

Targeted Metabolomics of the Arachidonic Acid Cascade – Current State and Challenges of LC-MS Analysis of Oxylipins

I. Willenberg^{1, #}, A.I. Ostermann^{1, #} and N.H. Schebb^{1, 2*}

¹University of Veterinary Medicine Hannover, Institute for Food Toxicology and Analytical Chemistry, Bischofsholer Damm 15, 30173 Hannover, Germany.

² University of Wuppertal, Institute of Food Chemistry, Gaußstraße 20, 42119 Wuppertal, Germany.

both authors contributed equally

*Corresponding author (Tel: +49 511 856 7780 ; Fax : +49 511 856 7409 ; E-mail: schebb@wwu.de)

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 as well as sensitive MS analysis. Currently, reversed phase LC in combination with detection on sensitive triple quadrupole instruments, operating in selected reaction monitoring (SRM) mode, is dominantly used for quantitative oxylipin analysis. A lack of standardized sample collection, handling and preparation procedures, degradation of the analytes during sample preparation and purity as well as availability of standards (internal standards) are the major problems of current targeted metabolomics approaches of the AA cascade. Major challenges for instrumental analytical methods are the detection of esterified oxylipins as well as separation and individual detection of oxylipin-isomers. Solving these issues are major tasks of current bio-analytical research which would help to elevate the knowledge of the biology of lipid mediators to a higher level.

Introduction:

Lipid mediators play an important role in biology. Particularly 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: i) constitutively expressed cyclooxygenase 1 (COX-1) and inducible COX-2, ii) 5-, 12- and 15-lipoxygenases (LOX) as well as iii) cytochrome P450 monooxygenases (CYP), particularly CYP2J and CYP2C, and non-enzymatically by (aut)oxidation. The initially formed products can be further converted by several other enzymes, for example by microsomal prostaglandin E synthase (mPGES) or by soluble epoxide hydrolase (sEH) leading to a pleiotrop 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-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 been unveiled yet, e.g. of 18-HEPE, being the major OH-FA metabolite in several cultured cells incubated with EPA [3].

More than half of the currently sold pharmaceuticals target directly the AA cascade [4], for example non-steroidal anti-inflammatory drugs (NSAID), aspirin and selective COX-2 inhibitors (e.g. celecoxib), or 5-LOX inhibitors and leukotriene (LT) antagonists. About 30 years after the

Nobel Prize to J. Vane, S. Bergström and B. Samuelsson for the discovery of the importance of prostaglandins (PGs), the biological roles of non-classical eicosanoids and oxylipins beyond PGs and LT becomes clearer. For example, multiple hydroxylated 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, though an epoxy-FA receptor has not been discovered. While both AA as well as DHA and EPA derived epoxides share the biological activity, the effects in cancer are different, with AA derived EpETrEs promoting angiogenesis, while n3-PUFA derived epoxides suppress tumor growth [7].

In order to investigate and understand the role of the large number of 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, such as AA, and n3-PUFA, such as EPA and DHA. By monitoring the activity of an enzyme or pathway of the cascade based on 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 unraveled [8]. Taking into account that many pharmaceuticals modulate the AA cascade, quantification of oxylipins is also crucial for the determination of *in vivo* target engagement of established drugs, such as aspirin, as well as new experimental drugs, such as sEH inhibitors. This article briefly summarizes the current state of LC-MS based targeted metabolomics of the AA cascade and focusses on the challenges of the quantification of oxylipins in biological matrices.

Instrumental analysis

Current targeted metabolomics LC-MS approaches of the AA cascade have impressive characteristics: One method allows the 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 show a similar performance, e.g. parallel quantification of 104 oxylipins with the use of 11 IS in 26 min and LODs between 1.8 and 340 pg on column [10], or quantification of 88 analytes (6 IS) in 21 min and LOQs between 0.06 and 15.96 pg on column [4,11,12]. For methods established in other labs the total number of covered analytes is not clear since the articles are focused on groups of analytes, e.g. resolvins, or only report oxylipins above the LOQ [13,14,5]. However, analysis times (of about 25 minutes) and sensitivities (LODs between 0.01 and 0.21 pg on column) are comparable [13,14,5].

