 4.72
10,11-DiHDPE	1.16	1.08	1.15	1.01	0.97	1.01	1.02	1.04	1.02	0.810 - 1.50
13,14-DiHDPE	0.55	0.48	0.56	0.48	0.45	0.45	0.46	0.48	0.48	0.386 - 0.717
16,17-DiHDPE	0.84	0.85	0.90	0.81	0.80	0.80	0.77	0.84	0.78	0.588 - 1.09
19,20-DiHDPE	4.35	4.15	4.41	4.19	4.26	4.21	4.15	4.04	3.99	3.04 - 5.64
9,10-DiHODE	1.65	1.49	1.61	1.70	1.69	1.66	1.65	1.64	1.66	1.15 - 2.14
12,13-DiHODE	1.09	0.99	1.31	1.28	1.15	1.24	1.16	1.18	1.11	0.762 - 1.41
15,16-DiHODE	43.6	41.4	45.3	45.5	47.3	43.5	42.7	43.6	45.2	30.5 - 56.6
9,10-DiHOME	27.9	28.1	27.0	27.4	26.6	26.4	26.5	26.9	27.1	19.5 - 36.2
12,13-DiHOME	29.8	31.3	29.4	31.6	32.0	28.5	27.0	26.2	26.2	20.8 - 38.7
9,10-DiH-stearic acid	30.5	29.0	31.1	35.3	32.9	25.1	24.4	27.6	28.0	21.3 - 39.6
8,15-DiHETE	<LLOQ	0.52	0.68	0.48	0.68	0.72	<LLOQ	1.20	0.99	0.363 - 0.675

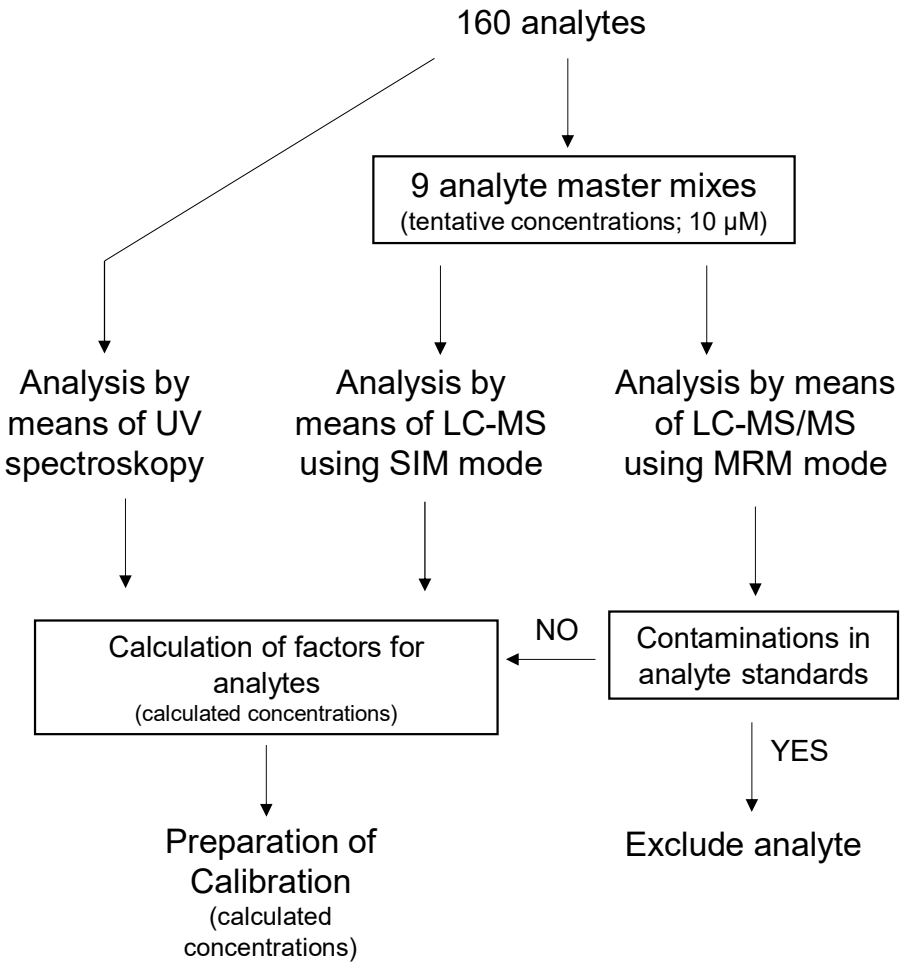


Fig. 8.1: Scheme to generate an oxylipin multi-analyte standard series with characterized purity and concentration.

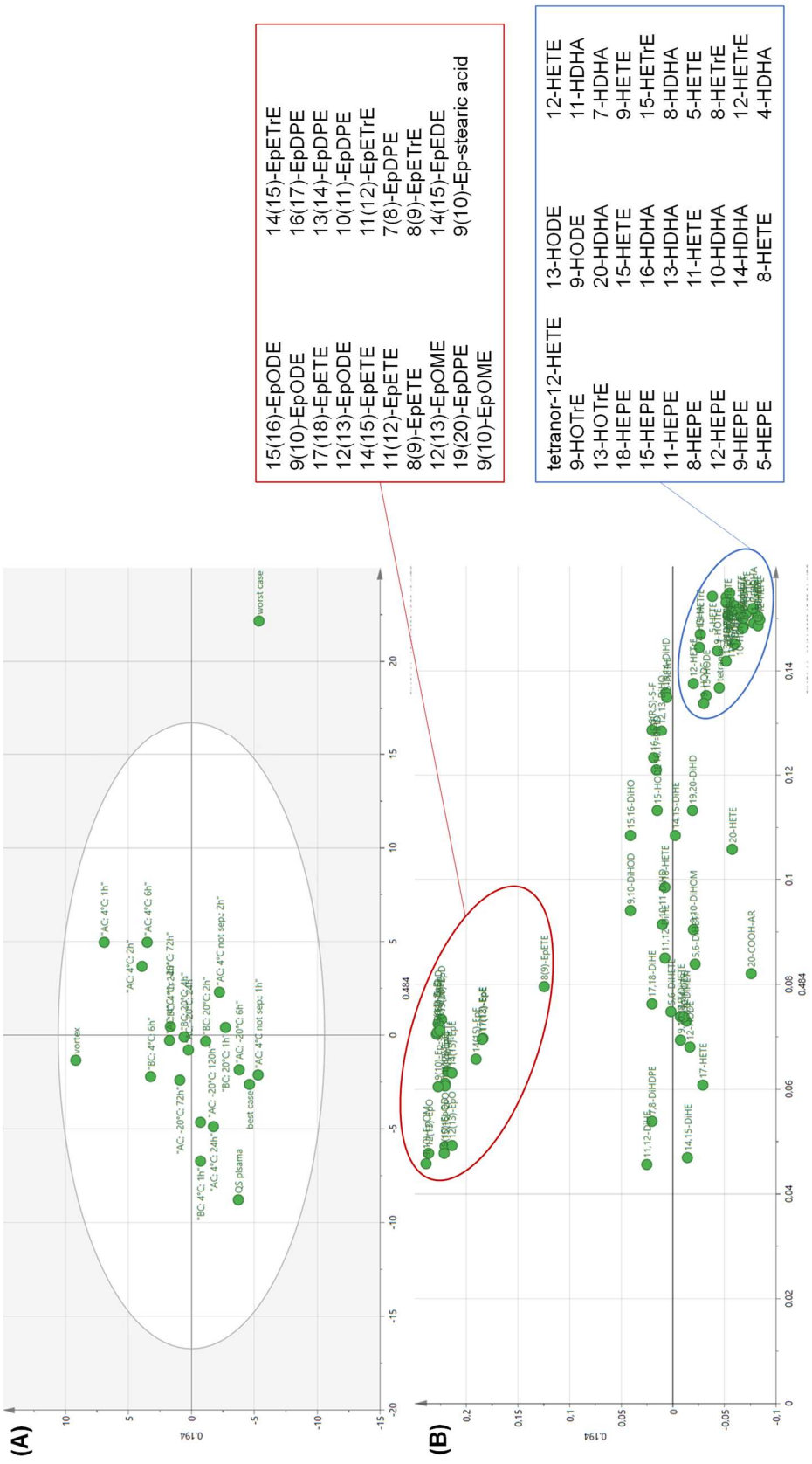


Fig. 8.2: Principal components analysis (PCA) model. **A)** The score plot for 1st (48% of variability) and 2nd (19% of variability) component shows the distribution of all storage conditions and identifies the worst case sample as main contributor to the variability. **B)** The loading plot shows to what extent the different storage conditions influence the oxylipin concentrations. "Vortex" influences the concentration of epoxy-PUFA (red box) and "worst case" has a great effect on the concentration of hydroxy-PUFA (blue box).

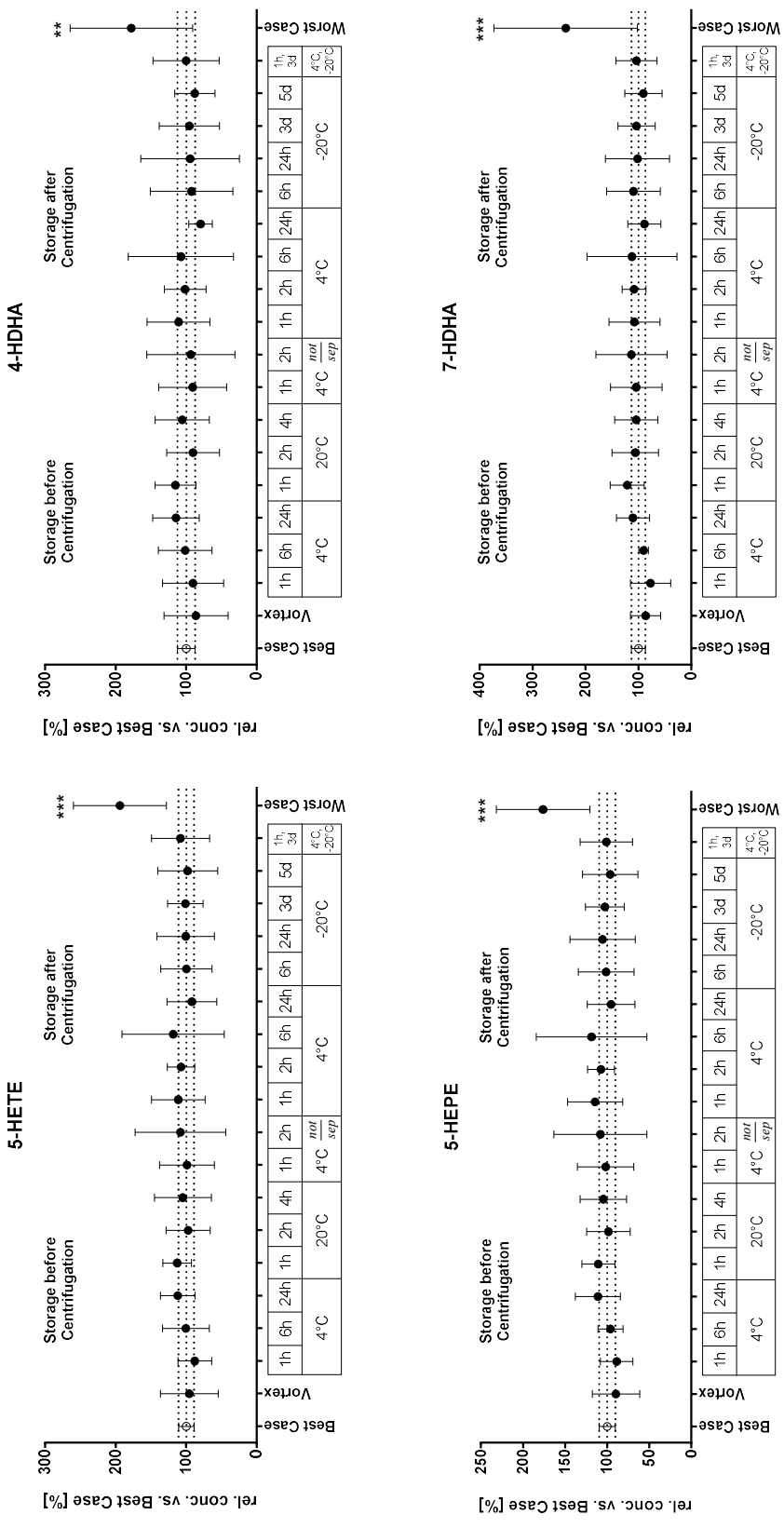


Fig. 8.3: Concentrations of total oxylipins derived from 5-LOX pathway during the different storage conditions and times. The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4; 12 for best case). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (** p<0.01; *** p<0.001).

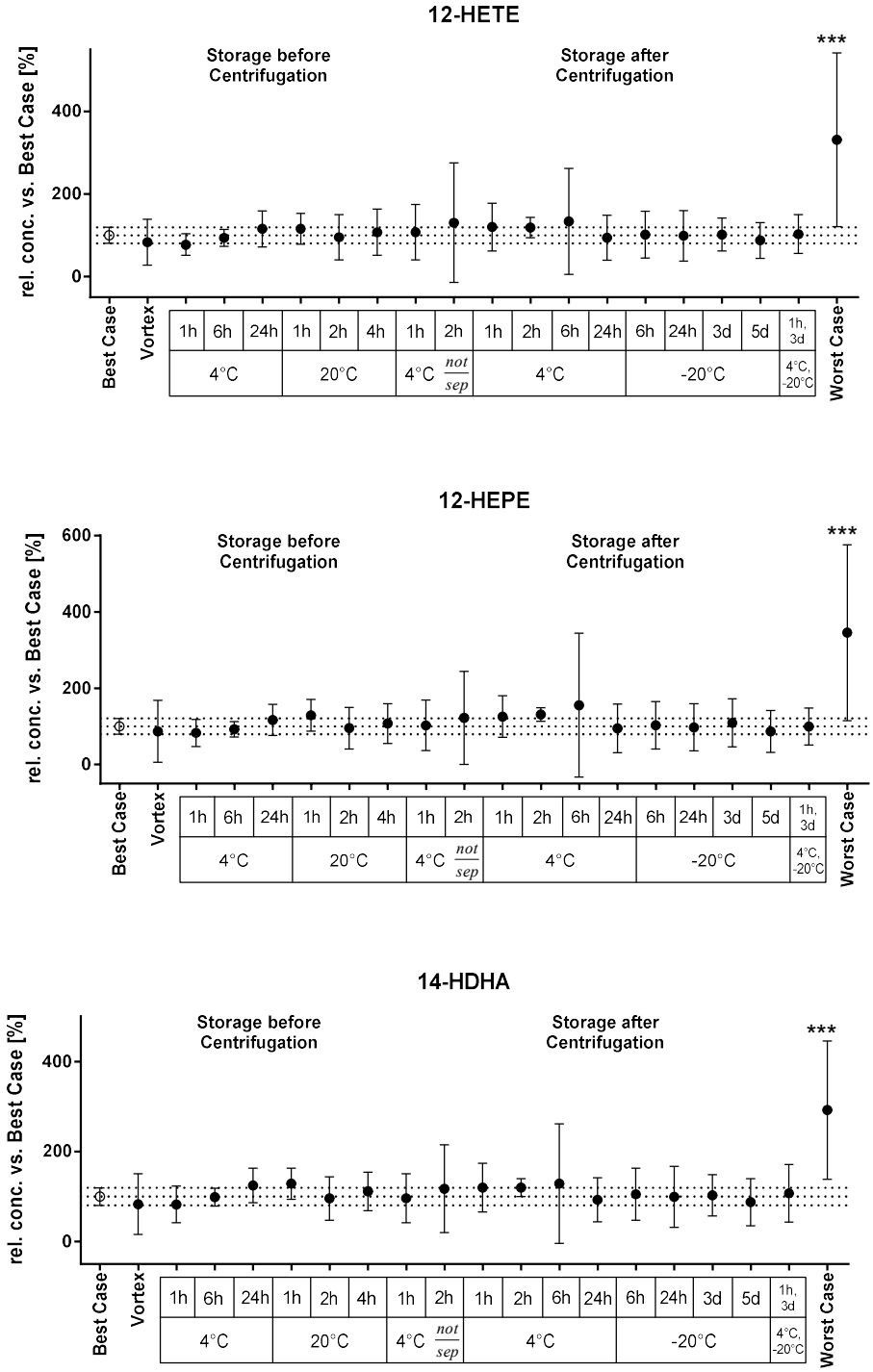


Fig. 8.4: Concentrations of total oxylipins derived from 12-LOX pathway during the different storage conditions and times. The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4, 12 for best case). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (*** $p<0.001$).

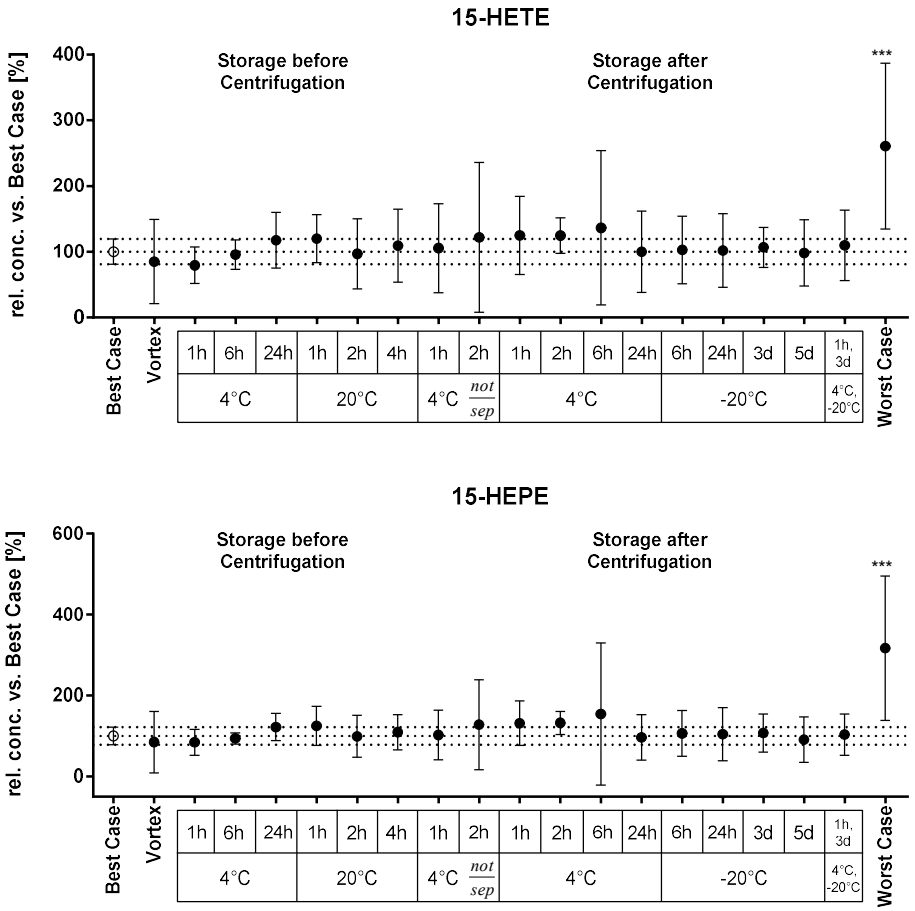


Fig. 8.5: Concentrations of total oxylipins derived from 15-LOX pathway during the different storage conditions and times. The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4; 12 for best case). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (*** $p<0.001$). Of note, the detection of 17-HDHA was not possible due to high baseline.

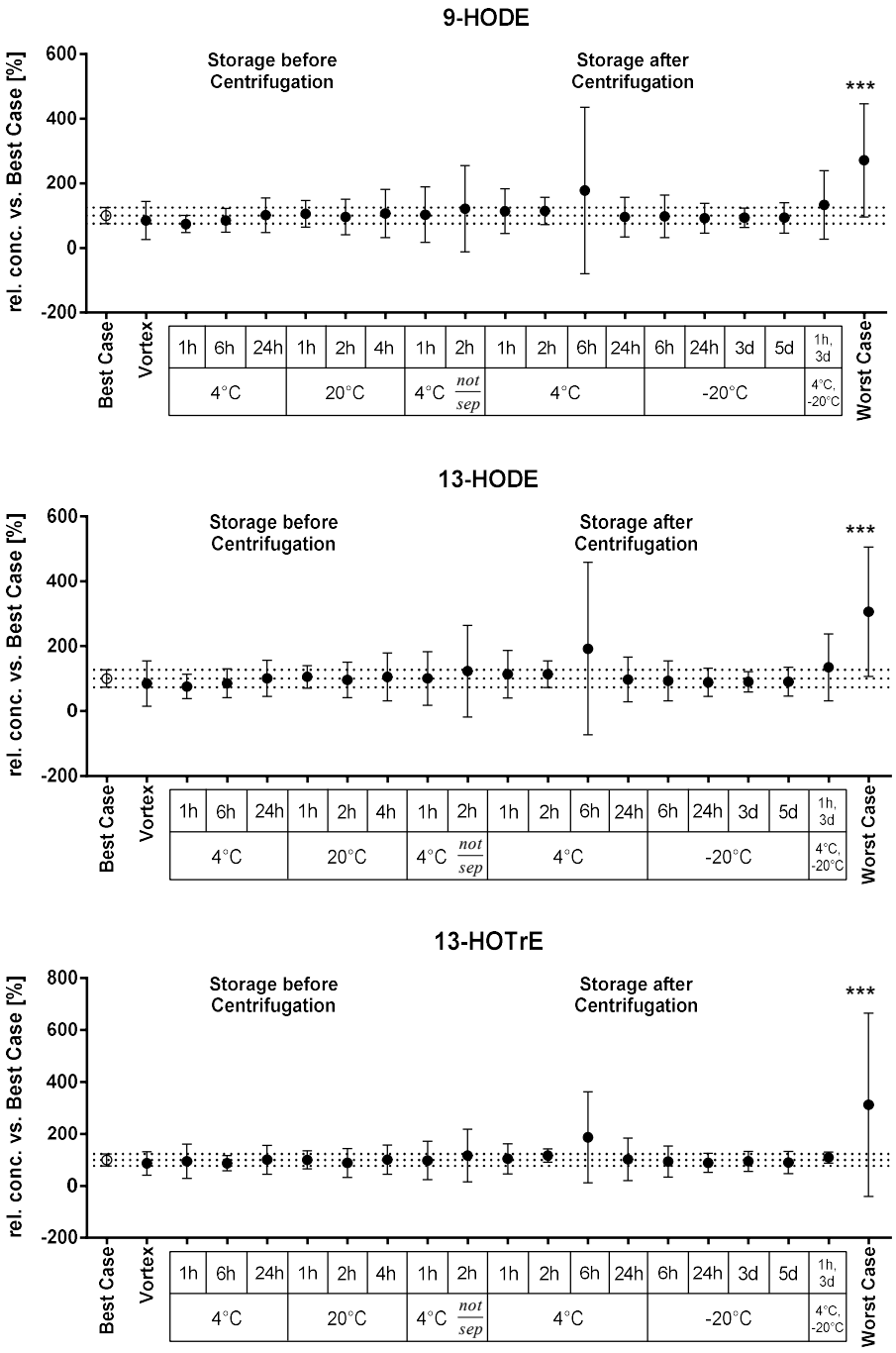


Fig. 8.6: Concentrations of total hydroxy-PUFA derived from LA and ALA during the different storage conditions and times. The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4; 12 for best case). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (*** $p<0.001$).

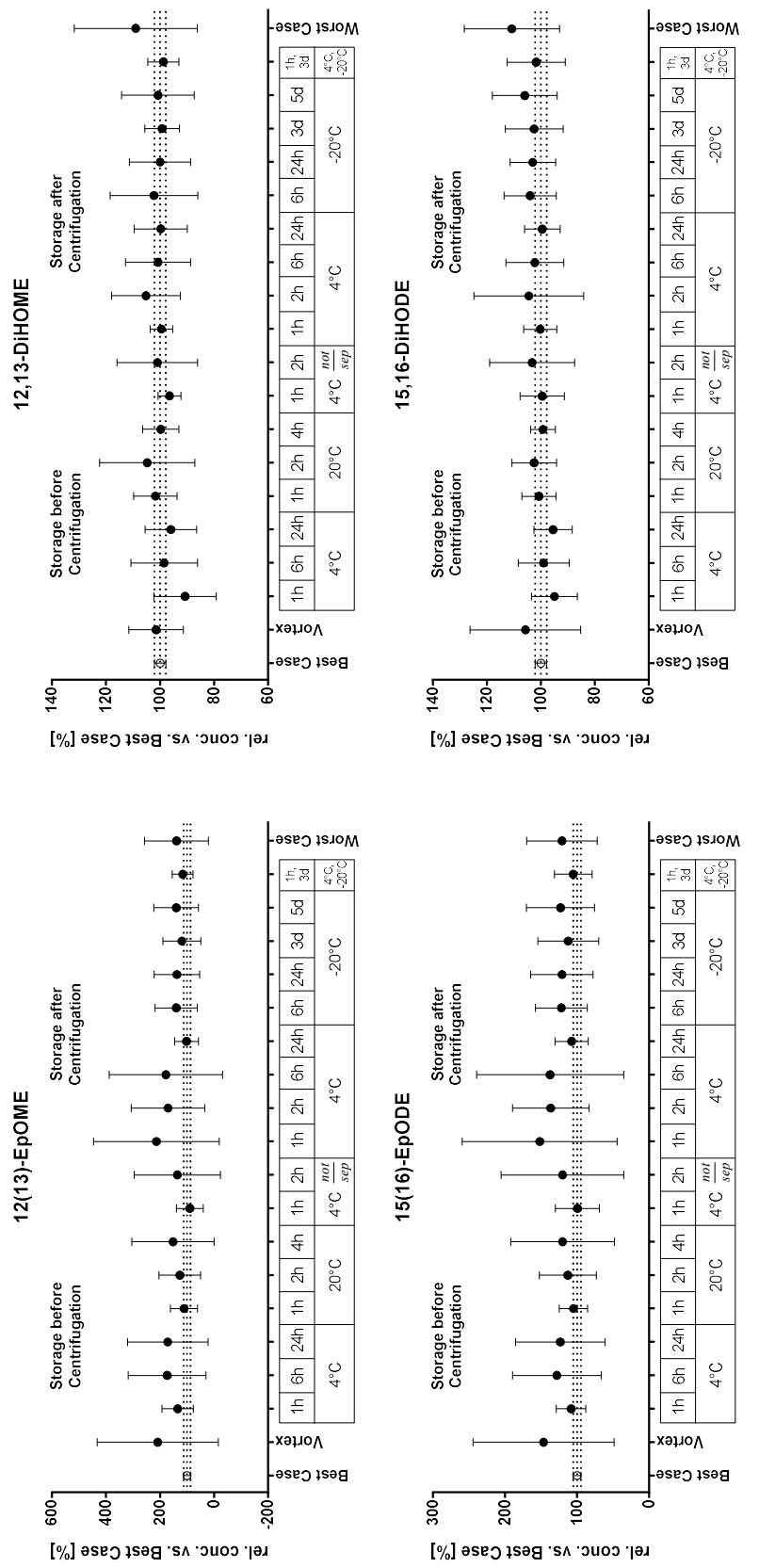


Fig. 8.7: Concentrations of total oxylipins derived from CYP pathway during the different storage conditions and times. On the left Ep-PUFA are presented and on the right the respective s*EH* metabolites (DiH-PUFA). The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4; 12 for best case). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test.

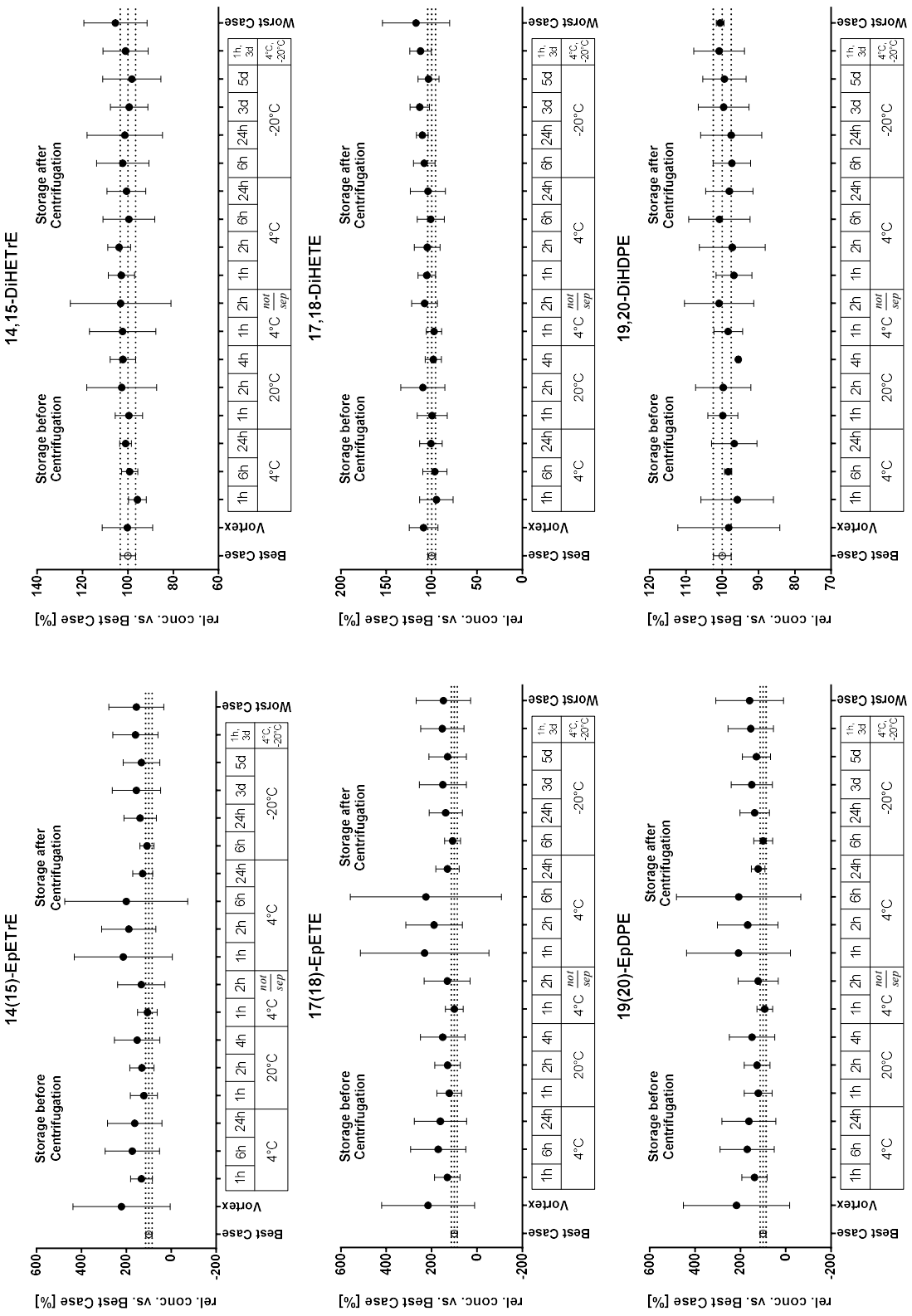


Fig. 8.7: Continued.

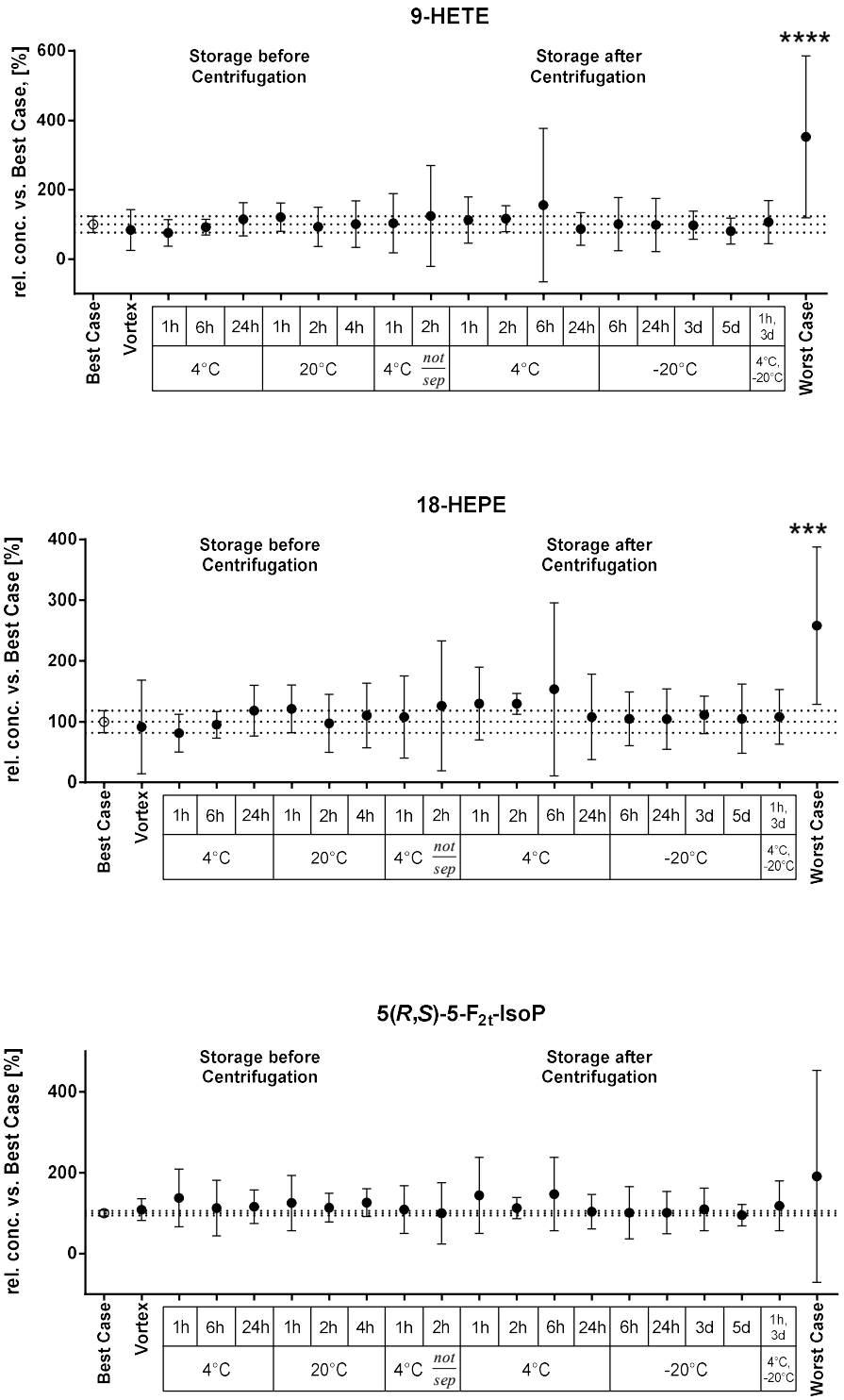


Fig. 8.8: Concentrations of total oxylipins during the different storage conditions and times. ARA derived 9-HETE and 5(*R,S*)-5-F_{2t}-IsoP can be formed during autoxidation. 18-HEPE derived from EPA might be formed autoxidatively or by acetylated COX-2. The relative concentrations were calculated against the baseline concentration (best case sample). Shown are mean ± 95% CI (n=4-12). The dotted lines mark the 95% CI of the best case sample. Statistical differences between baseline and different storage conditions were evaluated by one-way ANOVA followed by Tukey post-test (*** p<0.001).

