New technologies for the analysis of oxylipids : role and metabolism of oxylipids in rheumatic diseases

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Chapter 7

General discussion, conclusion and perspective

Discussion

Lipids are key players in many diseases and conditions, not only as membrane components and energy storage compartments but also as cell signaling molecules. For example, lipids and lipid mediators (LMs) play important roles in inflammation and inflammatory diseases but have been understudied compared to proteins (like cytokines and immunoglobulins). Also for other conditions and diseases, this under-representation of lipids compared to other metabolites holds true, with perhaps the exception of steroid hormones.

As is explained in the introduction of this thesis, lipids are a very diverse class of molecules, which has perhaps hindered their study, as the level of complexity is very high. With recent developments in separation techniques and mass spectrometry (MS), and the emerging of the field of lipidomics, the complexity has ceased to be as much of a hurdle, and increased interest in studying lipids as important factors in several diseases has emerged, e.g. in Alzheimer's disease [1], lipid metabolic disorder [2], and rheumatoid arthritis. [3, 4] In addition to general lipidomics, the subfields of oxylipid and LM (and there within, specialized pro-resolving mediator, SPM) analysis has also gained greater attention in inflammation and rheumatic diseases [5-7] as well as in parasitic diseases, as LM are important immunological signaling molecules. [8, 9]

To increase the quality of the acquired data the analytical platforms, used to perform these (oxy)lipid analyses, are still developing and evolving with the advances in technology. This applies to the extraction of the (target) analytes and/or interference of other analytes, the chromatographic separation, and finally the MS detection/resolution/specificity etc. For example, the bioactivity of oxylipids and LMs is highly stereospecific, and some difficulties have arisen in the chromatographic separation and resolution of these stereoisomers using classical reversed phase liquid chromatography tandem MS (RPLC-MS/MS), perhaps sometimes resulting in wrong conclusions being drawn, one isomere being confused with another. The work in this thesis was therefore focused on further development and application of analysis platforms for oxidized lipids; their identification, separation and levels in different matrices. All of which, work that can be further used in inflammatory or rheumatoid research.

Different approaches in the analysis were taken in the work described in this thesis; which we will now discuss based first on sample preparation, then separation, and finally detection:

In extracting the target analytes from the sample matrices, we utilized liquid-liquid extraction (LLE), solid-phase extraction (SPE) and single-solvent extractions of (oxy) lipids, in each project chosen with regards to target analyte and specificity, procedural ease, sample volume and analyte concentration, and necessary clean-up. In **Chapter 2** LLE extraction was chosen (based on the method described by Matyash et al.[10]) for an untargeted extraction of SF as it offers good coverage of lipid species. For the more targeted analysis and structural elucidation of classifying features and hydroxylated

fatty acids (hFAs), proteins were precipitated with isopropanol and the sample was reconstituted in the injection solvent after the eluent was dried down. The approaches in **Chapters 3-6** were targeted towards the more hydrophilic oxylipids and PUFAs, needing targeted extraction methods. To target oxylipids (**Chapter 3**), methanol (MeOH) protein precipitation of plasma and SF and dilution with water before injection provided a high-throughput method, easily adapted to 96-well plates and combined with short LC-MS/MS analysis times. The more hydrophobic lipids like PLs, MGs, and TGs were extracted to a smaller extent into MeOH than the oxylipids, and therefore not much clean-up was needed. FAs also have a lower recovery with this method than oxylipids, but the more hydrophilic PUFAs are extracted to a higher extent than the saturated FAs. A benefit of this method is its ease of procedure, which minimizes possible analyte loss. The drawback is, however, that low-concentration-analytes may not be detected, as the high injection volume may cause excessive dilution. In later chapters, plasma samples (**Chapter 5**) and part of the SF samples (**Chapter 6**) were precipitated with MeOH and the eluent dried down and reconstituted in a smaller MeOH/water volume before injection, to aid the target analyte-concentration. This however, can lead to overloading on the LC column for higher-concentration analytes, and protein clogging, so the analytical column care and cleaning is very important. This was a problem for some of the earliest measured samples in **Chapter 5**, where peak broadening was apparent, making e.g. TXB_2 difficult to quantify. In addition, for more complex sample matrices like tissue and SF, cell debris and other insoluble parts may also cause problems for the LC system and columns, as the cleaning-steps are at a minimum. Samples therefore need to be treated carefully after centrifuging so as not to resuspend these problematic proteins or cell debris. In **Chapter 6** MeOH precipitation and SPE of SF after storage was compared to MeOH precipitation immediately upon collection (and no SPE) in 20 randomly selected samples with rheumatic diseases (osteoarthritis, rheumatoid arthritis, and others) For 28 of the 37 detected analytes, the concentrations determined by the two methods correlated well, and in turn further analysis was restricted to these 28 analytes. Biological samples used in **Chapter 4** were murine peritoneal exudates. Cells were obtained by centrifuging the peritoneal lavage and ethanol used to extract oxylipids from the cells and precipitate proteins.

