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TRENDS

Targeted metabolomics of the arachidonic acid cascade: current state and challenges of LC–MS analysis of oxylipins

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Abstract Quantification of eicosanoids and oxylipins derived from other polyunsaturated fatty acids in biological samples is crucial for a better understanding of the biology of these lipid mediators. Moreover, a robust and reliable quantification is necessary to monitor the effects of pharmaceutical intervention and diet on the arachidonic acid (AA) cascade, one of today's most relevant drug targets. Low (sub-nanomolar) concentrations and a large number of structurally similar analytes, including regioisomers, require high chromatographic resolution and selective and sensitive mass spectrometry analysis. Currently, reversed-phase liquid chromatography in combination with detection on sensitive triple-quadrupole instruments, operating in selected reaction monitoring mode, is the main method of quantitative oxylipin analysis. A lack of standardized sample collection, handling, and preparation procedures, degradation of the analytes during sample preparation, and purity and availability of standards (internal standards) are the major problems of targeted metabolomics approaches for the AA cascade. Major challenges for instrumental analytical methods are the detection of esterified oxylipins, and separation and individual detection of oxylipin isomers. Solving these problems would help to further knowledge of the biology of lipid mediators, and is an important task for bio-analytical research.

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Abbreviations

AA	Arachidonic acid
AMPP	*N*-(4-aminomethylphenyl) pyridinium
BHT	Butylated hydroxytoluene
COX	Cyclooxygenase
CYP	Cytochrome P450 monooxygenase
DHA	Docosahexaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EpETrE, EET	Epoxyeicosatrienoic acid, epoxy-AA
FA	Fatty acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid; hydroxy-AA
HpETE	Hydroperoxyeicosatetraenoic acid; hydroperoxy-AA
HRMS	High-resolution MS
IS	Internal standard
LLE	Liquid–liquid extraction
LOQ	Limit of quantification
LOD	Limit of detection
LOX	Lipoxygenase
LT	Leukotriene
mPGES	Microsomal prostaglandin E synthase
MRM	Multiple reaction monitoring
NSAID	Non-steroidal anti-inflammatory drug
OH-FA	Hydroxy fatty acid
PFB	Pentafluorobenzyl
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
sEH	Soluble epoxide hydrolase

SOP	Standard operating procedure
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TxB	Thromboxane

Introduction

Lipid mediators have an important function in biology. In particular, eicosanoids (C20) and oxidative products of other long-chain polyunsaturated fatty acids (PUFA) regulate a large variety of cellular and physiological functions [1]. In mammals, these oxylipins are formed enzymatically via three pathways:

1. Constitutively expressed cyclooxygenase 1 (COX-1) and inducible COX-2;
2. 5, 12, and 15-lipoxygenases (LOX); and
3. Cytochrome P450 monooxygenases (CYP), particularly CYP2J and CYP2C.

They are also formed non-enzymatically, by (aut)oxidation.

The products initially formed can be further converted by several other enzymes, for example microsomal prostaglandin E synthase (mPGES) or soluble epoxide hydrolase (sEH), leading to a pleiotropy of oxylipins formed in the arachidonic acid (AA) cascade (Fig. 1). In several cases, the product pattern of the four processes overlaps. For example, 15-hydroxyeicosatetraenoic acid (HETE) is not only generated by 15-LOX, but also by COXs and autoxidation, with distinct differences in stereochemistry [2]. Moreover, the route of formation of several mediators has not yet been discovered; e.g. that of 18-HEPE, the major hydroxy fatty acid (OH-FA) metabolite in several cultured cells incubated with eicosapentaenoic acid (EPA) [3].

More than half of currently sold pharmaceuticals directly target the AA cascade [4], for example non-steroidal anti-inflammatory drugs (NSAID), including aspirin and selective COX-2 inhibitors (e.g. celecoxib), and 5-LOX inhibitors and leukotriene (LT) antagonists. Approximately 30 years after the Nobel Prize awarded to J. Vane, S. Bergström, and B. Samuelsson for the discovery of the importance of prostaglandins (PGs), the biological functions of non-classical eicosanoids and oxylipins other than PGs and LT are becoming clearer. For example, multiple hydroxylated docosahexaenoic acids (DHA) and EPA have been discovered as a new class of inflammation-resolving lipid mediators [5, 6]. The vasodilatory action of epoxy-FA (Fig. 1) on endothelial cells is well established and a large number of studies describe anti-inflammatory and analgesic effects, although an epoxy-FA receptor has not been discovered. Although AA, DHA and EPA-derived epoxides have similar biological activity, the effects in cancer are different, with AA-derived epoxyeicosatrienoic acids (EpETrEs) promoting angiogenesis, whereas n3-PUFA-derived epoxides suppress tumor growth [7].

To investigate and understand the function of the many different oxylipins in physiology, analytical methods are needed to quantify their levels in biological samples. The most promising strategy is the parallel quantification of a comprehensive pattern of products of the AA cascade derived from both n6-PUFA, for example AA, and n3-PUFA, for example EPA and DHA. By monitoring the activity of an enzyme or pathway of the cascade using several products instead of only one main product, the up and down-regulation of distinct pathways can be deduced with higher certainty. Moreover, routes of formation and crosstalk between the branches of the AA cascade can be determined [8]. Because many pharmaceuticals modulate the AA cascade, quantification of oxylipins is also crucial for the determination of in-vivo target engagement of established drugs, for example aspirin, and new experimental drugs, for example sEH inhibitors. This article briefly summarizes the current state of liquid chromatography–mass spectrometry (LC–MS)-based targeted metabolomics of the AA cascade and focuses on the challenges of the quantification of oxylipins in biological matrices.

Instrumental analysis

Current targeted metabolomics LC–MS approaches for the AA cascade have impressive characteristics. One method enables parallel analysis of 141 lipid mediators derived from different n6 and n3-FAs. Of these, 102 can be quantified against standards using 30 stable-isotope-labelled internal standards (IS) in a run time of only 25 min with high sensitivity (limit of detection (LOD) 0.1–1 pg on column) [9]. Several other approaches have similar performance, e.g. parallel quantification of 104 oxylipins with the use of 11 IS in 26 min with LODs of 1.8–340 pg on column [10], or quantification of 88 analytes (6 IS) in 21 min with limits of quantitation (LOQ) of 0.06–15.96 pg on column [4, 11, 12]. For methods established in other labs the total number of covered analytes is not clear because the articles focus on groups of analytes, e.g. resolvins, or only report oxylipins above the LOQ [5, 13, 14]. However, analysis times (of approximately 25 min) and sensitivities (LOD 0.01–0.21 pg on column) are comparable [5, 13, 14].

All of these methods use reversed-phase (RP) LC coupled to a highly sensitive triple-quadrupole (QqQ) MS instrument using negative electrospray ionization (ESI) of the slightly acidic analytes (fatty-acid derivatives). For the analysis of oxylipins in biological samples, the following challenges have to be addressed by the LC–MS method:

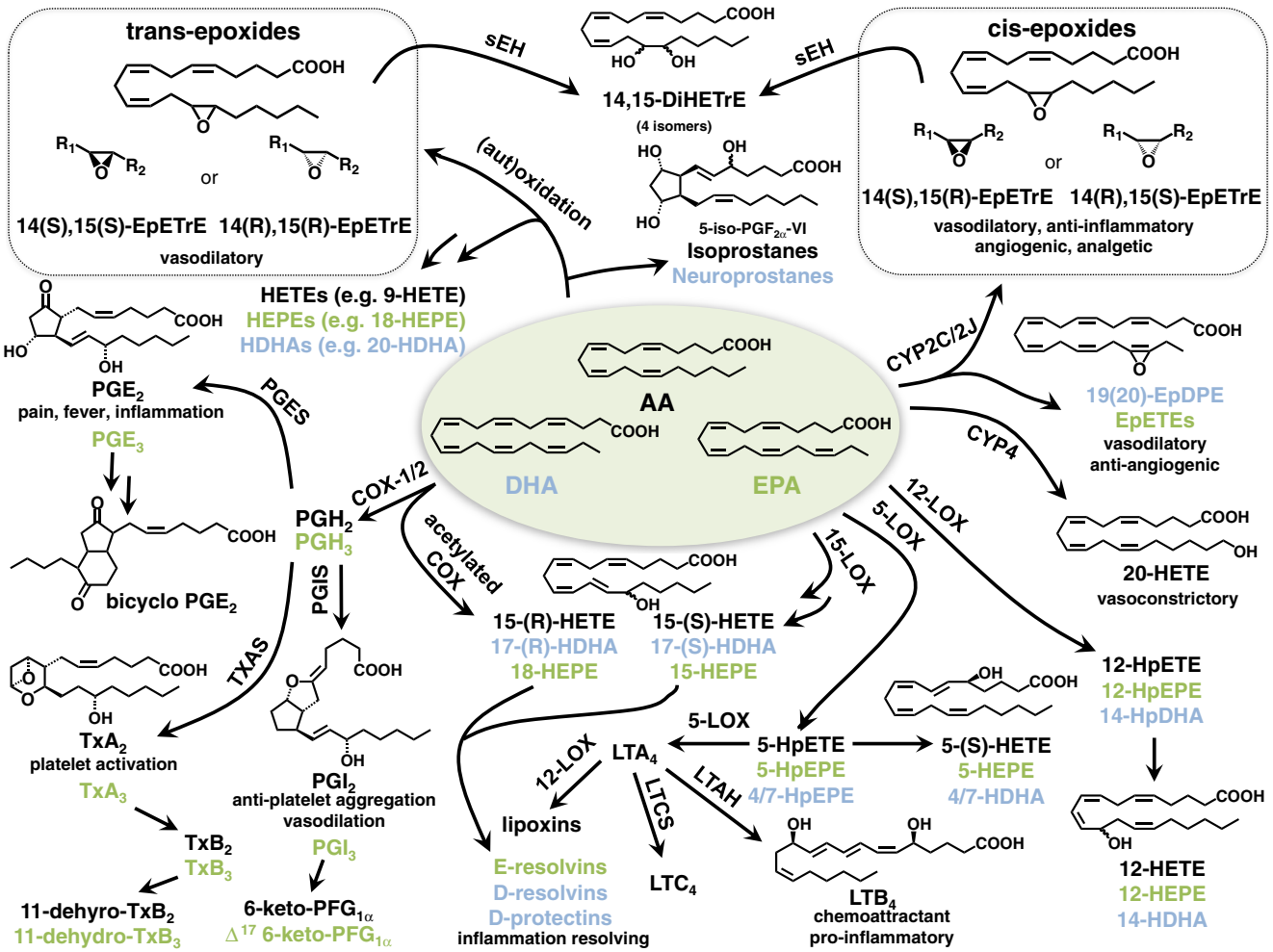

Fig. 1 Illustrative overview of the AA cascade, randomly highlighting a few metabolites to illustrate analytical challenges. It should be noted that the product pattern is not representative and the effects on biological function are highly simplified (for a more comprehensive figure of AA metabolites refer to Refs. [1, 10])