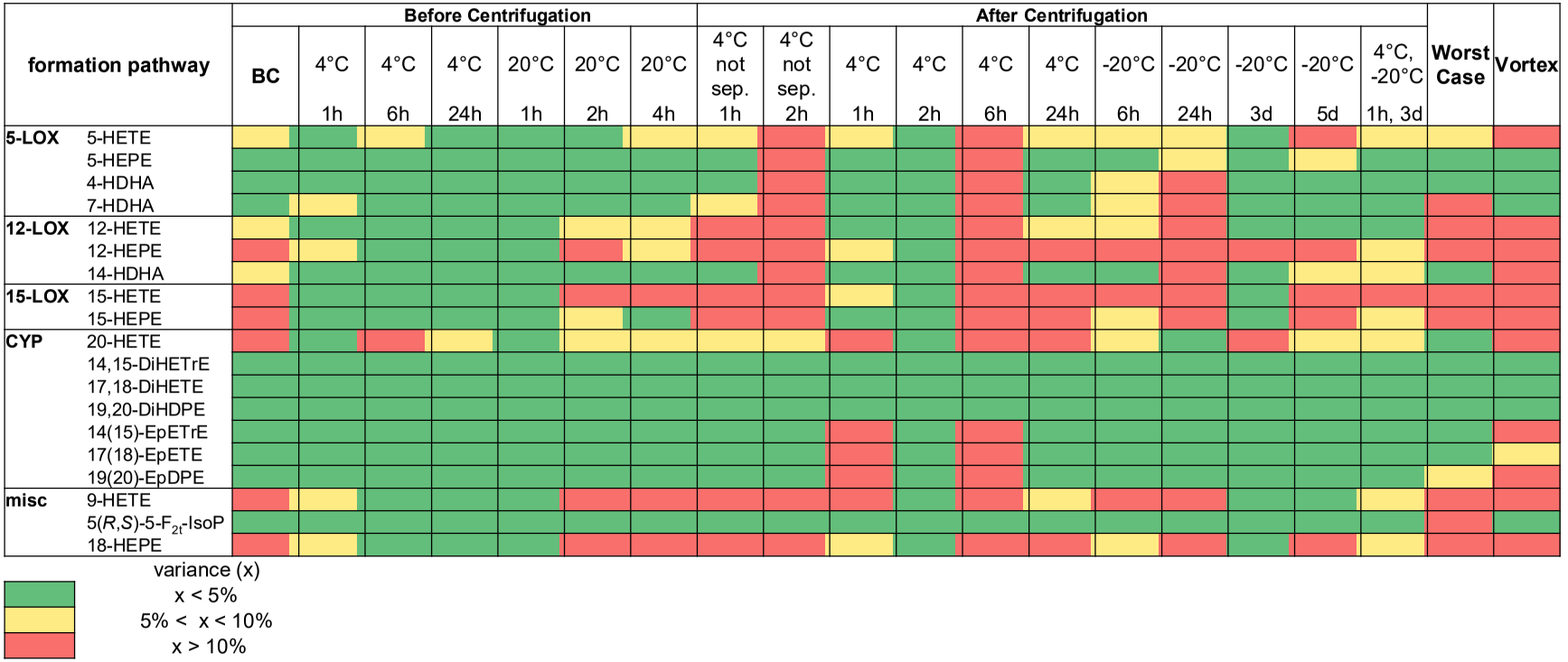


Fig. 8.9: Effects of the different storage conditions on the coefficient of variance (CV). The CV ($\frac{SD}{mean} * 100$) of the differently stored samples was compared to the interbatch CV of quality standard (QS) plasma (2 batches on 4 days) (CV_{sample}-CV_{QS}). When the difference of the sample variance is lower than 5% (CV_{sample}<CV_{QS}+5%) the sample is highlighted in green. Differences between 5% and 10% (CV_{QS}+5%<CV_{sample}<CV_{QS}+10%) are marked in yellow and higher than 10 % (CV_{sample}>CV_{QS}+10%) in red

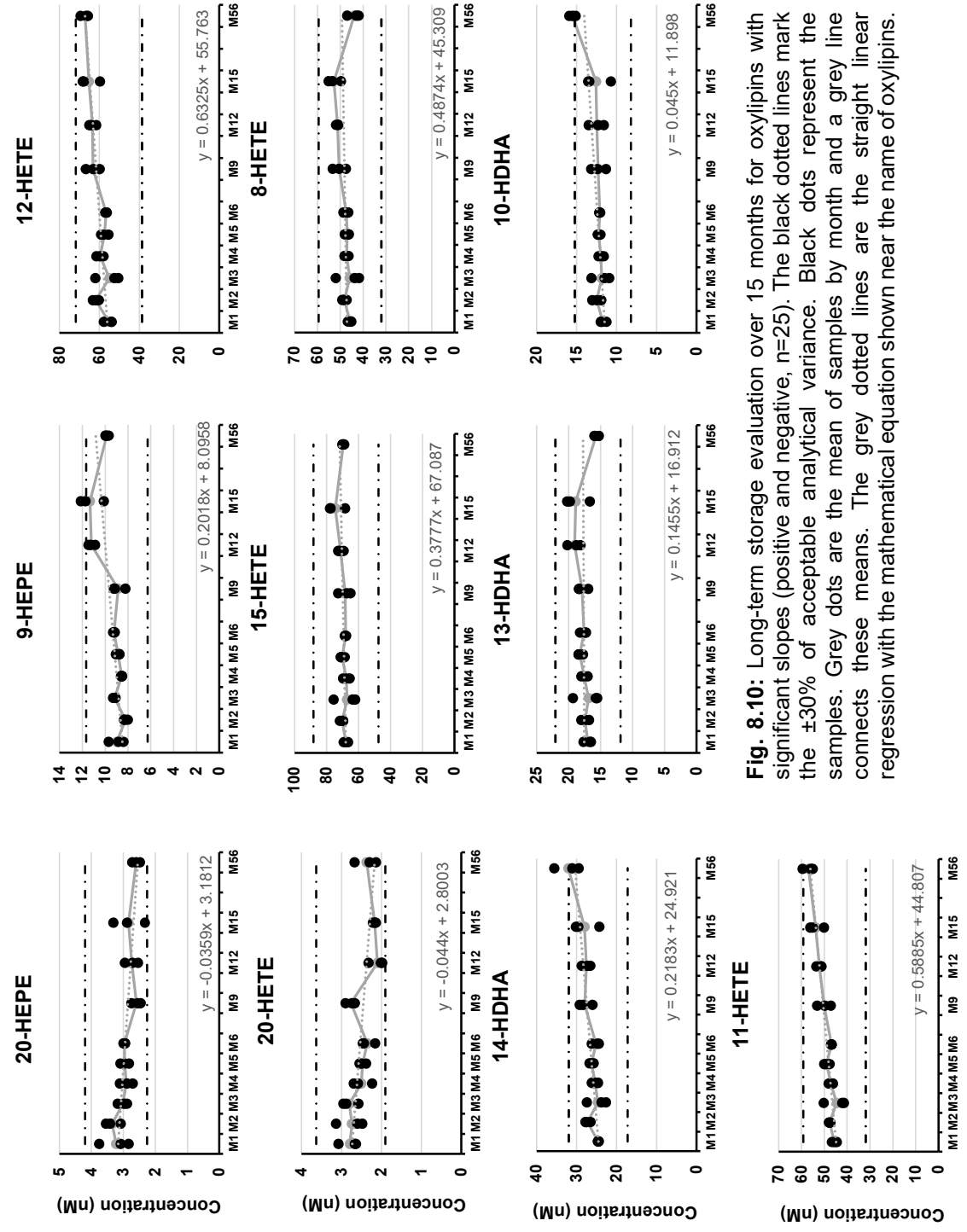


Fig. 8.10: Long-term storage evaluation over 15 months for oxylipins with significant slopes (positive and negative, n=25). The black dotted lines mark the ±30% of acceptable analytical variance. Black dots represent the samples. Grey dots are the mean of samples by month and a grey line connects these means. The grey dotted lines are the straight linear regression with the mathematical equation shown near the name of oxylipins.

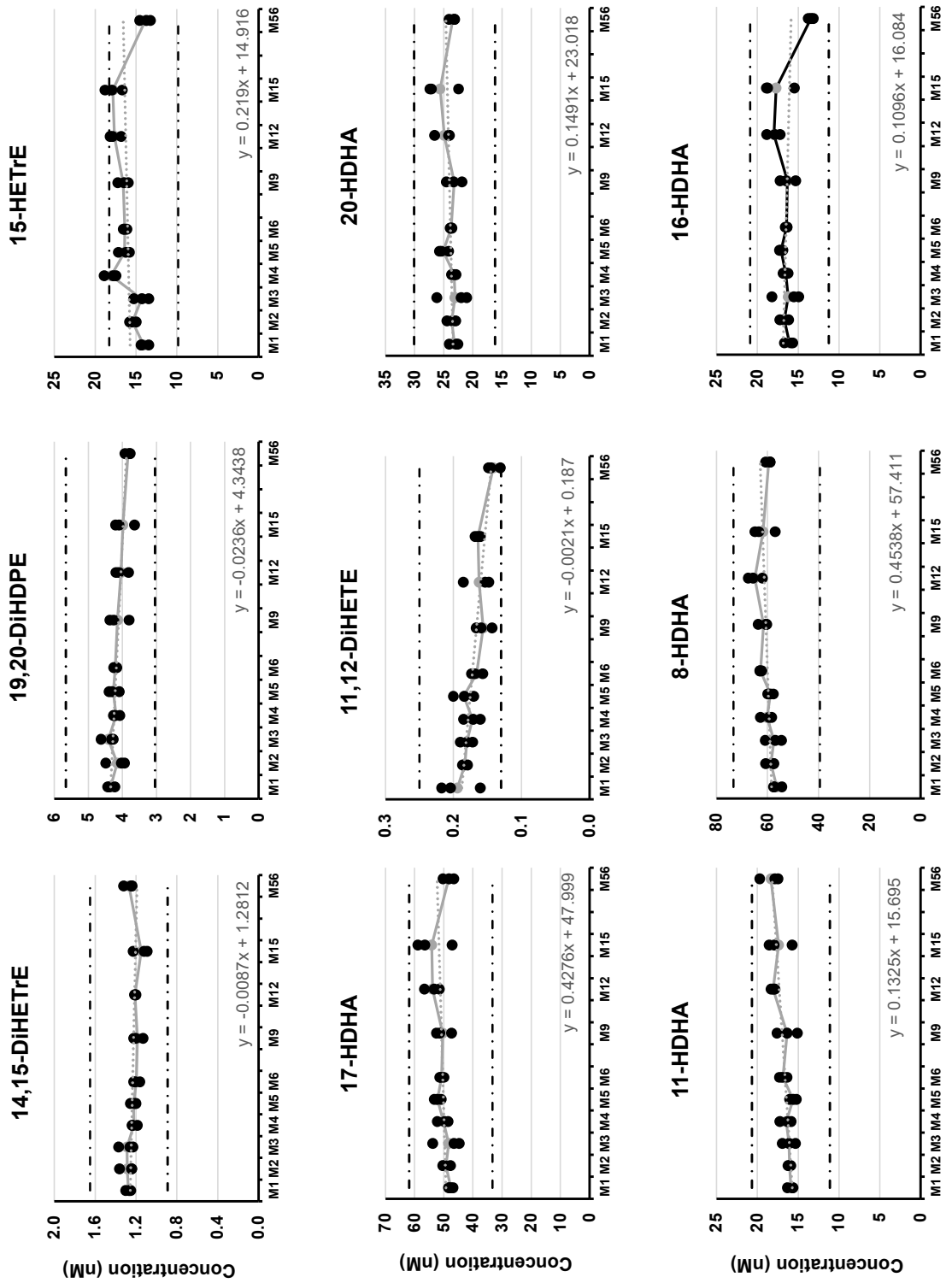


Fig. 8.10: Continued.

Chapter 4

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)
    - Moisten Hamilton syringe before use 1-2 times with MeOH
    - Get rid of any residual MeOH visible in the syringe by rapidly moving plunger up and down a few times
    - 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
    - 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
    - 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
    - one column volume EA/*n*Hex (75/25, *v/v*) with 1% HAc
    - 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*)
    - 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
    - Check pH in one sample per set using pH stripes with a 5.1-7.2 scale
    - 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
    - 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
    - One column volume water (ultrapure, 18 MΩ)
    - One column volume MeOH/H_2O (50/50, *v/v*)
    - 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 <u>clear (!)</u> 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

<u>Preparation of Antiox-Mix, eluent, buffers, etc.</u>

- 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.

Tab. 8.6: Critical separation pairs that require chromatographic separation.

Interference	Comment
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

Tab. 8.7: Interfering analytes.

Interference	Separate Integration possible?	Comment
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

Tab. 8.8: 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* 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 instrument	*LC*: Agilent 1260 Infinity II *MS*: SCIEX API 5500 QTRAP	*LC*: Agilent 1290 Infinity Analysis of samples in laboratory 3 *MS*: Agilent 6460 triple quadrupole	*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

Tab. 8.9 (pages 210-219): 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

Tab. 8.9: Continued. (Lab 1)

		Lab 1													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
isoprostanes	15-F_{1t}-IsoP	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	5(*R,S*)-5-F_{2c}-IsoP	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	5(*R,S*)-5-F_{2t}-IsoP	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	3.48	0.02
	15-F_{2t}-IsoP	<1.25	N/A	<1.25	N/A	25.06	2.15	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	15-oxo-15-F_{2t}-IsoP	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	13,14-dihydro-15-oxo-15-F_{2t}-IsoP	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	15-F_{3t}-IsoP	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A
prostaglandins	$PGF_{1\alpha}$	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	PGB_2	<0.5	N/A	<0.5	N/A	12.62	1.89	20.67	1.67	<0.5	N/A	<0.5	N/A	7.80	0.39
	6-keto-$PGF_{1\alpha}$	<6.4	N/A	<6.4	N/A	<6.4	N/A	<6.4	N/A	<6.4	N/A	<6.4	N/A	<6.4	N/A
	$PGF_{2\alpha}$	10.39	2.10	13.71	5.79	29.76	1.69	15.87	2.72	13.14	5.01	9.66	1.19	6.36	0.90
	PGJ_2	<0.135	N/A	<0.135	N/A	0.32	0.05	0.50	0.01	<0.135	N/A	<0.135	N/A	0.20	0.00
	PGB_3	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	Δ^{17}-6-keto-PGF_{1a}	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	$PGF_{3\alpha}$	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	1a,1b-dihomo-$PGF_{2\alpha}$	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
multiple hydroxylated-PUFA	LTB_3	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	5(*S*),12(*S*)-DiHETE	0.54	0.17	<0.25	N/A	2.41	0.10	<0.25	N/A	2.76	0.53	4.09	0.71	47.01	2.57
	5(*S*),15(*S*)-DiHETE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.74	0.18	7.31	0.45
	8(*S*),15(*S*)-DiHETE	<2.525	N/A	<2.525	N/A	<2.525	N/A	<2.525	N/A	<2.525	N/A	4.22	0.38	41.66	2.05
	LTB_4	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	6-trans-LTB_4	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	4.48	0.19
	6-trans-12-epi-LTB_4	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	8.40	0.20
	5(*S*),6(*R*)-DiHETE	<0.195	N/A	<0.195	N/A	<0.195	N/A	<0.195	N/A	<0.195	N/A	<0.195	N/A	0.78	0.21
	5(*S*),6(*S*)-DiHETE	<0.223	N/A	<0.223	N/A	<0.223	N/A	<0.223	N/A	<0.223	N/A	<0.223	N/A	0.57	0.04
	20-OH-LTB_4	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	20-COOH-LTB_4	<0.825	N/A	<0.825	N/A	<0.825	N/A	<0.825	N/A	<0.825	N/A	<0.825	N/A	<0.825	N/A
	18-COOH-dinor-LTB_4	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A
	LxA_4	<1.25	N/A	<1.25	N/A	60.36	1.88	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	17(*R,S*)-RvD4	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	RvD5	<1.25	N/A	<1.25	N/A	20.62	1.57	<1.25	N/A	<1.25	N/A	<1.25	N/A	2.64	0.13
	MaR2	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	0.62	0.10
	PDx	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	0.43	0.02	0.51	0.11	3.24	0.24
trihydroxylated ALA and LA	9,10,11-TriHOME	0.65	0.20	0.64	0.29	0.61	0.24	0.66	0.12	1.02	0.38	1.06	0.35	14.61	0.46
	9,10,13-TriHOME	1.71	0.36	<0.5	N/A	1.64	0.34	2.35	0.62	1.55	0.95	2.51	0.89	5.04	0.42
	9,12,13-TriHOME	5.28	1.34	3.77	0.78	4.81	0.34	6.20	0.82	5.44	0.85	6.65	0.22	15.31	1.70
	9,10,11-TriHODE	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	0.67	0.05
	9,10,13-TriHODE	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	9,12,13-TriHODE	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
hydroxy-PUFA	9-HODE	61.59	5.38	47.11	3.10	99.98	7.95	99.38	3.08	115.19	2.23	126.82	3.57	2842.28	147.34
	10-HODE	3.75	0.11	2.88	0.07	5.78	0.92	4.08	0.23	4.63	0.24	8.61	0.11	18.90	1.08
	12-HODE	1.97	0.05	1.52	0.12	3.31	0.63	2.31	0.13	2.67	0.26	4.88	0.16	10.32	0.19
	13-HODE	81.53	7.13	60.20	3.07	209.68	15.65	101.85	4.35	157.88	2.82	188.50	5.55	4251.82	223.90
	15-HODE	5.59	0.08	4.22	0.13	5.51	0.23	3.24	0.11	5.91	0.07	5.31	0.05	24.36	0.12
	9-HOTrE	2.48	0.13	1.35	0.03	3.36	0.34	2.22	0.18	3.87	0.21	5.39	0.06	95.08	1.54
	13-HOTrE	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	72.08	44.38
	13-gamma-HOTrE	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	6.20	0.38	111.21	11.29
	5-HETrE	2.00	0.33	1.12	0.10	2.22	0.13	1.55	0.04	1.38	0.00	4.02	0.06	18.80	0.87
	8-HETrE	3.69	0.32	<2.5	N/A	5.43	1.74	3.17	0.72	4.07	0.87	9.79	0.63	140.28	10.65
	12-HETrE	3.94	0.40	1.95	0.27	6.19	0.36	8.43	0.30	4.37	0.18	8.83	0.43	147.20	0.26
	15-HETrE	3.82	0.36	2.41	0.04	6.45	1.00	5.94	0.51	5.00	0.56	10.95	0.48	156.19	1.26
	5-HETE	13.93	0.35	13.33	0.94	18.89	2.20	18.83	1.51	15.05	0.82	26.57	0.20	433.84	22.76
	8-HETE	12.78	0.65	12.73	1.17	20.49	0.43	13.05	0.00	16.79	0.46	28.30	0.27	695.60	62.65
	9-HETE	16.87	1.34	14.27	0.10	27.31	3.63	12.72	1.40	22.98	2.01	41.61	0.58	1008.60	36.92
	11-HETE	19.14	0.84	16.26	1.61	31.24	0.27	86.92	0.60	24.95	1.12	42.81	2.18	983.45	108.86
	12-HETE	18.28	1.15	12.01	1.13	31.01	1.35	539.40	12.10	21.39	0.59	35.67	0.78	880.19	73.94
	15-HETE	7.60	0.24	6.85	0.37	17.64	1.84	29.42	3.25	10.04	0.23	18.90	0.09	369.79	32.73
	16-HETE	0.78	0.03	0.81	0.03	0.90	0.02	1.11	0.05	0.82	0.04	1.25	0.04	2.12	0.15
	17-HETE	<0.375	N/A	0.49	0.00	<0.375	N/A	<0.375	N/A	0.38	0.01	0.42	0.03	0.46	0.04
	18-HETE	0.55	0.06	1.08	0.05	0.63	0.03	0.54	0.00	0.65	0.00	0.73	0.07	0.77	0.01
	19-HETE	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A
	20-HETE	<2.5	N/A	<2.5	N/A	65.50	0.45	<2.5	N/A	<2.5	N/A	2.66	0.07	<2.5	N/A
	12-HHTrE	<5	N/A	<5	N/A	<5	N/A	1415.81	101.79	<5	N/A	<5	N/A	<5	N/A

Tab. 8.9: Continued. (Lab 1)

		Lab 1													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
hydroxy-PUFA	tetranor-12-HETE	0.24	0.01	0.30	0.00	0.25	0.00	0.43	0.00	0.24	0.01	0.40	0.01	2.14	0.10
	5-HEPE	1.50	0.02	3.49	0.23	2.02	0.18	1.05	0.08	1.98	0.05	3.17	0.09	34.32	1.95
	8-HEPE	0.71	0.03	1.80	0.11	1.13	0.16	0.32	0.00	1.06	0.03	1.84	0.00	37.17	1.01
	9-HEPE	2.36	0.08	5.75	0.34	4.07	0.31	0.90	0.06	4.05	0.07	6.56	0.05	124.15	6.32
	11-HEPE	1.26	0.02	2.88	0.36	2.01	0.19	1.58	0.09	1.94	0.04	3.41	0.04	63.31	3.25
	12-HEPE	2.57	0.05	4.82	0.43	4.41	0.26	9.75	0.21	3.69	0.03	6.48	0.03	123.61	7.68
	15-HEPE	1.99	0.04	4.08	0.52	22.97	1.33	1.29	0.01	3.32	0.17	5.71	0.12	106.57	4.57
	18-HEPE	3.23	0.07	7.60	0.53	27.38	2.05	3.61	0.26	5.07	0.13	9.73	0.35	40.38	8.08
	4-HDHA	6.47	0.26	7.87	0.40	9.85	1.09	8.86	0.73	9.12	0.39	13.16	0.48	153.20	1.12
	7-HDHA	4.73	0.20	6.52	0.11	6.23	0.65	3.73	0.04	6.23	0.14	8.65	0.06	129.35	1.29
	8-HDHA	3.91	0.12	4.74	0.41	6.09	0.57	2.62	0.05	5.98	0.06	7.91	0.20	130.56	1.89
	10-HDHA	4.51	0.32	5.31	0.62	6.72	0.30	3.46	0.01	6.28	0.13	8.81	0.01	137.84	0.10
	11-HDHA	5.80	0.09	6.30	0.75	9.11	1.09	4.38	0.26	9.16	0.31	13.02	0.25	219.23	0.54
	13-HDHA	4.78	0.31	5.72	0.77	7.64	0.05	4.97	0.09	7.48	0.50	10.25	0.50	176.22	10.52
	14-HDHA	6.11	0.29	3.87	0.47	9.68	0.31	11.22	0.18	5.93	0.04	7.91	0.28	138.36	0.31
	16-HDHA	4.06	0.19	4.46	0.48	6.30	0.45	3.11	0.05	6.45	0.16	8.71	0.18	140.92	5.80
	17-HDHA	<8.5	N/A	<8.5	N/A	15.54	1.06	<8.5	N/A	<8.5	N/A	9.59	0.11	150.81	1.98
	20-HDHA	7.55	0.24	8.00	0.73	12.56	1.23	9.62	1.00	10.51	0.38	16.48	0.84	207.66	2.80
epoxy-PUFA	9(10)-Ep-stearic acid	170.81	22.15	146.41	23.71	136.52	13.83	113.00	10.77	201.34	50.86	161.23	59.57	801.54	78.43
	9(10)-EpOME	145.30	25.91	142.94	32.59	127.22	18.03	152.93	31.56	185.02	48.53	133.38	46.07	244.46	102.32
	12(13)-EpOME	115.63	14.47	113.71	18.77	111.20	13.42	122.94	23.85	147.04	36.47	111.63	36.69	232.24	77.62
	9(10)-EpODE	5.28	1.06	3.53	0.54	4.36	0.66	2.26	0.24	7.23	1.93	5.87	1.93	6.35	2.55
	12(13)-EpODE	2.60	0.52	1.68	0.24	2.29	0.41	1.35	0.29	3.60	0.96	2.99	1.06	4.14	1.54
	15(16)-EpODE	21.11	2.17	11.42	0.92	19.47	1.72	13.58	1.75	26.14	4.19	19.61	4.01	51.58	9.11
	14(15)-EpEDE	7.31	1.26	5.65	0.65	6.02	0.41	5.07	0.59	8.30	1.59	9.15	2.76	9.09	3.59
	8(9)-EpETrE	28.65	4.76	38.99	5.65	26.77	1.80	34.57	3.10	33.25	6.37	32.59	5.37	35.56	12.71
	11(12)-EpETrE	38.62	8.01	52.66	4.90	36.03	1.20	47.17	4.08	43.28	6.45	42.86	7.41	45.68	15.57
	14(15)-EpETrE	56.72	11.33	79.74	6.32	75.57	1.46	71.95	8.57	68.17	11.52	66.29	13.47	71.36	24.17
	8(9)-EpETE	5.90	1.93	18.07	0.98	4.89	0.01	3.33	0.40	6.68	0.96	6.88	1.01	4.68	1.19
	11(12)-EpETE	6.02	1.60	19.55	0.85	5.73	0.35	3.54	0.53	7.97	1.38	8.16	1.90	4.36	1.36
	14(15)-EpETE	7.04	1.88	23.39	0.27	6.71	0.13	4.55	0.43	9.23	1.17	9.54	1.59	6.15	1.92
	17(18)-EpETE	11.67	3.36	38.80	1.22	66.11	2.83	6.38	0.94	14.84	2.67	14.87	2.83	9.32	3.78
	7(8)-EpDPE	8.85	1.62	12.35	1.08	7.69	0.15	8.02	0.62	12.37	2.06	9.22	1.67	6.77	2.38
	10(11)-EpDPE	10.62	2.37	14.14	2.00	8.90	0.41	8.89	0.84	13.52	2.26	10.78	1.85	7.38	2.19
	13(14)-EpDPE	10.14	2.25	14.67	2.09	9.70	0.76	9.72	1.30	14.70	3.25	12.19	3.33	7.86	2.94
	16(17)-EpDPE	10.05	2.29	14.89	1.69	9.75	0.36	9.61	1.34	14.69	3.02	12.00	2.99	7.90	2.68
	19(20)-EpDPE	15.76	2.84	23.07	0.94	49.39	2.79	15.44	1.91	24.81	3.92	19.03	3.74	11.82	2.94
vicinal dihydroxy-PUFA	9,10-DiH-stearic acid	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	9,10-DiHOME	18.68	0.90	14.61	0.22	39.27	1.49	24.85	0.02	25.26	0.17	11.58	0.19	41.09	1.85
	12,13-DiHOME	9.04	0.51	10.06	0.53	9.45	0.68	9.84	0.22	10.45	0.36	7.64	0.23	30.45	1.21
	9,10-DiHODE	0.43	0.03	0.49	0.04	0.44	0.01	0.39	0.01	0.41	0.01	0.35	0.03	2.39	0.06
	12,13-DiHODE	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	0.60	0.12
	15,16-DiHODE	13.90	0.49	13.34	0.42	13.63	0.69	16.95	0.28	10.91	0.03	9.68	0.57	18.73	0.61
	5,6-DiHETrE	16.12	0.28	23.98	0.26	16.43	0.79	19.72	0.37	14.64	0.06	17.72	0.36	16.39	1.04
	8,9-DiHETrE	3.57	0.10	4.59	0.03	3.59	0.13	3.98	0.02	3.17	0.04	3.99	0.01	2.93	0.37
	11,12-DiHETrE	<0.16	N/A	0.25	0.00	<0.16	N/A	<0.16	N/A	<0.16	N/A	0.17	0.01	<0.16	N/A
	14,15-DiHETrE	0.74	0.03	0.90	0.02	2.01	0.08	1.01	0.02	0.76	0.02	0.94	0.06	1.00	0.06
	5,6-DiHETE	1.94	0.13	6.68	0.24	2.19	0.27	<1.5	N/A	2.27	0.44	2.85	0.77	1.50	N/A
	8,9-DiHETE	0.37	0.01	0.97	0.01	0.39	0.03	0.28	0.01	0.38	0.02	0.62	0.09	0.29	0.02
	11,12-DiHETE	<0.25	N/A	0.39	0.00	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A
	14,15-DiHETE	<0.25	N/A	0.29	0.03	21.17	2.01	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A
	17,18-DiHETE	<0.55	N/A	1.68	0.07	0.75	0.05	<0.55	N/A	0.70	0.10	0.87	0.15	<0.55	N/A
	7,8-DiHDPE	3.26	0.14	4.87	0.12	3.37	0.32	4.06	0.11	2.68	0.05	2.90	0.08	<2.5	N/A
	10,11-DiHDPE	0.57	0.02	0.61	0.00	0.58	0.03	0.59	0.01	0.58	0.01	0.79	0.02	0.46	0.00
	13,14-DiHDPE	0.37	0.02	0.52	0.00	0.37	0.01	0.40	0.03	0.41	0.01	0.40	0.00	0.30	0.01
	16,17-DiHDPE	0.46	0.02	0.64	0.01	63.55	3.42	0.63	0.12	0.59	0.00	0.60	0.03	0.79	0.06
	19,20-DiHDPE	2.05	0.10	2.92	0.18	2.30	0.16	2.53	0.08	2.49	0.07	2.30	0.14	1.98	0.03
oxo-PUFA	9-oxo-ODE	12.36	2.57	8.68	2.00	29.67	7.38	48.38	12.03	19.04	0.54	31.68	3.07	133.56	4.12
	13-oxo-ODE	214.21	52.44	190.68	37.34	183.30	69.22	279.67	135.29	239.25	7.04	196.37	82.16	617.14	111.23
	9-oxo-OTrE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	13-oxo-OTrE	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	5-oxo-ETE	<2.5	N/A	<2.5	N/A	7.96	2.84	20.32	5.67	<2.5	N/A	8.81	1.73	4.87	1.15
	12-oxo-ETE	<2.05	N/A	2.22	0.20	3.40	0.50	7.00	0.97	<2.05	N/A	3.84	0.99	3.32	0.65
	15-oxo-ETE	45.35	12.55	59.00	9.16	32.37	20.83	55.00	40.51	51.41	0.55	37.54	18.90	64.21	19.83
	12-oxo-LTB_4	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	0.79	0.05
	17-oxo-n3DPA	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A
	4-oxo-DHA	<2.5	N/A	<2.5	N/A	4.20	1.85	8.48	1.55	<2.5	N/A	4.39	1.13	<2.5	N/A
	17-oxo-DHA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
other	20-COOH-ARA	7.99	1.33	9.74	0.40	6.86	0.24	8.18	1.20	8.06	0.60	9.77	1.89	9.80	1.59

Tab. 8.9: Continued. (Lab 2)