All of these methods employ reversed phase (RP) LC coupled to a highly sensitive triple-quadrupole (QqQ) (MS) instrument using negative electrospray ionization 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: (i) low (<nM) concentration of the analytes, (ii) huge concentration differences between the lowest and the highest abundant oxylipin within a single sample (>10³-fold), requiring a broad linear detector response [15,16], (iii) correct identification of the LC-MS peaks and (iv) 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 PGE₂ and PGD₂ (Fig. 1). While the sensitivity and linear detector response of modern QqQ-MS fulfill the demands of i-ii, the identity confirmation of the analytes (iii) requires obtaining meaningful fragment spectra [17,14]. Since all oxylipins can occur in form of several isomers, identification only by retention time and selected reaction monitoring (SRM) of the analyte's transition can lead to false conclusions. That is why many groups employ QTRAP instruments for oxylipin analysis, allowing operating the second

analytical quadrupole as linear ion trap to obtain high quality fragment spectra. Selective detection (iv) requires both, a highly efficient chromatographic separation and detection in SRM mode. This can be demonstrated for the analysis of 8-, 9- and 12-HETE (Fig. 1-2). Despite high chromatographic resolution using a modern $2\mu\text{m}$ particle filled column, 8- and 12-HETE coelute. As a consequence, quantification can only be achieved by detecting unique fragment ions in SRM. For 9- and 12-HETE it is the other way round: 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 today's LC-methods require rapid switching times and sensitivity at short dwell times of the MS. In order to keep cycle times of the MS short, but simultaneously providing enough data points per peak, all current methods use software assisted features like scheduled or dynamic multiple reaction monitoring (MRM). Nevertheless, narrow elution windows of a large number of analytes still require dwell times of less than 10 ms. In the future it will be interesting if other MS detector types will enter the field of targeted oxylipin analysis. However, since high resolution MS (HRMS) could improve signal to noise ratio, but does not allow distinguishing the numerous constitution isomers, detection in SRM mode is mandatory for this analysis. It will be interesting if modern qTOF instruments could provide sufficient sensitivity and linear detector response. For the orbitrap instruments, the major question is if the cycle time for SRM detection could be short enough for quantification of the narrow LC peaks. The first report about oxylipin analysis by using an orbitrap instrument is promising: The comparison of the quantitative performance between orbitrap and a QqQ-MS showed similar results with respect to linear detection ranges, with LODs for the orbitrap ranging between 10 and 30 pg of the oxylipins [18]. If these instruments prove to keep up with the rapid, sensitive and robust quantification of QqQ-MS, the combination of quantification with HRMS and continuous detection of fragment spectra would elevate instrumental oxylipin analysis to a new level.

Aside from improvements on side of the LC-MS instruments, **derivatization** could lead to better separation efficacy and particularly MS detection. Gelb and coworkers 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 enhance sensitivity on today's instruments, though they have a more efficient ion transmission in negative ESI compared to the instruments used by Gelb and coworkers.

Derivatization to pentafluorobenzyl (PFB) esters was used by Blair and Coworkers allowing 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 due to decreased background signals [2]. APCI is generally recognized to be less prone for matrix effects compared to ESI. Hence, a similar derivatization is promising to circumvent the massive problems occurring from ion suppression in RP-LC-ESI(-)-MS of oxylipins in biological samples [12]. New derivatization agents together with specific ionization, e.g. electrochemistry-assisted ionization [20], or novel ionization techniques, like dielectric barrier discharge ionization-MS [21], could further improve selectivity, sensitivity and robustness of oxylipin detection by mass spectrometry in the future.

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) as well as base (PGs) or acid (epoxy-FA) treatment. Yet, 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/protein precipitation with/by organic solvents. However, most analyses require pre-concentration. For this purpose liquid-liquid extractions

(LLE) [22] or most frequently solid phase extractions (SPE) [14,13,10,4,9] are used. By contrast to similar instrumental analyses of oxylipins, SPE protocols differ considerably from lab to lab. The employed stationary phases range from RP-18 [14] and mixed mode phases with RP-8 and anion exchange properties [13] to polymeric phases [9,10,4]. As it can be expected, these methods have a 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 utilizing anion exchange stationary phases [13] (carboxy acid moiety of the oxylipins) or polar and non-polar washing steps (water and n-hexane) prior elution of the medium to non-polar oxylipins by methyl formiate on the C-18 column [14]. It is interesting that the latter procedure on classical RP material overall outperforms the other protocols, with respect to recovery of IS, reduction of ion suppressing matrix and extraction efficacy of oxylipins from the biological matrix (Fig. 3). It would be interesting to explore if the performance could be further improved by using the elution procedure (water followed by n-hexane and elution with methyl formiate) on a modern polymeric stationary phase with embedded polar groups.