1. Low (<nmol L^{-1}) concentration of the analytes;
2. Huge concentration differences between the least and the most abundant oxylipin within a single sample (>10^3-fold), requiring a broad linear detector response [15, 16];
3. Correct identification of the LC–MS peaks; and
4. Simultaneous quantification of a multitude of chemically and structurally similar analytes, with numerous isomers present in all samples, e.g. regioisomers of hydroxy-FAs or PGE_2 and PGD_2 (Fig. 1).

Although the sensitivity and linear detector response of modern QqQ-MS fulfill the demands of 1 and 2, confirming the identity of the analytes (3) requires meaningful fragment spectra to be obtained [14, 17]. Because all oxylipins can occur in the form of several isomers, identification only by retention time and selected reaction monitoring (SRM) of the analyte's transition can lead to false conclusions. Therefore, many groups use QTRAP instruments for oxylipin analysis, enabling the second analytical quadrupole to be operated as a linear ion trap to obtain high-quality fragment spectra. Selective detection (4) requires both a highly efficient chromatographic separation and detection in SRM mode. This can be observed for the analysis of 8, 9, and 12-HETE (Figs. 1 and 2). Despite high chromatographic resolution using a modern <2 μm particle-filled column, 8 and 12-HETE coelute. In consequence, quantification can only be achieved by detecting unique fragment ions in SRM. For 9 and 12-HETE the opposite is true: the MS–MS spectra of both compounds are very similar and provide no specific SRM transitions. Therefore, these OH-FAs have to be separated chromatographically.

The narrow oxylipin peaks resulting from modern LC methods require rapid switching times and sensitivity at short dwell times of the MS. To keep cycle times of the MS short while simultaneously providing enough data points per peak, all current methods use software-assisted features, for example scheduled or dynamic multiple reaction monitoring (MRM). Nevertheless, the narrow elution windows of a large number of analytes still require dwell times of less than 10 ms.

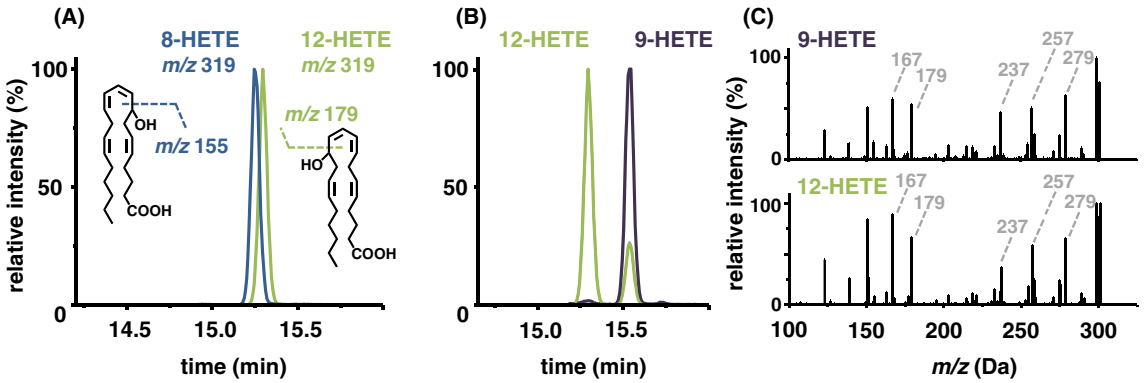

Fig. 2 Separation of regioisomers of hydroxy-AA (8, 9, and 12-HETE) by means of RP-18 LC and ESI-MS–MS. (**a**) Co-elution of 8 and 12-HETE, which can be detected independently on the basis of specific SRM transitions. (**b**) Chromatographic separation of 9 and 12-HETE. Both have interfering SRM transitions, resulting from almost-identical collision-induced-dissociation MS–MS spectra of their $[M-H^+]^-$ ions (**c**)

It will be interesting if other MS detector types become available for targeted oxylipin analysis. High-resolution MS (HRMS) could improve the signal-to-noise ratio but does not enable the numerous constitutional isomers to be distinguished, meaning detection in SRM mode is mandatory for this analysis. It will be interesting to determine whether modern quadrupole time-of-flight (qTOF) instruments can provide sufficient sensitivity and linear detector response. For orbitrap instruments, the main question is whether the cycle time for SRM detection could be short enough to enable quantification of the narrow LC peaks. The first report of oxylipin analysis using an orbitrap instrument is promising: the comparison of quantitative performance between the orbitrap and a QqQ-MS revealed similar results with respect to linear detection ranges, with LODs for the orbitrap in the range of 10–30 pg for oxylipins [18]. If these instruments prove able to match the rapid, sensitive, and robust quantification of QqQ-MS, the combination of quantification with HRMS and continous detection of fragment spectra would greatly enhance instrumental oxylipin analysis.

As well as improvements to LC–MS instruments, derivatization could lead to a better separation efficacy and, particularly, improved MS detection. Bollinger et al. introduced a derivatization strategy which converts the carboxy moiety of the oxylipins to an amide with positively charged *N*-(4-aminomethylphenyl) pyridinium (AMPP) [19], enabling detection in positive-ESI mode. This strategy could also improve sensitivity on today's instruments, although they have a more efficient ion transmission in negative-ESI mode than the instruments used by Bollinger et al.

Derivatization to pentafluorobenzyl (PFB) esters was used by Mesaros and Blair, enabling efficient normal-phase chiral separation of oxylipins (see below). Furthermore, dedicated ion formation by electron-capture atmospheric-pressure chemical ionization (APCI) increased both selectivity and sensitivity through decreasing background signals [2]. APCI is generally acknowledged to be less prone to matrix effects than ESI. Hence, a similar derivatization is promising to circumvent the massive problems resulting from ion suppression in RP-LC–ESI(−)-MS of oxylipins in biological samples [12]. New derivatization agents with specific ionization, e.g. electrochemistry-assisted ionization [20], or novel ionization techniques, for example dielectric-barrier-discharge ionization-MS [21], could further improve selectivity, sensitivity, and robustness of oxylipin detection by mass spectrometry.

Sample preparation

Extraction of free oxylipins from a biological matrix, like plasma or tissues, is not trivial. The analytes have a broad polarity range and are prone to degradation by autoxidation (all oxylipins) and by base (PGs) or acid (epoxy-FA) treatment. When analyte concentrations are well above the LOQ of the instrument—which is rarely the case—it is possible to directly inject the sample after dilution and/or protein precipitation with or by organic solvents. However, most analyses require pre-concentration. For this purpose liquid–liquid extractions (LLE) [22] or, most frequently, solid-phase extractions (SPE) [4, 9, 10, 13, 14] are used. In contrast to similar instrumental analyses of oxylipins, SPE procedures differ substantially from lab to lab. Stationary phases used range from RP-18 [14] and mixed-mode phases with RP-8 and anion-exchange properties [13] to polymeric phases [4, 9, 10]. As might be expected, these methods have dissimilar performance, leading to different oxylipin patterns for the same sample, particularly for epoxy-FAs (Fig. 3) [12]. The ESI-interfering matrix in plasma is most efficiently removed by specific SPE procedures using anion-exchange stationary phases [13] (carboxy acid moiety of the oxylipins) or polar and non-polar washing steps (water and *n*-hexane) before elution of the medium to non-polar oxylipins with methyl formate on the tC-18 column [14]. The latter procedure, on standard RP material, overall outperforms the other procedures with respect to recovery of IS, reduction of ion-