		Lab 2													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
isoprostanes	15-F_{1t}-IsoP	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	5(*R,S*)-5-F_{2c}-IsoP	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	5(*R,S*)-5-F_{2t}-IsoP	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	3.46	0.23
	15-F_{2t}-IsoP	<5	N/A	<5	N/A	22.48	0.81	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A
	15-oxo-15-F_{2t}-IsoP	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	13,14-dihydro-15-oxo-15-F_{2t}-IsoP	<100	N/A	<100	N/A	<100	N/A	<100	N/A	<100	N/A	<100	N/A	<100	N/A
	15-F_{3t}-IsoP	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A
prostaglandins	$PGF_{1\alpha}$	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	PGB_2	<5	N/A	<5	N/A	15.18	1.29	26.66	2.42	<5	N/A	<5	N/A	8.44	0.63
	6-keto-$PGF_{1\alpha}$	<12.8	N/A	<12.8	N/A	<12.8	N/A	<12.8	N/A	<12.8	N/A	<12.8	N/A	<12.8	N/A
	$PGF_{2\alpha}$	<5	N/A	<5	N/A	23.43	0.18	9.41	0.54	<5	N/A	<5	N/A	<5	N/A
	PGJ_2	<0.675	N/A	<0.675	N/A	<0.675	N/A	<0.675	N/A	<0.675	N/A	<0.675	N/A	<0.675	N/A
	PGB_3	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	1.01	0.17
	Δ^{17}-6-keto-PGF_{1a}	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	$PGF_{3\alpha}$	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A
	1a,1b-dihomo-$PGF_{2\alpha}$	<250	N/A	<250	N/A	<250	N/A	<250	N/A	<250	N/A	<250	N/A	<250	N/A
multiple hydroxylated-PUFA	LTB_3	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	5(*S*),12(*S*)-DiHETE	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	39.30	0.10
	5(*S*),15(*S*)-DiHETE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	7.01	0.75
	8(*S*),15(*S*)-DiHETE	<5.1	N/A	<5.1	N/A	<5.1	N/A	<5.1	N/A	<5.1	N/A	<5.1	N/A	38.68	0.78
	LTB_4	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	1.00	N/A
	6-trans-LTB_4	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	3.32	0.06
	6-trans-12-epi-LTB_4	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	6.49	0.12
	5(*S*),6(*R*)-DiHETE	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A
	5(*S*),6(*S*)-DiHETE	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A
	20-OH-LTB_4	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	20-COOH-LTB_4	<6.6	N/A	<6.6	N/A	<6.6	N/A	<6.6	N/A	<6.6	N/A	<6.6	N/A	<6.6	N/A
	18-COOH-dinor-LTB_4	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A
	LxA_4	<2.5	N/A	<2.5	N/A	52.29	1.51	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	17(*R,S*)-RvD4	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	RvD5	<2.5	N/A	<2.5	N/A	18.86	0.50	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	MaR2	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A
	PDx	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	2.64	0.26
trihydroxylated ALA and LA	9,10,11-TriHOME	0.59	0.04	<0.5	N/A	<0.5	N/A	0.62	0.09	0.89	0.02	0.83	0.09	17.20	1.33
	9,10,13-TriHOME	4.28	1.50	1.59	1.23	2.99	2.26	4.16	2.48	2.65	1.60	4.87	1.23	9.40	2.21
	9,12,13-TriHOME	11.93	4.75	6.79	3.65	8.82	5.20	11.36	5.27	10.31	6.26	13.90	3.58	28.97	0.72
	9,10,11-TriHODE	<0.25	N/A	0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	1.04	0.16
	9,10,13-TriHODE	<10	N/A	10.00	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	9,12,13-TriHODE	<0.75	N/A	0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A
hydroxy-PUFA	9-HODE	43.48	6.90	33.72	3.08	41.07	7.79	75.26	5.76	85.64	20.42	51.86	0.73	7322.53	5120.94
	10-HODE	3.56	0.68	2.15	0.38	3.28	0.37	3.10	0.07	4.36	0.16	5.82	0.19	42.83	31.89
	12-HODE	2.51	0.67	1.65	0.33	2.26	0.08	2.42	0.27	2.90	0.23	4.57	0.36	8.12	0.84
	13-HODE	61.88	8.80	47.75	3.79	127.24	19.77	77.20	3.20	121.14	25.83	77.40	2.77	11595.85	8059.53
	15-HODE	5.35	0.22	4.01	0.01	5.34	0.18	3.46	0.32	5.59	0.20	5.65	0.08	23.44	0.87
	9-HOTrE	1.76	0.12	1.05	0.22	1.60	0.09	1.95	0.18	2.61	0.16	3.12	0.16	92.56	0.06
	13-HOTrE	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	13-gamma-HOTrE	1.52	0.52	<1	N/A	1.45	0.07	1.55	0.23	2.79	0.44	3.71	0.84	118.83	4.54
	5-HETrE	1.08	0.09	0.48	0.09	0.93	0.07	0.92	0.04	0.60	0.05	1.82	0.15	10.36	0.45
	8-HETrE	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	63.08	1.21
	12-HETrE	<2.5	N/A	<2.5	N/A	<2.5	N/A	7.74	0.57	<2.5	N/A	3.09	0.27	121.79	3.86
	15-HETrE	1.91	0.38	1.33	0.22	1.74	0.23	4.17	0.02	2.16	0.17	3.49	0.27	120.33	3.92
	5-HETE	12.41	0.99	12.62	1.54	11.51	1.48	19.92	0.54	13.10	0.63	18.13	0.98	515.43	0.88
	8-HETE	8.16	1.55	9.14	3.01	7.06	0.69	9.95	1.30	8.11	1.65	11.45	0.74	588.01	20.54
	9-HETE	9.57	0.70	9.08	0.81	8.80	1.83	12.24	0.16	10.67	3.40	14.87	2.30	1005.22	61.62
	11-HETE	11.70	1.99	10.69	3.82	10.03	0.79	83.41	4.67	11.80	2.57	16.23	1.49	777.05	194.99
	12-HETE	10.94	1.74	8.22	3.23	11.00	0.82	532.15	8.40	8.96	1.83	12.37	1.25	811.25	15.48
	15-HETE	11.03	1.31	9.63	1.13	19.62	1.12	73.68	3.14	12.41	2.18	15.26	0.08	834.49	28.69
	16-HETE	<1	N/A	<1	N/A	<1	N/A	1.37	0.26	<1	N/A	<1	N/A	2.04	0.19
	17-HETE	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A	<1	N/A
	18-HETE	<1	N/A	1.34	0.22	<1	N/A	1.09	0.11	1.00	N/A	1.13	0.06	1.00	N/A
	19-HETE	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	20-HETE	<10	N/A	<10	N/A	54.84	1.42	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	12-HHTrE	<2.5	N/A	<2.5	N/A	<2.5	N/A	1472.24	59.75	<2.5	N/A	<2.5	N/A	<2.5	N/A

Tab. 8.9: Continued. (Lab 2)

		Lab 2													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
hydroxy-PUFA	tetranor-12-HETE	0.20	0.01	0.25	0.06	0.18	0.01	0.45	0.01	0.20	0.02	0.32	0.00	2.02	0.13
	5-HEPE	1.99	0.03	4.05	0.22	1.76	0.17	1.48	0.16	2.46	0.05	3.19	0.17	58.39	1.51
	8-HEPE	0.79	0.08	2.15	0.25	0.72	0.03	0.57	0.05	0.86	0.03	1.29	0.01	66.22	0.97
	9-HEPE	2.04	0.20	3.64	0.27	1.66	0.45	1.43	0.04	2.28	0.31	3.43	0.45	124.32	4.14
	11-HEPE	0.73	0.12	1.75	0.30	0.63	0.14	1.61	0.00	0.87	0.09	1.24	0.05	66.96	0.84
	12-HEPE	1.65	0.25	3.28	0.48	1.56	0.27	11.34	0.49	1.51	0.24	2.54	0.25	138.03	3.49
	15-HEPE	<10	N/A	<10	N/A	22.53	1.29	<10	N/A	<10	N/A	<10	N/A	117.45	3.31
	18-HEPE	<5	N/A	<5	N/A	21.57	0.09	<5	N/A	<5	N/A	<5	N/A	86.37	17.74
	4-HDHA	3.97	0.31	4.69	0.75	3.51	0.83	6.12	0.48	4.83	0.02	5.51	0.08	117.73	4.36
	7-HDHA	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	83.31	5.02
	8-HDHA	2.03	0.18	2.59	0.19	1.82	0.28	1.87	0.09	2.43	0.09	3.17	0.10	95.79	2.80
	10-HDHA	2.78	0.51	3.26	1.05	2.14	0.11	2.81	0.53	2.91	0.05	3.94	0.54	123.19	3.50
	11-HDHA	3.04	0.55	3.78	0.68	2.64	0.40	3.74	0.37	3.95	0.00	4.99	0.20	173.96	3.12
	13-HDHA	2.82	0.24	3.33	1.11	2.58	0.29	3.92	0.58	3.37	0.29	4.47	0.37	147.38	1.24
	14-HDHA	4.11	0.52	<2.75	N/A	4.69	0.18	10.41	0.94	3.04	0.08	3.48	0.26	126.80	0.84
	16-HDHA	2.37	0.25	2.46	0.16	2.00	0.42	2.62	0.12	2.95	0.12	3.37	0.34	128.76	1.44
	17-HDHA	<8.5	N/A	<8.5	N/A	9.69	1.07	<8.5	N/A	<8.5	N/A	<8.5	N/A	145.79	0.20
	20-HDHA	4.69	0.77	4.74	0.72	4.28	0.51	7.35	0.11	5.39	0.38	6.41	0.24	196.10	1.42
epoxy-PUFA	9(10)-Ep-stearic acid	97.60	12.15	61.63	18.31	74.16	14.62	74.80	15.46	78.96	1.52	83.81	28.77	458.79	125.67
	9(10)-EpOME	102.08	5.59	83.98	16.30	91.74	5.81	115.68	5.36	113.95	15.71	82.40	14.63	120.52	2.19
	12(13)-EpOME	91.98	1.57	75.09	9.59	87.42	2.26	96.58	4.02	99.74	18.95	75.70	13.76	136.88	2.62
	9(10)-EpODE	2.83	0.20	1.71	0.22	2.42	0.04	1.50	0.16	3.40	0.67	2.64	0.54	2.47	0.14
	12(13)-EpODE	1.69	0.03	0.86	0.14	1.40	0.09	1.00	0.02	2.12	0.49	1.65	0.34	2.09	0.01
	15(16)-EpODE	15.24	1.04	<9.24	N/A	13.86	1.11	11.77	0.48	18.33	1.35	13.15	3.14	36.07	3.13
	14(15)-EpEDE	3.51	0.26	2.24	0.42	3.15	0.29	2.54	0.06	2.97	0.29	3.97	0.19	3.29	0.14
	8(9)-EpETrE	18.82	0.74	19.82	2.90	<5	N/A	23.10	0.62	20.73	5.44	19.45	1.43	16.05	0.27
	11(12)-EpETrE	24.16	1.48	24.55	5.82	<0.75	N/A	30.69	1.99	27.65	9.73	23.28	2.05	19.46	0.70
	14(15)-EpETrE	37.40	1.82	45.04	1.33	59.02	3.88	47.25	0.95	42.22	9.99	37.30	0.28	30.23	3.42
	8(9)-EpETE	<5	N/A	9.33	0.10	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	11(12)-EpETE	3.64	0.31	10.74	0.31	3.55	0.52	2.13	0.17	4.57	1.32	4.25	0.45	1.67	0.23
	14(15)-EpETE	4.19	0.51	12.83	0.73	3.72	0.28	2.83	0.36	5.90	2.03	4.73	0.13	2.16	0.12
	17(18)-EpETE	<10	N/A	19.45	0.66	61.26	6.11	<10	N/A	<10	N/A	<10	N/A	<10	N/A
	7(8)-EpDPE	<8.7	N/A	<8.7	N/A	<8.7	N/A	<8.7	N/A	<8.7	N/A	<8.7	N/A	<8.7	N/A
	10(11)-EpDPE	5.62	0.25	5.72	1.11	<0.25	N/A	5.50	0.28	6.93	1.93	5.38	0.42	3.43	0.11
	13(14)-EpDPE	5.86	0.48	7.03	0.57	5.56	0.45	5.72	0.20	7.05	1.61	5.30	0.39	2.79	0.04
	16(17)-EpDPE	5.27	0.46	6.92	0.02	4.92	0.39	5.09	0.08	6.98	1.75	4.93	0.44	2.77	0.20
	19(20)-EpDPE	9.39	0.35	11.50	0.13	40.78	1.41	8.94	0.67	12.53	2.19	10.23	0.33	<5	N/A
vicinal dihydroxy-PUFA	9,10-DiH-stearic acid	60.34	3.92	45.64	4.37	56.41	6.45	57.21	11.98	45.40	0.68	42.98	1.95	39.15	6.29
	9,10-DiHOME	16.97	0.60	13.33	0.33	37.14	0.64	23.43	0.92	25.27	0.78	11.32	0.18	40.23	0.02
	12,13-DiHOME	9.11	1.09	9.02	0.21	8.88	0.13	9.58	0.15	10.53	0.66	6.70	0.13	28.65	0.80
	9,10-DiHODE	0.53	0.04	0.53	0.06	0.46	0.02	0.49	0.05	0.47	0.04	0.40	0.04	2.37	0.01
	12,13-DiHODE	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	15,16-DiHODE	15.03	1.15	13.66	0.87	15.05	0.87	19.10	0.72	12.87	0.17	10.38	0.58	19.37	0.77
	5,6-DiHETrE	14.74	0.08	19.59	0.47	13.14	0.17	17.33	0.05	12.18	0.51	16.06	1.35	13.94	1.04
	8,9-DiHETrE	3.53	0.19	4.30	0.16	3.35	0.15	4.14	0.15	3.15	0.09	3.99	0.01	6.51	3.50
	11,12-DiHETrE	1.18	0.08	1.17	0.04	13.63	0.07	1.45	0.03	1.07	0.00	1.47	0.11	1.28	0.11
	14,15-DiHETrE	0.84	0.04	0.93	0.02	2.19	0.09	1.25	0.02	0.86	0.01	0.93	0.03	1.10	0.02
	5,6-DiHETE	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	8,9-DiHETE	<0.5	N/A	1.17	0.01	<0.5	N/A	<0.5	N/A	0.82	0.23	1.30	0.01	<0.5	N/A
	11,12-DiHETE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.87	0.05
	14,15-DiHETE	0.75	0.44	0.74	0.06	26.97	0.40	0.67	0.10	1.04	0.30	1.04	0.23	0.48	0.02
	17,18-DiHETE	<1.1	N/A	1.92	0.33	<1.1	N/A	<1.1	N/A	1.53	0.11	1.49	0.07	<1.1	N/A
	7,8-DiHDPE	3.23	0.04	4.22	0.38	2.83	0.02	3.63	0.24	2.61	0.02	2.72	0.16	1.65	0.32
	10,11-DiHDPE	0.55	0.01	0.51	0.01	0.46	0.01	0.51	0.02	0.52	0.02	0.71	0.03	0.44	0.01
	13,14-DiHDPE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	16,17-DiHDPE	<0.5	N/A	0.65	0.04	62.77	0.98	0.67	0.00	0.59	0.00	0.59	0.02	0.88	0.09
	19,20-DiHDPE	2.39	0.18	3.06	0.03	2.42	0.29	3.01	0.16	2.78	0.03	2.52	0.03	2.20	0.00
oxo-PUFA	9-oxo-ODE	12.05	4.33	8.12	1.93	9.48	0.85	23.85	4.06	14.64	1.66	12.83	0.22	91.41	2.29
	13-oxo-ODE	194.94	129.71	156.95	108.81	113.05	12.63	739.47	58.96	105.64	4.98	125.42	9.73	669.94	28.07
	9-oxo-OTrE	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A	<0.75	N/A
	13-oxo-OTrE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	1.45	0.00
	5-oxo-ETE	<5	N/A	<5	N/A	<5	N/A	12.66	1.90	<5	N/A	<5	N/A	<5	N/A
	12-oxo-ETE	<4.1	N/A	<4.1	N/A	<4.1	N/A	6.68	1.08	<4.1	N/A	<4.1	N/A	<4.1	N/A
	15-oxo-ETE	39.34	27.23	52.82	46.20	21.18	3.28	132.22	5.55	19.57	1.47	17.48	9.94	54.35	15.18
	12-oxo-LTB_4	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.59	0.03
	17-oxo-n3DPA	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A	<25	N/A
	4-oxo-DHA	<2.5	N/A	<2.5	N/A	<2.5	N/A	5.01	0.83	<2.5	N/A	<2.5	N/A	<2.5	N/A
	17-oxo-DHA	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A	<50	N/A
other	20-COOH-ARA	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A	<10	N/A

Tab. 8.9: Continued. (Lab 3)

		Lab 3													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
isoprostanes	15-F_{1t}-IsoP	<2.58	N/A	<2.58	N/A	<2.58	N/A	<2.58	N/A	<2.58	N/A	<2.58	N/A	<2.58	N/A
	5(*R*,*S*)-5-F_{2c}-IsoP	<2.62	N/A	<2.62	N/A	<2.62	N/A	<2.62	N/A	<2.62	N/A	<2.62	N/A	3.57	0.43
	5(*R*,*S*)-5-F_{2t}-IsoP	0.39	0.07	<0.28	N/A	<0.28	N/A	0.79	0.40	0.51	0.13	0.71	0.02	3.96	0.89
	15-F_{2t}-IsoP	<0.9	N/A	<0.9	N/A	26.54	4.96	<0.9	N/A	<0.9	N/A	<0.9	N/A	<0.9	N/A
	2,3-dinor-15-(*R*,*S*)-15-F_{2t}-IsoP	<0.22	N/A	<0.22	N/A	<0.22	N/A	<0.22	N/A	<0.22	N/A	<0.22	N/A	<0.22	N/A
	15-oxo-15-F_{2t}-IsoP	<2.38	N/A	<2.38	N/A	<2.38	N/A	<2.38	N/A	<2.38	N/A	<2.38	N/A	<2.38	N/A
	13,14-dihydro-15-oxo-15-F_{2t}-IsoP	120.33	17.43	253.71	93.89	217.56	147.86	208.06	164.80	289.16	175.90	241.03	211.95	202.64	105.64
	15-F_{3t}-IsoP	<25.98	N/A	<25.98	N/A	<25.98	N/A	<25.98	N/A	<25.98	N/A	<25.98	N/A	<25.98	N/A
prostaglandins	$PGF_{1\alpha}$	<2.30	N/A	<2.30	N/A	<2.30	N/A	<2.30	N/A	<2.30	N/A	<2.30	N/A	<2.30	N/A
	PGB_2	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.5	N/A	0.5	N/A	<0.5	N/A
	6-keto-$PGF_{1\alpha}$	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	$PGF_{2\alpha}$	<0.69	N/A	7.15	4.15	18.32	5.19	9.11	0.95	2.06	1.10	0.7	N/A	0.70	0.00
	PGJ_2	<0.20	N/A	<0.20	N/A	<0.20	N/A	<0.20	N/A	<0.20	N/A	<0.20	N/A	<0.20	N/A
	PGB_3	<0.2911	N/A	<0.2911	N/A	<0.2911	N/A	<0.2911	N/A	<0.2911	N/A	<0.2911	N/A	0.82	0.21
	Δ^{17}-6-keto-PGF_{1a}	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	$PGF_{3\alpha}$	<8.78	N/A	<8.78	N/A	<8.78	N/A	<8.78	N/A	<8.78	N/A	<8.78	N/A	<8.78	N/A
	1a,1b-dihomo-$PGF_{2\alpha}$	<52.09	N/A	<52.09	N/A	<52.09	N/A	<52.09	N/A	<52.09	N/A	<52.09	N/A	<52.09	N/A
multiple hydroxylated-PUFA	LTB_3	<0.69	N/A	<0.69	N/A	<0.69	N/A	<0.69	N/A	<0.69	N/A	<0.69	N/A	<0.69	N/A
	5(*S*),12(*S*)-DiHETE	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	27.13	3.03
	5(*S*),15(*S*)-DiHETE	<0.85	N/A	<0.85	N/A	<0.85	N/A	<0.85	N/A	<0.85	N/A	<0.85	N/A	5.86	0.20
	8(*S*),15(*S*)-DiHETE	<2.56	N/A	<2.56	N/A	<2.56	N/A	<2.56	N/A	<2.56	N/A	<2.56	N/A	34.00	5.32
	LTB_4	<0.41	N/A	<0.41	N/A	<0.41	N/A	<0.41	N/A	<0.41	N/A	<0.41	N/A	<0.42	N/A
	6-trans-LTB_4	<0.42	N/A	<0.42	N/A	<0.42	N/A	<0.42	N/A	<0.42	N/A	<0.42	N/A	2.95	0.27
	6-trans-12-epi-LTB_4	<0.27	N/A	<0.27	N/A	<0.27	N/A	<0.27	N/A	<0.27	N/A	<0.27	N/A	5.11	0.23
	5(*S*),6(*R*)-DiHETE	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A
	5(*S*),6(*S*)-DiHETE	<2.04	N/A	<2.04	N/A	<2.04	N/A	<2.04	N/A	<2.04	N/A	<2.04	N/A	<2.04	N/A
	20-OH-LTB_4	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A	<0.76	N/A
	20-COOH-LTB_4	<2.81	N/A	<2.81	N/A	<2.81	N/A	<2.81	N/A	<2.81	N/A	<2.81	N/A	<2.81	N/A
	18-COOH-dinor-LTB_4	<25.52	N/A	<25.52	N/A	<25.52	N/A	<25.52	N/A	<25.52	N/A	<25.52	N/A	<25.52	N/A
	LxA_4	<0.51	N/A	<0.51	N/A	54.41	5.13	<0.51	N/A	<0.51	N/A	<0.51	N/A	<0.51	N/A
	17(*R*,*S*)-RvD4	<0.50	N/A	<0.50	N/A	<0.50	N/A	<0.50	N/A	<0.50	N/A	<0.50	N/A	<0.50	N/A
	RvD5	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	0.03
	MaR2	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A	<0.78	N/A
	PDx	<2.36	N/A	<2.36	N/A	<2.36	N/A	<2.36	N/A	<2.36	N/A	<2.36	N/A	<2.36	N/A
trihydroxylated ALA and LA	9,10,11-TriHOME	1.67	0.20	4.07	3.21	5.49	0.62	6.14	1.28	7.96	7.87	7.32	2.83	24.17	0.20
	9,10,13-TriHOME	4.11	0.39	6.86	5.91	13.99	0.82	17.12	3.09	15.45	16.67	13.21	8.88	15.95	0.52
	9,12,13-TriHOME	13.39	1.59	29.28	6.33	56.32	11.74	60.71	6.81	65.99	48.54	57.51	11.17	58.84	12.08
	9,10,11-TriHODE	<0.1	N/A	1.73	2.06	<0.1	N/A	<0.1	N/A	4.36	5.95	2.70	3.40	1.11	1.04
	9,10,13-TriHODE	<5.39	N/A	<5.39	N/A	15.47	13.13	<5.39	N/A	<5.39	N/A	<5.39	N/A	13.00	2.89
	9,12,13-TriHODE	0.51	0.07	3.64	4.61	0.70	0.01	0.53	0.03	0.80	0.64	<0.51	N/A	0.67	0.06
hydroxy-PUFA	9-HODE	38.07	4.86	28.68	0.15	54.27	29.58	79.48	10.34	69.57	4.68	64.60	16.00	2836.65	55.31
	10-HODE	6.48	2.18	4.73	0.43	56.04	70.56	11.57	4.34	7.34	0.54	23.48	12.44	25.51	3.73
	12-HODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	13-HODE	54.27	6.73	52.20	13.99	134.95	55.36	86.29	17.08	96.88	7.07	95.67	15.83	2681.84	48.31
	15-HODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	9-HOTrE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	13-HOTrE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	13-gamma-HOTrE	<2.14	N/A	<2.14	N/A	<2.14	N/A	<2.14	N/A	<2.14	N/A	<2.15	N/A	109.99	13.80
	5-HETrE	1.17	0.04	0.70	0.32	0.98	0.07	1.15	0.05	0.66	0.06	1.93	0.28	12.35	0.51
	8-HETrE	<4.68	N/A	<4.68	N/A	<4.68	N/A	<4.68	N/A	<4.68	N/A	<4.68	N/A	96.32	24.46
	12-HETrE	1.57	0.10	1.08	0.33	2.22	0.44	8.02	0.73	1.25	0.17	2.82	0.20	145.03	9.14
	15-HETrE	1.54	0.10	<0.68	N/A	1.81	0.14	4.27	0.49	1.82	0.30	3.66	0.30	139.84	5.10
	5-HETE	12.81	0.45	12.33	0.87	12.99	1.65	22.11	2.83	11.96	3.49	20.73	1.77	637.64	0.51
	8-HETE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	9-HETE	4.65	0.92	6.41	1.20	6.74	0.65	11.12	3.73	7.73	0.78	11.73	0.82	972.63	30.24
	11-HETE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	12-HETE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	15-HETE	7.47	0.35	8.12	2.12	18.17	1.20	78.56	2.31	11.60	0.90	15.76	0.35	852.10	0.50
	16-HETE	<0.97	N/A	<0.97	N/A	<0.97	N/A	1.39	0.16	<0.97	N/A	<0.97	N/A	1.98	0.25
	17-HETE	<2.99	N/A	<2.99	N/A	<2.99	N/A	<2.99	N/A	<2.99	N/A	<2.99	N/A	<2.99	N/A
	18-HETE	0.74	0.64	1.34	0.45	<0.8	N/A	<0.8	N/A	<0.8	N/A	<0.8	N/A	<0.8	N/A
	19-HETE	<10.82	N/A	<10.82	N/A	<10.82	N/A	<10.82	N/A	<10.82	N/A	<10.82	N/A	<10.82	N/A
	20-HETE	<5.68	N/A	<5.68	N/A	52.12	7.43	<5.68	N/A	<5.68	N/A	<5.68	N/A	<5.68	N/A
	12-HHTrE	1.48	0.12	<0.96	N/A	1.36	0.32	1807.70	360.65	<0.95	N/A	3.68	1.52	<0.95	N/A

Tab. 8.9: Continued. (Lab 3)

		Lab 3													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
hydroxy-PUFA	tetranor-12-HETE	<0.26	N/A	<0.26	N/A	<0.26	N/A	0.44	0.09	<0.26	N/A	0.39	0.10	2.02	0.39
	5-HEPE	1.69	0.17	3.41	0.58	2.48	1.24	1.62	0.03	2.52	0.49	3.51	1.00	58.54	3.47
	8-HEPE	0.51	0.03	1.37	0.06	0.86	0.29	0.57	0.16	0.90	0.12	1.30	0.28	61.50	4.30
	9-HEPE	<5.07	N/A	<5.07	N/A	<5.07	N/A	<5.07	N/A	<5.07	N/A	<5.07	N/A	119.54	2.96
	11-HEPE	0.40	0.02	1.01	0.08	0.84	0.38	1.70	0.21	0.99	0.30	1.36	0.41	71.84	1.37
	12-HEPE	1.19	0.16	<0.84	N/A	2.96	1.76	12.29	0.18	<0.84	N/A	<0.84	N/A	147.17	0.81
	15-HEPE	<4.54	N/A	<4.54	N/A	21.07	1.18	<4.54	N/A	<4.54	N/A	<4.54	N/A	116.51	0.77
	18-HEPE	<10.14	N/A	<10.14	N/A	23.30	2.82	<10.14	N/A	<10.14	N/A	<10.14	N/A	85.02	35.84
	4-HDHA	2.90	0.28	3.75	0.72	3.54	0.79	6.34	1.57	4.86	0.28	5.53	0.67	129.00	10.61
	7-HDHA	<2.617	N/A	4.16	1.10	<2.62	N/A	3.41	0.13	2.82	0.14	4.41	1.22	100.11	0.85
	8-HDHA	1.59	0.11	2.25	0.08	2.02	0.45	2.23	0.53	2.38	0.10	3.35	0.40	113.40	1.92
	10-HDHA	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	11-HDHA	2.18	0.22	2.66	0.34	3.02	0.33	4.06	1.59	4.02	0.25	5.40	0.33	205.63	3.88
	13-HDHA	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	14-HDHA	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	16-HDHA	1.42	0.27	1.64	0.02	1.88	0.24	2.96	1.03	2.76	0.29	3.56	0.65	123.84	9.20
	17-HDHA	<18.03	N/A	<18.03	N/A	<18.03	N/A	<18.03	N/A	<18.03	N/A	<18.03	N/A	137.02	9.81
	20-HDHA	3.16	0.12	3.43	0.50	4.06	0.11	7.53	1.83	5.66	0.99	6.14	0.85	178.74	2.78
epoxy-PUFA	9(10)-Ep-stearic acid	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	9(10)-EpOME	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	12(13)-EpOME	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	9(10)-EpODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	12(13)-EpODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	15(16)-EpODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	14(15)-EpEDE	2.95	0.23	2.70	0.70	3.48	0.48	3.22	0.97	3.87	1.64	4.75	1.29	3.78	0.20
	8(9)-EpETrE	13.29	1.17	19.29	0.82	17.52	5.23	22.41	3.99	13.04	1.47	21.96	6.53	18.77	3.58
	11(12)-EpETrE	12.94	1.76	21.33	4.90	20.55	9.46	24.05	9.30	16.36	3.38	24.92	13.15	20.71	7.41
	14(15)-EpETrE	22.08	8.13	35.05	11.82	47.48	8.45	40.02	15.30	31.87	13.10	33.46	11.24	31.64	6.00
	8(9)-EpETE	2.79	0.58	7.58	0.93	5.02	2.50	2.72	0.91	3.84	0.16	7.41	5.09	3.53	1.33
	11(12)-EpETE	1.95	0.48	6.19	0.26	3.63	1.83	1.74	0.48	3.95	2.37	4.17	1.92	1.99	0.58
	14(15)-EpETE	<2.75	N/A	7.02	0.89	<2.75	N/A	<2.75	N/A	<2.75	N/A	<2.75	N/A	<2.75	N/A
	17(18)-EpETE	<5.5	N/A	23.71	14.58	49.76	4.36	<5.5	N/A	<5.5	N/A	<5.5	N/A	<5.5	N/A
	7(8)-EpDPE	4.40	0.40	6.08	0.51	5.43	0.83	5.69	1.37	4.73	1.69	6.61	1.69	3.18	0.59
	10(11)-EpDPE	3.43	0.10	5.43	0.51	5.33	1.95	4.51	1.10	4.64	0.14	6.24	2.50	3.70	0.72
	13(14)-EpDPE	3.46	0.80	5.17	0.73	5.21	2.13	5.02	1.59	6.18	3.07	5.44	2.29	3.05	0.66
	16(17)-EpDPE	3.14	0.78	5.06	1.03	5.21	2.74	5.07	1.94	5.30	1.70	5.26	2.19	3.18	0.84
	19(20)-EpDPE	6.32	0.68	13.58	7.45	31.87	4.13	8.15	1.41	8.56	1.05	9.70	3.03	6.49	1.39
vicinal dihydroxy-PUFA	9,10-DiH-stearic acid	134.34	18.72	199.18	129.94	218.75	100.65	331.00	73.53	301.86	243.21	301.75	206.75	99.45	9.21
	9,10-DiHOME	21.40	2.28	25.16	11.07	43.15	5.64	39.14	9.47	38.45	14.29	26.98	12.55	53.09	1.25
	12,13-DiHOME	9.60	0.92	15.26	7.11	16.25	7.91	20.18	8.62	22.58	15.58	20.65	14.40	35.02	2.60
	9,10-DiHODE	<0.4	N/A	<0.4	N/A	<0.4	N/A	0.46	0.03	<0.4	N/A	<0.4	N/A	2.57	0.31
	12,13-DiHODE	<10.26	N/A	<10.26	N/A	<10.26	N/A	<10.26	N/A	<10.26	N/A	<10.26	N/A	<10.26	N/A
	15,16-DiHODE	14.95	0.77	13.05	0.27	14.58	2.11	19.40	1.40	13.21	2.12	12.44	2.89	21.16	1.63
	5,6-DiHETrE	14.13	0.14	18.79	1.07	14.97	2.15	18.76	1.30	12.28	0.78	16.79	0.73	14.12	0.99
	8,9-DiHETrE	3.36	0.11	3.92	0.11	3.18	0.24	3.88	0.03	2.98	0.09	4.10	0.08	2.93	0.04
	11,12-DiHETrE	1.17	0.09	3.19	2.87	12.25	2.48	1.55	0.00	1.19	0.24	1.52	0.20	1.27	0.05
	14,15-DiHETrE	0.85	0.07	1.16	0.30	1.93	0.34	1.25	0.00	0.82	0.05	1.06	0.10	1.01	0.18
	5,6-DiHETE	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	8,9-DiHETE	<0.57	N/A	0.85	0.01	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A
	11,12-DiHETE	0.18	0.04	0.32	0.08	<0.12	N/A	<0.12	N/A	0.31	0.04	0.41	0.02	0.16	0.03
	14,15-DiHETE	0.31	0.03	5.49	7.07	<0.28	N/A	0.37	0.06	0.43	0.08	0.68	0.05	0.37	0.03
	17,18-DiHETE	<1.19	N/A	1.62	0.28	<1.19	N/A	<1.19	N/A	<1.19	N/A	1.49	0.02	<1.19	N/A
	7,8-DiHDPE	2.74	0.30	3.77	0.11	2.81	0.60	3.26	0.10	<0.45	N/A	2.74	0.37	1.52	0.08
	10,11-DiHDPE	0.49	0.01	0.50	0.07	0.55	0.13	0.51	0.06	0.52	0.08	0.80	0.15	0.46	0.03
	13,14-DiHDPE	0.32	0.03	0.40	0.05	0.36	0.09	0.47	0.14	0.42	0.13	<0.24	N/A	0.28	0.02
	16,17-DiHDPE	<0.72	N/A	<0.72	N/A	57.12	0.49	<0.72	N/A	<0.72	N/A	<0.72	N/A	0.84	0.03
	19,20-DiHDPE	2.16	0.23	2.43	0.17	2.31	0.17	2.67	0.13	3.00	0.81	2.48	0.51	2.21	0.43
oxo-PUFA	9-oxo-ODE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	13-oxo-ODE	108.77	26.96	164.09	74.80	219.89	61.55	586.99	227.34	201.97	94.79	244.22	102.51	<0.527	N/A
	9-oxo-OTrE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	13-oxo-OTrE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	5-oxo-ETE	<5.02	N/A	<5.02	N/A	<5.02	N/A	20.92	0.37	<5.02	N/A	<5.02	N/A	<5.02	N/A
	12-oxo-ETE	<2.42	N/A	<2.42	N/A	<2.42	N/A	10.79	4.14	<2.42	N/A	<2.42	N/A	3.06	0.21
	15-oxo-ETE	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	12-oxo-LTB_4	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A	<0.57	N/A
	17-oxo-n3DPA	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
	4-oxo-DHA	<2.56	N/A	<2.56	N/A	<2.56	N/A	8.51	0.72	<2.56	N/A	<2.56	N/A	<2.56	N/A
	17-oxo-DHA	ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue		ISTD issue	
other	20-COOH-ARA	<10.98	N/A	<10.99	N/A	<10.99	N/A	<10.99	N/A	<10.99	N/A	<10.99	N/A	<10.99	N/A

Tab. 8.9: Continued. (Lab 4)