It can thus be stated that no single extraction protocol is good for all samples, but it has to be chosen based on variables like sample volume, target analyte(s), and analyte concentration range. In addition, it is important, as seen in **Chapter 6**, where two extraction methods were compared, that different extraction methods may not result in the same recoveries for all target analytes, and therefore comparison of analyte concentration between datasets is not only dependent on the analysis method itself, but can also be dependent on the extraction protocol chosen. Therefore the signals of samples with similar levels of low abundant analytes may end up slightly above the lower limit of

quantitation (LLOQ) of an analysis platform or the analytes may not be detected, due to differences in recoveries between extraction protocols.

LC is a good and very common separation method for most lipids. It is robust, reproducible, and has a high resolving power. In the work presented in this thesis, we chose to use C18 RPLC for untargeted lipidomics as its separation efficiency for the lipidome is greater than that of normal phase or HILIC. For targeted oxylipid analysis, C18 RPLC was also chosen, as it separates based on the fatty acid moiety. Using different polarity solvents and gradients, a range of lipids can thus be separated based on hydrophobicity. Collecting fractions for further separation or analysis is also easy with LC as you can collect fractions of the mobile phase before it reaches the MS, contrary to e.g. GC where the separation happens in the gas phase. In **Chapter 2** a gradient of water/ACN/iPA with ammonium formate and formic acid was used for untargeted analysis, while a gradient of water and MeOH with acetic acid was used for the hFAs analysis. Working towards a targeted platform for hFA and oxylipid analysis, MeOH/water gradients with acetic acid were used (**Chapters 3-6**). As the separation achieved with C18 RPLC is not sufficient for the analysis of all oxylipid stereoisomers, and hFA *R*- and *S*-enantiomers are not separated at all, additional separation techniques were used in **Chapters 4** and **5** for identification and to gain more stereochemical information. Ion mobility, or in this case differential mobility spectrometry (DMS), gives an additional separation dimension which we used to separate isomers overlapping on an RP-C_{18} column (**Chapter 4**). This is extremely important given that the bioactivity of the molecule is dependent on its stereochemistry. To our knowledge, at that time, DMS coupled to MS had not been used in the separation of LMs (other than prostanoids) before. Using this technology we separated isomers that are hard to separate on a C_{18} column, among them an important separation of LTB_4 and its isomer 5*S*,12*S*-diHETE. A highly advantageous part of using the DMS technology in oxylipid analysis, compared to other IM-MS techniques, is that it works analogue to ion trap (IT)-MS in selecting the ions that are let through, and is thus highly compatible with the targeted SRM methods used in oxylipid analysis.