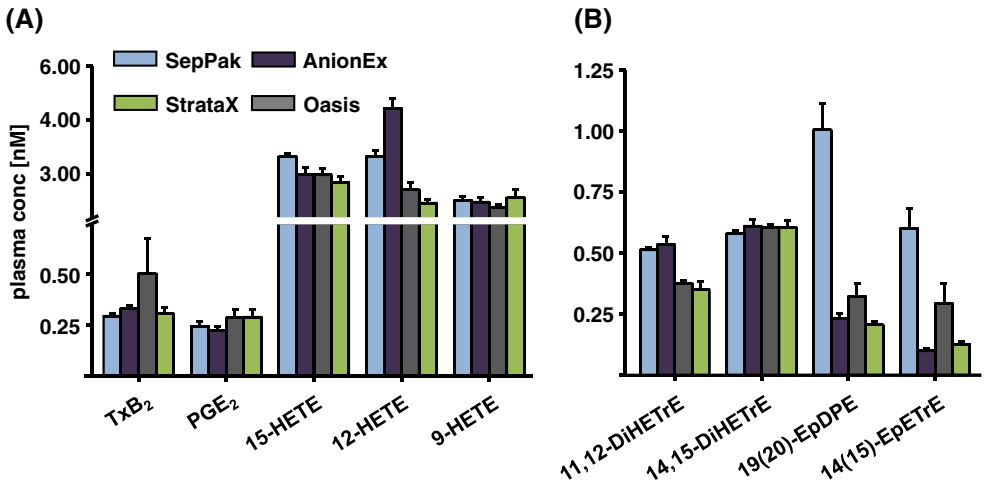

Fig. 3 Concentrations of selected oxylipins in combined human EDTA plasma, obtained using different well-established solid-phase-extraction (*SPE*) methods for sample preparation. (**a**) Prostanoides and hydroxy-FA of AA, and (**b**) dihydroxy-FA and epoxy-FA of AA and DHA. All results are shown as mean±SD (n=5). The following procedures were used: SepPak: tC18 phase, washing with water and *n*-hexane, elution with methyl formiate [14]; AnionEx: C8 phase with anion-exchange properties, washing with methanol–water (*v*/*v*, 1:1), elution with ethyl acetate–*n*-hexane (75:25) acidified with 1% acetic acid [13]; StrataX: polymeric phase with polar groups, washing with 10 % methanol, elution with methanol [9]; Oasis: polymeric phase with polar groups, washing with 5 % methanol acidified with 0.1 % acetic acid, subsequent elution with methanol and ethyl acetate [4]. Instrumental analysis was performed as described in Refs. [4, 11]. The complete evaluation of the performance of the procedures can be found in Ref. [12]

suppressing matrix, and extraction efficacy of oxylipins from the biological matrix (Fig. 3).

An adequate sample preparation which eliminates as much matrix as possible is essential, because insufficient removal of interfering matrix compounds is highly problematic for quantitative oxylipin analysis. In all methods, a single internal standard (IS) is used for a whole group of structurally similar analytes eluting at different retention times. Thus the IS cannot compensate for all matrix effects, leading to matrix-dependent over and under-calculations of the oxylipin concentration. In addition to further optimization of sample-preparation procedures, the availability and use of more heavy-isotope-labeled IS would much improve robustness, accuracy, and precision of oxylipin analysis in biological samples.

(Biological) variation of oxylipin concentration

Reports on human serum and plasma oxylipin concentrations document strong interindividual variations between samples [15, 23]. These variations impede the recognition of biologically significant differences in lipid-mediator levels between groups of different (patho)physiological conditions. Moreover, significant effects of pharmacological or nutritional intervention can vanish within high standard deviations and/or standard errors. Part of the variation is obviously based on biological differences, e.g. different habits of human subjects; for example, physical exercise elicits changes in systemic levels of epoxy and dihydroxy-FAs [24]. Little is known about the changes in oxylipins during the circadian rhythm; however, it is clear that nutritional status strongly affects the levels of circulating lipids and lipid mediators. Even a single moderate dose of n3-PUFA causes changes in plasma hydroxy, epoxy, and dihydroxy-FA levels [25]. Thus, sample collection from human subjects should be as standardized as possible (e.g. fixed fasting period, time of day, and physical activity).

Another major problem contributing to poor precision in the analysis of biological samples is the formation and degradation of oxylipins after sample collection. Even short storage of blood before further processing has massive effects on the plasma concentration of several oxylipins (Fig. 4). Moreover, if the sample sits for a few minutes in the centrifuge, after centrifugation and before the plasma is collected and frozen, the levels of some oxylipins are significantly reduced (Electronic Supplementary Material, ESM). Prolonged storage in the freezer can also lead to degradation and loss of analytes, as revealed for several DHA and EPA-derived resolvins and prostanoides [5]. In a few studies COX and sEH inhibitors, as well as protease and esterase inhibitors, are added to the samples to prevent enzymatic formation and/or degradation [26]. More frequently antioxidants, for example radical-scavenging butylated hydroxytoluene (BHT) and chelating ethylenediaminetetraacetic acid (EDTA), are used to prevent oxylipin degradation or formation (e.g. 11-HETE, 9-HETE, isoprostanes) by autoxidation during sample preparation [4, 10, 11, 26]. However, the benefit of these procedures has not yet been systematically evaluated for a comprehensive set of oxylipins. Overall, artificial (ex-vivo) formation and/or degradation of the lipid mediators is one of the major challenges of

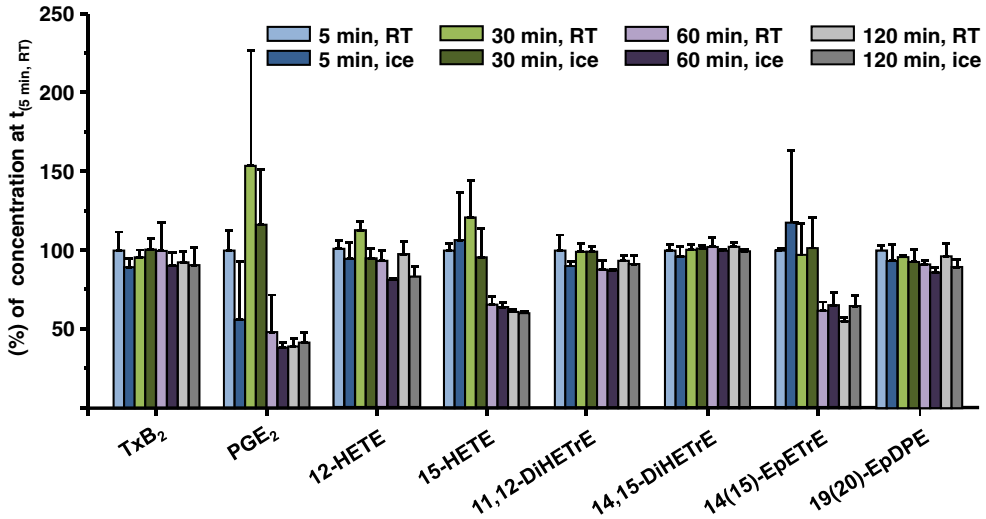

Fig. 4 Ex-vivo degradation and formation of oxylipins in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, combined, and left for 5 min, 30 min, 60 min, or 120 min either at room temperature (RT) or on ice. After centrifugation (1200*g*, 15 min, 4 °C), the plasma was immediately frozen (−80 °C) and the oxylipin concentration was analyzed within five days (Oasis SPE [4, 11, 12]). The resulting concentrations after different periods of time in sample preparation are compared with those with direct sample preparation ($t_{(5min,\ RT)}$). The results clearly reveal that after 60 min storage of whole blood the levels of several oxylipins are greatly reduced (e.g. 15-HETE and 14(15)-EpETrE), whereas other analytes are formed ex-vivo (e.g. PGE_2). Values are shown as mean±SD (n=4). More results about the effect of storage during sample preparation of human blood can be found in the ESM

analysis of biological samples, which can only be addressed by strict standard operating procedures (SOPs) including rapid sample processing and optimized storage conditions (−80 °C, short time). For a few oxylipins, ex-vivo formation and/or degradation can be excluded and/or measured by additional determination of their endogenously formed metabolites, e.g. 11-dehydro-TxB_2 [27] with TxB_2, or bicyclo-prostaglandin E_2 as a stable degradation product of PGE_2 [28].

Plasma or serum?

Both plasma and serum should be regarded as appropriate matrices for quantitative oxylipin analysis of circulating oxylipins [5, 16]. For plasma, the anticoagulant should be chosen carefully: EDTA seems to be the best choice because, for example, heparin is known to cause artifacts [29]. For serum it has to be kept in mind that coagulation is in part mediated by the AA cascade and causes massive (ex-vivo) formation of several oxylipins, including TxB_2 and 12-HETE (Fig. 1). Moreover, detectability of low-concentration mediators, for example resolvins, is improved [5]. Regarding variability, it remains to be evaluated whether plasma or serum enables the determination of oxylipin concentrations in blood with higher precision.

Accuracy and inter-lab comparability

In addition to high intersample variation, huge differences between mean concentrations of lipid mediators were found in different studies, e.g. for human plasma or serum (summarized in [15]). This indicates that the accuracy of current methods is a further problem. Because of the above-summarized difficulties of oxylipin analysis and their nature as endogenously formed lipid mediators (biomarkers), validation procedures suggested for drugs, e.g. by the European Medicines Agency (EMA), are not or are only in part applicable. With respect to accuracy, current methods have been used to determine, for example, the recovery in spiked plasma [10] or in (matrix-free) saline phosphate buffer [4]. Interestingly, others failed to recover oxylipins from buffer, probably because of low solubility of oxylipins in the aqueous solution [10]. Regardless of how reliable recovery rates in (spiked) quality-control samples are determined, the quantification relies on the availability and purity of reference standards. In most cases these compounds have to be synthetized or are obtained commercially (currently from a single company). For the latter, the affordable quantities are so low that purity cannot be checked by standard chemical methods. Thus, a mistake in the concentration provided by the manufacturer leads directly to systemic errors. Matching LC–MS response from batch to batch and comparing peak areas of regioisomers in selected ion monitoring mode under isocratic LC conditions are the only possible ways to verify manufacturer information. To improve quality of results it will be important to agree on criteria which have to be fulfilled for a fit-for-purpose validation in oxylipin analysis. With an exchange of samples, shared standards between groups, and inter-laboratory tests, this would help improve comparability.