		Lab 4													
	concentration	**Pool 1 - B**		**Pool 2 - n3**		**Pool 3 - S**		**Pool 4 - Ca**		**Pool 5 - Ob-H**		**Pool 6 - Ob+H**		**Pool 7 - B2**	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
isoprostanes	15-F_{1t}-IsoP	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	5(*R,S*)-5-F_{2c}-IsoP	2.01	0.48	<1.25	N/A	2.39	0.51	2.09	0.51	1.86	0.02	3.08	0.67	5.95	0.29
	5(*R,S*)-5-F_{2t}-IsoP	0.97	0.27	0.76	0.12	0.82	0.11	0.96	0.17	0.80	0.08	1.43	0.29	5.43	0.28
	15-F_{2t}-IsoP	<0.25	N/A	<0.25	N/A	26.17	0.66	0.63	0.08	<0.25	N/A	<0.25	N/A	0.67	0.07
	2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	15-oxo-15-F_{2t}-IsoP	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	13,14-dihydro-15-oxo-15-F_{2t}-IsoP	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	15-F_{3t}-IsoP	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A
prostaglandins	$PGF_{1\alpha}$	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	PGB_2	1.39	0.00	<1.25	N/A	4.65	0.96	15.30	1.65	<1.25	N/A	2.92	1.55	3.61	0.46
	6-keto-$PGF_{1\alpha}$	<1.6	N/A	<1.6	N/A	<1.6	N/A	<1.6	N/A	<1.6	N/A	<1.6	N/A	<1.6	N/A
	$PGF_{2\alpha}$	<0.5	N/A	<0.5	N/A	23.97	2.17	9.71	0.45	<0.5	N/A	<0.5	N/A	<0.5	N/A
	PGJ_2	<0.675	N/A	<0.675	N/A	1.04	0.05	3.99	0.38	<0.68	N/A	<0.6775	N/A	0.85	0.06
	PGB_3	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	Δ^{17}-6-keto-PGF_{1a}	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	$PGF_{3\alpha}$	31.78	36.93	14.42	10.86	5.57	3.49	5.00	1.61	13.62	11.48	41.49	54.27	14.04	16.25
	1a,1b-dihomo-$PGF_{2\alpha}$	0.98	0.78	0.84	0.46	0.56	0.24	0.47	0.05	1.11	0.37	1.78	1.50	1.39	0.80
multiple hydroxylated-PUFA	LTB_3	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A	<0.375	N/A
	5(*S*),12(*S*)-DiHETE	3.04	1.00	1.25	0.08	1.77	0.16	1.10	0.01	8.10	3.39	8.20	0.16	80.90	0.80
	5(*S*),15(*S*)-DiHETE	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	<12.5	N/A	13.70	0.05
	8(*S*),15(*S*)-DiHETE	3.33	1.43	2.00	0.04	2.45	0.01	1.55	0.05	5.61	1.29	8.19	1.49	57.45	1.72
	LTB_4	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A
	6-trans-LTB_4	0.61	0.22	<0.25	N/A	<0.25	N/A	<0.25	N/A	0.59	0.03	0.98	0.03	9.03	0.70
	6-trans-12-epi-LTB_4	0.79	0.27	<0.38	N/A	<0.38	N/A	<0.38	N/A	1.31	0.17	1.81	0.04	16.82	1.15
	5(*S*),6(*R*)-DiHETE	<0.975	N/A	<0.975	N/A	<0.975	N/A	<0.975	N/A	<0.975	N/A	<0.975	N/A	<0.975	N/A
	5(*S*),6(*S*)-DiHETE	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.446	N/A	<0.45	N/A
	20-OH-LTB_4	0.28	0.05	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A
	20-COOH-LTB_4	<1.65	N/A	<1.65	N/A	<1.65	N/A	<1.65	N/A	<1.65	N/A	<1.65	N/A	<1.65	N/A
	18-COOH-dinor-LTB_4	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A	<5	N/A
	LxA_4	<2.5	N/A	<2.5	N/A	48.80	1.70	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	17(*R,S*)-RvD4	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	RvD5	<1.25	N/A	<1.25	N/A	15.33	6.08	<1.25	N/A	<1.25	N/A	<1.25	N/A	5.89	0.86
	MaR2	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	2.68	0.02
	PDx	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.79	0.15	4.83	0.48
trihydroxylated ALA and LA	9,10,11-TriHOME	0.85	0.32	0.80	0.26	0.99	0.33	1.17	0.06	4.40	3.06	2.23	0.16	16.70	7.92
	9,10,13-TriHOME	2.82	1.06	1.85	0.51	3.99	1.97	36.47	47.01	3.18	0.60	4.90	1.69	11.14	0.55
	9,12,13-TriHOME	6.87	3.08	2.87	0.91	9.69	4.77	8.02	0.11	6.30	1.15	10.51	2.40	32.67	2.92
	9,10,11-TriHODE	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A
	9,10,13-TriHODE	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A	<1.25	N/A
	9,12,13-TriHODE	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A	<0.125	N/A
hydroxy-PUFA	9-HODE	309.51	107.98	173.06	14.09	234.68	23.26	259.24	29.16	411.67	37.02	584.61	13.60	4524.45	370.22
	10-HODE	4.12	1.18	2.62	0.33	4.33	0.94	3.36	0.63	5.54	0.13	9.07	0.51	21.58	1.86
	12-HODE	2.17	0.61	1.33	0.16	2.25	0.48	1.69	0.42	2.79	0.09	4.95	0.22	11.46	1.61
	13-HODE	495.67	160.74	273.67	16.97	426.00	41.96	368.00	31.58	618.17	49.73	937.33	27.34	6731.67	502.05
	15-HODE	5.62	0.51	4.29	0.41	5.66	0.75	3.53	0.05	5.45	0.21	5.87	0.37	28.21	1.99
	9-HOTrE	13.20	4.21	4.38	0.53	8.49	0.57	5.53	0.25	17.85	1.15	30.26	0.72	117.70	25.88
	13-HOTrE	18.16	5.51	5.40	0.78	11.00	1.42	5.49	0.25	24.62	0.49	43.35	2.47	27.22	1.01
	13-gamma-HOTrE	17.65	6.38	8.27	0.04	10.14	0.82	6.88	0.58	19.95	1.34	56.15	9.69	225.17	77.55
	5-HETrE	5.98	1.43	3.00	0.07	5.06	3.09	3.66	1.16	3.94	1.03	12.76	6.88	16.91	2.84
	8-HETrE	24.78	6.81	10.05	2.80	15.60	3.11	12.29	1.33	26.35	1.48	50.38	7.88	211.33	29.70
	12-HETrE	31.64	10.28	11.40	3.35	23.52	0.59	23.57	1.51	31.12	1.39	70.63	1.31	252.33	63.64
	15-HETrE	26.67	5.65	11.83	1.18	19.02	0.73	15.50	2.40	26.02	4.36	57.07	3.87	236.67	9.90
	5-HETE	121.26	24.96	84.56	14.06	78.51	17.34	89.37	5.28	130.67	7.26	206.09	3.05	1001.00	36.30
	8-HETE	104.12	33.87	73.25	10.82	66.47	10.14	72.45	18.31	132.50	17.68	216.83	28.52	927.00	104.65
	9-HETE	189.33	49.57	115.67	11.79	110.42	2.24	108.45	13.03	190.83	30.88	342.75	9.55	1515.00	54.21
	11-HETE	145.17	39.45	99.43	6.46	98.92	1.34	161.50	19.09	181.67	53.27	303.92	10.72	1393.33	84.85
	12-HETE	146.89	41.13	97.97	12.49	100.43	14.47	624.83	63.88	175.00	15.08	284.42	25.57	1208.33	139.06
	15-HETE	129.31	37.21	92.91	11.30	97.17	11.51	122.32	12.65	154.44	22.40	228.98	13.64	1070.67	124.45
	16-HETE	1.33	0.16	1.40	0.09	1.31	0.11	1.68	0.06	1.31	0.08	1.96	0.34	3.27	0.02
	17-HETE	<0.25	N/A	0.49	0.21	<0.25	N/A	0.37	0.03	<0.25	N/A	<0.25	N/A	0.46	0.13
	18-HETE	0.87	0.18	1.68	0.29	0.94	0.20	0.88	0.01	0.96	0.17	<1.1	N/A	1.10	0.28
	19-HETE	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A
	20-HETE	<2.5	N/A	<2.5	N/A	61.98	5.54	<2.5	N/A	<2.5	N/A	3.86	0.41	<2.5	N/A
	12-HHTrE	2.48	0.28	0.79	0.21	2.39	0.33	1356.67	42.43	0.81	0.33	2.78	0.24	0.59	0.03

Tab. 8.9: Continued. (Lab 4)

		Lab 4													
	concentration	**Pool 1 - B**		**Pool 2 - n3**		**Pool 3 - S**		**Pool 4 - Ca**		**Pool 5 - Ob-H**		**Pool 6 - Ob+H**		**Pool 7 - B2**	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
hydroxy-PUFA	tetranor-12-HETE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	0.81	0.30	2.18	0.34
	5-HEPE	17.20	2.71	25.37	2.56	11.32	0.57	6.97	0.37	22.33	3.80	35.73	4.68	100.20	0.42
	8-HEPE	15.95	5.26	22.66	2.04	9.93	1.17	6.00	0.01	23.83	5.61	40.33	5.31	106.00	10.47
	9-HEPE	29.82	9.93	44.17	6.84	19.02	2.19	11.39	0.46	40.93	9.24	71.28	3.79	197.17	17.68
	11-HEPE	16.23	3.84	22.99	2.41	10.34	0.45	7.75	0.70	22.28	4.97	41.52	9.88	105.61	0.88
	12-HEPE	36.16	9.26	52.38	3.70	25.12	0.35	25.97	2.97	50.97	6.08	86.58	6.48	227.83	14.85
	15-HEPE	27.60	8.08	40.42	3.79	40.75	1.01	10.55	1.60	37.22	4.83	69.28	9.45	185.00	2.83
	18-HEPE	31.91	8.19	48.98	3.94	45.85	2.19	14.95	2.33	46.43	1.46	76.23	17.87	63.83	6.08
	4-HDHA	63.39	19.65	39.35	9.69	41.83	7.26	35.52	3.94	77.77	8.77	120.65	18.60	230.00	34.41
	7-HDHA	38.91	5.36	29.72	1.30	20.88	0.45	15.90	1.93	45.02	5.63	70.97	1.32	188.83	2.12
	8-HDHA	32.97	3.27	21.86	1.85	18.25	0.41	13.57	0.07	43.10	7.20	54.69	12.40	162.96	14.24
	10-HDHA	34.82	6.36	28.48	0.02	20.45	1.25	18.23	3.21	50.20	7.07	67.94	2.13	195.33	3.77
	11-HDHA	62.20	8.36	48.13	0.80	39.45	3.65	30.53	1.18	76.77	18.15	125.87	12.21	321.67	24.51
	13-HDHA	40.77	9.19	32.58	0.02	24.85	0.31	21.22	4.50	60.52	17.70	81.09	3.05	233.83	51.62
	14-HDHA	38.95	7.48	29.16	1.23	28.84	0.88	26.79	1.83	52.96	8.26	72.65	3.44	207.90	4.15
	16-HDHA	33.17	8.97	24.08	1.96	20.13	2.97	15.72	2.00	45.93	7.12	58.79	3.88	184.67	10.84
	17-HDHA	38.33	10.21	25.90	1.48	27.77	2.79	16.80	2.69	52.36	9.30	68.96	4.73	199.01	7.53
	20-HDHA	48.20	8.17	35.72	2.90	31.80	0.14	29.20	0.52	64.13	4.95	87.33	8.31	288.50	1.65
epoxy-PUFA	9(10)-Ep-stearic acid	729.44	556.14	347.00	185.73	414.83	297.69	225.15	157.71	531.83	210.95	832.08	586.31	508.50	14.85
	9(10)-EpOME	840.44	493.78	452.33	125.87	466.17	255.27	397.17	266.11	774.17	449.48	774.83	278.36	498.17	3.06
	12(13)-EpOME	911.43	513.87	579.05	131.69	530.70	222.73	353.91	235.73	916.98	527.62	999.62	403.78	667.23	36.63
	9(10)-EpODE	32.42	29.40	16.65	11.95	19.63	16.70	4.93	3.18	30.33	21.78	33.77	14.66	13.64	5.88
	12(13)-EpODE	24.58	22.58	10.13	6.42	12.55	8.09	2.97	1.94	20.70	11.27	25.45	12.09	14.94	6.59
	15(16)-EpODE	136.82	111.63	64.22	35.00	88.73	54.12	18.30	7.21	113.57	47.38	152.42	56.45	131.87	48.27
	14(15)-EpEDE	134.70	105.69	74.35	12.47	79.03	36.11	13.44	9.10	98.12	5.92	184.75	89.45	78.62	13.32
	8(9)-EpETrE	302.89	220.41	314.83	58.22	184.17	28.05	73.45	35.14	321.83	147.31	310.83	76.13	212.50	1.18
	11(12)-EpETrE	881.89	434.33	921.83	81.79	541.83	37.48	85.15	45.47	863.33	136.71	959.58	192.45	715.33	10.84
	14(15)-EpETrE	1160.56	633.74	1045.67	86.27	616.33	87.21	141.43	87.54	923.00	206.48	1262.50	293.45	765.67	88.15
	8(9)-EpETE	66.22	61.84	167.00	31.58	30.80	6.84	12.50	N/A	93.32	47.35	78.58	13.75	59.15	12.89
	11(12)-EpETE	111.21	49.34	2.50	N/A	96.98	31.61	5.67	3.36	155.38	40.94	219.92	62.81	80.13	30.03
	14(15)-EpETE	192.56	171.83	518.33	137.65	97.20	18.38	6.77	2.95	162.35	24.54	224.75	47.02	101.65	33.02
	17(18)-EpETE	299.23	275.78	880.67	288.50	239.83	78.02	13.16	6.43	244.50	51.62	356.17	100.64	137.98	56.59
	7(8)-EpDPE	177.09	121.01	185.00	4.71	95.68	3.04	18.23	9.81	188.50	38.42	175.33	17.44	74.97	7.12
	10(11)-EpDPE	173.00	121.47	161.00	0.94	90.93	14.47	16.26	9.63	189.30	74.53	164.75	39.95	89.13	4.86
	13(14)-EpDPE	168.00	63.17	271.67	8.49	159.83	83.67	16.65	13.98	274.33	16.97	282.50	108.19	95.65	1.72
	16(17)-EpDPE	263.00	186.67	207.00	17.44	135.32	45.28	18.17	12.40	211.67	31.58	236.50	47.38	87.57	1.37
	19(20)-EpDPE	259.89	168.81	245.33	8.01	149.00	6.13	35.38	17.98	257.17	100.17	273.33	44.31	146.00	5.66
vicinal dihydroxy-PUFA	9,10-DiH-stearic acid	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	9,10-DiHOME	18.34	2.09	14.68	0.35	39.25	3.51	24.75	0.35	25.58	0.97	12.27	0.52	43.35	4.78
	12,13-DiHOME	11.28	0.65	11.83	0.33	10.52	0.49	10.88	0.26	13.55	2.43	9.89	1.02	34.60	1.74
	9,10-DiHODE	0.53	0.10	0.47	0.05	0.41	0.06	0.38	0.04	0.37	0.09	0.33	0.04	2.23	0.52
	12,13-DiHODE	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	15,16-DiHODE	13.94	1.33	13.55	0.31	14.00	0.24	17.85	1.01	11.07	2.55	9.44	0.45	17.37	4.24
	5,6-DiHETrE	318.44	180.49	310.33	10.37	168.67	5.66	25.92	0.54	219.17	39.83	325.33	61.28	244.00	27.81
	8,9-DiHETrE	3.92	0.60	5.00	0.73	3.97	0.78	4.44	0.51	3.10	0.01	4.79	0.07	4.83	0.14
	11,12-DiHETrE	1.79	0.09	2.13	0.13	15.88	1.04	1.50	0.03	1.62	0.09	2.24	0.07	1.81	0.16
	14,15-DiHETrE	2.51	0.51	3.04	0.21	3.24	0.18	1.21	0.03	2.42	0.59	3.35	0.37	2.50	0.16
	5,6-DiHETE	49.53	28.11	121.55	1.63	25.93	0.11	1.83	0.35	91.90	12.16	81.40	10.32	30.44	7.16
	8,9-DiHETE	0.58	0.18	1.29	0.03	0.46	0.01	<0.375	N/A	1.05	0.07	1.22	0.17	0.86	0.33
	11,12-DiHETE	0.38	0.11	1.03	0.03	0.28	0.01	0.15	0.01	0.82	0.10	0.72	0.14	0.29	0.07
	14,15-DiHETE	0.45	0.15	1.21	0.11	24.53	0.94	0.25	0.09	0.91	0.42	0.95	0.22	0.62	0.07
	17,18-DiHETE	1.34	0.34	3.91	0.20	1.06	0.13	0.50	0.04	2.04	0.89	2.42	0.35	1.11	0.17
	7,8-DiHDPE	3.19	0.02	4.73	0.15	3.01	0.42	3.93	0.08	2.20	0.29	2.81	0.14	1.81	0.08
	10,11-DiHDPE	0.69	0.05	0.79	0.03	0.64	0.01	0.61	0.04	0.65	0.06	1.00	0.08	0.61	0.01
	13,14-DiHDPE	0.65	0.16	0.82	0.02	0.51	0.05	0.44	0.04	0.62	0.08	0.78	0.12	0.48	0.01
	16,17-DiHDPE	0.88	0.14	1.04	0.02	65.63	2.07	0.56	0.04	0.93	0.09	1.01	0.07	0.89	0.04
	19,20-DiHDPE	2.83	0.34	3.79	0.12	2.72	0.02	2.80	0.19	3.43	0.54	3.02	0.10	2.37	0.13
oxo-PUFA	9-oxo-ODE	22.92	10.01	8.24	2.45	29.60	10.18	43.70	18.86	16.60	8.44	20.07	1.04	84.88	16.24
	13-oxo-ODE	543.56	189.68	228.00	81.08	551.67	311.60	496.17	188.80	411.67	71.65	609.00	57.98	1578.33	256.92
	9-oxo-OTrE	<0.375	N/A	<0.375	N/A	<0.375	N/A	0.55	0.14	<0.375	N/A	<0.37675	N/A	1.28	0.35
	13-oxo-OTrE	1.32	0.44	<0.25	N/A	0.47	0.07	0.30	0.04	1.36	0.13	2.72	0.26	11.95	1.63
	5-oxo-ETE	4.31	0.85	<2.5	N/A	<2.5	N/A	18.81	10.68	<2.5	N/A	<2.5	N/A	5.39	1.73
	12-oxo-ETE	545.89	203.82	256.18	51.89	289.94	81.08	253.04	97.12	451.75	124.57	969.65	153.65	2416.27	197.14
	15-oxo-ETE	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A	<0.25	N/A
	12-oxo-LTB_4	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	1.30	0.29
	17-oxo-n3DPA	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A
	4-oxo-DHA	2.52	0.69	2.10	0.70	2.96	1.73	7.22	3.21	2.55	1.06	<1.25	N/A	<1.25	N/A
	17-oxo-DHA	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A	<125	N/A
other	20-COOH-ARA	6.18	0.94	9.25	1.52	6.58	1.20	7.46	0.16	6.59	0.33	8.70	1.34	9.46	2.42

Tab. 8.9: Continued. (Lab 5)

		Lab 5													
	concentration	Pool 1 - B		Pool 2 - n3		Pool 3 - S		Pool 4 - Ca		Pool 5 - Ob-H		Pool 6 - Ob+H		Pool 7 - B2	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
isoprostanes	15-F_{1t}-IsoP	<0.23	N/A	<0.23	N/A	<0.23	N/A	<0.23	N/A	<0.23	N/A	<0.23	N/A	<0.23	N/A
	5(*R,S*)-5-F_{2c}-IsoP	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A
	5(*R,S*)-5-F_{2t}-IsoP	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	0.19	0.01
	15-F_{2t}-IsoP	<0.43	N/A	<0.43	N/A	2.30	0.24	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A
	2,3-dinor-15-(*R,S*)-15-F_{2t}-IsoP	<0.1	N/A	<0.1	N/A	<0.1	N/A	<0.1	N/A	<0.1	N/A	<0.1	N/A	<0.1	N/A
	15-oxo-15-F_{2t}-IsoP	<1.51	N/A	<1.51	N/A	<1.51	N/A	<1.51	N/A	<1.51	N/A	<1.51	N/A	<1.51	N/A
	13,14-dihydro-15-oxo-15-F_{2t}-IsoP	<0.42	N/A	<0.42	N/A	1.60	0.08	0.85	0.06	<0.42	N/A	<0.42	N/A	<0.42	N/A
	15-F_{3t}-IsoP	<7.55	N/A	<7.55	N/A	<7.55	N/A	<7.55	N/A	<7.55	N/A	<7.55	N/A	<7.55	N/A
prostaglandins	$PGF_{1\alpha}$	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A
	PGB_2	<0.59	N/A	<0.59	N/A	0.85	0.06	1.41	0.09	<0.59	N/A	<0.59	N/A	<0.59	N/A
	6-keto-$PGF_{1\alpha}$	<0.448	N/A	<0.448	N/A	<0.448	N/A	<0.448	N/A	<0.448	N/A	<0.448	N/A	<0.448	N/A
	$PGF_{2\alpha}$	<0.38	N/A	<0.38	N/A	1.85	0.03	1.00	0.10	<0.38	N/A	<0.38	N/A	<0.38	N/A
	PGJ_2	<0.0756	N/A	<0.0756	N/A	<0.0756	N/A	<0.0756	N/A	<0.0756	N/A	<0.0756	N/A	<0.0756	N/A
	PGB_3	<0.16	N/A	<0.16	N/A	<0.16	N/A	<0.16	N/A	<0.16	N/A	<0.16	N/A	<0.16	N/A
	Δ^{17}-6-keto-PGF_{1a}	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A	<0.5	N/A
	$PGF_{3\alpha}$	<2.26	N/A	<2.26	N/A	<2.26	N/A	<2.26	N/A	<2.26	N/A	<2.26	N/A	<2.26	N/A
	1a,1b-dihomo-$PGF_{2\alpha}$	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
multiple hydroxylated-PUFA	LTB_3	<0.33	N/A	<0.33	N/A	<0.33	N/A	<0.33	N/A	<0.33	N/A	<0.33	N/A	<0.33	N/A
	5(*S*),12(*S*)-DiHETE	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A
	5(*S*),15(*S*)-DiHETE	<1.41	N/A	<1.41	N/A	<1.41	N/A	<1.41	N/A	<1.41	N/A	<1.41	N/A	3.73	1.35
	8(*S*),15(*S*)-DiHETE	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	<0.39	N/A	1.00	0.61
	LTB_4	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A	<0.38	N/A
	6-trans-LTB_4	<0.15	N/A	<0.15	N/A	<0.15	N/A	0.21	0.00	<0.15	N/A	<0.15	N/A	3.03	0.78
	6-trans-12-epi-LTB_4	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A
	5(*S*),6(*R*)-DiHETE	<0.546	N/A	<0.546	N/A	<0.546	N/A	<0.546	N/A	<0.546	N/A	<0.546	N/A	<0.546	N/A
	5(*S*),6(*S*)-DiHETE	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	<0.17	N/A	1.12	0.15
	20-OH-LTB_4	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A	<0.32	N/A
	20-COOH-LTB_4	<2.40	N/A	<2.40	N/A	<2.40	N/A	<2.40	N/A	<2.40	N/A	<2.40	N/A	<2.40	N/A
	18-COOH-dinor-LTB_4	<1.46	N/A	<1.46	N/A	<1.46	N/A	<1.46	N/A	<1.46	N/A	<1.46	N/A	<1.46	N/A
	LxA_4	<0.08	N/A	<0.08	N/A	4.41	0.22	<0.08	N/A	0.19	0.04	<0.08	N/A	<0.08	N/A
	17(*R,S*)-RvD4	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A
	RvD5	<0.36	N/A	<0.36	N/A	13.27	1.59	<0.36	N/A	<0.36	N/A	<0.36	N/A	1.74	0.34
	MaR2	<0.45	N/A	<0.45	N/A	<0.45	N/A	<0.45	N/A	<0.45	N/A	<0.45	N/A	<0.45	N/A
	PDx	<0.28	N/A	<0.28	N/A	<0.28	N/A	<0.28	N/A	0.38	0.06	0.40	0.05	2.26	0.54
trihydroxylated ALA and LA	9,10,11-TriHOME	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	1.63	0.26
	9,10,13-TriHOME	<0.15	N/A	<0.15	N/A	<0.15	N/A	<0.15	N/A	<0.15	N/A	<0.15	N/A	0.26	0.08
	9,12,13-TriHOME	0.17	0.02	<0.13	N/A	0.13	0.00	0.14	0.01	0.19	0.02	0.16	0.03	0.66	0.18
	9,10,11-TriHODE	<0.07	N/A	<0.07	N/A	<0.07	N/A	<0.07	N/A	<0.07	N/A	<0.07	N/A	<0.07	N/A
	9,10,13-TriHODE	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A	<0.43	N/A
	9,12,13-TriHODE	<0.05	N/A	<0.05	N/A	<0.05	N/A	<0.05	N/A	<0.05	N/A	<0.05	N/A	<0.05	N/A
hydroxy-PUFA	9-HODE	75.10	5.17	74.08	12.38	99.01	18.21	138.56	30.57	109.17	0.22	106.81	1.00	1548.73	237.45
	10-HODE	5.84	0.84	5.26	1.62	8.53	0.99	9.60	5.28	6.77	2.71	8.21	0.57	24.44	2.69
	12-HODE	3.81	0.63	3.27	0.99	4.88	1.01	5.87	3.21	4.37	1.72	4.66	0.38	9.40	0.95
	13-HODE	100.65	10.26	94.59	16.11	179.19	40.97	154.48	42.30	144.22	2.91	131.24	4.21	1759.65	263.86
	15-HODE	<0.56	N/A	<0.56	N/A	<0.56	N/A	<0.56	N/A	<0.56	N/A	<0.56	N/A	<0.56	N/A
	9-HOTrE	2.54	0.22	1.81	0.44	3.00	1.03	2.68	0.60	3.46	0.08	4.17	0.28	90.97	15.76
	13-HOTrE	3.29	0.37	2.66	0.47	4.00	1.20	3.17	0.96	4.81	0.32	5.31	0.18	18.32	6.49
	13-gamma-HOTrE	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	<2.5	N/A	67.05	19.89
	5-HETrE	1.73	0.16	<1.29	N/A	1.68	0.10	1.53	0.05	1.77	0.24	2.47	0.41	12.93	6.92
	8-HETrE	4.14	0.62	3.73	0.49	4.83	1.33	4.93	0.94	3.47	0.48	6.16	0.31	128.95	22.86
	12-HETrE	2.76	0.22	2.56	0.36	4.08	1.19	9.33	1.31	2.56	0.43	5.04	1.21	158.95	40.46
	15-HETrE	3.84	0.55	3.18	0.42	4.77	1.39	7.34	1.29	3.61	0.83	7.03	1.37	170.22	28.72
	5-HETE	16.11	2.20	22.34	2.87	18.10	5.04	30.00	4.71	15.86	2.14	23.17	3.20	467.31	74.64
	8-HETE	15.90	2.80	23.53	3.55	19.34	6.79	28.48	9.40	14.14	2.00	24.90	5.47	<0.69	N/A
	9-HETE	12.58	2.06	16.22	3.87	14.85	4.84	152.33	1.93	10.57	3.17	16.85	3.22	389.42	50.87
	11-HETE	16.26	2.73	22.66	3.84	20.15	7.53	93.57	14.83	14.33	1.97	25.81	5.88	<0.06	N/A
	12-HETE	17.16	2.74	19.77	3.39	21.62	7.05	516.95	22.26	12.96	1.58	22.65	5.33	810.82	206.83
	15-HETE	20.43	2.98	28.35	4.70	34.80	8.35	69.05	14.98	20.38	1.86	31.29	5.20	575.31	104.72
	16-HETE	0.39	0.01	0.42	0.01	0.38	0.03	0.65	0.01	0.39	0.02	0.48	0.01	1.29	0.48
	17-HETE	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A	<0.34	N/A
	18-HETE	0.76	0.03	1.40	0.09	0.76	0.04	1.05	0.20	0.92	0.14	0.96	0.06	0.90	0.06
	19-HETE	<5.89	N/A	<5.89	N/A	<5.89	N/A	<5.89	N/A	<5.89	N/A	<5.89	N/A	<5.89	N/A
	20-HETE	<1.68	N/A	<1.68	N/A	40.14	2.03	<1.68	N/A	<1.68	N/A	<0.16	N/A	<0.68	N/A
	12-HHTrE	0.95	0.06	<0.36	N/A	0.87	0.11	894.08	115.54	0.44	0.01	1.30	0.01	<0.36	N/A

Tab. 8.9: Continued. (Lab 5)

		Lab 5													
	concentration	**Pool 1 - B**		**Pool 2 - n3**		**Pool 3 - S**		**Pool 4 - Ca**		**Pool 5 - Ob-H**		**Pool 6 - Ob+H**		**Pool 7 - B2**	
	[nmol/L]	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
hydroxy-PUFA	tetranor-12-HETE	<2.42	N/A	<2.42	N/A	<2.42	N/A	<2.42	N/A	<2.42	N/A	<2.42	N/A	<2.42	N/A
	5-HEPE	1.67	0.16	4.91	0.57	1.86	0.40	1.51	0.21	2.01	0.02	2.88	0.35	44.11	11.00
	8-HEPE	0.77	0.16	2.94	0.63	1.01	0.37	0.77	0.25	0.81	0.06	1.41	0.39	45.01	15.59
	9-HEPE	1.39	0.24	5.18	1.12	1.82	0.61	1.37	0.37	1.60	0.09	2.53	0.41	86.40	31.14
	11-HEPE	0.79	0.16	2.99	0.74	1.05	0.42	1.37	0.34	0.86	0.11	1.55	0.38	45.36	13.47
	12-HEPE	1.47	0.24	4.85	1.07	2.08	0.57	6.09	0.22	1.51	0.06	2.60	0.46	86.76	27.99
	15-HEPE	1.61	0.29	5.58	1.41	16.19	1.76	1.88	0.56	1.73	0.16	2.82	0.53	78.85	19.06
	18-HEPE	1.25	0.19	4.25	0.90	9.02	1.09	1.69	0.35	1.41	0.13	2.26	0.35	5.99	0.62
	4-HDHA	12.59	2.10	19.78	3.86	14.08	4.52	20.36	5.02	14.31	2.80	18.62	4.75	201.50	38.46
	7-HDHA	15.67	2.76	27.96	4.78	18.52	6.59	21.53	7.33	16.71	3.33	22.47	6.93	260.50	145.51
	8-HDHA	10.83	1.99	17.09	2.92	13.13	4.62	13.68	4.32	12.25	2.35	17.07	4.41	210.68	70.01
	10-HDHA	6.26	1.29	9.77	1.57	7.51	2.93	8.51	2.81	6.44	0.91	9.95	3.02	148.50	3.47
	11-HDHA	16.34	3.31	26.55	5.00	20.45	7.78	26.02	8.94	19.54	5.30	28.37	9.87	343.87	102.76
	13-HDHA	7.40	1.54	12.70	2.31	9.49	4.09	11.83	4.03	8.43	1.16	12.66	4.16	238.07	5.94
	14-HDHA	5.62	1.07	7.31	1.35	7.76	2.59	11.74	3.03	5.20	0.79	7.62	2.48	144.34	16.48
	16-HDHA	6.93	1.58	11.30	2.55	8.71	3.45	9.30	3.50	7.87	1.29	11.39	3.33	143.96	18.55
	17-HDHA	2.63	0.52	4.11	0.99	7.84	1.66	3.90	1.29	2.92	0.66	4.25	1.18	95.05	29.15
	20-HDHA	9.73	1.70	14.36	2.75	11.87	4.26	15.95	3.96	11.14	2.05	14.73	3.36	196.39	25.34
epoxy-PUFA	9(10)-Ep-stearic acid	115.45	16.46	71.82	3.09	110.44	19.63	67.15	7.70	113.53	35.17	113.38	42.98	683.21	516.66
	9(10)-EpOME	133.87	19.65	94.73	9.53	131.51	10.66	117.36	15.68	152.49	38.60	154.19	29.46	218.74	7.68
	12(13)-EpOME	109.07	17.52	77.65	8.10	112.81	12.63	97.56	17.67	122.90	37.27	128.32	25.53	192.12	5.36
	9(10)-EpODE	0.95	0.18	0.50	0.07	0.93	0.08	0.38	0.03	1.36	0.27	1.59	0.04	1.83	0.97
	12(13)-EpODE	1.54	0.33	0.71	0.12	1.46	0.02	0.77	0.12	2.14	0.31	2.35	0.06	3.08	0.63
	15(16)-EpODE	2.09	0.21	0.97	0.03	2.09	0.03	1.46	0.07	2.52	0.19	2.32	0.03	10.51	6.67
	14(15)-EpEDE	6.22	1.49	3.33	0.63	5.67	0.67	4.13	1.08	6.50	2.47	9.79	3.56	8.54	2.54
	8(9)-EpETrE	16.67	0.73	25.49	2.00	24.22	6.59	23.40	2.65	21.03	2.76	32.53	0.90	31.16	1.03
	11(12)-EpETrE	24.79	1.54	31.40	2.33	27.41	5.73	29.03	1.39	26.58	9.09	35.32	9.84	34.87	0.25
	14(15)-EpETrE	35.94	7.73	35.05	9.64	55.13	3.94	39.99	13.18	37.57	13.55	50.68	15.31	47.32	12.12
	8(9)-EpETE	2.27	0.02	7.47	0.73	3.02	1.01	1.76	0.22	3.18	0.26	5.35	0.13	2.82	0.26
	11(12)-EpETE	3.01	0.66	7.57	1.92	2.95	0.42	1.88	0.61	3.74	1.43	5.02	1.32	2.99	1.16
	14(15)-EpETE	8.34	1.93	20.59	5.66	8.25	1.38	4.54	1.69	10.46	4.39	13.73	4.52	5.34	0.67
	17(18)-EpETE	3.69	0.86	9.27	2.29	33.51	3.63	1.86	0.52	4.86	1.56	6.34	1.50	4.29	1.94
	7(8)-EpDPE	4.54	0.24	6.76	0.24	6.26	1.56	5.16	0.45	7.16	0.85	9.19	0.91	5.21	0.11
	10(11)-EpDPE	11.18	1.20	18.46	2.49	11.49	3.45	7.20	0.81	12.00	5.21	10.25	4.04	5.04	1.43
	13(14)-EpDPE	8.10	1.98	8.48	1.74	7.44	1.24	6.12	2.44	10.48	3.97	10.74	3.26	4.85	0.23
	16(17)-EpDPE	10.51	2.13	10.98	2.40	10.34	1.30	8.44	2.86	13.47	4.76	14.67	4.57	6.84	0.67
	19(20)-EpDPE	20.62	4.16	21.48	4.17	79.83	1.54	15.99	4.12	28.42	9.16	30.75	8.17	10.91	2.22
vicinal dihydroxy-PUFA	9,10-DiH-stearic acid	56.14	3.17	48.16	0.63	62.27	5.19	63.38	1.82	58.52	6.41	57.57	2.13	27.05	10.52
	9,10-DiHOME	15.37	0.39	12.08	0.13	36.45	1.34	23.01	1.61	23.18	1.13	9.97	0.05	38.25	4.66
	12,13-DiHOME	3.67	0.23	2.90	0.22	2.56	0.04	3.62	0.36	9.09	1.48	9.21	1.15	16.43	9.24
	9,10-DiHODE	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.06	N/A	<0.0602	N/A	0.18	0.06
	12,13-DiHODE	<0.12	N/A	<0.12	N/A	<0.12	N/A	<0.12	N/A	<0.12	N/A	<0.12	N/A	<0.12	N/A
	15,16-DiHODE	0.41	0.01	0.41	0.01	0.51	0.04	0.69	0.00	0.41	0.02	0.37	0.00	0.84	0.33
	5,6-DiHETrE	29.89	1.34	42.35	1.16	30.61	0.22	47.88	0.01	27.16	1.98	33.74	2.18	19.97	4.97
	8,9-DiHETrE	1.04	0.04	1.10	0.01	14.15	0.46	1.90	0.29	0.94	0.04	1.22	0.05	1.09	0.16
	11,12-DiHETrE	3.11	0.07	3.99	0.05	3.20	0.05	5.20	0.21	2.71	0.40	3.58	0.19	3.10	0.50
	14,15-DiHETrE	0.70	0.02	0.82	0.06	1.84	0.25	1.39	0.46	0.72	0.05	0.76	0.03	0.78	0.07
	5,6-DiHETE	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A	<0.04	N/A
	8,9-DiHETE	0.35	0.01	0.60	0.02	<0.25	N/A	<0.24	N/A	0.61	0.04	1.21	0.22	0.27	0.04
	11,12-DiHETE	0.21	0.01	0.31	0.02	0.16	0.01	0.16	0.02	0.41	0.00	0.82	0.09	0.17	0.01
	14,15-DiHETE	0.23	0.04	0.29	0.10	12.49	0.59	0.29	0.12	0.46	0.21	0.76	0.44	0.14	0.01
	17,18-DiHETE	0.49	0.08	1.21	0.01	0.54	0.03	0.35	0.04	1.28	0.37	1.87	0.31	0.53	0.17
	7,8-DiHDPE	5.16	0.15	7.73	0.38	5.14	0.11	8.59	0.48	4.27	0.65	4.50	0.29	1.74	0.38
	10,11-DiHDPE	1.28	0.08	1.35	0.05	1.38	0.03	1.98	0.35	1.34	0.14	1.86	0.09	0.72	0.28
	13,14-DiHDPE	0.81	0.04	1.06	0.09	0.82	0.08	1.36	0.36	0.87	0.05	0.81	0.05	0.50	0.20
	16,17-DiHDPE	0.37	0.02	0.49	0.01	53.17	1.74	0.60	0.08	0.43	0.01	0.47	0.02	0.64	0.09
	19,20-DiHDPE	1.58	0.05	2.23	0.12	1.66	0.09	2.39	0.27	1.95	0.01	1.69	0.04	1.56	0.12
oxo-PUFA	9-oxo-ODE	38.07	10.35	22.08	0.17	36.40	0.22	180.55	66.36	50.94	4.75	37.09	15.76	233.53	59.50
	13-oxo-ODE	205.98	16.27	148.82	20.65	204.54	34.58	315.68	130.62	198.16	132.93	219.16	137.85	669.50	248.00
	9-oxo-OTrE	<0.4	N/A	<0.4	N/A	<0.4	N/A	0.95	0.08	<0.4	N/A	<0.4	N/A	<0.4	N/A
	13-oxo-OTrE	0.32	0.01	0.20	0.00	0.32	0.04	0.31	0.12	0.36	0.21	0.43	0.20	1.13	0.71
	5-oxo-ETE	6.57	1.71	7.25	0.25	6.48	0.96	82.56	37.17	7.30	0.40	6.36	3.40	8.04	2.70
	12-oxo-ETE	24.18	2.17	22.66	4.28	22.59	2.92	28.81	17.32	20.67	15.74	29.27	21.23	37.50	15.70
	15-oxo-ETE	44.52	3.62	43.70	7.39	42.13	10.16	41.30	30.39	38.93	32.68	58.13	41.94	84.40	43.41
	12-oxo-LTB_4	0.14	0.00	<0.12	N/A	<0.12	N/A	<0.12	N/A	<0.12	N/A	0.21	0.03	0.37	0.11
	17-oxo-n3DPA	24.35	5.49	23.85	8.71	22.88	5.59	26.96	15.82	31.33	19.48	34.27	20.22	16.40	0.01
	4-oxo-DHA	7.70	0.50	10.74	0.23	9.27	0.44	58.77	25.43	9.16	2.34	9.87	7.02	4.45	1.75
	17-oxo-DHA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
other	20-COOH-ARA	8.86	1.53	11.98	2.53	8.71	2.01	10.56	3.30	9.59	2.66	11.21	3.00	<11.50	5.79