As well as using additional ion mobility separation we took advantage of the ease with which fractions of samples can be collected on LC platforms, in both **Chapters 2** and **5**. There fractions collected were run on a second different column or again on the same column with a different gradient, for 2D-separation. Chiral chromatography was also utilized to separate LM isomers and to separate PUFA-derived hFA enantiomers in the second separation dimension in **Chapter 5**. This enantiomer separation is no-less important than the separation of bioactive LMs from less-bioactive isomers, as the enzymatic products of these PUFAs have a preferred chiral configuration, mostly being *S*, while non-enzymatic oxidation products are racemic products with an *R* to *S* ratio of 1. Thus utilizing chiral chromatography after traditional RPLC we can gain further insight into whether a hFA is enzymatically made or not.

In this thesis we have utilized MS in all chapters and all aspects of the research. Advances in MS, making it possible to gain data that can identify structural isomers, is very valuable to the complexity of (oxy)lipid analysis. We took advantage of both direct infusion (DI) and analyte separation prior to the MS; but used only one ionization method, electrospray ionication (ESI). As mentioned in **Chapter 1**, ESI is a soft ionization method that ionizes complex lipids well. PUFAs and oxylipids ionize reasonably well in negative mode ESI, despite the generally lower ionization efficiency for FAs. The lipidomes' complexity is well exemplified by the high resolution 'lipid fingerprint' achieved with the 15T DI-ESI-FTICRMS (direct infusion electrospray ionization Fourier transform ion-cyclotron mass spectrometry) offered in **Chapter 2**. This analysis gave a master list of almost 1300 masses detected in at least one of the samples. In order to gain information about the specific lipids driving the clustering of the samples, the obtained accurate masses were used for initial structure hypothesis, and MS/MS and MS3 approaches with an IT-MS were used for the identification of structural isomers. Here, the combined use of the two platforms together did not result in redundant data and was highly beneficial. The IT-MS had in addition the advantageous feature to be able to switch back and forth from ESI+ to ESI-, thus being capable of measuring each sample in both ionization modes within a single run: a very useful feature for lipidomics analysis, as some lipids ionize only in one of the modes.

For the oxylipid analyses described in **Chapters 3-6** a QTrap®, triple quadrupole (QqQ) linear iontrap (LIT) hybrid mass spectrometer, was used. The data was mostly collected in the QqQ-based SRM mode, utilizing known, species specific, fragmentation patterns of oxylipids in addition to known chromatographic retention times. A highly beneficial feature of the QTrap is that while in SRM mode, focusing on given transitions (from parent ion to daughter ion masses), it can collect enhanced product ion (EPI) MS/MS spectra using the LIT. The parent ion-linked MS/MS spectra from these scans can then be library-matched to known product ion scans for qualitative identification, or structural elucidation of unknown compounds that share a SRM transition of a different compound. Examples of this can be found in **Chapter 3**, where using this approach, 19,20-diHDPA and LTE_4 were identified in post-mortem SF, LTB_4 in activated polymorphonuclear cells, and pro-resolving RvE2 was found in activated whole blood that had been incubated with EPA. As a transition needs to be known before detecting an analyte with SRM and the method preferably optimized with regard to collision energy (CE) (as seen in Supplementary Table S1 in **Chapter 3**) a downside to having targeted SRM methods is that you should preferably be in possession of a standard of the analyte for optimization. As the lab was just starting on its journey in targeted oxylipid analysis with the work described in **Chapter 3** our library of standards grew since then, as can be seen in comparison of the lipid lists in **Chapters 3** and **5**.

The analyte extraction, separation, and detection are essential elements for the generation

of good, reliable data, which in turn are the basis of proper sample analysis. However, all these considerations and work are inconsequential if the samples are not properly collected, handled and stored, and the data does not mirror the true state of the sample at the time of collection.