The free, the bound, and the total… – or the analytical challenge of detecting esterified oxylipins

Although a substantial portion of oxylipins is incorporated in lipids, it is believed that their paracrine and autocrine action is mainly mediated by their free, i.e. non-esterified, form [16]. The esterified (bound) oxylipins can readily be liberated, e.g. by phospholipases [30]. In plasma, the concentrations of esterified epoxy-FAs and hydroxy-FAs exceed the concentrations of the free ones by approximately 50 to 350-fold and 10 to 40-fold, respectively [13, 16]. Esterified oxylipins are commonly quantified after saponification (base hydrolysis) [13, 16, 31–33]. For this purpose, the samples are incubated with sodium hydroxide (1.00–3.75 mol L^{-1}); either overnight at 4 °C [31], or at 60 °C for 20–30 min [13, 16]. Other methods incubate the extracted lipids with 0.1 mol L^{-1} sodium carbonate at 4 °C overnight [33], or perform transesterification of lipids to methyl esters (0.35 mol L^{-1} sodium methoxide solution for 60 min at 60 °C) with subsequent hydrolysis by the addition of water (60 min) [32]. The performance of the different cleavage procedures has not been systematically compared. However, alkaline treatment degrades a large number of oxylipins—particularly PGs, e.g. β-hydroxy-keto prostanoids including PGE_2, PGD_2, or thromboxanes [31, 32]—and thus information on their concentration is lost. Harsh alkaline treatment could even lead to the formation of conjugated FAs from PUFA [34], and thus is prone to produce artifacts of the polyunsaturated analytes. In contrast, a moderate saponification could lead to incomplete liberation of esterified oxylipins. All current cleavage techniques yield a combined sum of free and esterified oxylipins, and provide no information on how the analytes are bound in the samples. Yet it is highly relevant to know whether a mediator is bound to the sn2-position of a phospholipid of the cell membrane, and thus rapidly releasable by phospholipases upon inflammatory stimuli, or if it is bound in a triacylglyceride (fat) with an unknown biological fate. One way of addressing this problem would be to separate the different lipid classes of a lipid extract before hydrolysis, as commonly performed for fatty-acid analysis [35]. Another possibility is direct detection of the esterified oxylipins, as recently successfully performed, e.g. for C16:0/12-HETE-phosphatidylethanolamine [36]. Combining all oxylipins and possible lipids results in a fairly large number of analytes. Thus, the integration of targeted oxylipin metabolomics in lipidomics is one of the biggest challenges for analytical chemistry of oxylipins. However, the low (total) concentration of the lipid mediators makes it doubtful whether the sensitivity of today's instruments is sufficient for their detection if they are distributed in several individually detected lipids.

Detection of stereoisomers

A major task for instrumental analytical methods, and one which it is important to address, is differentiation between stereochemical configurations. Cis–trans isomers of epoxides (Fig. 1) can be well resolved by RP chromatography. The enzymatically formed cis-isomers elute first, followed by the trans-epoxides formed by (aut)oxidation [37] (the same SRM transition as the cis-isomer, eluting 1–3 min later). Although quite large peaks of trans-epoxides are observed for biological samples, particularly after conjugate cleavage, these metabolites are not included in most current methods. Thus the concentrations of trans-epoxides are not evaluated, which makes it impossible to assess their effect as lipid mediators. In particular, the important question of whether and to what extent trans-epoxy-FAs contribute to the biological effects attributed to cis-epoxy-FAs cannot be evaluated.

The robust and efficient RP chromatography fails to separate formed enantiomers. Thus, for example, the lipid mediator referred to as 14(15)-EpETrE (or 14(15)-EET) is in fact four compounds, which are generated via CYP conversion and (aut)oxidation processes and can be hydrolyzed by sEH at isomer-specific rates [37] (Fig. 1). In addition to the epoxides almost all oxylipins are chiral, however, the specific biological activity of most sterioisomers is not known.

Whereas enzymatic routes form products at a distinct enantiomer ratio, aut(oxidation) processes result in the formation of racemates. Therefore, chiral separation can be very helpful to differentiate between the routes of formation. Moreover, in several cases different enzymatic routes of formation can be distinguished on the basis of the enantiomer ratio, e.g. (±)15-HETE (Fig. 1). Whereas the formation of 15-(S)-H(p)ETE is catalyzed by 15-LOX, 15(R)-H(p)ETE is formed by aspirin-acetylated COX-2 [2]. As revealed for this example, chiral separation would greatly assist in distinguishing the route of formation of the lipid mediators, which is poorly understood for several oxylipins, e.g. for the dominantly formed and bioactive 18-HEPE (Fig. 1). Several chiral chromatographic separation methods have been developed, as recently summarized by Mesaros and Blair [19]. However, because chiral LC cannot achieve the robustness and (overall) separation power of RP chromatography, it is comparatively rarely used. Thus, targeted oxylipin metabolomics would greatly benefit from progress in chiral LC–MS approaches [38]. A promising further tool for enantiomer separation might be ion-mobility spectrometry using a chiral modifier [39] at the front end of the MS or (chiral) supercritical fluid chromatography. Of all challenges for instrumental analytical chemistry mentioned in this article, the integration of chiral separation into routinely used targeted metabolomics techniques would have the largest effect on our understanding of the biology of oxylipins. With the data resulting from these methods, one could not only monitor the activity of distinct enzymatic and (aut)oxidative pathways in-vivo, but also identify the biologically most active isomers.

Outlook

Highly sensitive LC–MS methods have been developed which enable an impressive understanding of the biological importance of the lipid mediators formed in the AA cascade. However, numerous questions remain to be answered, e.g., determining the mechanisms of the effects of dietary n3-PUFA intake on human health. Comprehensive investigation of hydroxy-n3-PUFAs, resolvins, and n3-epoxides by methods summarized in this article could enable researchers to address this question.

With today's ultra-high-performance liquid chromatography and high-end QqQ-MS, the instrumentation for highly sensitive and specific detection of oxylipins is available. Moreover, new HRMS instruments may bring about much progress in targeted lipid-mediator analysis; with these instruments it is easy to generate peaks, areas, and numbers. However, it will still be a challenge to obtain meaningful results, i.e. accurate concentrations in biological samples. In particular, the optimization of sample collection, stabilization, and preparation seem to be required. With respect to instrumental analysis, the greatest challenges are differential detection of stereoisomers and analysis of esterified oxylipins. To achieve this progress in targeted oxylipin metabolomics, it is crucial that analytical chemistry is regarded as an integral part of medical and biological research. Thoroughly developed methods and their continuous improvement require time and (grant) money. Although not all method developments may address fundamental scientific questions (for example the detection of esterified oxylipins), improvements to sample preparation, a fit-for-purpose validation, and interlab comparison seem to be of particularly high importance for the field.

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Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Targeted metabolomics of the arachidonic acid cascade: current state and challenges of LC-MS analysis of oxylipins

Ina Willenberg, Annika I. Ostermann, Nils H. Schebb

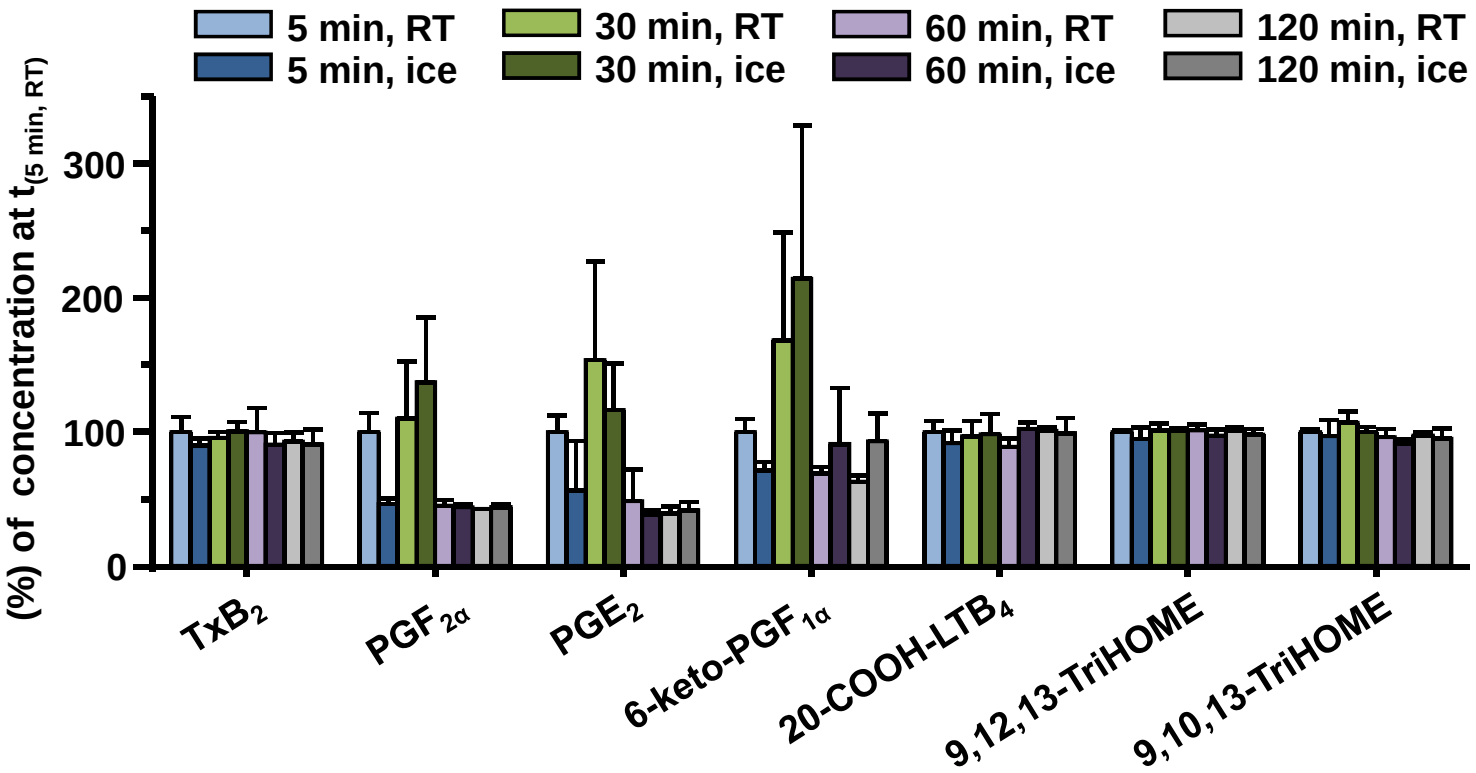

Fig. S1: *Ex vivo* degradation/formation of TxB_2, prostaglandins (PGs) and trihomes in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE, [4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5\ min,\ RT)}$). In comparison to 5 min storage at RT the concentration of the PGs was significantly decreased already 5 min after storage on ice. After 30 min (ice and RT) the PG levels were increased before they finally decreased again after 60 min. The concentrations of TxB_2, 20-COOH-LTB_4 and trihomes were stable up to 120 min either on ice or at RT. Shown are mean ± SD (n=4).