Tab. 8.10: Consensus value estimates for oxylipins quantified in seven QC plasmas. Shown are MEDM, u and COD for oxylipins with acceptable consensus value.

	QC Plasma 1			QC Plasma 1 (without Lab4)			QC Plasma 2			QC Plasma 3			QC Plasma 4			QC Plasma 5			QC Plasma 6			QC Plasma 7		
	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)
5(*R*,*S*)-5-F_{2t}-IsoP																						3.5	1.0	29.7
15-F_{2t}-IsoP										25.1	6.1	24.2												
PGB_2													18.0	7.1	39.6							7.8	2.2	27.6
$PGF_{2\alpha}$										23.4	6.2	26.6	9.4	2.7	28.3									
5(*S*),12(*S*)-DiHETE																						43.2	15.0	34.8
5(*S*),15(*S*)-DiHETE																						7.0	2.1	29.3
8(*S*),15(*S*)-DiHETE																						38.7	11.3	29.3
LxA4										52.3	13.2	25.2												
RvD5										17.1	2.5	14.8												
(N)PD1																						4.4	1.2	26.6
PDx																			0.5	0.2	31.3	2.9	0.7	25.1
9,10,11-TriHOME																						16.7	4.5	26.9
9,10,13-TriHOME	3.5	0.9	25.9	4.1	1.2	28.8																		
9,10,11-TriHODE																						1.0	0.2	18.6
9-HODE				52.5	12.8	24.4																		
10-HODE	4.1	0.9	22.7	4.8	1.2	24.2	2.9	1.0	33.9							5.5	0.9	15.9				24.4	5.4	22.0
12-HODE	2.3	0.6	23.8	2.5	0.7	29.9	1.6	0.6	38.9	2.8	0.9	30.8				2.8	0.6	19.4	4.8	0.1	2.9	9.9	1.0	10.0
13-HODE				71.7	15.3	21.4				179	70.0	39.1												
15-HODE	5.6	0.1	2.2				4.2	0.1	2.8	5.5	0.1	2.1	3.5	0.1	3.5	5.6	0.2	3.3	5.6	0.2	3.8	24.4	2.1	8.4
9-HOTrE				2.5	0.4	14.5																93.8	8.7	9.2
13-gamma-HOTrE																						111	32.8	29.5
5-HETrE				1.4	0.3	23.6																12.9	2.4	18.4
8-HETrE																						129	32.1	24.9
12-HETrE				2.8	0.8	30.7																147	29.0	19.7
15-HETrE				2.9	1.0	34.2																156	25.8	16.5
5-HETE				13.4	1.1	8.4																515	138	26.9
8-HETE				12.8	2.9	23.1																696	136	19.6
9-HETE				11.1	3.5	31.9																1005	198	19.7
11-HETE				16.3	2.9	17.6							90.2	25.6	28.4							983	245	24.9
12-HETE				17.2	3.2	18.8							536	33.2	6.2							846	130	15.4
15-HETE													73.7	17.2	23.3							834	178	21.3
16-HETE										0.9	0.3	38.3	1.4	0.2	17.5							2.0	0.4	18.3
18-HETE	0.7	0.1	10.9	0.7	0.1	12.5	1.3	0.1	8.5	0.8	0.1	15.4	1.0	0.2	17.4	0.9	0.1	15.1	1.0	0.1	15.3	0.9	0.1	13.7
20-HETE										54.8	5.9	10.7												
12-HHTrE													1416	176	12.4				2.8	0.9	33.1			
tetranor-12-HETE													0.4	0.0	2.1				0.4	0.2	39.0	2.1	0.1	3.2
5-HEPE				1.7	0.1	7.6																58.4	13.7	23.4
8-HEPE				0.7	0.1	11.5																61.5	15.2	24.8
9-HEPE				2.0	0.4	19.0																124.2	22.2	17.9
11-HEPE				0.8	0.2	28.3																67.0	12.0	18.0
12-HEPE				1.6	0.4	25.4							11.3	4.3	37.8							138.0	28.5	20.6
15-HEPE										22.5	5.3	23.7										116.5	21.3	18.3
18-HEPE										23.3	7.4	31.9										63.8	22.0	34.5
4-HDHA																						153.2	32.9	21.5
7-HDHA										18.5	6.4	34.7										129.3	48.0	37.1
8-HDHA																						130.6	29.4	22.5

Tab. 8.10: Continued.

	QC Plasma 1			QC Plasma 1 (without Lab4)			QC Plasma 2			QC Plasma 3			QC Plasma 4			QC Plasma 5			QC Plasma 6			QC Plasma 7		
	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)	MEDM	*u*	COD (%)
10-HDHA				4.5	1.2	27.7																143	20.5	14.3
11-HDHA																						219	53.1	24.2
13-HDHA				4.8	1.7	35.9																205	34.5	16.8
14-HDHA				5.6	0.8	14.9																141	24.9	17.6
16-HDHA																						141	13.4	9.5
17-HDHA																						146	19.6	13.5
20-HDHA				6.1	2.2	35.8																196	24.9	12.7
9(10)-Ep-stearic acid				115.4	30.7	26.6																596	120	20.2
9(10)-EpOME				133.9	17.9	13.4																		
12(13)-EpOME				109.1	9.7	8.9																		
12(13)-EpODE				1.7	0.5	27.9																		
15(16)-EpODE													12.7	4.6	36.0									
14(15)-EpEDE				4.9	1.6	33.7																		
8(9)-EpETrE				17.7	4.3	24.3																		
11(12)-EpETrE				24.5	6.3	25.6																		
14(15)-EpETrE				36.7	8.7	23.7																		
8(9)-EpETE													2.7	0.6	22.1									
11(12)-EpETE				3.3	1.1	33.0																		
14(15)-EpETE				7.0	1.7	23.7							4.5	1.0	21.4									
10(11)-EpDPE				8.1	3.0	36.5							7.2	2.7	37.9									
13(14)-EpDPE				7.0	2.1	29.7																		
16(17)-EpDPE				7.7	2.8	36.9							8.4	3.1	36.3									
19(20)-EpDPE				12.6	4.8	38.2																		
9,10-DiHOME	18.3	1.3	7.2	17.8	1.8	10.1	14.6	3.1	20.9	39.3	1.5	3.8	24.8	4.0	16.3	25.3	3.6	14.3	11.6	4.2	36.1	41.1	3.3	8.1
12,13-DiHOME	9.1	1.6	17.8	9.1	1.9	21.4	10.1	2.6	25.5	9.4	2.6	27.1	9.8	3.1	31.8	10.5	3.2	30.5	9.2	3.3	35.5	30.4	4.3	14.2
9,10-DiHODE	0.5	0.0	8.8				0.5	0.0	4.2	0.4	0.0	4.2	0.4	0.0	9.5	0.4	0.0	9.4	0.4	0.0	7.6	2.4	0.6	24.8
15,16-DiHODE	13.9	3.7	26.8	14.4	5.0	34.4	13.3	3.5	25.9	14.0	3.7	26.2	17.9	4.7	26.3	11.1	3.1	27.9	9.7	2.7	27.8	18.7	4.9	26.0
5,6-DiHETrE				15.4	5.2	33.6							19.7	7.3	37.0									
8,9-DiHETrE	3.5	0.7	19.3	3.4	0.9	24.7	4.3	0.9	20.8				4.0	0.6	14.7	3.1	0.6	18.5	4.0	0.8	20.0			
11,12-DiHETrE										12.9	3.7	28.9				1.4	0.5	35.2				1.5	0.6	37.0
14,15-DiHETrE				0.8	0.1	7.2				2.0	0.3	16.5	1.3	0.1	6.0									
8,9-DiHETE	0.4	0.1	28.1				1.0	0.2	17.3							0.7	0.2	28.7	1.2	0.2	17.9			
11,12-DiHETE																			0.7	0.2	24.2			
14,15-DiHETE										22.8	4.1	18.1	0.3	0.1	39.1	0.7	0.2	35.7	0.9	0.1	15.0	0.4	0.1	31.8
17,18-DiHETE							1.7	0.6	36.6	0.7	0.2	26.7				1.4	0.4	26.2	1.5	0.3	23.1			
7,8-DiHDPE	3.2	0.5	16.9	3.2	0.7	22.4	4.7	0.9	18.7	3.0	0.6	18.9	3.9	1.3	33.0	2.6	0.6	23.4	2.8	0.5	16.1	1.7	0.1	5.2
10,11-DiHDPE	0.6	0.2	32.6				0.6	0.2	35.1	0.6	0.2	38.2				0.6	0.2	35.7	0.8	0.3	34.4	0.5	0.1	18.4
13,14-DiHDPE	0.5	0.2	35.3				0.7	0.2	33.3	0.4	0.1	32.2				0.5	0.2	29.8	0.8	0.2	24.5	0.4	0.1	24.1
16,17-DiHDPE							0.6	0.2	24.5	62.8	3.5	5.6	0.6	0.0	5.1	0.6	0.1	23.7	0.6	0.2	27.1	0.8	0.1	7.1
19,20-DiHDPE	2.2	0.3	12.6	2.1	0.2	10.3	2.9	0.4	12.7	2.3	0.2	9.0	2.7	0.2	5.6	2.8	0.3	12.3	2.5	0.3	11.0	2.2	0.2	9.0
9-oxo-ODE										29.6	7.8	26.4							25.9	8.3	32.2			
13-oxo-ODE							164.1	21.0	12.8				496	124	24.9	202.0	62.6	31.0						
5-oxo-ETE																						5.4	1.4	25.7
15-oxo-ETE	44.5	2.7	6.0	44.5	2.7	6.0	52.8	5.8	11.0	32.4	7.7	23.7				38.9	12.2	31.4	37.5	14.6	38.9	64.2	12.0	18.7
12-oxo-LTB4																						0.7	0.3	37.6
20-COOH-ARA	8.0	1.1	13.4				9.7	1.2	12.2	6.9	0.9	13.8	8.2	1.3	16.0	8.1	1.1	13.4	9.8	0.9	9.7	9.8	0.9	9.1

Tab. 8.11: 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).**

	Number of oxylipins with a consensus value						
	QC plasma 1	QC plasma 2	QC plasma 3	QC plasma 4	QC plasma 5	QC plasma 6	QC plasma 7
Number of quantified oxylipins	78	78	86	82	78	84	96
Number of oxylipins with consensus value	17	19	29	29	21	23	60
% of oxylipins with consensus value	21.8	24.4	33.7	35.4	26.9	27.4	62.5

	Number of oxylipins with a consensus value (without Lab 4)						
	QC plasma 1	QC plasma 2	QC plasma 3	QC plasma 4	QC plasma 5	QC plasma 6	QC plasma 7
Number of quantified oxylipins	64	66	74	75	68	69	86
Number of oxylipins with consensus value	47	41	54	45	43	43	70
% of oxylipins with consensus value	73.4	62.1	73.0	60.0	63.2	62.3	81.4

Tab. 8.12: 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 sample from MS1; Δ intra-MS3: difference of concentration between the lower and the higher sample from MS3. MS effect was considered consistent when Δ inter-MS > Δ intra-MS1 and Δ intra-MS3.

	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 & intra-MS3	
9,10,13-TriHOME	2.16	0.21	0.68	yes	0.5	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	1.6	-	-
12-HETrE	1.13	0.43	0.5	yes	16.48	1.31	14.68	yes	yes
15-HETrE	0.09	0.21	0.94	-	0.18	1.5	1.46	-	-
5-HETE	2.52	1.22	4.2	-	0.35	5.4	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	0.5	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	1.3	-	2.3	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	2.5	-	-
16-HDHA	0.48	0.2	1.02	-	0.74	2.08	2.21	-	-
20-HDHA	0.24	0.08	1.74	-	1	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	3.9	-	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	2.3	-	-
10(11)-EpDPE	1.24	0.79	1.61	-	2.25	3.32	1.68	-	-
13(14)-EpDPE	1.3	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	1.8	-	-
12,13-DiHOME	2.15	0.37	3.19	-	2.77	0.29	2.21	-	-
15,16-DiHODE	0.09	1.15	3.4	-	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	0.8	-	0.26	0.04	0.02	-	-
13-oxo-ODE	21.24	30.04	36.09	-	31.53	41.99	46.88	-	-

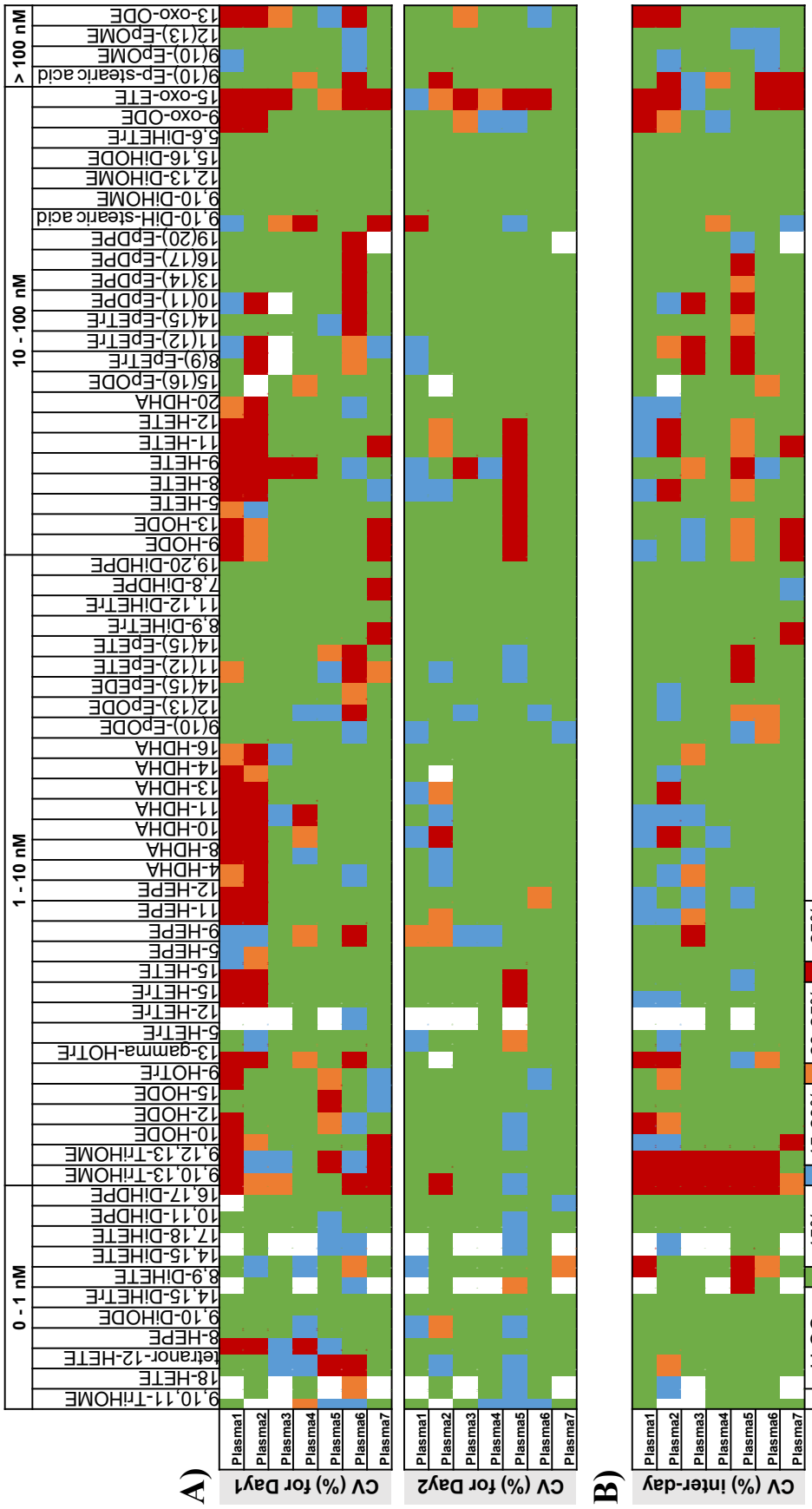


Fig. 8.11: Intra-day **(A)** and inter-day **(B)** variability of the oxylipin analysis in laboratory 2. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD ($\mathbf{CV} = \frac{SD}{mean} * \mathbf{100}$). Shown are the CVs of 69 oxylipins analyzed in the seven QC plasmas in laboratory 2 displayed in four different colors.

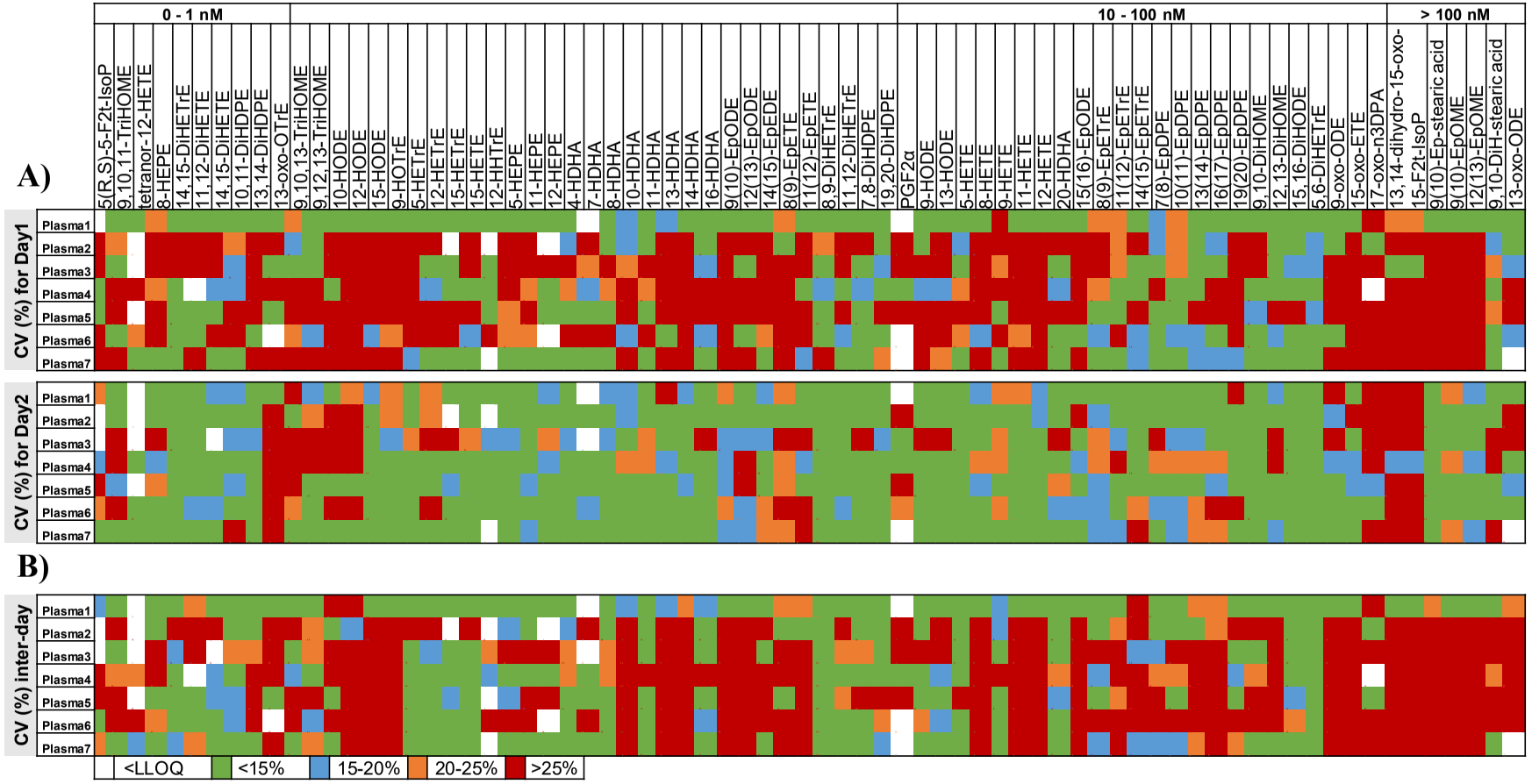


Fig. 8.12: Intra-day **(A)** and inter-day **(B)** variability of the oxylipin analysis in laboratory 3. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD ($\mathbf{CV} = \frac{SD}{mean} * \mathbf{100}$). Shown are the CVs of 73 oxylipins analyzed in the seven QC plasmas in laboratory 3 displayed in four different colors.

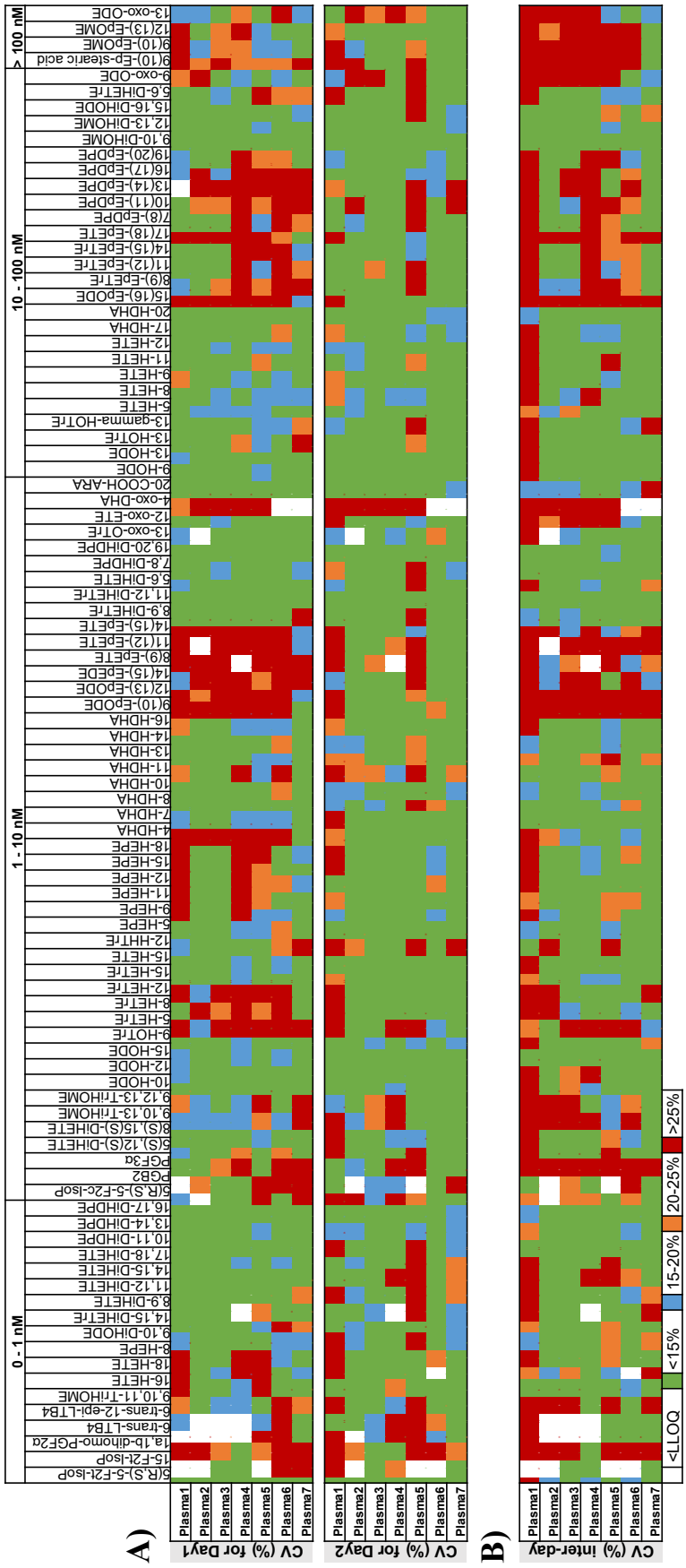


Fig. 8.13: Intra-day **(A)** and inter-day **(B)** variability of the oxylipin analysis in laboratory 4. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients of variance (CV) were calculated using the mean concentration and SD ($\mathbf{CV} = \frac{SD}{mean} * \mathbf{100}$). Shown are the CVs of 94 oxylipins analyzed in the seven QC plasmas in laboratory 4 displayed in four different colors.

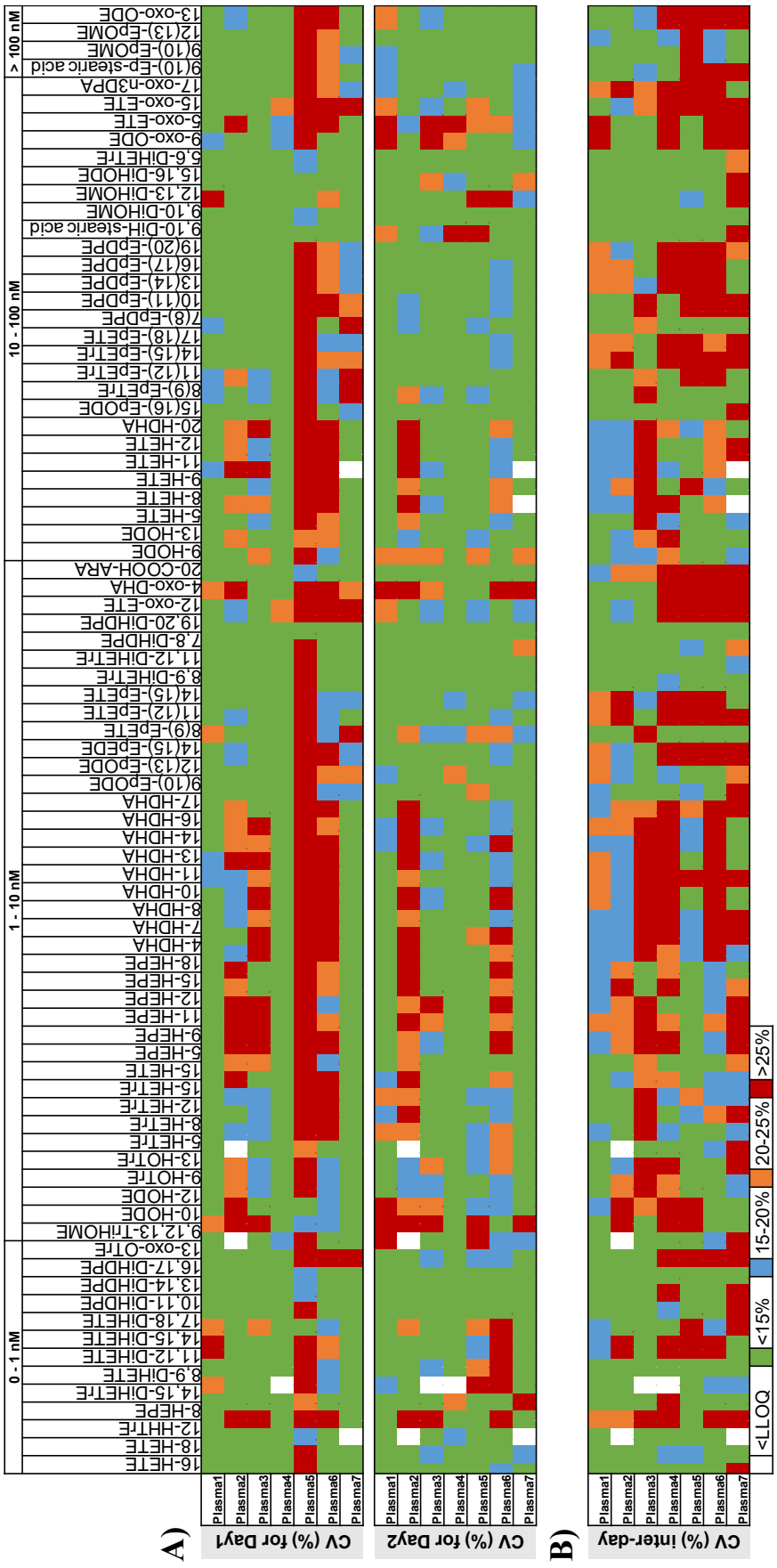


Fig. 8.14: Intra-day **(A)** and inter-day **(B)** variability of the oxylipin analysis in laboratory 5. Oxylipin analysis in the seven QC plasmas was carried out in triplicates on two consecutive days and the variability within each day as well as the inter-day variability were determined. The coefficients 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.