As shown in **Chapter 5**, the use of different anticoagulants in the preparation of plasma can affect the measured concentration of oxylipids. This might be due to possible activation of platelets and leukocytes at the time of blood collection, which will change the plasma oxylipid profile. It has been shown in both human [11, 12] and canine [13] plasma that different anticoagulants activate platelets to a different extent. The results in **Chapter 5** show that for a few of the measured analytes, the ratio of their concentration in EDTA plasma to heparin plasma was very low at baseline. The difference in platelet activation would account for the low EDTA/heparin ratio of platelet products 12-LOX and TXB_2. The 12-LOX isoform that is expressed by platelets gives 12*S*-HETE [14] and the chiral data supports this hypothesis of the origin of the measured 12-HETE and TXB_2 in heparin plasma. (Supplementary Figure S63 of **Chapter 5**, not shown in this thesis but available in the online published edition of the paper on which the chapter is based on). Studies have determined a mixture of citrate, theophylline, adenosine, and dipyridamole (CTAD) or a combination of CTAD and EDTA to limit platelet activation [11-13, 15, 16], and therefore these might be studied further with respect to lipid/oxylipid analysis.

When it comes to the low EDTA/heparin ratio of the other anlytes shown in **Chapter 5** at baseline, 5-HETE, LTB_4, LTD_4, and LTE_4 it would, similar to the platelet activation, maybe be most easily explained by a heparin-derived leukocyte activation at the time of blood collection resulting in a difference in 5-LOX activation and consequent LTA4H and LTC4S activity (as platelets do not express 5-LOX). Contrary to this theory, we do see from the chiral data that the difference in levels at that time most likely does not stem from increased activity of 5-LOX in the heparin plasma, and therefore is not likely to be due to leukocyte activation. If maintaining the activation-of-blood-by-heparin theory, this would then point to increased PLA_2 activity, increasing the available AA pool for conversion. And while the PLA_2 activity could be, at least in part, from PMNs [17] (even though they should be expressing 5-LOX if activated) it could also, as well as the LTC4S activity [18, 19], stem from activation of platelets by heparin [20, 21]; bringing us back to the explanation that the difference seen at baseline between heparin and EDTA plasma is due to heparin-activation of platelets. But contradictory to that, the substrate of LTC4S and LTA4H is LTA_4, made by 5-LOX activity, which could only be explained by the activation of leukocytes. [19, 22] Another explanation for this apparent contradiction, that was not addressed in the chapter itself, is EDTA's chelating effect: By removing the Ca^{2+} needed for PLA_2 and LOX activation and/or the Fe^{+3} present in the LOX active state. [23] This chelating would then further exaggerate the effect of heparin on platelet activation by inhibiting 12-LOX activity in the EDTA samples, whereas effects on 15-LOX products

would not be expected, as 15-LOX expressing cells are only present in blood in very low abundance. In addition, matrix effect in the ionization of metabolites in the EDTA plasma could play a role, [24, 25], as well as the effect of heparin on PLA_2 activity, either enhancing it [17] or inhibiting [26] Of course the possibility of a combination of all these events is also plausible: Activated platelets in heparin blood, EDTA chelating affecting LOX activity, heparin affecting PLA_2 activity, and matrix effect.

For further determination and conclusions on the possible effects of different anticoagulants on levels of oxylipids at baseline level, the experiment from **Chapter 5** would have to be repeated and enzyme activity monitored, as all we have from this experiment are the metabolites and no actual data on the enzymes. Post-column infusion of metabolites, as we did in **Chapter 3**, to assess the matrix effect in the plasma from different anti-coagulants would also be needed. Therefore no conclusions on the reason for this difference can be drawn at this time, matrix effect or otherwise, but the difference was clearly observed.

In addition to plasma, the importance of methods in collecting, and pre-analytical sample handling and treatment, also holds true for other sample matrices, e.g. treatment of SF with hyaluronidase as mentioned in **Chapter 6**, as well as other kinds of biological samples.