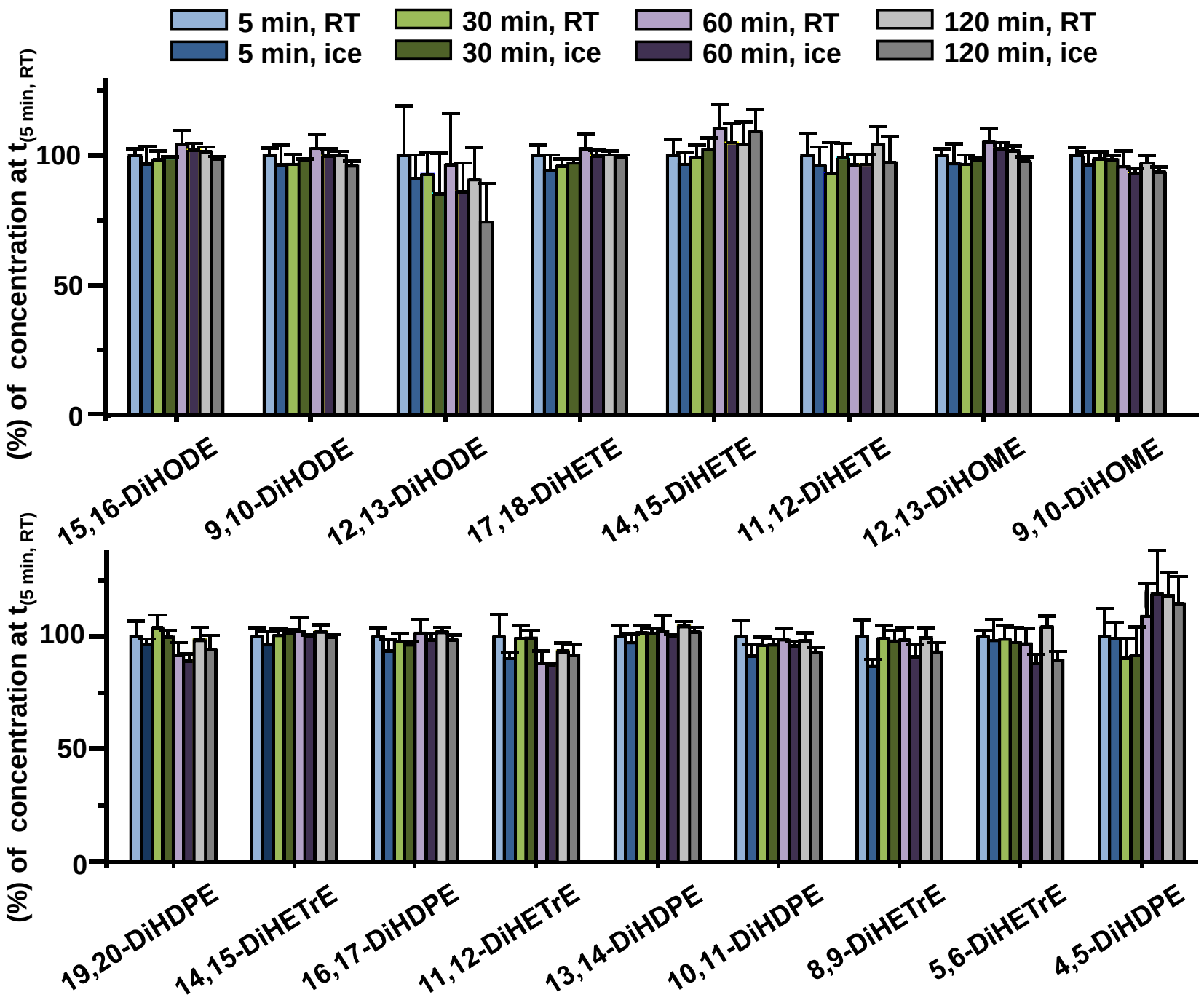

Fig. S2: *Ex vivo* degradation/ formation of diols in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE, [4])The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5\ min,\ RT)}$). Shown are mean ± SD (n=4).

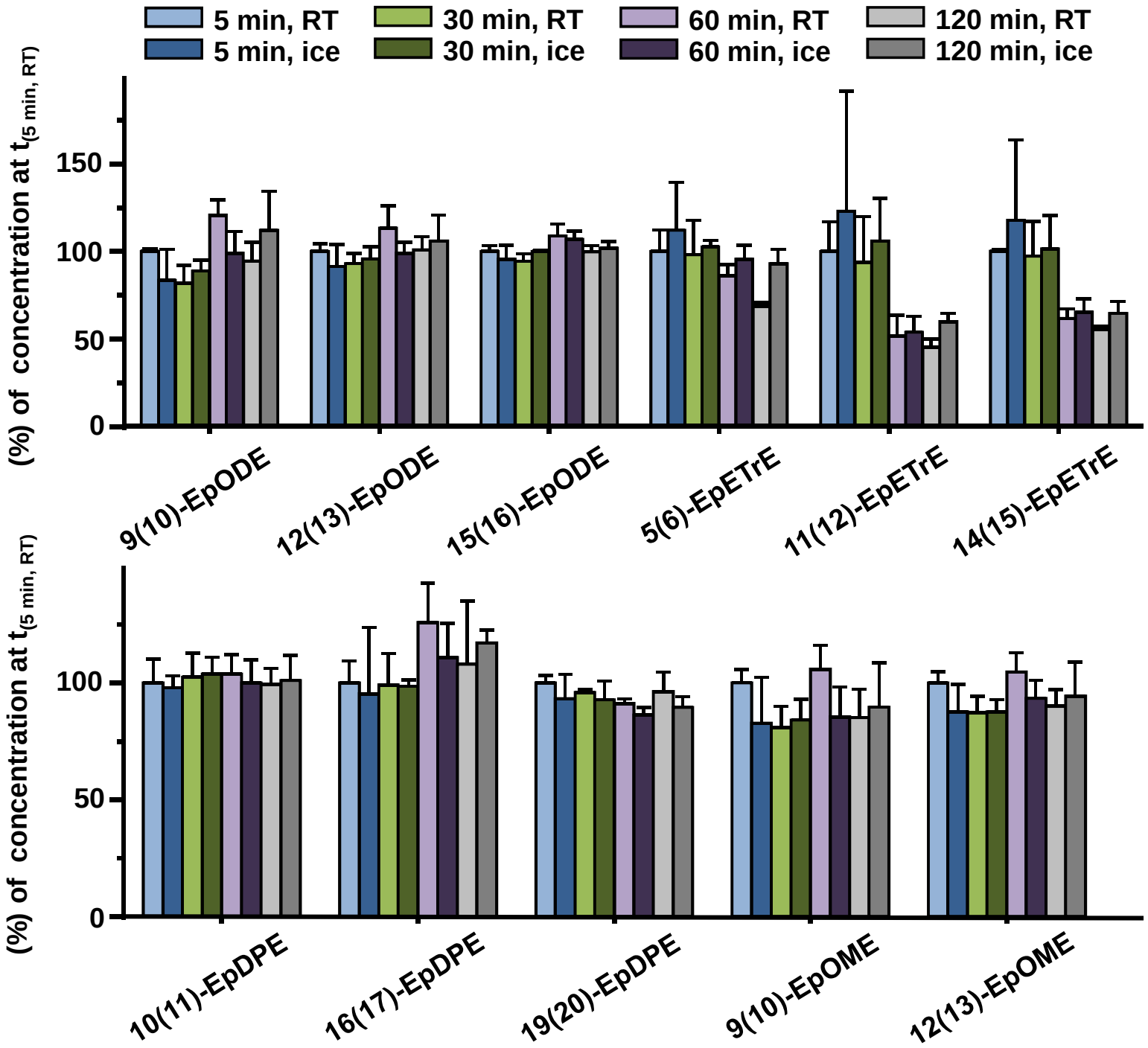

Fig. S3: *Ex vivo* degradation/ formation of epoxides in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE, [4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5\ min,\ RT)}$). The concentrations of 11(12)- and 14(15)-EpETrE were halved after 60 min of storage either on ice or at RT. The other epoxides were not affected up to 120 min of storage. Shown are mean ± SD (n=4).