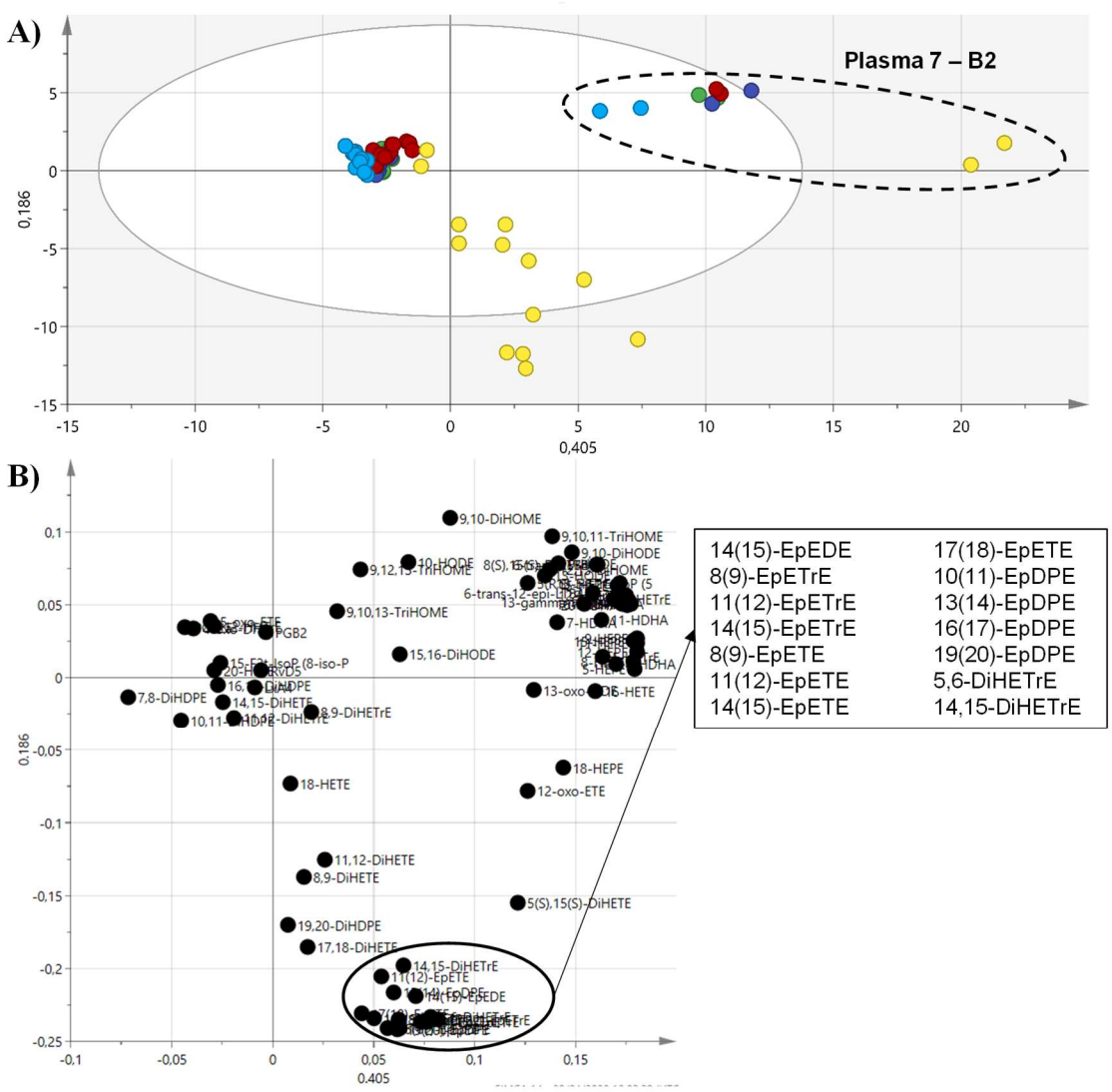


Fig. 8.15: Principal components analysis (PCA) including epoxy-PUFA. The model was built with 75 samples and 74 oxylipins (R^2X=0.695 and Q^2=0.633). A) The score plot shows that the main variability is related to the type of plasma, QC plasma 7 – B2 **(dotted circle)**. In the 2nd component, laboratory 4 distinguishes itself. B) The loading plot shows that the discrimination of laboratory 4 is related to epoxy-PUFA **(solid circle)**.

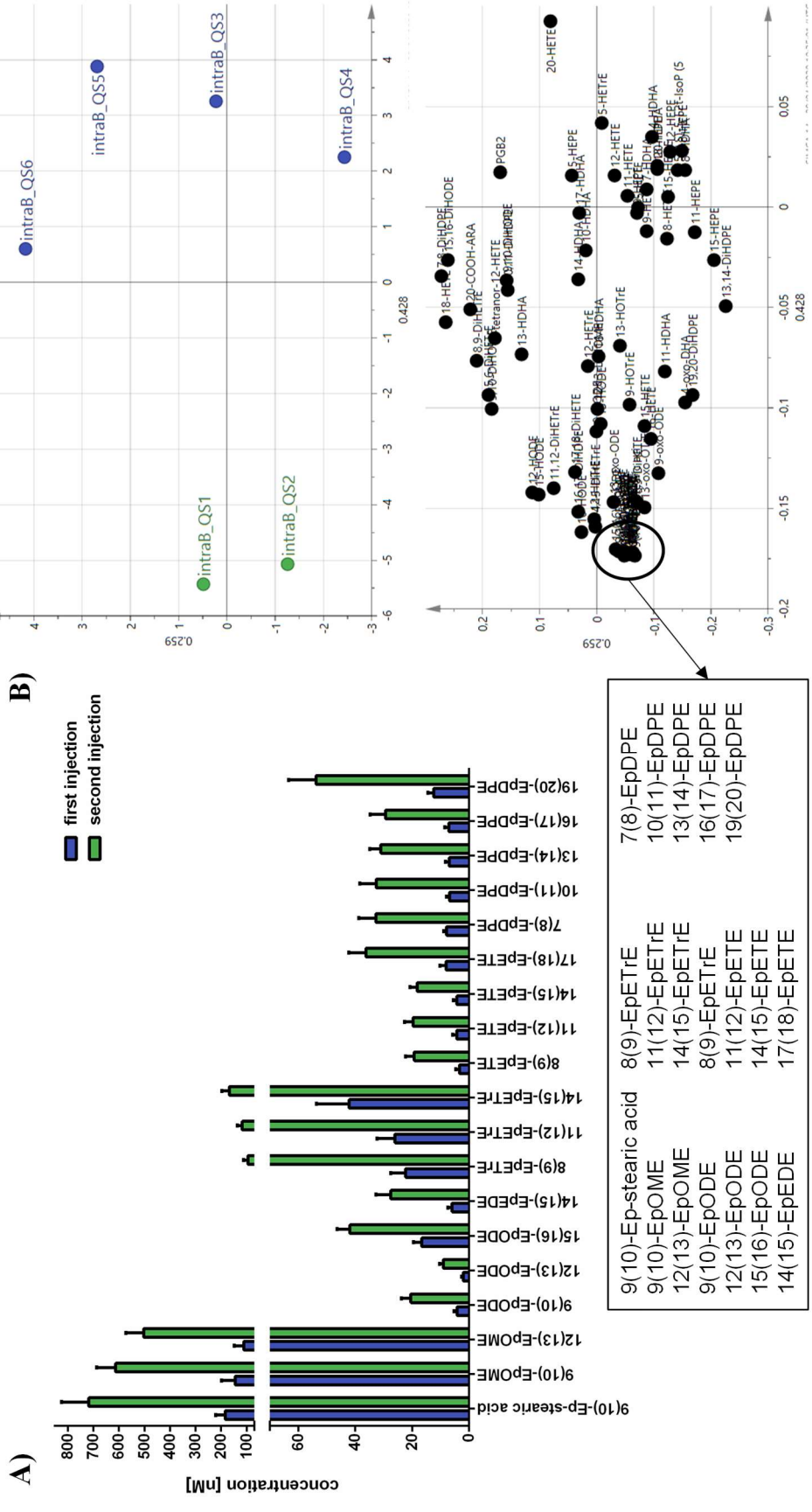
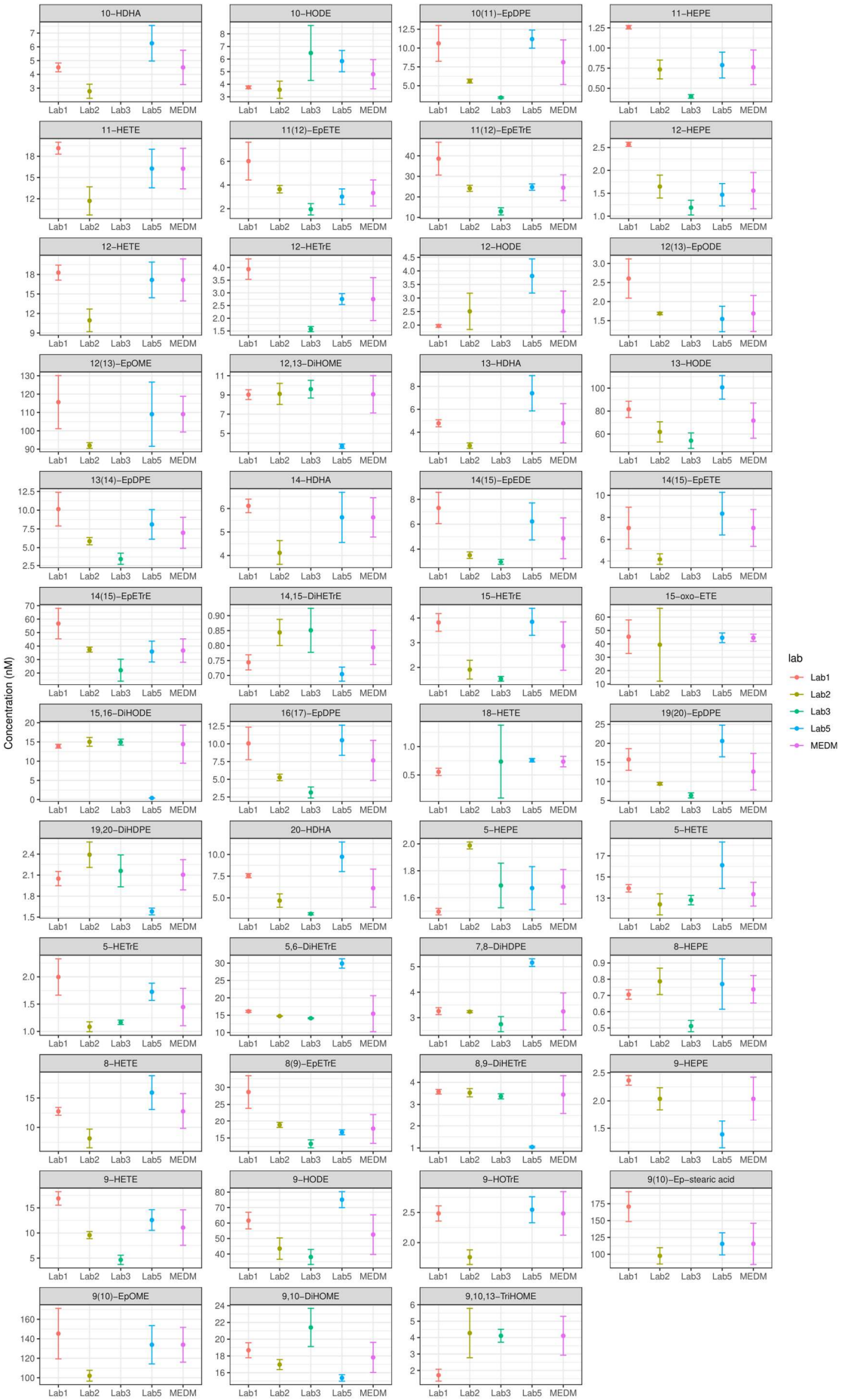


Fig. 8.16: Effects of sample re-injection on the concentration of epoxy-PUFA. **A)** Apparent concentrations of epoxy-PUFA in plasma 1. Concentrations derived from re-injected samples are marked in green and once injected samples are depicted in blue. **B)** Partial least square discriminant analysis (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.

Fig. 8.17: 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%).


10−HDHA
10−HODE
10(11)−EpDPE
11−HEPE
11−HETE
11(12)−EpETE
11(12)−EpETrE
12−HEPE
12−HETE
12−HETrE
12−HODE
12(13)−EpODE
12(13)−EpOME
12,13−DiHOME
13−HDHA
13−HODE
13(14)−EpDPE
14−HDHA
14(15)−EpEDE
14(15)−EpETE
14(15)−EpETrE
14,15−DiHETrE
15−HETrE
15−oxo−ETE
15,16−DiHODE
16(17)−EpDPE
18−HETE
19(20)−EpDPE
19,20−DiHDPE
20−HDHA
5−HEPE
5−HETE
5−HETrE
5,6−DiHETrE
7,8−DiHDPE
8−HEPE
8−HETE
8(9)−EpETrE
8,9−DiHETrE
9−HEPE
9−HETE
9−HODE
9−HOTrE
9(10)−Ep−stearic acid
9(10)−EpOME
9,10−DiHOME
9,10,13−TriHOME
Lab1
Lab2
Lab3
Lab5
MEDM
Concentration (nM)
lab
Lab1
Lab2
Lab3
Lab5
MEDM

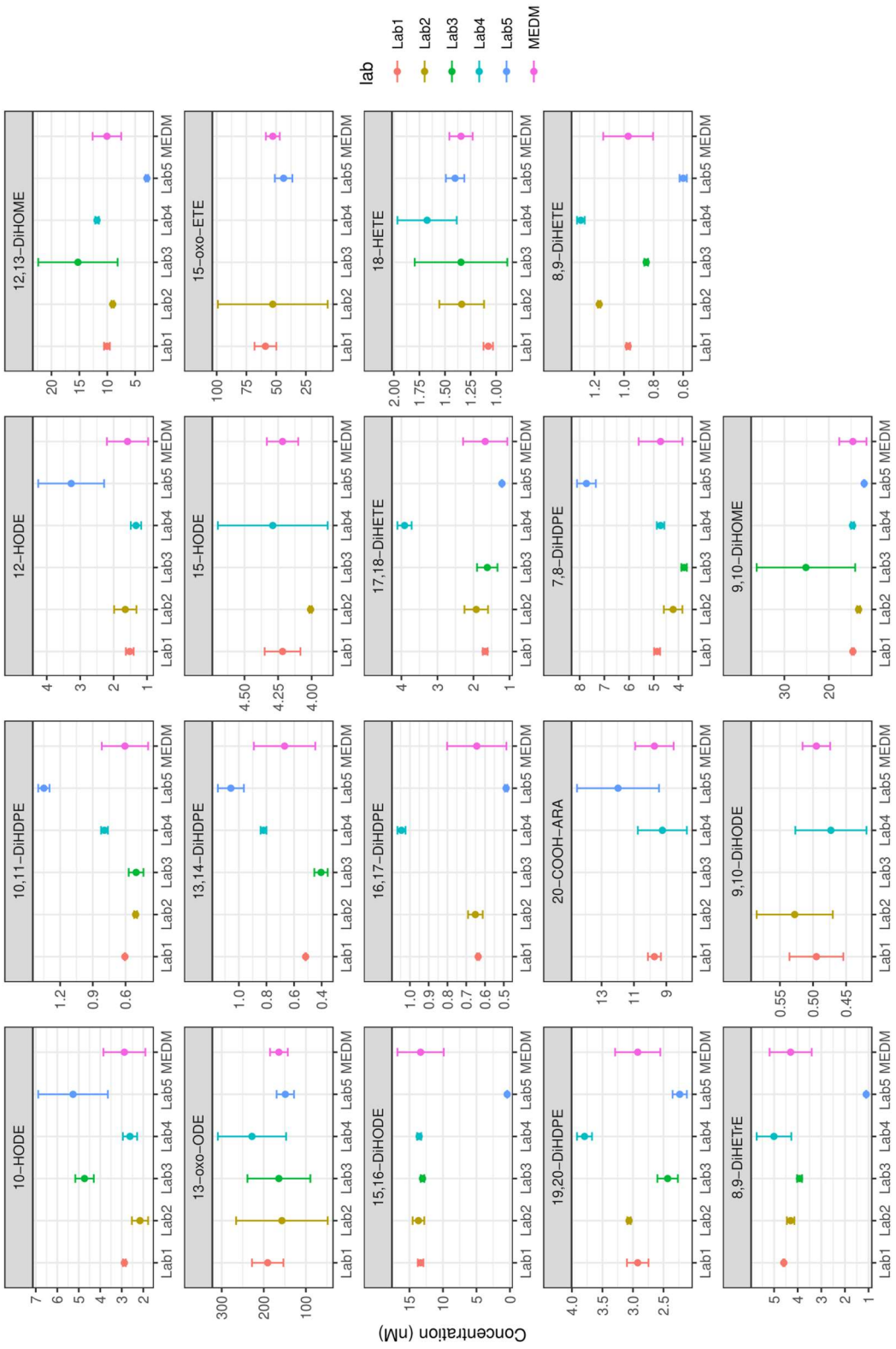


Fig. 8.18: MEDM location plots for QC plasma 2. Plots were created for oxylipins quantified in QC plasma 2 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 19 oxylipins with acceptable consensus value (COD < 40%).

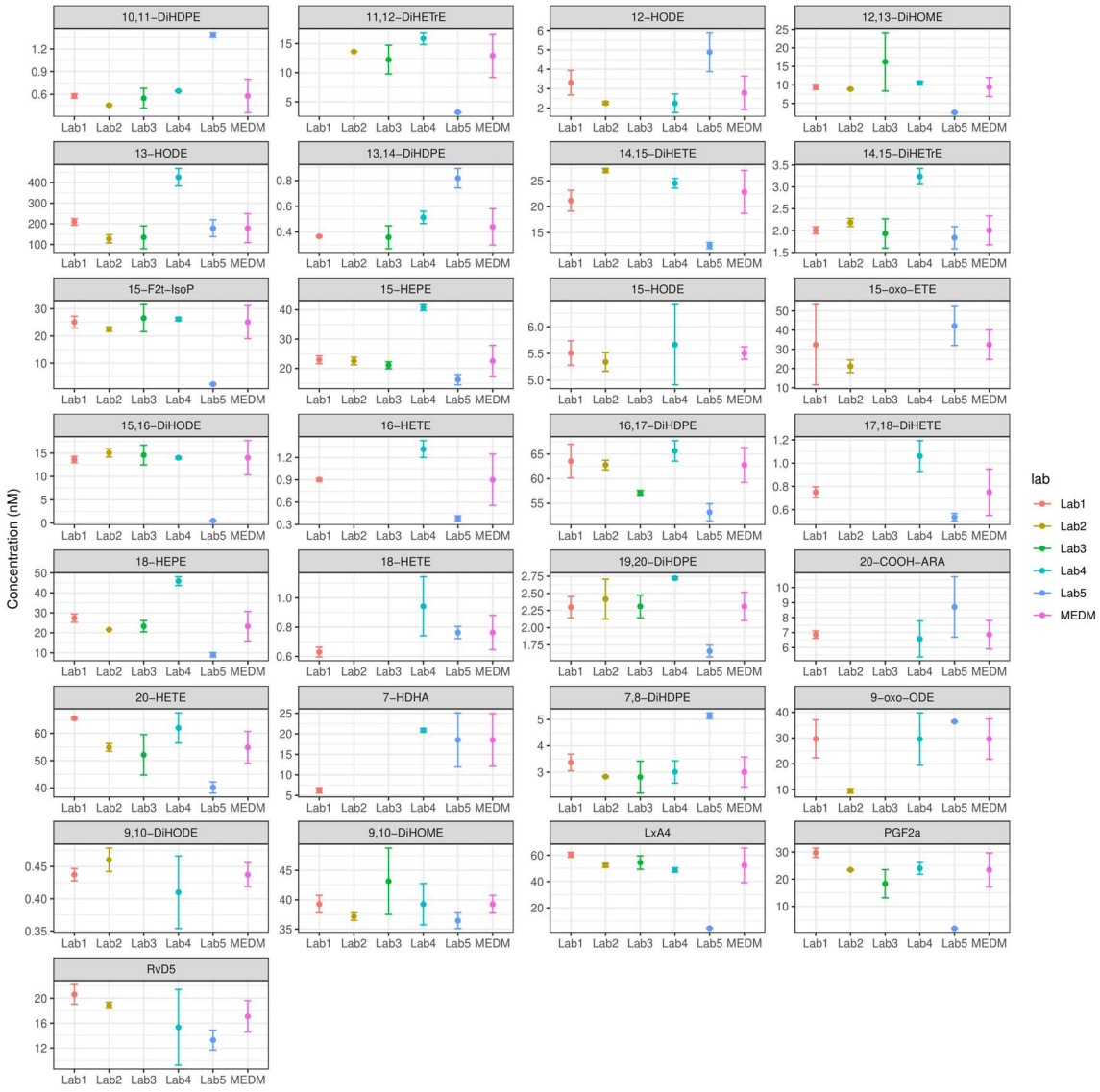


Fig. 8.19: MEDM location plots for QC plasma 3. Plots were created for oxylipins quantified in QC plasma 3 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 29 oxylipins with acceptable consensus value (COD < 40%).

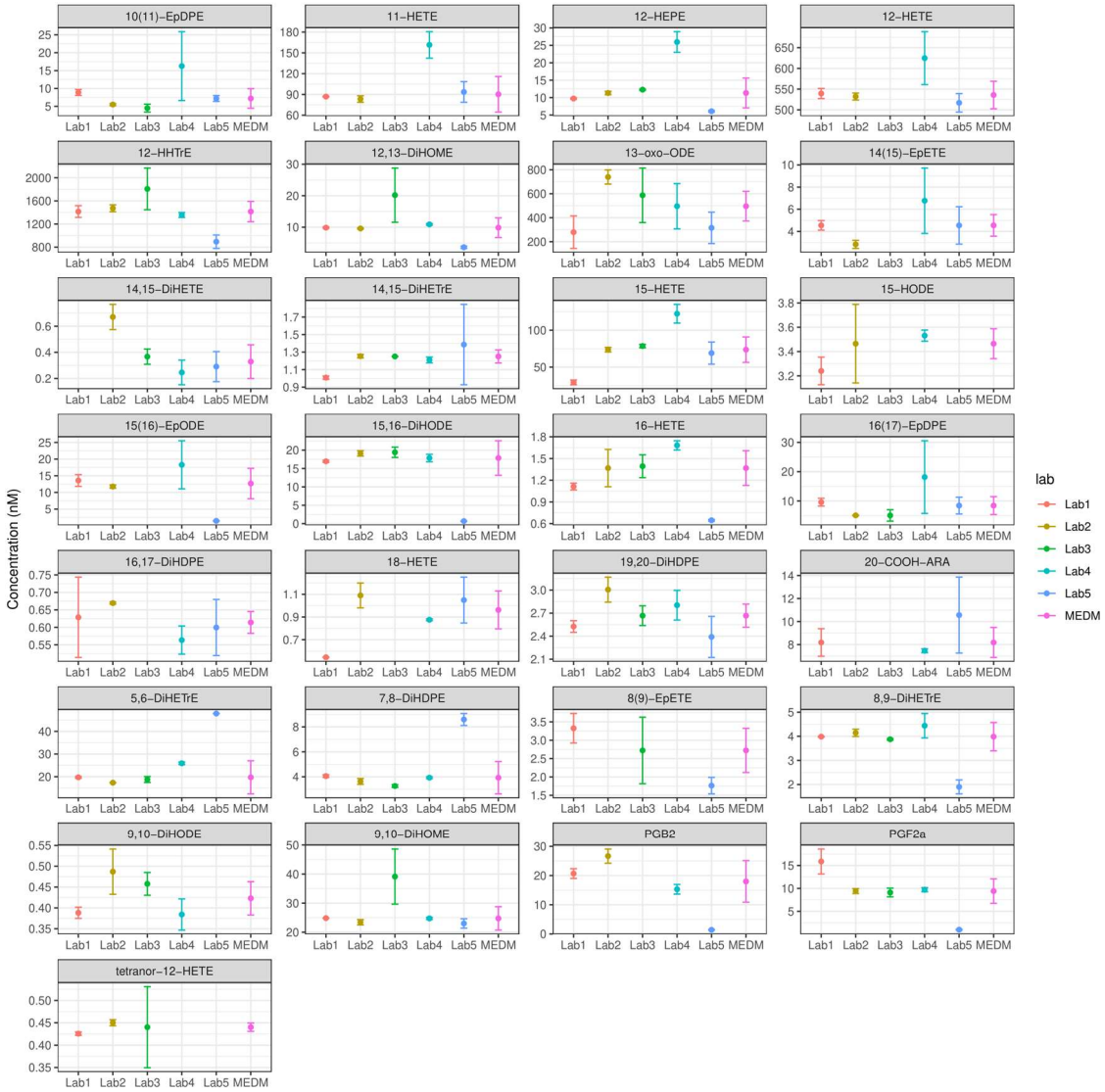


Fig. 8.20: MEDM location plots for QC plasma 4. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 29 oxylipins with acceptable consensus value (COD < 40%).

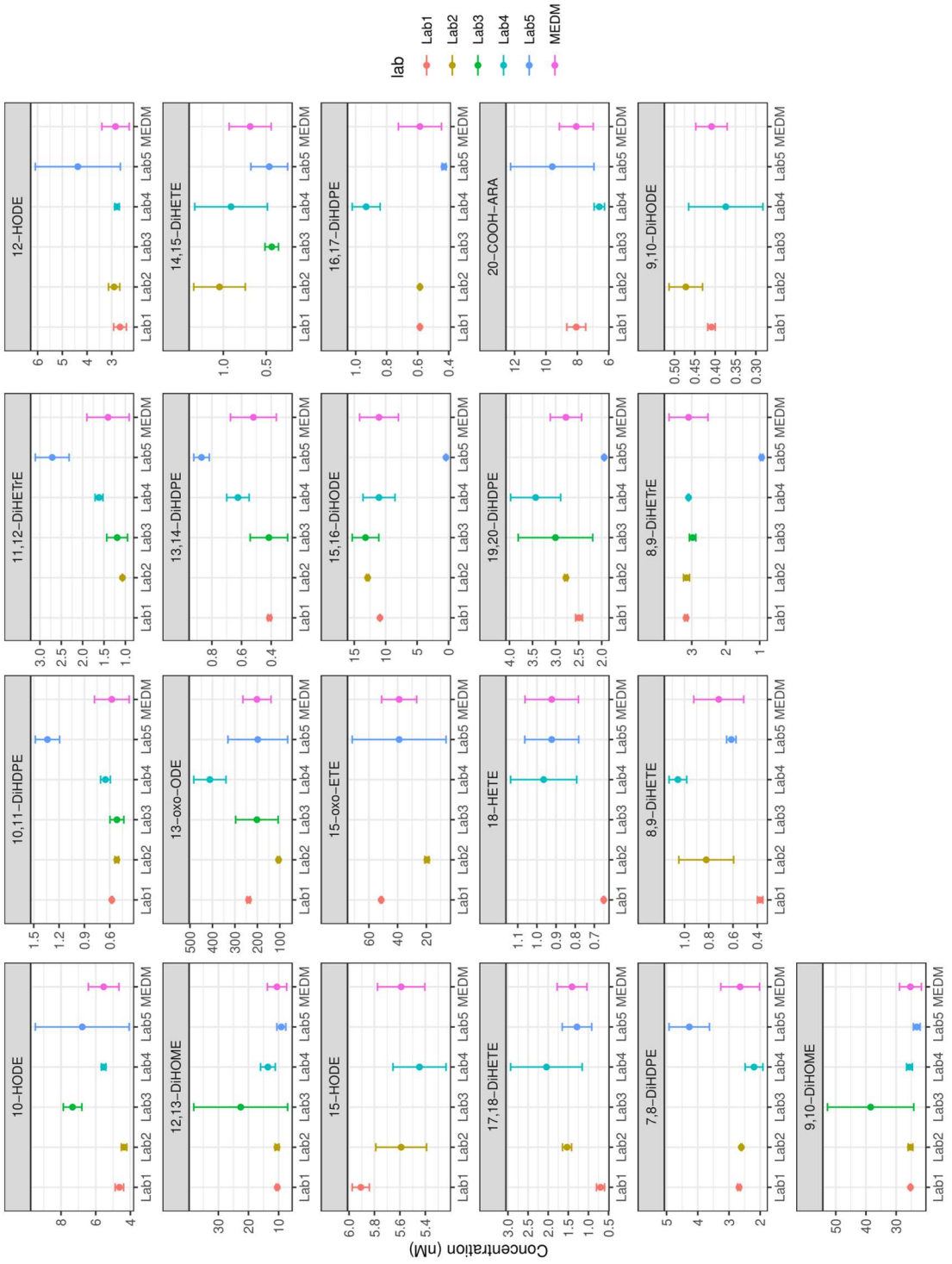


Fig. 8.21: MEDM location plots for QC plasma 5. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 21 oxylipins with acceptable consensus value (COD < 40%).

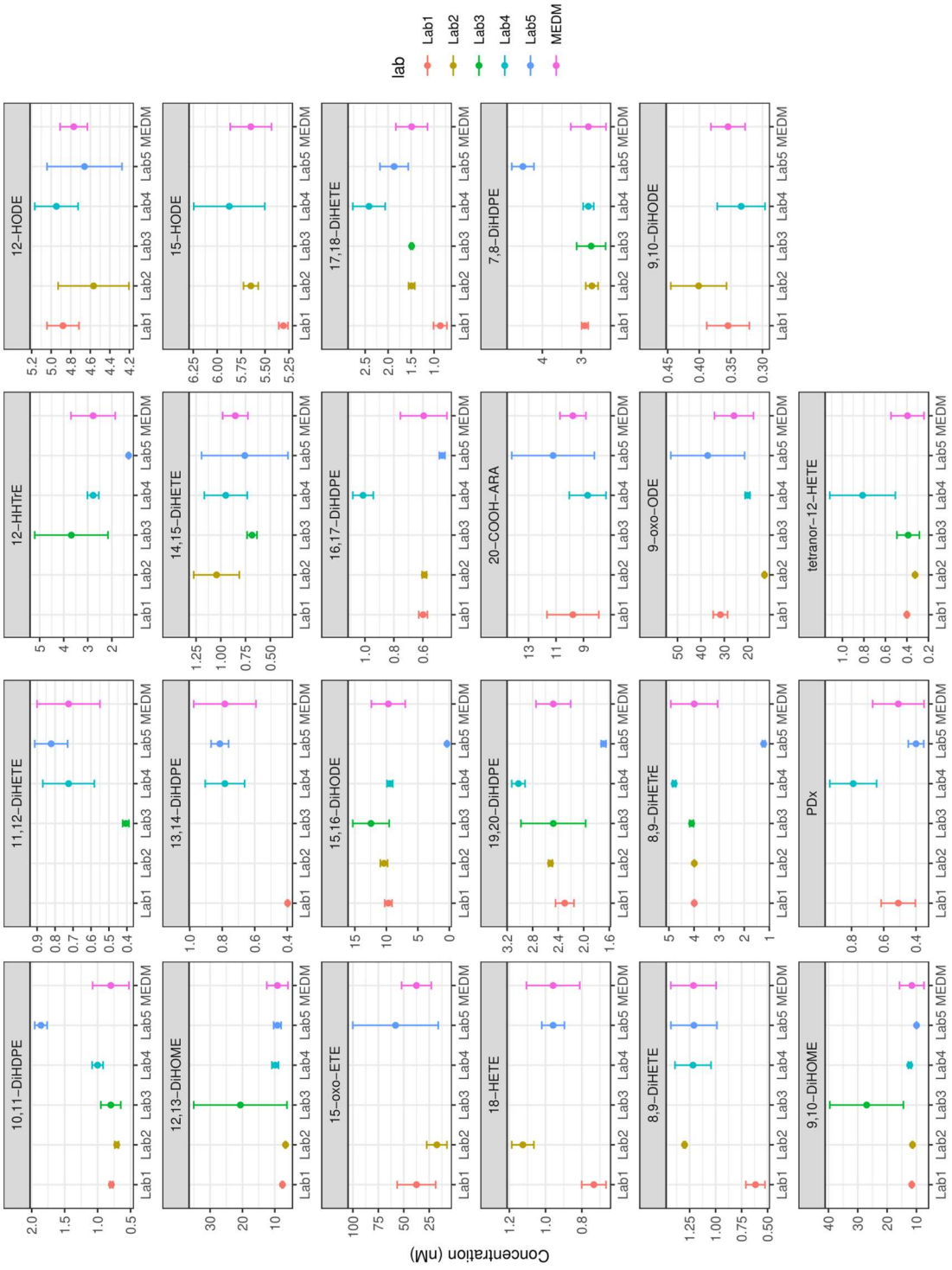


Fig. 8.22: MEDM location plots for QC plasma 6. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 23 oxylipins with acceptable consensus value (COD < 40%).