What we also see with regard to the sample collecting and preparation, is that the goal of the study must be used to determine the best method of collection and handling. When platelet products are the targeted analytes, EDTA, CTAD or a combination (as mentioned here above) would perhaps be preferred over heparin plasma to avoid the *ex vivo* activation. In addition, EDTA chelating effect might inactivate residual enzymes in the plasma, but might cause ion suppression at the same time. In other cases heparin, citrate or some other anticoagulant might be preferred; each with their respective pros and cons, heparin possibly affecting PLA_2 activity etc. In addition to different anticoagulants for the preparation of plasma, serum could be used for blood analysis, which has not been touched upon prior in this thesis. The blood is spun down after the coagulation has occurred and has the benefit over plasma, that there is nothing added to the blood. Therefore, matrix effect or cell activation by added anticoagulants is not an issue and the problem of comparison between different anticoagulants between studies is avoided. However, coagulation of blood is a form of its activation, and thus the oxylipid profile of serum will not mirror the oxylipid profile of the blood just prior to collection, especially with regard to platelet oxylipid products. Colas et al. showed that in commercially available serum multiple SPMs were detected while in commercial lithium heparin plasma, only few were present. [27] Both were pools of plasma from a 100 individuals, even though these are not the same individuals. This indicates that the activation by the coagulation event in serum changes the oxylipid profile so drastically (and not only with regard to platelet products like TXA_2) that it would not be useful in oxylipid analysis unless the research specifically

targets wounds or coagulation.

In addition to the handling and preparation of samples, proper storage plays a huge role in minimizing *ex vivo* changes and maintaining basal levels of analytes in samples through time. As discussed here above, the difference in 12-HETE levels at baseline between EDTA and heparin in **Chapter 5** seems to be mostly a result of difference in platelet activation in the whole blood upon collection. From the chiral data however it seems that the increase in 12-HETE levels over time seen at -20 °C is not further activation of platelets in neither the EDTA aliquots nor the heparin, but of non-enzymatical nature. Contrary to 12-HETE, the *S/R* ratio of 5-HETE increases over time at -20 °C but only in heparin plasma, indicating an possible increase in residual 5-LOX activity during storage; while in the EDTA plasma there were no clear changes in *S/R* ratio over time, perhaps due to EDTA chelating effect on residual 5-LOX. For the third HETE that was analyzed on the chiral platform, 15-HETE, the *S/R* ratio changes very little over time but does seem to approach 1 over time for in both EDTA and heparin plasma. This indicates, similar to 12-HETE, that the predominant contribution over time of 15-HETE in both EDTA and heparin plasma, and 5-HETE in EDTA plasma, is non-enzymatic, non-stereospecific, due perhaps to the increased pool of AA. As of now we do not have an explanation for this increased *S/R* ratio of 5-HETE over time in heparin plasma. That is, if it's due to an active 5-LOX enzyme in the plasma aliquots, [28, 29] the origin of that enzyme in the plasma is not certain since leukocytes should not be present and 5-LOX is not in soluble form, but membrane bound. [30]

In addition to an increase in levels of analytes that were already measured above the limit of detection at baseline, what we also see is the 'appearance' of other oxylipids over time in aliquots stored at -20 °C. While many of those oxylipids could be formed *in vivo* via 5-LOX pathways, it is clear from the chromatograms (like those seen in Figures 8 and 9 in **Chapter 5**) that many other isoforms have formed as well, making it more likely to be due to non-stereospecific non-enzymatic pathways. In addition, this effect is seen in both the heparin and EDTA plasma, while the chiral data (as mentioned above) would indicate only possible 5-LOX activity in the heparin plasma. After one year of storage the only very few were detected in the aliquots stored at -80 °C, as seen in the supplementary figures of **Chapter 5**, but more could possibly be detected in samples older than that. What we see for sure, is that samples kept for not even half a year at -20 °C already have a changed oxylipid profile, that does not mirror the profile at the time of collection. After 1 year at -80 °C some changes are seen and after more than a year at -80 °C more detectable species will most likely appear. That would give 'false positives' in the analysis if the focus is on the presence of these appearing analytes. This could then perhaps explain why different results are often obtained with regards to detection of certain SPMs by different labs or in different studies. In **Chapters 3** and **6**, no endogenous SPMs were found in plasma or in the soluble part of the SF, only the markers of their precursors,