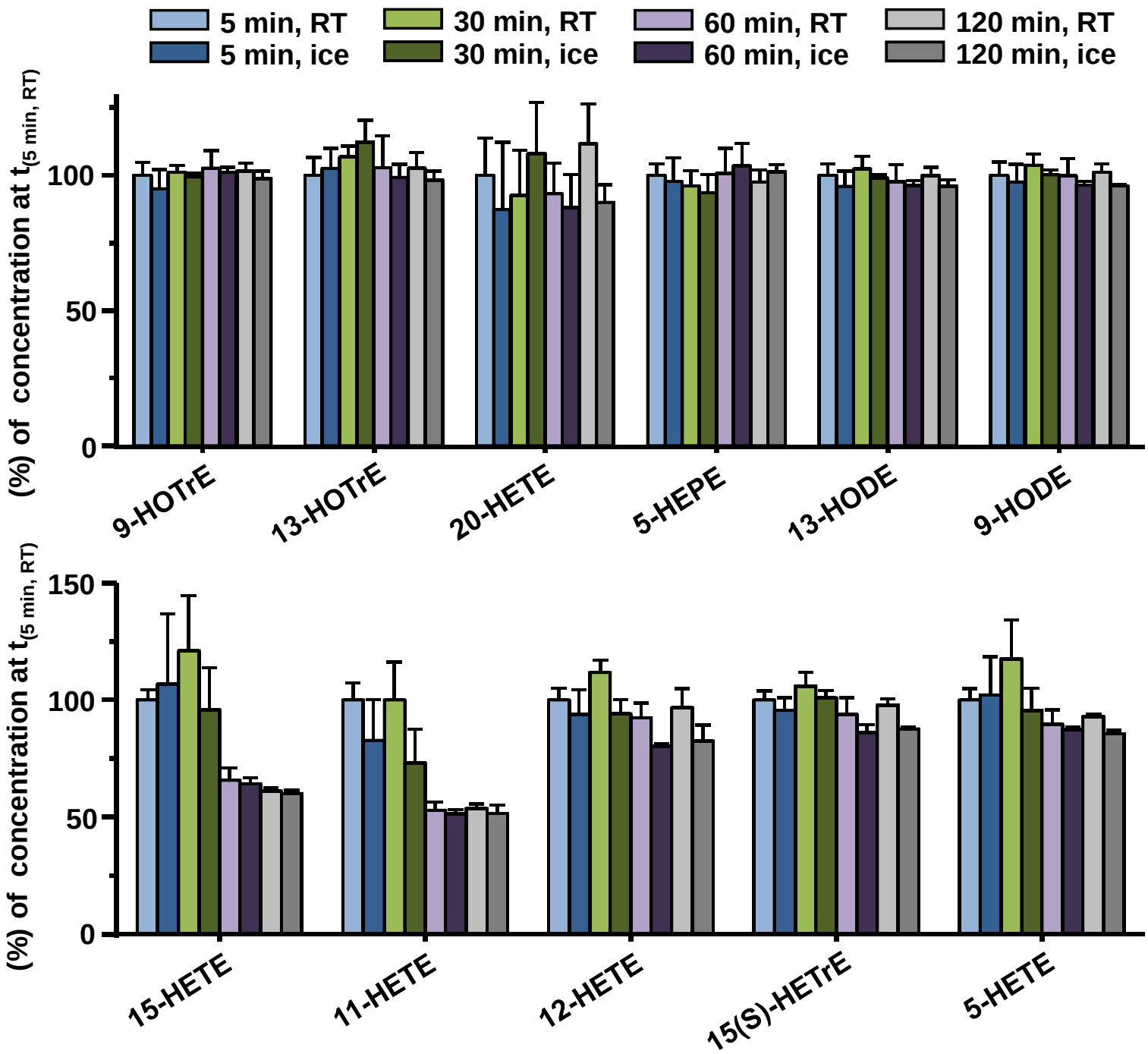

Fig. S4: *Ex vivo* degradation/ formation of alcohols in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE, [4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5\ min,\ RT)}$). While concentrations of 11- and 15-HETE halved after 60 min on ice or at RT, the levels of further alcohols were stable up to 120 min. Shown are mean ± SD (n=4).

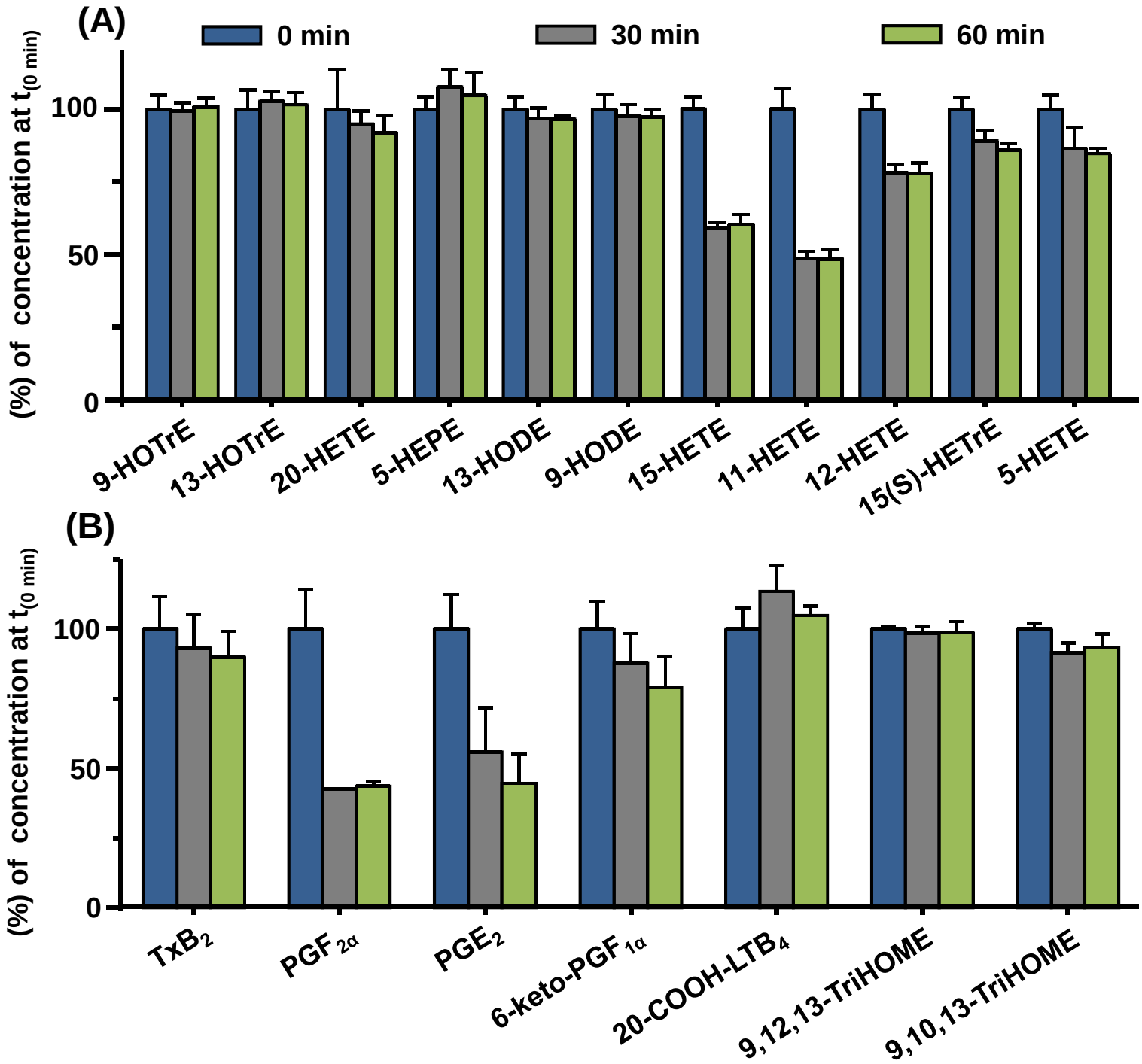

Fig. S5: *Ex vivo* degradation/ formation of alcohols **(A)** as well as TxB_2, prostaglandins and trihomes **(B)** in freshly centrifuged human plasma. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and centrifuged (1200 x g, 15 min, 4 °C) after 5 min at room temperature (RT). The resulting plasma was left with the cell pellet for 0 min, 30 min or 60 min on ice before freezing at -80 °C till analysis. Oxylipin concentrations were analyzed within 5 days (Oasis SPE, [4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(0\ min)}$). While the concentrations of 11- and 15-HETE halved in the first 30 min after centrifugation, most other alcohols showed no losses **(A)**. The levels of PGE_2 and $PGF_{2\alpha}$ significantly decreased in the first 30 min of storage while TxB_2, 20-COOH-LTB_4, 6-keto-$PGF_{1\alpha}$ and the trihomes showed no changes up to 60 min **(B)**. Shown are mean ± SD (n=4).