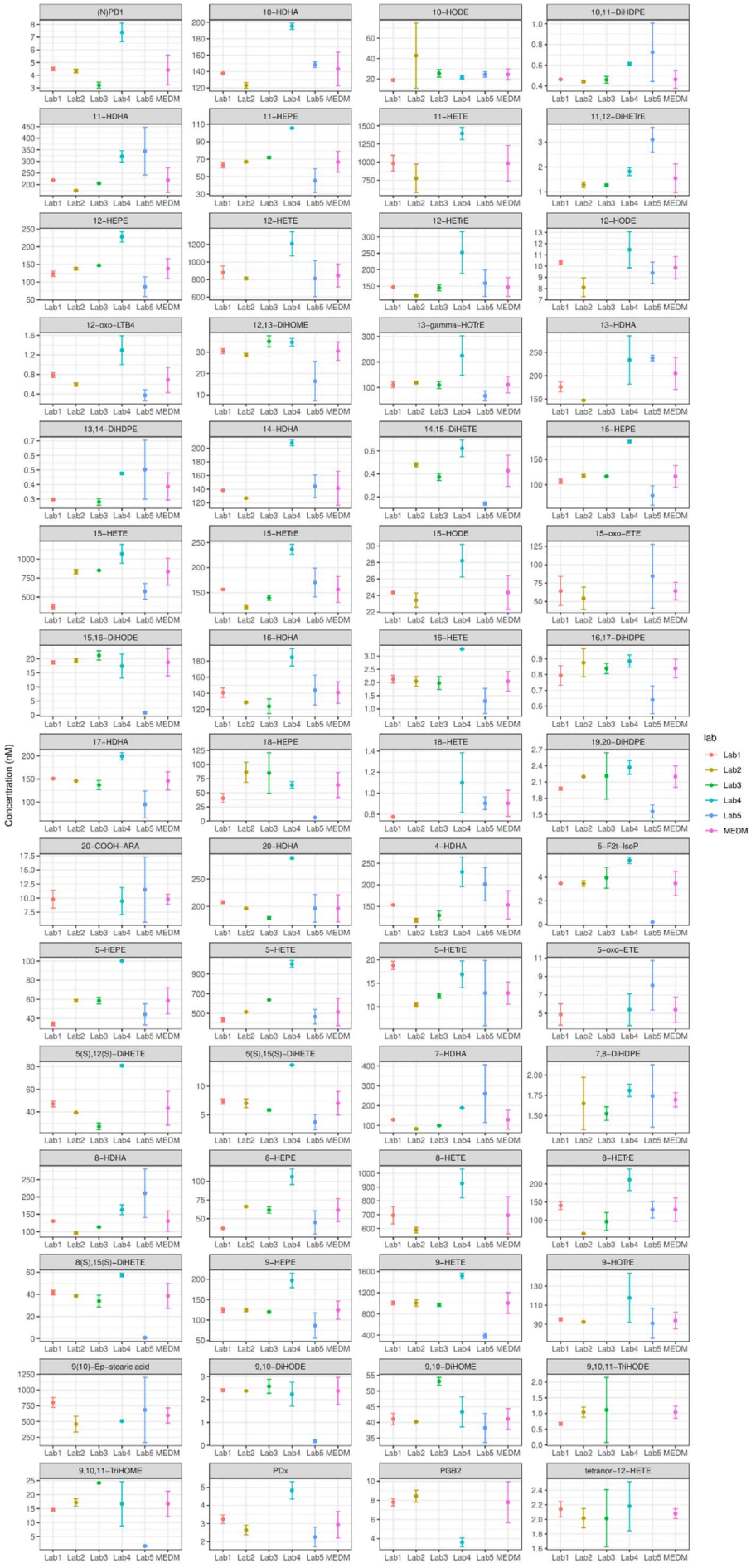


Fig. 8.23: MEDM location plots for QC plasma 7. Plots were created for oxylipins quantified in QC plasma 4 by all laboratories. Shown are the mean concentrations ± SD determined in each laboratory and MEDM ± *u* for 60 oxylipins with acceptable consensus value (COD < 40%).

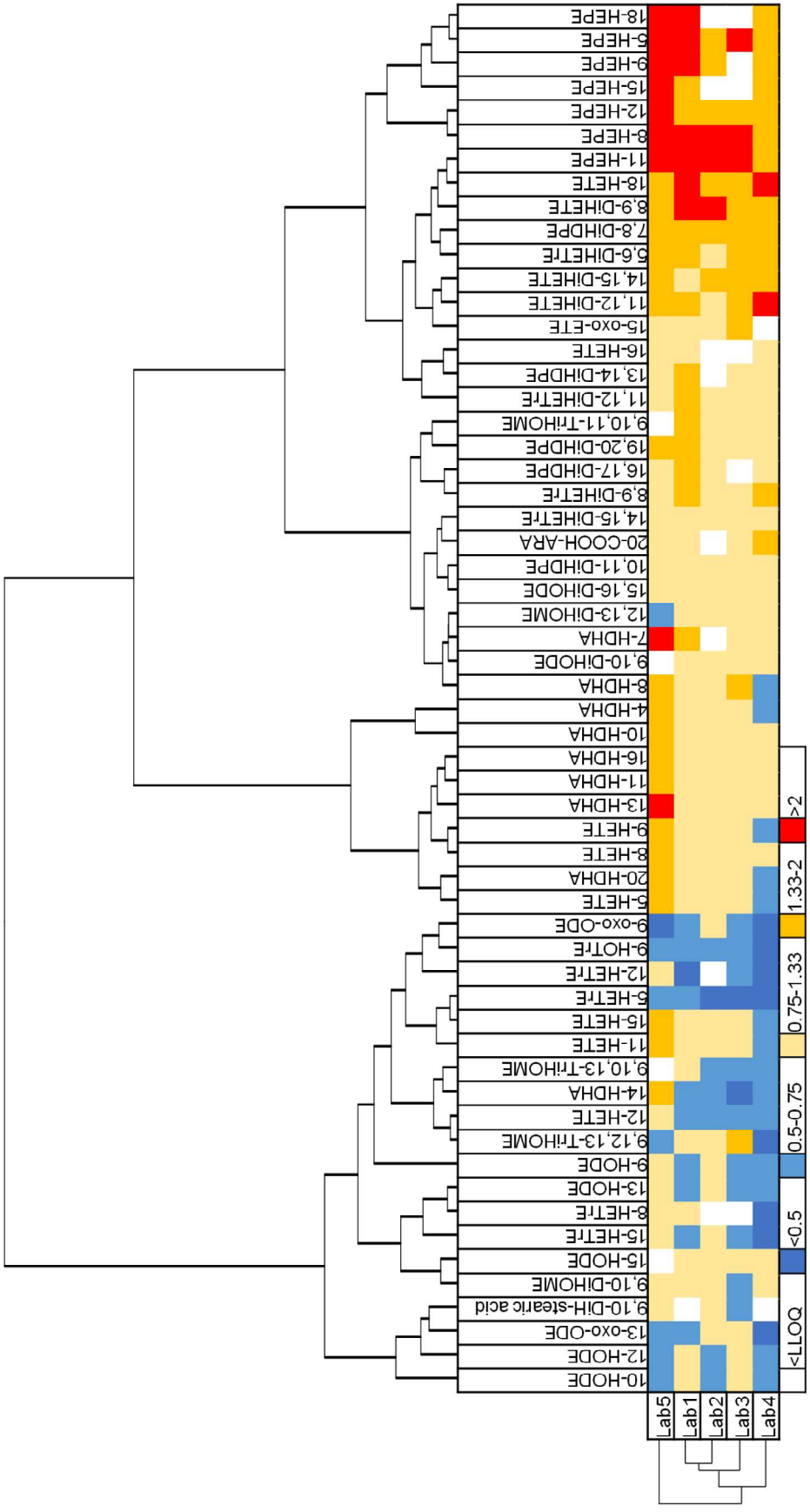


Fig. 8.24: Heatmap of ratios between the QC plasma 2 – n3 against QC plasma 1 - B. The heatmap is displayed in six different colors according to the range of ratio. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.

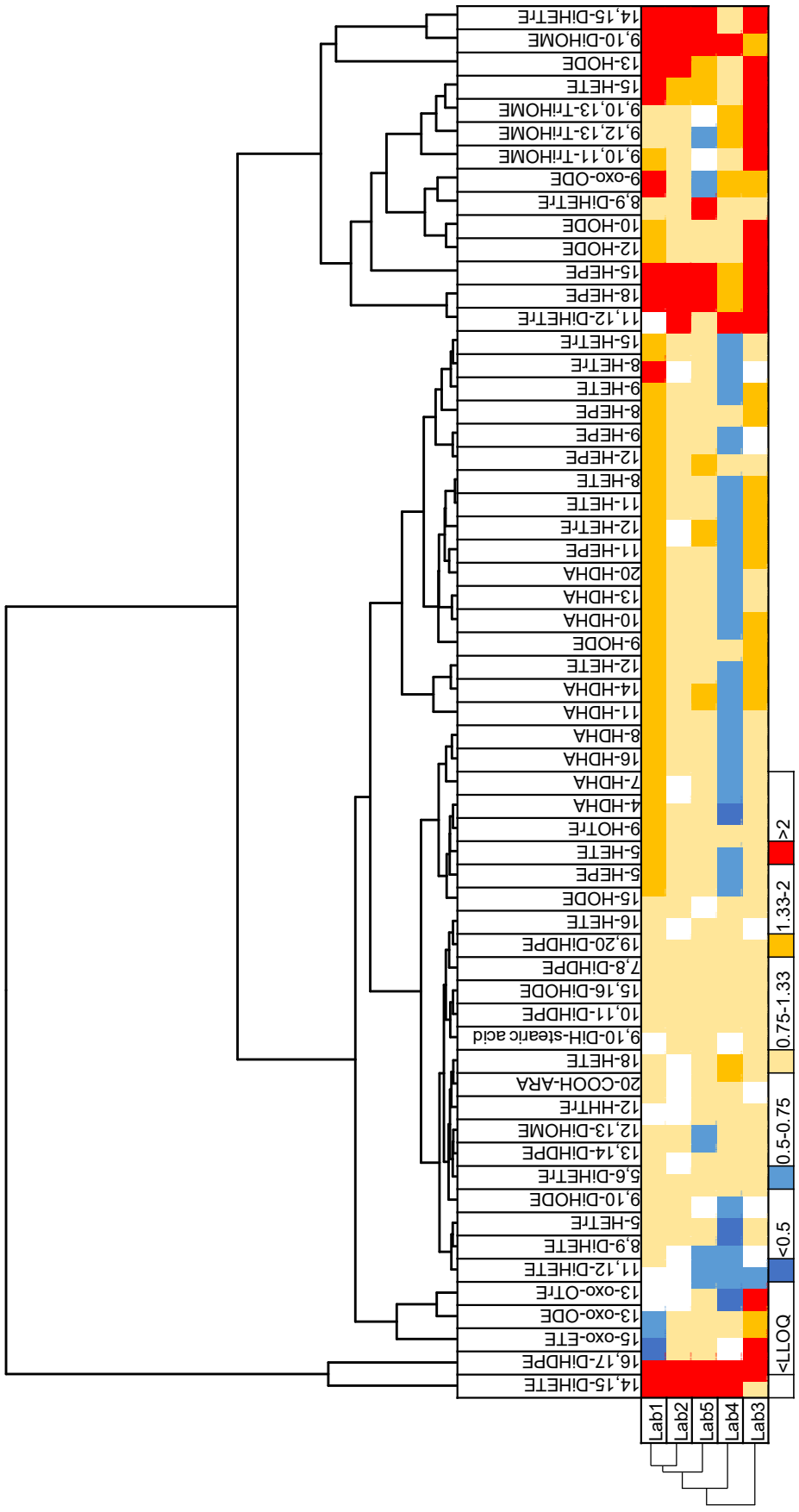


Fig. 8.25: Heatmap of ratios between the QC plasma 3 – S against QC plasma 1 - B. The heatmap is displayed in six different colors according to the range of ratios. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.

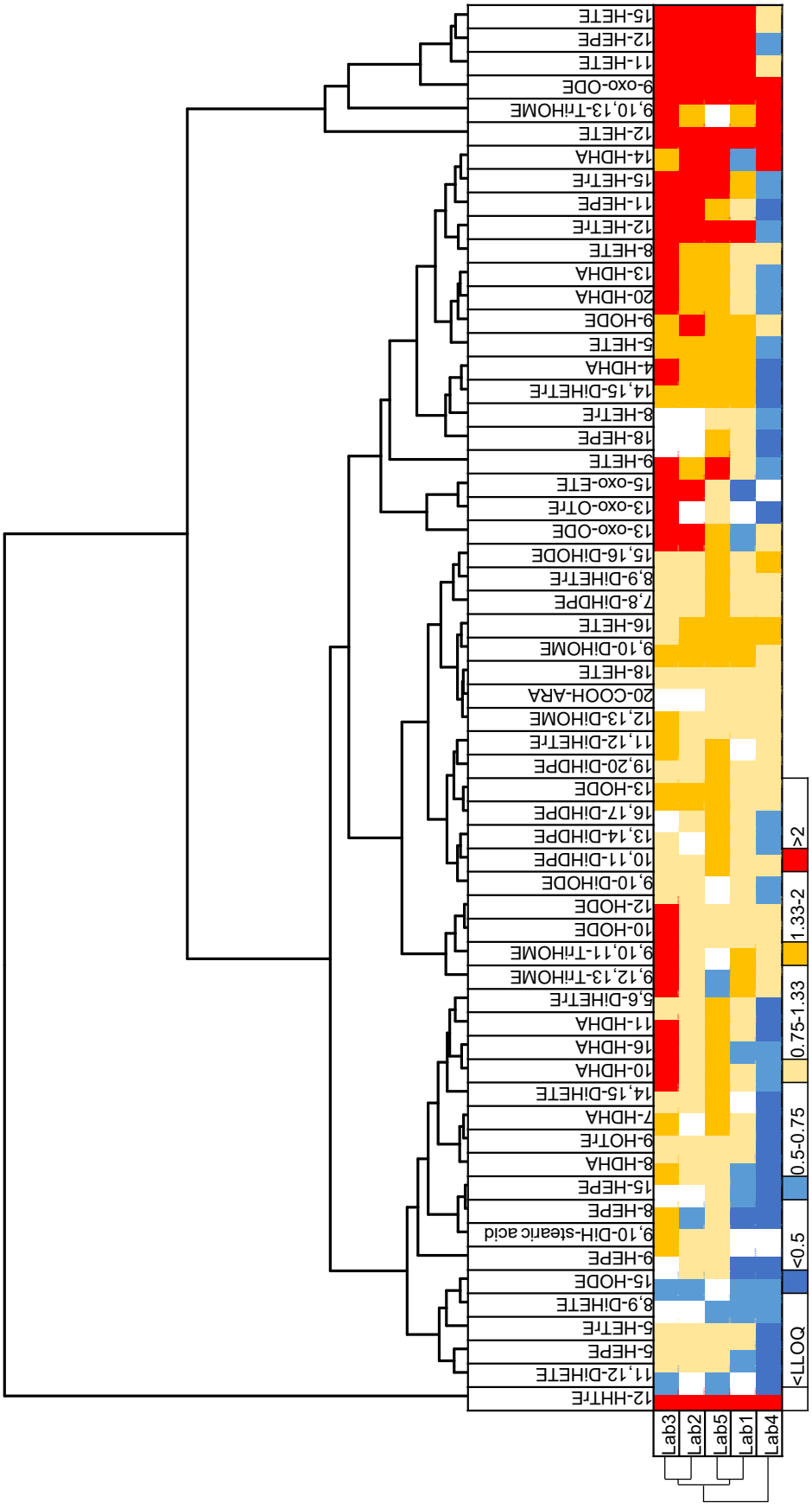


Fig. 8.26: Heatmap of ratios between the QC plasma 4 – Ca against QC plasma 1 - B. The heatmap is displayed in six different colors according to the range of ratio. Hierarchical clustering is based on the similarities between laboratories and between oxylipins.

A)

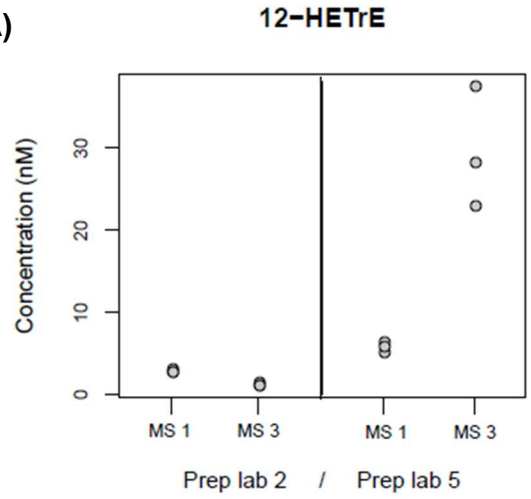
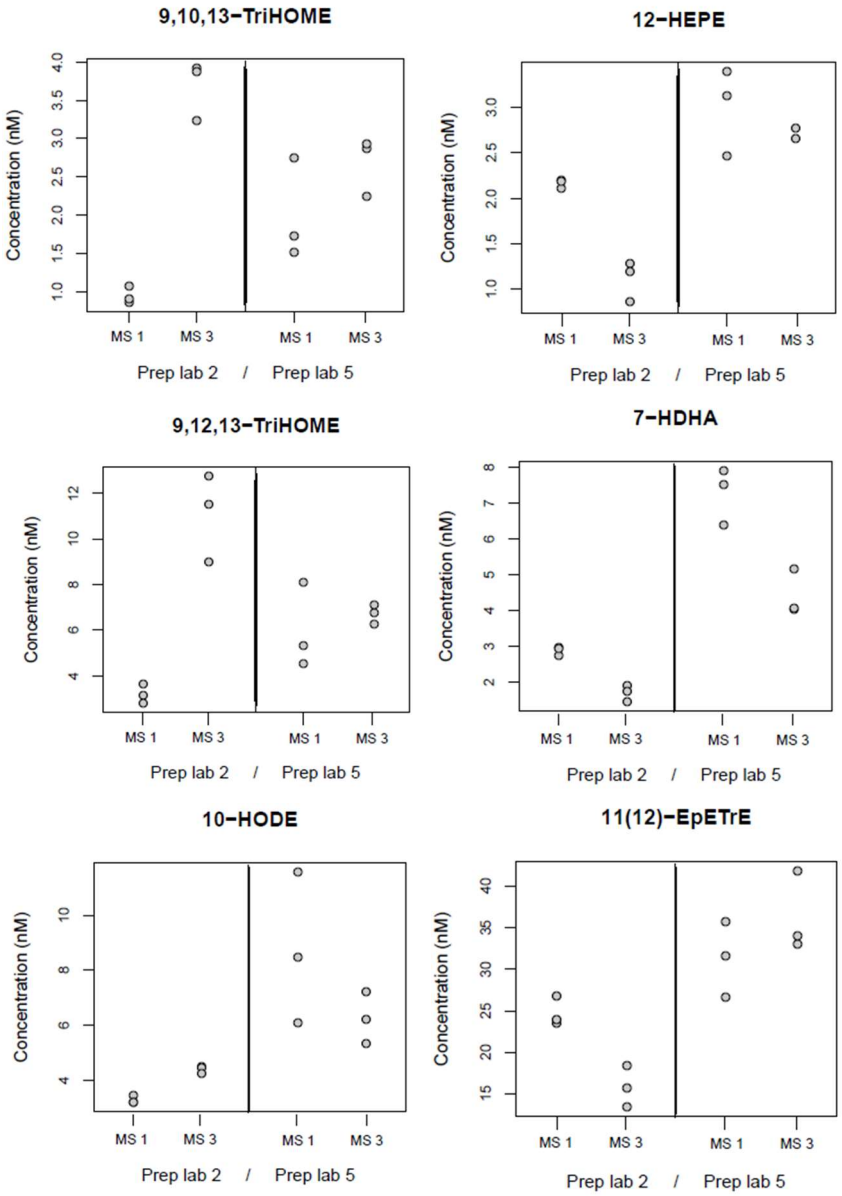


Fig. 8.27: Variability of the LC-MS/MS platform. QC plasma 1 – B extracts were prepared in Lab 2 and 5 (Prep lab 2 and Prep lab 5) and analyzed by means of LC-MS in Lab 1 and 3 (MS1 and MS3). 42 oxylipins above LLOQ were investigated and for most of these oxylipins no significant effects were observable. Shown are selected oxylipins with a significant MS-platform effect (A) and with MS-platform effect only for samples prepared in laboratory 2 (B).

B)



Chapter 5

Selection of peptides

For analysis of proteins using a quantitative targeted proteomics method, unique proteotypic peptides (PTPs) [1] are selected to ensure that the target protein is unambiguously identified and can be detected.

The following human proteins were selected to characterize macrophage differentiation and to quantify LXR:

- Oxysterols receptor LXR-alpha (LXRα; Uniprot accession no.: Q13133)
- Oxysterols receptor LXR-beta (LXRβ; Uniprot accession no.: P55055)
- Peroxisome proliferator-activated receptor gamma (PPARγ; Uniprot accession no.: P37231)
- Interleukin-1 receptor type 2 (IL-1RII; Uniprot accession no.: P27930)
- Toll-like receptor 2 (TLR2; Uniprot accession no.: O60603)
- Toll-like receptor 4 (TLR4; Uniprot accession no.: O00206)
- Inducible Nitric oxide synthase (iNOS; Uniprot accession no.: P35228)

The detailed procedure is described here exemplarily for iNOS (gene: NOS2). The peptides for the other proteins were selected accordingly.

An in silico tryptic digestion using ExPASy peptide mass and peptide cutter [2] was carried out, whereby 128 peptides with lengths between 2 and 43 amino acids (aa) were initially obtained. These peptides were analyzed for their suitability as PTPs based on certain criteria. Peptides shorter than 7 (low probability of unique sequence) and longer than 22 aa (poor MS ionization or out of measuring range) were discarded [3] and 58 peptides remained (Tab. 8.13).

These peptides were checked for uniqueness in the human proteome using the NextProt peptide uniqueness checker [4] and NCBI Blast [5] databases. Two of the checked peptides were not unique, as they are also found in the sequence of other proteins. The theoretical cleavage probability for the C-terminal cleavage

site of the peptides was calculated using ExPASy peptide cutter [2] and additionally the overall cleavage probability was predicted using the decision tree CP-DT [6]. Peptides with a cleavage probability <90% or a total cleavage probability <70% were excluded, leaving 48 peptides.

The remaining peptides were screened for posttranslational modifications (PTMs) (Uniprot [7], Phosphosite Plus [8]), such as phosphorylation or glycosylation, or naturally occurring variants resulting from single nucleotide polymorphisms (SNPs) [7]. Alternative splice variants of the corresponding protein were also considered [7] and 33 peptides remained. Furthermore, the presence of certain aa (cysteine (C), asparagine (N) and glutamine (Q, especially N-terminal), methionine (M), tryptophan (W)) may be probe to chemical modifications [9-11], such that a maximum of two of these aa are tolerated in a peptide. Finally, 18 peptides were left.

In order to also ensure that the selected peptides elute within an optimal detection window of the chromatographic method, an estimated retention time was calculated using Sequence Specific Retention Calculator (SSRCalc [12]; gradient delay time: 1.5 min, acetonitrile gradient: 1.05 %B/min, "100 Å C18 column, 0.1% Formic Acid 2015"). After these calculations, 4 peptides remained (Tab. 8.14) and they were ordered as standards as well as corresponding heavy labeled peptides were obtained from JPT Peptides (Berlin, Germany).

For the selected peptides, product ion spectra were recorded and 3-5 of the most intensive fragments per peptide were selected to achieve the highest possible sensitivity. The SRMAtlas database [13] was used to assist the characterization of the fragments. The collision energy was optimized for all transitions. Constant values were used for all other parameters, e.g. 40 V for the declustering potential, which were optimized for the detection of peptides [3]. The same fragments and potentials were selected for both unlabeled and heavy labeled peptides with the terminal arginine and lysine being uniformly labeled by 13C and 15N isotopes.

After optimization of the transitions, the peptides were spiked into primary M0 macrophage lysates at a concentration of 50 nM. Two of the selected peptides showed poor sensitivity and matrix interference. Finally, for the two remaining peptides, two transitions (one quantifier and one qualifier) each, which are particularly sensitive and had only few matrix interferences, were selected (Tab. 8.15). In addition, the ratio between the two transitions is monitored to ensure peak identity and thus reliable quantification of the peptides in the samples.

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Tab. 8.13: Selection of iNOS peptides from in silico tryptic digest. Selection of peptides was based on peptide length (7-22 aa), uniqueness, cleavage probability and prediction, known posttranslational modifications (PTMs), single nucleotide polymorphism (SNPs), number of unfavored aa and predicted retention time (tR). Shown are only peptides with lengths between 7-22 aa. Peptides selected for the method are shown in bold.

peptide sequence	position	$[M+H]^+$	peptide length [aa]	uniqueness [1]	C-terminal cleavage probability [%] [2]	overall cleavage probability [%] [3]	SNPs [4]	PTM [5]	unfavoured aa [6]	predicted tR [min] [7]
DPELFEIPPDLVLEVAMEHPK	315-335	2418.2	21	unique	100	84			1 x M	-
APCATSSPVTQDDLQYHNLS	31-51	2275.1	21	unique	100	88		S37: p; S50: p	1 x N; 2 x Q; 1 x C	-
EIETTGTYQLTGDELIFATK	172-191	2230.1	20	unique	100	86	177:G → V		1 x Q	-
LLLVVTSTFGNGDCPGNGE	585-604	2021.0	20	unique	95	89			2 x N; 1 x C	-
SEALAWDLGALFSCAFNPK	550-568	2040.0	19	unique	100	87	552:A → G		1 x N; 1 x W; 1 x C	-
DHTPTEIHLTVAVVTYHTR	915-933	2190.1	19	unique	100	94	933:R → G			-
GDPANVEFTQLCIDLGWKPK	279-298	2231.1	18	unique	100	12			1 x N; 1 x Q; 1 x W; 1 x	-
FTNSPTFLEVLEEFPSLR	873-890	2126.1	18	unique	100	92			1 x N	30.0
QNVTIMDHHSAAESFMK	429-445	1945.9	17	unique	100	93	439:A → T		2 x M; 1 x N; 1 x Q	-
DQAVVEINIAVLHSFQK	412-428	1911.0	17	unique	100	90	423:L → I		1 x N; 2 x Q	-
YHEDIFGAVFPYEAK	1123-1137	1785.8	15	unique	91	81				18.8
NWGSGMTFQDTLHHK	89-103	1758.8	15	unique	100	87			1 x M; 1 x N; 1 x Q; 1 x	-
LEALDESGSYWVSDK	803-817	1698.8	15	unique	74	77	805:A → D		1 x W	-
LNEEQVEDYFFQLK	1105-1118	1801.9	14	unique	100	79			1 x N; 2 x Q	11.1
LYTSNVTWDPHHYR	697-710	1788.8	14	unique	100	90			1 x N; 1 x W	-
VSAGFLLSQLPILKPR	891-906	1739.1	14	unique	50	17		S892: p	1 x Q	-
QQNESPQPLVETGK	52-65	1554.8	14	unique	93	84			1 x N; 3 x Q	-
YAVFGLGSSMYPR	621-633	1447.7	13	unique	100	94			1 x M	17.6
FDVVPLVLQANGR	302-314	1427.8	13	unique	100	86			1 x N; 1 x Q	19.5
VAVQPSSLEMSAL	1141-1153	1331.7	13	unique	-	-			1 x M; 1 x Q	19.8
IQWSNLQVFDAR	204-215	1476.8	12	unique	100	94			1 x N; 2 x Q; 1 x W	-
LEALCQPSEYSK	859-870	1367.7	12	unique	100	77			1 x Q; 1 x C	-
YAGYQMPDGSIR	267-278	1357.6	12	unique	100	93			1 x M; 1 x Q	10.9
EPGHLYVCGDVR	1075-1086	1344.6	12	unique	100	75			1 x C	-
LVQDSQPLDLSK	711-722	1342.7	12	unique	100	90			2 x Q	12.3
GVLHAVHTAYSR	1036-1047	1310.7	12	unique	100	87				7.7
VVDGPTPHQTVR	791-802	1305.7	12	unique	100	93	800:T → A		1 x Q	5.2
FCAFAHDIDQK	634-644	1294.6	11	unique	100	91			1 x Q; 1 x C	-
LAQVATEEPER	846-856	1242.6	11	unique	100	92			1 x Q	7.5
VTILFATETGK	**539-549**	**1179.7**	**11**	**unique**	**91**	**87**				**15.3**
FHQYAMNGEK	13-22	1224.5	10	unique	83	78			1 x M; 1 x N; 1 x Q	-
AVLFACMLMR	521-530	1154.6	10	unique	100	84			2 x M; 1 x C	-
QNLQSPTSSR	741-750	1117.6	10	unique	92	86	747:T → A	S745: p	1 x N; 2 x Q	-
LDATPLSSPR	74-83	1056.6	10	unique	100	85		T77: p; S81: p		-
SCLGSIMTPK	114-123	1036.5	10	unique	100	76			1 x M; 1 x C	-
QQLASEVLR	1062-1070	1043.6	9	unique	100	95			2 x Q	-
YNILEEVGR	**389-397**	**1092.6**	**9**	**unique**	**96**	**93**			**1 x N**	**15.1**
YSTNNGNIR	233-241	1038.5	9	unique	100	94		S234: p	3 x N	-
SAITVFPQR	242-250	1018.6	9	P29474; P35228	100	88			1 x Q	-
AACETFDVR	679-687	1011.5	9	unique	100	94	679:A → S		1 x C	-
THVWQDEK	498-505	1042.5	8	unique	92	80			1 x Q; 1 x W	5.5
EMFEHICR	222-229	1064.5	8	unique	100	87			1 x M; 1 x C	-
ALSSMHAK	723-730	844.4	8	unique	88	89			1 x M	3.3
VYVQDILR	1054-1061	1005.6	8	unique	100	85		Y1055: p	1 x Q	-
VWNAQLIR	259-266	999.6	8	unique	100	94	266:R → H		1 x N; 1 x Q; 1 x W	-
LSCLEEER	577-584	978.5	8	unique	100	87		S578: p	1 x C	-
SWAVQTFK	671-678	966.5	8	unique	86	97	676:T → I		1 x Q; 1 x W	-
FYSISSSR	907-914	946.5	8	unique	84	90	913:S → P	Y1055: p	x C	-
DINNNVEK	23-30	945.5	8	unique	94	81	23:D → G		3 x N	-
MTLVFGCR	1010-1017	926.5	8	P29474; P35228	91	87			1 x M; 1 x C	-
DFCDVQR	382-388	882.4	7	unique	100	95			1 x Q; 1 x C	-
IEEHLAR	159-165	867.5	7	unique	100	89				4.3
LHDSQHK	997-1003	864.4	7	unique	100	85			1 x Q	3.4
QHIQIPK	690-696	863.5	7	unique	100	86			2 x Q	-
YMQNEYR	446-452	1003.4	7	unique	100	92			1 x M; 1 x N; 1 x Q	-
MGLETHK	399-405	815.4	7	unique	100	86			1 x M	3.3
DVAHTLK	1090-1096	783.4	7	unique	100	86				3.9
SPESLVK	67-73	759.4	7	unique	100	73				6.4

[1])from BLAST [5] and NeXtprot [4]; [2] calculated from peptide cutter [2] (≥ 90%); [3] calculated from CP-DT [6] (≥ 70%); [4] SNPs from Uniprot [7]; [5] PTMs from Uniprot [7] and Phosphosite Plus [8] (p – phosphorylation); [6] unfavored aa (C, M, N, Q, W; max. 2); [7] predicted retention time (tR) from SSRCalc [12] (3 – 30 min); -: not evaluated

Tab. 8.14: Selected proteotypic peptides (PTPs) for LXRα, LXRβ, PPARγ, IL-1RII, TLR2, TLR4, iNOS. The selection is based on peptide length (7-22 aa), uniqueness, cleavage probability or prediction, occurrence of single nucleotide polymorphisms (SNPs), variation in splice variants or posttranslational modifications (PTMs), unfavored amino acids and predicted retention time (Tab. 8.13)

peptides	position	$[M+H]^+$	length [aa]	uniqueness [1]	C-terminal cleavage probability [2] [%]	overall cleavage probability [3] [%]	SNPs [4]	variation in splice variants [5]	PTMs [6]	unfavored aa [7]	pred. tR [8] [min]
Oxysterols receptor LXR-alpha (LXRα; Q13133; gene: NR1H3)											
EECVLSEEQIR *	165-175	1334.6	11	unique	100	86	-	-	-	1 x Q	12.0
MLGNELCSVCGDK *	92-104	1368.6	13	unique	93	83	-	-	-	1 x M; 1 x N	12.6
TSAIEVMLLETSR	292-304	1449.8	13	unique	86	95	-	-	-	1 x M	20.1
Oxysterols receptor LXR-alpha (LXRα; Q13133; gene: NR1H3) / Oxysterols receptor LXR-beta (LXRβ; P55055; gene: NR1H2)											
TLSSVHSEQVFALR	416-429	1573.8	14	no	100	88	-	-	-	1 x Q	13.8
Oxysterols receptor LXR-beta (LXRβ; P55055; gene: NR1H2)											
VEALQQPYVEALLSYTR	391-407	1980.0	17	unique	100	77	-	-	-	2 x Q	21.6
VTPWPLGADPQSR	248-260	1423.7	13	unique	84	87	-	-	-	1 x W; 1 x Q	13.7
YNHETECITFLK	319-330	1497.7	12	unique	86	92	-	-	-	1 x N; 1 x H	10.4
Peroxisome proliferator-activated receptor gamma (PPARγ; P37231, gene: PPARG)											
LNHPESSQLFAK	451-462	1370.7	12	unique	95	93	-	missing in isoform 3	-	1 x N; 1 x Q; 1 x H	9.4
HLYDSYIK	245-252	1038.5	8	unique	100	92	-	missing in isoform 3	-	1 x H	9.1
TDPVVADYK	86-94	1007.5	9	unique	100	90	-	-	-	-	9.2
Interleukin-1 receptor type 2 (IL-1RII; P27930; gene: IL1R2)											
EDLHMDFK	318-325	1034.5	8	unique	100	90	-	missing in isoform 2	-	1 x M; 1 x H	9.8
TVPGEEETR	77-85	1017.5	9	unique	100	91	-	-	-	-	4.2
TISASLGSR	245-253	891.5	9	unique	100	86	-	-	-	-	7.9
Toll-like receptor 2 (TLR2; O60603; gene: TLR2)											
TVFVLSENFVK	699-709	1282.7	11	unique	100	87	-	-	-	1 x N	19.3
EQLDSFHTLK	517-526	1217.6	10	unique	100	92	-	-	-	1 x Q; 1 x H	11.4
VGNMDTFTK	156-164	1012.5	9	unique	100	90	-	-	-	1 x N; 1 x M	9.1
Toll-like receptor 4 (TLR4; O00206; gene: TLR4)											
DFIPGVAIAANIIHEGFHK	711-729	2049.1	19	unique	100	77	-	-	-	1 x N; 2 x H	21.6
NLDLSFNPLR	58-67	1188.6	10	unique	100	92	-	-	-	2 x N	17.4
LVLGEFR	258-264	833.5	7	unique	100	90	-	-	-	-	13.4
Nitric oxide synthase, inducible (iNOS; P35228; gene: NOS2)											
VTILFATETGK	539-549	1179.7	11	unique	91	87	-	-	-		15.3
YNILEEVGR	389-397	1092.6	9	unique	86	92	-	-	-	1 x N	15.1

Tab. 8.15: Selected transitions for **(A)** unlabeled and **(B)** heavy labeled (lys: U-$^{13}C_6$; U-$^{15}N_2$; arg: U-$^{13}C_6$; U-$^{15}N_4$) peptide data for LXRα, LXRβ, PPARγ, IL-1RII, TLR2, TLR4, iNOS. Shown are Q1 and Q3 *m/z*, optimized collision energies (CE), area ratios of qualifier to quantifier transitions and retention time (tR, mean ± range, within one analytical batch, n = 20)). (A) Linear calibration range, limit of detection (LOD) and limit of quantification (LLOQ), as well as the transitions of the corresponding internal standards (IS) are shown for quantifier transitions (bold). Accuracy of calibrators was within a range of ± 15% (20% for LLOQ). The heavy labeled peptides are spiked at a concentration of 25 nM for all peptides (concentration in vial).