like 17-HDHA and 18-HEPE, contrary to earlier published results by our lab. [3] Along those lines, contrary results have been reported on the presence of SPM in human plasma: Skarke et al. described not finding RvE1 even after fish oil supplementation [31], while Oh et al. reported finding RvE2 in plasma without any supplementation and RvE1 after EPA and aspirin supplementation. [32] Interestingly, Skarke et al. also describe the detection of PD1 and MaR1 in the plasma after supplementation, but as can be seen by comparison of their multipeak chromatograms to the chromatograms in **Chapter 5**, these are most likely isomers. [31]

In addition to these possibly false positives when it comes to describing the endogenous presence of SPMs, **Chapter 5** also clearly shows that samples kept for different amounts of time at -20 °C without MeOH cannot be compared with regard to levels; as can be seen by the continuous rise in analyte levels over time. Thus, collecting samples over time and storing them at -20 °C until analysis is questionable in oxylipid analysis. Even storage at -80 °C may not be enough to circumvent this effect, unless MeOH is added prior to the storage, as we see for e.g. TXB_2.

Conclusion and perspective.

Lipids are a very diverse and complex class of molecules. This includes multiple key players in many diseases and conditions. Oxylipids (and LMs and SPMs especially) play key roles in inflammation and rheumatic diseases, and with the added knowledge of their relevance and their highly stereospecific bioactivity, the importance of good analytical methods has become increasingly more important. We have shown in this thesis multiple methods in sample preparation, and the importance of sample handling and storage prior to analysis. It is important to take these into view when results, that might even seem conflicting, are compared.

As discussed above, we have showed that enzyme activity can possibly change the levels of analytes over time, in addition to possible *ex vivo* synthesis of compounds not present at basal conditions (or were below the LLOQ). In addition to this, radical non-enzymatic oxidation (as shown by the chiral data and the multiple isomers of SPMs that appear) and peroxidation are also a problem in storage of lipids. This becomes more important when the storage time increases. Samples should however be kept as cold as possible/practical for short-term storage as well, in order to minimize all reactions rates. For long-term storage, keeping samples at as low a temperature as possible, in addition to precipitating the proteins out of the solution, provides fairly easy/accessible solutions and will maintain the sample integrity for much longer periods of time. Comparing data from samples treated differently before the analysis can result in conclusions that are not based on a proper comparison.

After sample work-up, prior to reaching the MS, the separation of oxylipids stereoisomers (that cannot be identified based on mass or fragmentation alone) is

important, and was achieved in this research with both C18 RPLC and chiral columns, and novel additional ion mobility separation. However, due to the great diversity of lipids, a single analysis platform may not be sufficient, and multiple (often complementary) methods may have to be utilized in sample handling, analyte extraction and separation, and detection. Decisions on which method(s) to use depend of course on the technology available, but also on the target analyte and goal of the research. The benefit of multi-platform approaches in e.g. the identification of structural isomers with tandem MS combined with high resolution MS. All these factors are important in all (oxy)lipid and LMs analysis.

Since its emergence early this century, the field of lipidomics has grown immensely. In the last few years the field of LM and SPM analysis has especially been gaining momentum, as evidence of the involvement of these compounds in health, disease, and therepeutics has emerged. However, as discussed above, analyzing LM and SPM presence in disease has no meaning if we are not sure that the analytes we measure were there *in vivo*. In addition to that, one could claim that detecting certain analytes in human samples after supplementation of precursors does not indicate that these molecules are present there *in situ*, only that the cells are capable of synthesizing them when the substrate is supplied. But the field of LM and SPM research has also focused on the therapeutic use of SPMs and their stable analogs, and much has to be further gained and researched in that area. Therefore the development of good reliable methods will continue to be necessary, as well as further research on sample handling and storing.

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