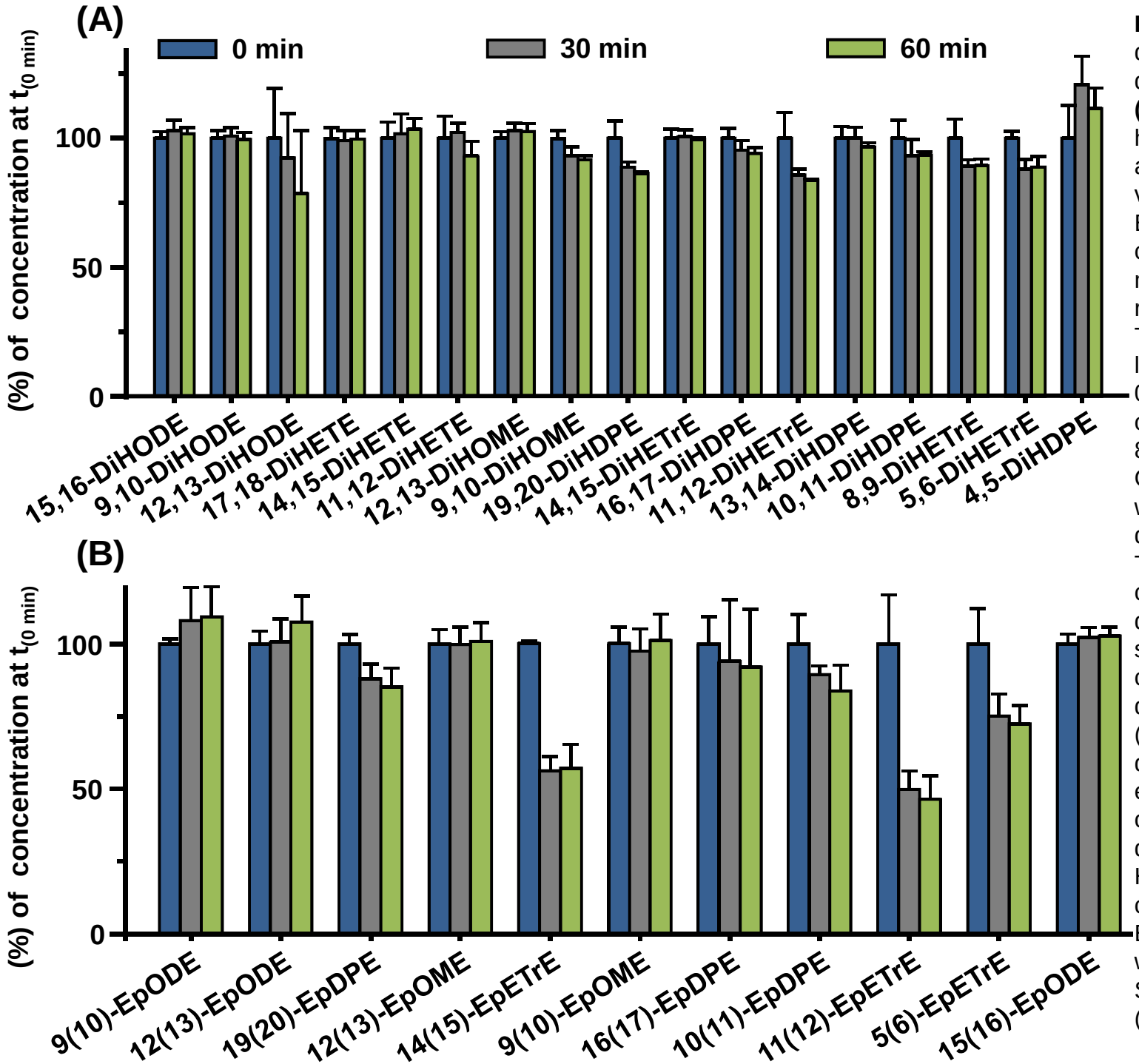

Fig. S6: *Ex vivo* degradation/ formation of diols **(A)** and epoxides **(B)** in freshly centrifuged human plasma. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and centrifuged (1200 x g, 15 min, 4 °C) after 5 min at room temperature (RT). The resulting plasma was left with the cell pellet for 0 min, 30 min or 60 min on ice before freezing at -80 °C till analysis. Oxylipin concentrations were analyzed within 5 days (Oasis SPE, [4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(0\ min)}$). The detected diols **(A)** and most of the epoxides **(B)** showed no or only minor losses during this storage. However, the concentrations of all EpETrEs decreased within the first 30 min. Shown are mean ± SD (n=4).

Tab. S1: Concentrations of oxylipins in plasma obtained from whole blood after different storage conditions. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE, [4 ,11-12]). Shown are mean ± SD (n=4).

analyte	5 min RT (pM)			5 min, ice (pM)			30 min RT (pM)			30 min, ice (pM)			60 min RT (pM)			60 min, ice (pM)			120 min RT (pM)			120 min, ice (pM)		
	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD
TxB_2	392.2	±	45.3	350.7	±	22.3	375.3	±	17.7	394.4	±	27.4	391.2	±	70.5	354.8	±	33.3	363.0	±	27.3	355.9	±	42.6
$PGF_{2\alpha}$	164.6	±	23.5	76.2	±	6.9	180.6	±	70.7	225.3	±	79.7	74.2	±	7.4	72.2	±	3.9	< LOQ (70.3)	±	-	72.5	±	3.4
PGE_2	27.4	±	3.4	15.4	±	10.1	42.1	±	20.1	31.9	±	9.5	13.2	±	6.4	10.5	±	0.9	10.7	±	1.4	11.4	±	1.7
6-keto-$PGF_{1\alpha}$	206.3	±	20.7	147.3	±	12.5	347.0	±	166.3	441.4	±	236.2	142.2	±	10.9	187.8	±	85.5	129.6	±	9.6	192.0	±	42.5
20-COOH-LTB4	310.8	±	23.7	284.7	±	28.6	300.5	±	37.1	305.4	±	46.0	276.5	±	18.1	317.7	±	14.1	313.4	±	8.2	307.8	±	34.6
9,12,13-TriHOME	42610.0	±	433.4	40336.2	±	3574.8	43035.5	±	2310.0	42953.5	±	822.2	43074.5	±	1888.0	41452.0	±	1849.6	42914.7	±	964.1	41698.5	±	1757.0
9,10,13-TriHOME	1990.1	±	36.6	1926.4	±	246.7	2124.8	±	172.9	1987.2	±	77.1	1911.2	±	117.7	1819.4	±	63.2	1937.0	±	41.7	1886.4	±	158.0
9-HOTrE	2200.9	±	103.5	2092.3	±	158.1	2226.5	±	57.1	2187.5	±	31.0	2260.8	±	143.5	2224.8	±	40.8	2235.3	±	66.2	2173.2	±	59.4
13-HOTrE	1634.2	±	108.0	1677.5	±	121.6	1744.5	±	68.1	1835.2	±	131.4	1679.2	±	194.0	1624.0	±	76.5	1676.4	±	95.9	1606.6	±	52.0
20-HETE	785.1	±	108.0	685.9	±	195.1	726.4	±	132.4	848.6	±	146.9	731.7	±	89.3	692.6	±	93.9	876.9	±	115.0	707.1	±	51.0
5-HEPE	134.8	±	5.8	131.9	±	11.5	129.5	±	7.6	126.2	±	9.0	135.8	±	12.5	139.5	±	11.5	131.5	±	6.0	136.6	±	3.4
13-HODE	27428.5	±	1177.5	26318.2	±	1528.5	28053.2	±	1255.3	27127.2	±	404.2	26768.7	±	1707.2	26375.3	±	485.4	27401.5	±	873.0	26364.3	±	623.0
9-HODE	30861.3	±	1546.6	30095.2	±	2043.4	32013.5	±	1276.3	30922.3	±	591.4	30790.2	±	1985.8	29731.1	±	459.0	31222.3	±	965.3	29639.9	±	173.8
15-HETE	1233.2	±	52.2	1315.9	±	369.7	1492.0	±	287.9	1180.6	±	223.9	809.7	±	65.1	792.0	±	30.7	753.2	±	19.7	741.4	±	17.9
11-HETE	665.3	±	48.8	550.6	±	115.6	665.4	±	106.8	487.1	±	94.8	352.2	±	23.3	342.4	±	10.7	356.9	±	13.1	343.4	±	23.3
12-HETE	988.5	±	49.1	926.5	±	104.3	1103.9	±	52.0	929.6	±	60.1	912.7	±	62.8	794.4	±	8.4	955.6	±	79.5	814.4	±	66.7
15(S)-HETrE	394.6	±	16.0	376.9	±	21.1	418.1	±	23.1	398.2	±	12.9	370.1	±	28.4	339.3	±	13.4	386.2	±	9.4	345.6	±	3.1
5-HETE	1190.5	±	56.7	1215.9	±	195.1	1398.8	±	198.6	1137.9	±	110.8	1067.4	±	74.0	1038.7	±	14.8	1105.9	±	9.1	1018.8	±	16.9
15,16-DiHODE	25001.4	±	668.0	24183.1	±	1704.3	24578.8	±	907.2	24783.0	±	77.5	26114.0	±	1384.9	25527.1	±	674.5	25368.4	±	466.1	24684.7	±	248.9
9,10-DiHODE	707.7	±	20.4	682.8	±	53.7	683.7	±	27.6	694.8	±	4.9	727.3	±	38.2	706.6	±	19.2	708.4	±	11.6	679.5	±	14.0
12,13-DiHODE	675.3	±	130.2	616.6	±	60.0	626.6	±	58.2	575.9	±	106.2	650.4	±	135.4	581.6	±	74.5	612.2	±	83.5	502.4	±	100.5
17,18-DiHETE	341.3	±	14.2	321.4	±	21.0	326.8	±	10.5	331.2	±	5.5	350.5	±	18.9	340.7	±	7.7	342.2	±	5.5	339.7	±	2.9
14,15-DiHETE	48.4	±	3.1	46.7	±	2.2	48.1	±	2.3	49.4	±	2.3	53.5	±	4.3	50.7	±	3.6	50.5	±	4.3	52.8	±	4.1
11,12-DiHETE	37.1	±	3.2	35.7	±	2.7	34.6	±	4.4	36.8	±	2.1	35.7	±	1.6	35.8	±	1.5	38.7	±	2.5	36.1	±	3.7
12,13-DiHOME	22190.9	±	591.2	21501.0	±	1736.6	21442.2	±	789.9	21819.0	±	133.1	23326.1	±	1229.5	22776.8	±	525.6	22621.4	±	379.2	21702.3	±	377.9
9,10-DiHOME	10992.6	±	341.1	10604.9	±	560.8	10854.3	±	310.8	10817.8	±	168.8	10511.4	±	692.7	10224.2	±	206.2	10684.2	±	315.0	10294.3	±	228.7
19,20-DiHDPE	3911.3	±	261.2	3769.8	±	102.3	4057.0	±	227.6	3896.6	±	113.7	3584.6	±	220.5	3485.0	±	119.1	3857.0	±	215.3	3685.7	±	241.5