(A) unlabeled peptides

gene / protein	peptide	transitions	Q1 *m/z*	Q3 *m/z*	tR [min]	rel. ratio to quantifier [%]	CE (V)	IS Transitions	calibration range [nM]	LOD [pM]	LLOQ [pM]
NR1H3 / Oxysterols receptor LXR-alpha											
	EECVLSEEQIR	**$M^{2+} \rightarrow y_7^+$**	696.3	874.5	12.04 ± 0.08		35	$M^{2+} \rightarrow y_7^+$	0.08 - 1060	50	80
		$M^{2+} \rightarrow y_6^+$	696.3	761.4		82	35				
	MLGNELCSVCGDK	**$M^{2+} \rightarrow y_6^+$**	741.8	665.3	13.38 ± 0.12		40	$M^{2+} \rightarrow y_6^+$	0.45 - 897	230	450
		$M^{2+} \rightarrow y_7^+$	741.8	825.3		47	40				
	TSAIEVMLLETSR	**$M^{2+} \rightarrow y_7^+$**	725.4	849.5	21.41 ± 0.15		35	$M^{2+} \rightarrow y_7^+$	0.87 - 1167	530	870
		$M^{2+} \rightarrow y_5^+$	725.4	605.2		47	30				
NR1H3 / Oxysterols receptor LXR-alpha ; NR1H2 / Oxysterols receptor LXR-beta											
	TLSSVHSEQVFALR	**$M^{3+} \rightarrow b_2^+$**	525.3	215.1	14.15 ± 0.07		20	$M^{3+} \rightarrow b_2^+$	0.45 - 1270	300	450
		$M^{3+} \rightarrow y_{12}^+$	525.3	680.4		20	25				
NR1H2 / Oxysterols receptor LXR-beta											
	VEALQQPYVEALLSYTI	**$M^{3+} \rightarrow y_5^+$**	660.7	639.4	23.63 ± 0.08		30	$M^{3+} \rightarrow y_5^+$	0.61 - 339	250	610
		$M^{3} \rightarrow y_4^+$	660.7	526.3		63	25				
	VTPWPLGADPQSR	**$M^{2+} \rightarrow y_4^+$**	712.4	487.3	16.71 ± 0.10		45	$M^{2+} \rightarrow y_4^+$	0.47 - 1050	280	470
		$M^{2+} \rightarrow y_9^{++}$	712.4	940.5		61	40				
	YNHETECITFLK	**$M^{3+} \rightarrow y_5^+$**	518.9	621.4	13.75 ± 0.06		25	$M^{3+} \rightarrow y_5^+$	3.0 - 1220	1800	3000
		$M^{3} \rightarrow y_6^+$	518.9	781.4		97	25				
PPARG / Peroxisome proliferator-activated receptor gamma											
	LNHPESSQLFAK	**$M^{3+} \rightarrow y_2^+$**	457.6	218.2	10.35 ± 0.11		30	$M^{3+} \rightarrow y_2^+$	2.7 - 1002	1500	2700
		$M^{3+} \rightarrow y_{11}^{++}$	457.6	629.3		43	27				
	HLYDSYIK	**$M^{3+} \rightarrow y_2^+$**	346.9	260.2	12.18 ± 0.08		17	$M^{3+} \rightarrow y_2^+$	5.7 - 1230	3000	5700
		$M^{3+} \rightarrow b_2^+$	346.9	251.2		58	23				
	TDPVVADYK	**$M^{2+} \rightarrow y_7^+$**	504.3	791.4	9.07 ± 0.05		21	$M^{2+} \rightarrow y_7^+$	0.03 - 1035	10	28
		$M^{2+} \rightarrow b_2^+$	504.3	217.1		49	21				
IL1R2 / Interleukin-1 receptor type 2											
	TVPGEEETR	**$M^{2+} \rightarrow y_7^{++}$**	509.3	409.2	5.42 ± 0.07		21	$M^{2+} \rightarrow y_7^{++}$	0.05 - 559	24	54
		$M^{2+} \rightarrow b_2^+$	509.3	201.1		10	21				
	TISASLGSR	**$M^{2+} \rightarrow y_7^+$**	446.3	677.4	8.54 ± 0.12		22	$M^{2+} \rightarrow y_7^+$	0.01 - 1110	4	11
		$M^{2+} \rightarrow y_5^+$	446.3	519.3		18	25				
TLR2 / Toll-like receptor 2											
	TVFVLSENFVK	**$M^{2+} \rightarrow y_7^+$**	641.9	836.5	19.35 ± 0.11		29	$M^{2+} \rightarrow y_7^+$	0.5 - 1054	280	500
		$M^{2+} \rightarrow b_2^+$	641.9	201.1		77	27				
	EQLDSFHTLK	**$M^{3+} \rightarrow y_8^{++}$**	406.5	480.8	11.49 ± 0.07		18	$M^{3+} \rightarrow y_8^{++}$	0.12 - 1200	75	120
		$M^{3+} \rightarrow y_6^{++}$	406.5	366.7		20	19				
	VGNMDTFTK	**$M^{2+} \rightarrow y_2^+$**	506.7	248.2	10.15 ± 0.08		34	$M^{2+} \rightarrow y_2^+$	0.25 - 1050	100	250
		$M^{2+} \rightarrow b_3^+$	506.7	271.1		88	26				
TLR4 / Toll-like receptor 4											
	NLDLSFNPLR	**$M^{2+} \rightarrow y_8^+$**	594.8	961.5	20.19 ± 0.12		26	$M^{2+} \rightarrow y_8^+$	0.24 - 1140	109	243
		$M^{2+} \rightarrow y_6^+$	594.8	733.4		62	29				
	LVLGEFR	**$M^{2+} \rightarrow b_2^+$**	417.3	213.2	15.48 ± 0.10		18	$M^{2+} \rightarrow b_2^+$	0.1 - 1080	75	98
		$M^{2+} \rightarrow y_2^+$	417.3	322.2		58	29				
NOS2 / Nitric oxide synthase, inducible											
	VTILFATETGK	**$M^{2+} \rightarrow y_8^+$**	590.3	866.5	16.82 ± 0.08		27	$M^{2+} \rightarrow y_8^+$	0.028 - 1006	15	28
		$M^{2+} \rightarrow y_9^+$	590.3	979.6		68	23				
	YNILEEVGR	**$M^{2+} \rightarrow y_6^+$**	546.8	702.4	16.57 ± 0.07		27	$M^{2+} \rightarrow y_6^+$	0.069 - 964	43	69
		$M^{2+} \rightarrow y_5^+$	546.8	589.3		73	28				

(B) heavy labeled peptides

Gene / Protein	Peptide	Transitions	Q1 *m/z*	Q3 *m/z*	tR [min]	Rel. Ratio to quantifier [%]
NR1H3 / Oxysterols receptor LXR-alpha						
	EECVLSEEQIR	$M^{2+} \rightarrow y_7^+$	701.3	884.5	12.04 ± 0.08	
		$M^{2+} \rightarrow y_6^+$	701.3	771.4		82
	MLGNELCSVCGDK	$M^{2+} \rightarrow y_6^+$	745.8	673.3	13.38 ± 0.12	
		$M^{2+} \rightarrow y_7^+$	745.8	833.3		46
	TSAIEVMLLETSR	$M^{2+} \rightarrow y_7^+$	730.4	859.5	21.41 ± 0.15	
		$M^{2+} \rightarrow y_5^+$	730.4	615.2		44
NR1H3 / Oxysterols receptor LXR-alpha ; NR1H2 / Oxysterols receptor LXR-beta						
	TLSSVHSEQVFALR	$M^{3+} \rightarrow b_2^+$	528.6	215.1	14.15 ± 0.07	
		$M^{3+} \rightarrow y_{12}^+$	528.6	685.4		20
NR1H2 / Oxysterols receptor LXR-beta						
	VEALQQPYVEALLSYTI	$M^{3+} \rightarrow y_5^+$	664.0	649.4	23.63 ± 0.08	
		$M^3 \rightarrow y_4^+$	664.0	536.3		71
	VTPWPLGADPQSR	$M^{2+} \rightarrow y_4^+$	717.4	497.3	16.71 ± 0.10	
		$M^{2+} \rightarrow y_9^{++}$	717.4	950.5		63
	YNHETECITFLK	$M^{3+} \rightarrow y_5^+$	521.6	629.4	13.75 ± 0.06	
		$M^3 \rightarrow y_6^+$	521.6	789.4		97
PPARG / Peroxisome proliferator-activated receptor gamma						
	LNHPESSQLFAK	$M^{3+} \rightarrow y_2^+$	460.2	226.2	10.35 ± 0.11	
		$M^{3+} \rightarrow y_{11}^{++}$	460.2	633.3		57
	HLYDSYIK	$M^{3+} \rightarrow y_2^+$	349.5	268.2	12.18 ± 0.08	
		$M^{3+} \rightarrow b_2^+$	349.5	251.2		58
	TDPVVADYK	$M^{2+} \rightarrow b_2^+$	508.3	217.1	9.07 ± 0.05	
		$M^{2+} \rightarrow y_7^+$	508.3	799.4		50
IL1R2 / Interleukin-1 receptor type 2						
	TVPGEEETR	$M^{2+} \rightarrow b_2^+$	514.3	201.1	5.42 ± 0.07	
		$M^{2+} \rightarrow y_7^{++}$	514.3	414.2		11
	TISASLGSR	$M^{2+} \rightarrow y_7^+$	451.3	687.4	8.54 ± 0.12	
		$M^{2+} \rightarrow y_5^+$	451.3	529.3		10
TLR2 / Toll-like receptor 2						
	TVFVLSENFVK	$M^{2+} \rightarrow y_7^+$	645.9	844.5	19.35 ± 0.11	
		$M^{2+} \rightarrow b_2^+$	645.9	201.1		69
	EQLDSFHTLK	$M^{3+} \rightarrow y_8^{++}$	409.2	484.8	11.49 ± 0.07	
		$M^{3+} \rightarrow y_6^{++}$	409.2	370.7		21
	VGNMDTFTK	$M^{2+} \rightarrow y_2^+$	510.7	256.2	10.15 ± 0.08	
		$M^{2+} \rightarrow b_3^+$	510.7	271.1		86
TLR4 / Toll-like receptor 4						
	NLDLSFNPLR	$M^{2+} \rightarrow y_8^+$	599.8	971.5	20.19 ± 0.12	
		$M^{2+} \rightarrow y_6^+$	599.8	743.4		62
	LVLGEFR	$M^{2+} \rightarrow b_2^+$	422.3	213.2	15.48 ± 0.10	
		$M^{2+} \rightarrow y_2^+$	422.3	332.2		35
NOS2 / Nitric oxide synthase, inducible						
	VTILFATETGK	$M^{2+} \rightarrow y_8^+$	594.3	874.5	16.82 ± 0.08	
		$M^{2+} \rightarrow y_9^+$	594.3	987.6		67
	YNILEEVGR	$M^{2+} \rightarrow y_6^+$	551.8	712.4	16.57 ± 0.07	
		$M^{2+} \rightarrow y_5^+$	551.8	599.3		72

Tab. 8.16: Oxylipin concentrations and 15-LOX levels in M2-like macrophages. Primary blood monocytic cells were differentiated to M2-like macrophages with 10 ng/mL M-CSF for 8 days and incubated with IL-4 for the final 48 h. M2-like macrophages were incubated with 1 µM T09 for different periods of time to investigate the time-dependent correlation of 15-LOX abundance and activity (top) and with different T09 concentrations for 24 h to determine the dose-dependent correlation (bottom). The results are shown as mean ± SEM (n=3-5).

	oxylipin concentrations							**15-LOX level**
	12-HETE	15-HETE	5,15-diHETE	5,12-diHETE	8,15-diHETE	5,15-diHEPE	7,17-diHDHA	
Dose dependent incubations [pmol/mg protein]								
Ctrl	2.44 ± 0.81	30.1 ± 16.9	0.77 ± 0.53	0.27 ± 0.09	0.17 ± 0.09	0.11 ± 0.04	0.53 ± 0.35	0.65 ± 0.20
0.3 µM T09	4.56 ± 1.78	50.0 ± 28.5	1.02 ± 0.57	0.23 ± 0.09	0.75 ± 0.14	0.16 ± 0.07	1.35 ± 0.96	0.77 ± 0.09
1 µM T09	5.75 ± 2.30	64.9 ± 30.8	1.81 ± 0.77	0.30 ± 0.10	0.98 ± 0.27	0.19 ± 0.04	2.16 ± 0.39	1.06 ± 0.07
3 µM T09	7.53 ± 2.72	85.2 ± 30.7	3.20 ± 0.43	0.39 ± 0.06	1.42 ± 0.45	0.25 ± 0.06	3.60 ± 1.04	1.50 ± 0.12
10 µM T09	10.1 ± 3.96	127 ± 52.5	4.59 ± 0.46	0.46 ± 0.16	1.68 ± 0.77	0.35 ± 0.07	7.07. ± 2.37	1.74 ± 0.16
Time dependent incubations [pmol/mg protein]								
Ctrl	15.5 ± 2.19	184 ± 29.1	9.18 ± 1.13	0.47 ± 0.10	3.30 ± 0.42	0.28 ± 0.06	10.73 ± 1.02	3.42 ± 0.54
3 h	22.7 ± 2.23	256 ± 39.8	25.6 ± 4.92	1.00 ± 0.38	7.73 ± 0.38	0.55 ± 0.12	21.1 ± 3.67	4.99 ± 0.94
6 h	26.4 ± 3.26	277 ± 33.7	28.3 ± 2.76	1.23 ± 0.16	9.60 ± 1.41	0.99 ± 0.27	25.5 ± 0.81	5.12 ± 0.57
18 h	27.1 ± 3.35	280 ± 24.9	33.7 ± 1.30	1.76 ± 0.43	12.9 ± 1.91	0.98 ± 0.20	33.1 ± 1.30	5.66 ± 1.09
24 h	32.5 ± 3.44	327 ± 15.9	45.9 ± 8.24	2.17 ± 0.21	14.0 ± 2.47	0.87 ± 0.19	40.4 ± 1.65	6.35 ± 0.84
30 h	33.5 ± 8.86	476 ± 213	59.6 ± 19.8	1.11 ± 0.39	10.4 ± 0.88	0.83 ± 0.34	56.9 ± 17.3	4.45 ± 2.00
48 h	29.3 ± 2.86	364 ± 100	57.3 ± 6.37	0.64 ± 0.12	10.15 ± 3.15	0.64 ± 0.45	39.5 ± 5.28	3.79 ± 1.30

Tab. 8.17: Oxylipin concentrations and 15-LOX levels in M2-like macrophages. Primary blood monocytic cells were differentiated to M2-like macrophages with 10 ng/mL M-CSF for 8 days and incubated with IL-4 for the final 48 h. For 24 h, M2-like macrophages were incubated with known LXR antagonist (GSK2033), FXR agonists (fexaramine and hyodeoxycholic acid (HDCA)) and RXR agonists (bexarotene and 9-*cis* retinoic acid (9-RA)). In order to find an endogenous ligand inducing 15-LOX activity the cells were also treated with different sterols. The results are shown as mean ± SEM (n=3-5).

	Oxylipin concentrations				15-LOX level
	[pmol/mg protein]				
	12-HETE	**15-HETE**	**5,15-diHETE**	**7,17-diHDHA**	**15-LOX**
Ctrl	5.96 ± 2.10	85.2 ± 35.8	3.88 ± 1.87	6.10 ± 3.75	0.92 ± 0.13
GSK2033 (1 µM)	13.6 ± 3.77	163 ± 49.3	23.4 ± 9.12	24.5 ± 18.2	1.73 ± 0.59
Fexaramine (1 µM)	15.7 ± 8.60	206 ± 113	25.9 ± 1.19	44.9 ± 8.60	1.77 ± 0.56
HDCA (10 µM)	1.52 ± 0.69	18.8 ± 7.91	0.58 ± 0.27	2.03 ± 0.23	1.09 ± 0.77
Bexarotene (1 µM)	27.7 ± 8.06	309 ± 88.2	42.1 ± 18.4	49.5 ± 20.8	2.25 ± 0.46
9-RA (10 µM)	13.7 ± 9.16	266 ± 19.1	21.2 ± 4.21	27.5 ± 4.64	0.82 ± 0.26
22(R)-OH Chol (10 µM]	14.6 ±5.74	172 ± 62.6	26.2 ± 13.5	30.7 ± 14.6	1.94 ± 0.20
24(S)-OH Chol (5 µM)	23.9 ± 17.7	190 ± 138	20.1 ± 16.8	78.5 ± 53.8	0.57 ± 0.13
25-OH Chol (10 µM)	3.92 ± 1.78	50.3 ± 24.8	4.95 ± 3.35	6.28 ± 3.79	1.10 ± 0.39
24(S).25-Ep Chol (1 µM)	7.26 ± 2.98	101 ± 36.6	8.77 ± 5.12	10.0 ± 5.06	1.91 ±0.30
24(S).25-Ep Chol (10 µM)	30.8 ± 20.1	238 ± 96.9	21.9 ± 8.94	27.9 ± 10.9	2.13 ± 0.40
7-keto Chol (10 µM)	19.0 ± 15.2	190 ± 138	20.1 ± 6.78	78.5 ± 5.76	1.04 ± 0.69
Desmosterol (1 µM)	53.2 ± 8.53	817 ± 308	100 ± 0.73	287 ± 4.64	2.62 ± 0.69

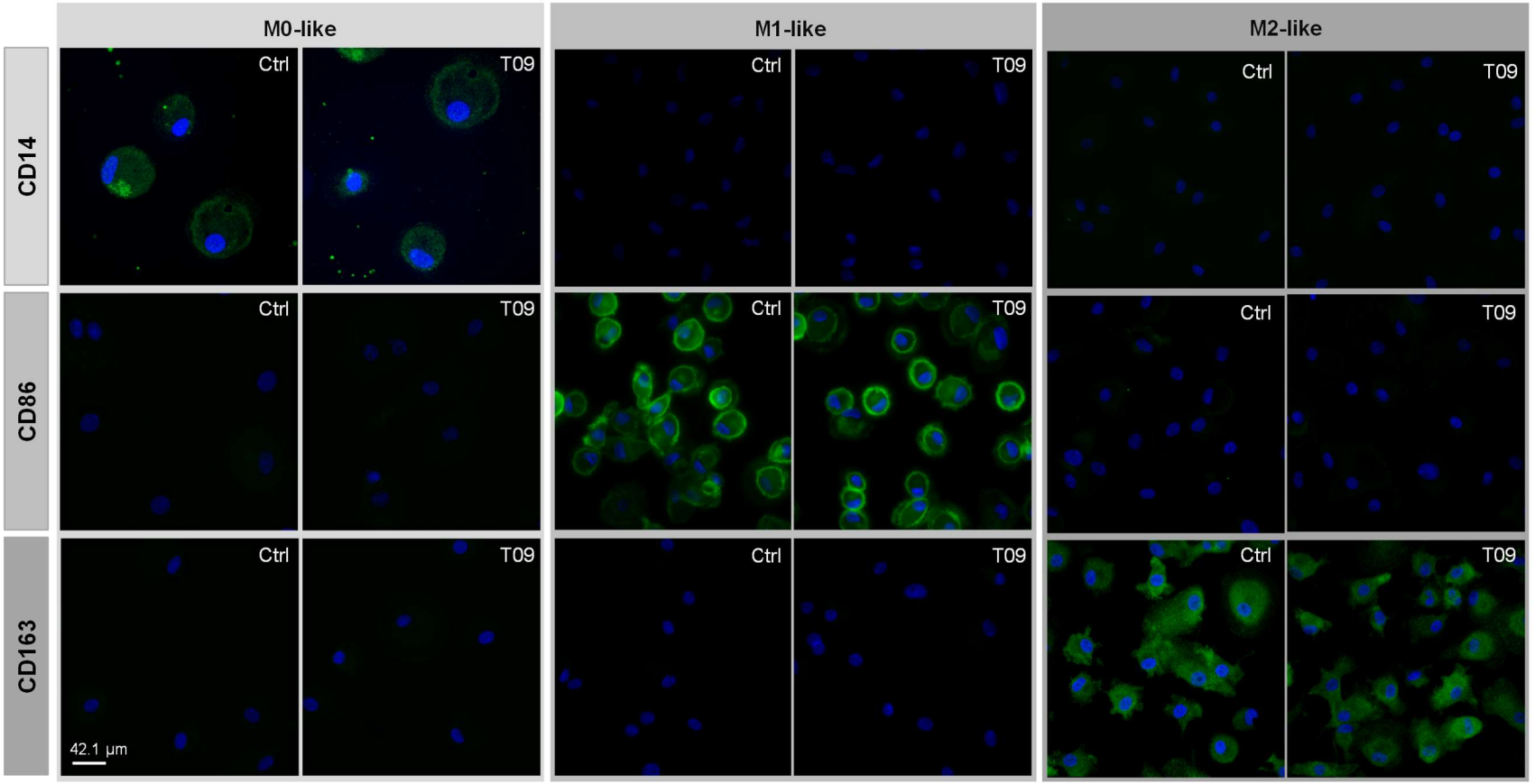

Fig. 8.28: Immunostaining of macrophage surface markers. Primary human blood monocytic cells were treated with 10 ng/mL GM-CSF (M1 type) and M-CSF (M2 type) for 8 days as well as 10 ng/mL IFNγ (M1 type) or IL-4 (M2 type) for the final 2 days. Additionally, M1 cells were challenged with 100 ng/mL LPS for the final 6 h. For M0 type, the adhered monocytes were left untreated for 8 days. The three different macrophage phenotypes were incubated w/ or w/o the synthetic LXR agonist T09 (1 µM) for the final three hours. The three macrophage phenotypes were immunostained for specific macrophage marker CD14, CD86, or CD163 (all mouse anti-human; Bio-Rad Laboratories, Feldkirchen, Germany) using goat anti-mouse IgG Alexa Fluor secondary antibodies from Thermo Scientific, Langenselbold, Germany (green). Nuclei were counterstained with Hoechst (blue). Immunostaining shows that CD14 is only expressed in M0-like, CD86 in M1-like and CD 163 in M2-like macrophages. T09 has no influence on the expression of the surface markers.

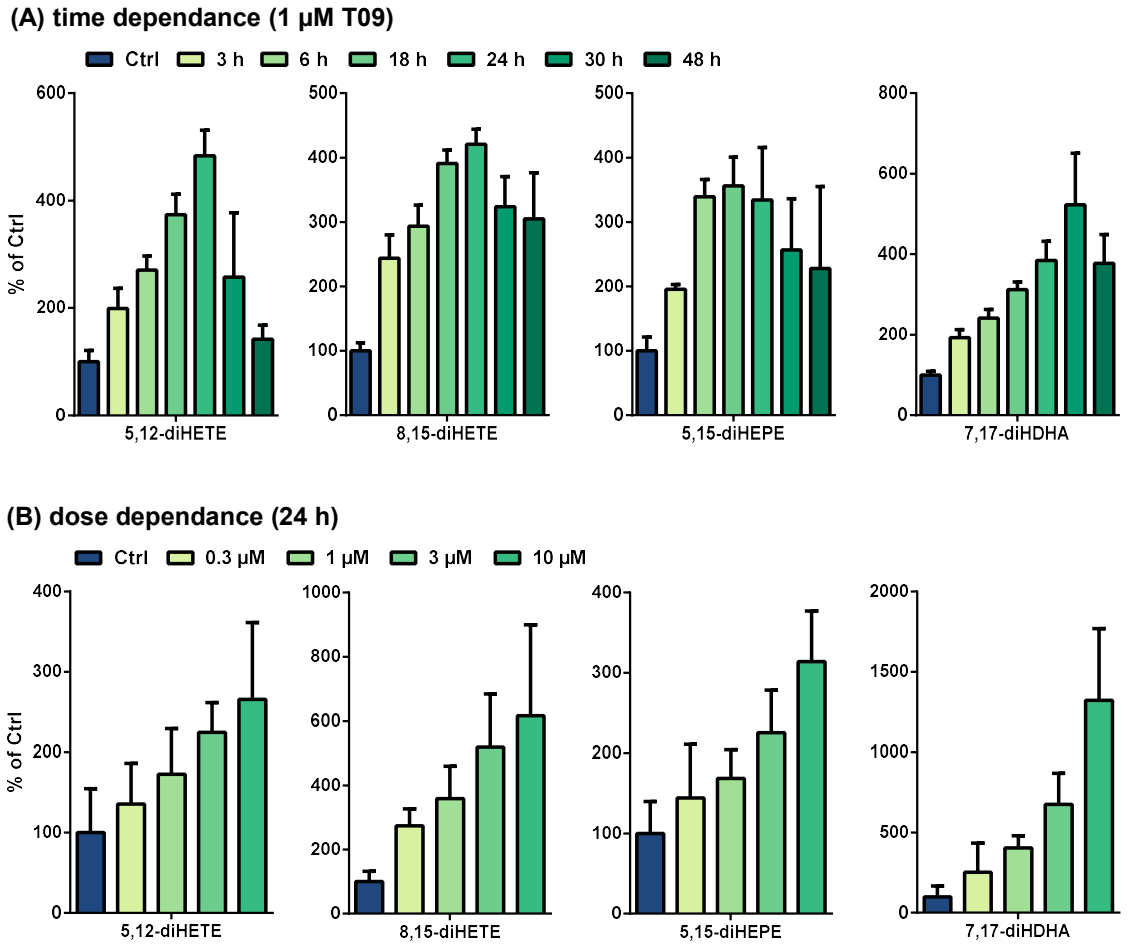

Fig. 8.29: Time- and dose-dependent 15-LOX activity. **(A)** M2-like macrophages were incubated with 1 µM T09 for different periods of time to investigate the time-dependent of 15-LOX activity. **(B)** For the evaluation of the dose-dependence, M2-like macrophages were treated with different T09 concentrations for 24 h. Shown are relative amounts of multiple hydroxylated oxylipins 5,12-diHETE, 8,15-diHETE (ARA-derived), 5,15-diHEPE (EPA-derived) and 7,17-diHDHA (DHA-derived) (mean ± SEM, n=3-6). All incubations were repeated on two different days. Results are shown as % of Ctrl (0.1% DMSO). The concentrations determined in the cells can be found in the Tab. 8.16.

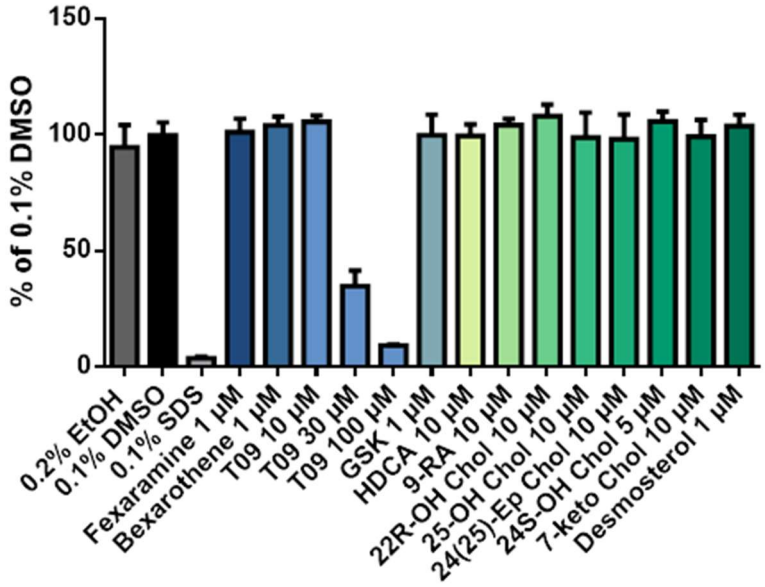

Fig. 8.30: Cell viability assay of the test compounds in M2-like macrophages. Cell viability was determined by resazurin assay [14]. The cells were incubated with the different test compounds at the indicated concentrations for 24 h. DMSO and ethanol (EtOH) served as vehicle control and sodium dodecyl sulfate (SDS) as positive control. Dehydrogenase activity was measured as resorufin formation from resazurin (5 µg/mL) by fluorometric readout at 590 nm after excitation at 560 nm [14]. Shown are mean ± SD for n = 6-12 technical replicates from a pool of 4 donors.

Abbreviations

9-RA	9-*cis* retinoic acid
aa	amino acid
ACN	acetonitrile
AdA	adrenic acid
ALA	α-linolenic acid
ALOX	gene of the lipoxgenase enzyme
ANOVA	analysis of variance
ARA	arachidonic acid, C20:4n6
BC	blood cell(s)
BCA	bicinchoninic acid
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAD	collision activated dissociation
CD	cluster of differentiation
CE	collision energy
CI	confidential intervall
CID	collision induced dissociation
COX	cyclooxygenase
CP-DT	cleavage prediction with decision trees
CV	coefficient of variation
CXP	collision cell exit potential
CYC1	cytochrome c1
CYP	cytochrome P450 monooxygenases
DGLA	dihomo-gamma linolenic acid, C20:3n6
DHA	docosahexaenoic acid, C22:6n3
diH	dihydroxy
DiHDHA	dihydroxy docosahexaenoic acid
DiHDPE	dihydroxy docosapentaenoic acid
DiHEPE	dihydroxy eicosapentaenoic acid
DiHETE	dihydroxy eicosatetraenoic acid
DiHETrE	dihydroxy eicosatrienoic acid

DiHODE	dihydroxy octadecadienoic acid
DiHOME	dihydroxy octadecenoic acid
DiOH-FA	dihydroxy fatty acid(s)
DP	declustering potential
DPA	docosapentaenoic acid, C22:5n3/C22:5n6
EA	ethyl acetate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	entrance potential
EPA	eicosapentaenoic acid, C20:5n3
EpDPE	epoxy docosapentaenoic acid
EpETE	epoxy eicosatetraenoic acid
EpETrE	epoxyeicosatrienoic acid
Ep-FA	epoxy fatty acid
EpODE	epoxy octadecadienoic acid
EpOME	epoxyoctadecenoic acid
ESI	electrospray ionization
FA	Fatty acid
FIA	flow injection analysis
FITC	fluorescein isothiocyanat
FLAP	five lipoxygenase activating protein
FWHM	full width at half maximum
FXR	farnesoid X receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLA	γ-linolenic acid
GM-CSF	granulocyte/macrophage colony-stimulating factor
H(p)ETE	hydro(pero)xyeicosatetraenoic acid
HDCA	hyodeoxycholic acid
HDHA	hydroxydocosahexaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HETrE	hydroxy eicosatrienoic acid
HHT/HHTrE	hydroxy heptadecatrienoic acid
HOAc	acetic acid
HODE	hydroxy-octadecadienoic acid

Hoechst	Bisbenzimid H 33258
HOTrE	hydroxy-octadecatrienoic acid
HpEPE	hydroperoxy eicosapentaenoic acid
HpETE	hydroperoxy eicosatetraenoic acid
HPLC	high performance liquid chromatography
IFN	interferon
IL	interleukin
IL-1RII	interleukin 1 receptor type II
iNOS	inducible nitric oxide synthase
IS	internal standard
IsoP	isoprostane
LA	linoleic acid, C18:2n6
LC	liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
LC-UV	liquid chromatography with ultraviolet detection
LLOQ	lower limit of quantification
LM	lipid mediator
LO	lipoxygenase
LOD	limit of detection
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
Lx	lipoxin
LXR	liver X receptor
m/z	mass-to-charge ratio
MAPEG	membrane associated proteins in eicosanoid and glutathione metabolism
MaR	maresin(s)
M-CSF	macrophage colony-stimulating factor
MeOH	methanol
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MS	mass spectrometry
n3/n6/n9	omega-3/6/9
n3-DPA	n3-docosapentaenoic acid

NF-κB	nuclear factor κB
NIST SRM	National Institute of Standards and Technology standard reference material
NSAIDs	non-steroidal anti-inflammatory drugs
nsSNP	single nucleotide polymorphism
OA	oleic acid
OH-FA	hydroxy fatty acid(s)
oxoETE	oxo-eicosatetraenoic acid
oxoODE	oxo-octadecadienoic acid
oxoOTrE	oxo-octadecatrienoic acid
P/S	penicillin/streptomycin
PBMC	peripheral blood monocytic cells
PBS	phosphate buffered saline
PCA	principal component analysis
PG	prostaglandin
PLA	phospholipase A
PLS-DA	partial least squares discriminate analysis
PMNL	polymorphonuclear leukocyte
PPAR	peroxisome proliferator activated receptor
PPIB	peptidyl-prolyl cis-trans isomerase B
PTGS	genes of the prostaglandin G/H synthase proteins
PTM	postranslational modification
PTP	proteotypic peptide
PUFA	polyunsaturated fatty acids
qPCR	quantitative polymerase chain reaction
QS	quality standard
RBC	red blood cells
RP	reversed phase
RT	room temperature
Rv	resolvin

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Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

List of Publications

Publications in peer-reviewed journals

Within the scope of this thesis

Mainka M., Pfaff R., Hartung N. M., Schebb N. H. (2022) Sterol derivatives specifically increase anti-inflammatory oxylipin formation in M2-like macrophages by LXR mediated induction of 15-lipoxygenase LOX, submitted for publication. *Submitted for publication.*

Mainka M., George S., Angioni C., Ebert R., Goebel T., Kampschulte N., Krommes A., Weigert A., Thomas D., Schebb N. H., Steinhilber D., Kahnt A. S. (2022) On the biosynthesis of specialized pro-resolving mediators in human neutrophils and the influence of cell integrity. Biochim Biophys Acta Mol Cell Biol Lipids. 1867(3) pp. 159093; doi: 10.1016/j.bbalip.2021.159093.

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