Tab. S1: Continued

analyte	5 min RT (pM)			5 min, ice (pM)			30 min RT (pM)			30 min, ice (pM)			60 min RT (pM)			60 min, ice (pM)			120 min RT (pM)			120 min, ice (pM)		
	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD	mean		SD
14,15-DiHETrE	742.7	±	27.2	714.1	±	46.3	745.7	±	25.5	752.1	±	12.9	758.8	±	46.8	742.1	±	4.3	760.2	±	20.0	739.9	±	9.4
16,17-DiHDPE	329.3	±	12.3	307.7	±	17.7	322.2	±	11.2	316.5	±	5.5	334.0	±	20.1	323.3	±	10.2	336.4	±	6.1	323.5	±	7.5
11,12-DiHETrE	701.3	±	70.4	631.8	±	20.8	694.9	±	39.8	696.4	±	21.9	616.4	±	40.3	612.9	±	4.9	655.4	±	25.0	641.5	±	36.7
13,14-DiHDPE	299.4	±	13.9	290.9	±	11.5	304.4	±	10.5	303.8	±	6.2	306.7	±	20.7	299.8	±	2.1	313.3	±	6.0	305.2	±	6.0
10,11-DiHDPE	264.8	±	18.7	242.1	±	13.4	254.2	±	9.9	254.6	±	7.6	260.6	±	13.4	253.9	±	4.6	260.0	±	9.3	246.5	±	5.0
8,9-DiHETrE	347.4	±	25.7	300.9	±	11.3	344.2	±	20.4	340.4	±	15.8	341.7	±	20.0	316.0	±	18.6	345.6	±	14.7	323.5	±	14.9
5,6-DiHETrE	232.7	±	6.0	228.2	±	22.0	229.7	±	14.6	226.3	±	15.7	225.1	±	16.1	204.6	±	9.6	242.4	±	11.4	208.2	±	9.0
4,5-DiHDPE	425.5	±	53.6	421.3	±	30.5	383.9	±	38.6	389.8	±	53.7	463.6	±	63.1	505.9	±	82.8	503.1	±	42.6	487.2	±	51.1
9(10)-EpODE	171.4	±	2.8	143.1	±	30.7	140.4	±	17.6	152.3	±	10.5	207.0	±	14.6	169.8	±	20.8	162.0	±	18.3	191.8	±	38.7
12(13)-EpODE	203.4	±	8.9	186.0	±	25.8	189.4	±	11.8	194.9	±	13.7	230.4	±	25.7	201.4	±	12.9	205.2	±	15.5	215.5	±	30.2
19(20)-EpDPE	352.9	±	11.2	329.2	±	36.2	338.1	±	4.7	327.4	±	28.8	321.7	±	7.5	304.0	±	11.6	339.8	±	29.6	315.9	±	15.9
12(13)-EpOME	4378.6	±	211.1	3834.2	±	521.0	3813.0	±	321.7	3834.9	±	234.5	4584.6	±	364.3	4087.4	±	337.5	3937.9	±	306.8	4131.8	±	635.3
14(15)-EpETrE	175.7	±	2.0	206.9	±	80.5	170.8	±	34.8	178.2	±	33.7	108.6	±	9.9	114.9	±	13.7	96.9	±	3.6	113.7	±	11.4
9(10)-EpOME	2206.0	±	128.8	1826.3	±	429.4	1785.6	±	199.6	1855.9	±	196.5	2334.7	±	225.2	1886.9	±	283.3	1878.1	±	268.1	1975.9	±	420.4
16(17)-EpDPE	49.0	±	4.7	46.8	±	13.9	48.6	±	6.6	48.3	±	1.4	61.7	±	8.3	54.3	±	7.1	52.9	±	13.3	57.4	±	2.6
10(11)-EpDPE	123.7	±	12.4	121.2	±	6.1	126.7	±	12.5	128.3	±	9.0	128.4	±	10.2	123.7	±	12.1	123.0	±	8.4	125.0	±	13.4
11(12)-EpETrE	184.0	±	30.9	226.3	±	125.6	172.9	±	47.8	195.1	±	44.8	95.3	±	21.4	99.2	±	16.7	83.7	±	8.5	110.4	±	8.4
5(6)-EpETrE	542.6	±	65.9	608.5	±	149.0	532.4	±	106.4	556.4	±	19.4	466.7	±	35.8	518.7	±	43.5	373.0	±	10.5	504.6	±	45.5
15(16)-EpODE	4523.0	±	156.4	4323.6	±	368.6	4272.9	±	191.2	4521.4	±	34.5	4922.4	±	315.7	4832.8	±	219.6	4510.5	±	155.0	4603.0	±	171.0

Tab. S2: Concentrations of oxylipins in freshly centrifuged plasma which was stored on ice for different periods of time. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and centrifuged (1200 x g, 15 min, 4 °C) after 5 min at room temperature (RT). The resulting plasma was left with the cell pellet for 0 min, 30 min or 60 min on ice before freezing at -80 °C till analysis. Oxylipin concentrations were analyzed within 5 days (Oasis SPE [4, 11-12]). Shown is mean (n=4) with SD.

analyte	0 min (pM)			5 min (pM)			30 min (pM)		
	mean		SD	mean		SD	mean		SD
TxB_2	392.2	±	45.3	365.4	±	47.3	352.2	±	37.1
$PGF_{2\alpha}$	164.6	±	23.5	70.3	±	0.0	71.7	±	2.8
PGE_2	27.4	±	3.4	15.3	±	4.4	12.2	±	2.9
6-keto-$PGF_{1\alpha}$	206.3	±	20.7	180.7	±	22.4	163.0	±	23.4
20-COOH-LTB4	310.8	±	23.7	352.6	±	28.7	326.0	±	10.3
9,12,13-TriHOME	42610.0	±	433.4	41995.3	±	934.9	42088.5	±	1699.4
9,10,13-TriHOME	1990.1	±	36.6	1821.6	±	67.5	1860.0	±	91.9
9-HOTrE	2200.9	±	103.5	2189.0	±	62.2	2219.0	±	64.5
13-HOTrE	1634.2	±	108.0	1678.9	±	55.4	1659.7	±	70.7
20-HETE	785.1	±	108.0	745.3	±	35.1	720.7	±	47.7
5-HEPE	134.8	±	5.8	145.1	±	8.3	141.2	±	10.4
13-HODE	27428.5	±	1177.5	26550.6	±	995.9	26497.1	±	339.6
9-HODE	30861.3	±	1546.6	30123.0	±	1227.5	30064.0	±	728.3
15-HETE	1233.2	±	52.2	730.2	±	21.5	743.1	±	43.6
11-HETE	665.3	±	48.8	323.5	±	17.0	322.1	±	21.4
12-HETE	988.5	±	49.1	772.1	±	26.9	769.1	±	36.9
15(S)-HETrE	394.6	±	16.0	351.8	±	14.2	338.3	±	8.8
5-HETE	1190.5	±	56.7	1029.5	±	83.2	1008.1	±	21.3
15,16-DiHODE	25001.4	±	668.0	25775.9	±	949.5	25459.6	±	570.7
9,10-DiHODE	707.7	±	20.4	714.3	±	23.1	704.6	±	19.2
12,13-DiHODE	675.3	±	130.2	623.6	±	115.9	530.4	±	164.6
17,18-DiHETE	341.3	±	14.2	338.1	±	13.0	340.4	±	11.1
14,15-DiHETE	48.4	±	3.1	49.3	±	3.7	50.1	±	2.0

analyte	0 min (pM)			5 min (pM)			30 min (pM)		
	mean		SD	mean		SD	mean		SD
11,12-DiHETE	37.1	±	3.2	38.0	±	1.4	34.6	±	2.1
12,13-DiHOME	22190.9	±	591.2	22866.3	±	656.9	22778.2	±	692.7
9,10-DiHOME	10992.6	±	341.1	10260.8	±	383.8	10085.2	±	155.4
19,20-DiHDPE	3911.3	±	261.2	3473.1	±	74.6	3381.9	±	21.4
14,15-DiHETrE	742.7	±	27.2	746.9	±	20.4	739.9	±	4.6
16,17-DiHDPE	329.3	±	12.3	314.2	±	12.7	310.1	±	7.2
11,12-DiHETrE	701.3	±	70.4	601.7	±	15.8	586.5	±	4.1
13,14-DiHDPE	299.4	±	13.9	300.0	±	12.6	289.2	±	5.0
10,11-DiHDPE	264.8	±	18.7	246.7	±	17.2	247.7	±	3.6
8,9-DiHETrE	347.4	±	25.7	309.9	±	8.1	310.3	±	8.6
5,6-DiHETrE	232.7	±	6.0	204.8	±	8.4	206.8	±	9.6
4,5-DiHDPE	425.5	±	53.6	514.3	±	46.3	474.5	±	34.0
9(10)-EpODE	171.4	±	2.8	185.2	±	19.5	187.3	±	17.7
12(13)-EpODE	203.4	±	8.9	204.6	±	16.4	218.7	±	18.3
19(20)-EpDPE	352.9	±	11.2	310.5	±	17.5	300.6	±	22.9
12(13)-EpOME	4378.6	±	211.1	4366.2	±	267.7	4414.4	±	287.8
14(15)-EpETrE	175.7	±	2.0	98.9	±	8.3	100.5	±	14.3
9(10)-EpOME	2206.0	±	128.8	2150.1	±	170.4	2230.9	±	202.2
16(17)-EpDPE	49.0	±	4.7	46.0	±	10.3	45.0	±	9.8
10(11)-EpDPE	123.7	±	12.4	110.7	±	3.7	103.6	±	11.0
11(12)-EpETrE	184.0	±	30.9	91.8	±	11.5	85.4	±	14.8
5(6)-EpETrE	542.6	±	65.9	407.8	±	40.9	392.4	±	34.7
15(16)-EpODE	4523.0	±	156.4	4620.1	±	155.2	4641.5	±	141.1