An adequate sample preparation which eliminates as much matrix as possible is essential since 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 the sample preparation procedures, the availability and use of more heavy isotope labeled IS would largely improve robustness, accuracy and precision of oxylipin analysis in biological samples.

(Biological) variation of oxylipin concentration

Current reports about human serum and plasma oxylipin concentrations document strong (interindividual) variations between samples from different subjects [15,23]. These variations hamper 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/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 the 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 the 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 carried out as standardized as possible (e.g. fixed fasting period, day time, 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 (Figure 4). Moreover, if the sample sits for few minutes in the centrifuge, after centrifugation and before the plasma is collected and frozen, the levels of some oxylipins are significantly reduced (Supplementary Information, SI). Furthermore, prolonged storage in the freezer can also lead to degradation and loss of the analytes as shown for several DHA and EPA derived resolvins and prostanoides [5]. In few studies COX and sEH inhibitors as well as protease and esterase inhibitors are added to the samples to prevent enzymatic formation/degradation [26]. More frequently antioxidants, such as 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 [26,11,10,4]. However, the benefit of these procedures has not been systematically evaluated for a comprehensive set of oxylipins so far. Overall, artificial (*ex vivo*) formation/degradation of

the lipid mediators is one of the major challenges in the analysis of biological samples which can only be addressed by tight standard operating procedures (SOPs) including rapid sample processing and optimized storage conditions (-80 °C, short time). Only for few oxylipins, *ex vivo* formation/degradation can be excluded/measured by additional determination of their endogenously formed metabolites, e.g. 11-dehydro-TxB₂ [27] together with TxB₂ or bicycloprostaglandin E₂ as stable degradation product of PGE₂ [28].

Plasma or serum?

Both, plasma as well as serum should be regarded as appropriate matrices for quantitative oxylipin analysis of circulating oxylipins [5,16]. In case of plasma, the anticoagulant should be chosen carefully: EDTA seems to be the best choice since 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 such as TxB₂ and 12-HETE (Fig. 1). Moreover, detectability of low concentrated mediators, such as resolvins is improved [5]. Regarding variability, it remains to be evaluated if plasma or serum allows the determination of oxylipin concentrations in blood with a higher precision.

Accuracy and inter-lab comparability

In addition to high intersample variations, huge differences between the mean concentrations of the 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 as suggested for drugs, e.g. by European Medicines Agency (EMA), are not or only in part applicable. With respect to accuracy, current methods determined 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 the oxylipins in the aqueous solution [10]. Regardless how rigid 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 synthesized or are obtained commercially (currently from a single company). For the latter, the affordable quantities are so low that purity cannot be checked by classical chemical methods. Thus, a mistake in the concentration provided by the manufacturer directly leads to systematic 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 possibilities to verify the plausibility of manufacturer information. In order to improve quality of the results in the future it would be important to agree on criteria which have to be fulfilled for a fit-for-purpose-validation in oxylipin analysis. Together with an exchange of samples, standards between groups and inter-laboratory tests this would help to improve comparability.

The free, the bound, and the total...

– or the analytical challenge in detecting esterified oxylipins

Although a significant 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 about 50 to 350-fold and 10 to 40-fold, respectively [16,13]. Esterified oxylipins are commonly quantified following saponification (base hydrolysis) [13,31,32,16,33]. For this purpose, the samples are incubated with sodium hydroxide (1.00-

3.75 M) 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 M sodium carbonate at 4 °C overnight [33] or perform transesterification of lipids to methyl esters (0.35 M sodium methoxide solution for 60 min at 60 °C) following hydrolysis by the addition of water (60 min) [32]. The performance of the different cleavage-protocols has not been systematically compared up to now. However, alkaline treatment degrades a large number of oxylipins, particularly PGs, e.g. β -hydroxy-keto prostanoids, such as PGE₂, PGD₂, or thromboxanes [32,31] 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. On the contrary, a moderate saponification could lead to incomplete liberation of esterified oxylipins. All current cleavage techniques lead to the sum of free and esterified oxylipins and provide no information on how the analytes are bound in the samples. Yet, it is highly relevant, if a mediator is bound to the sn2-position of a phospholipid of the cell membrane, rapidly releasable by phospholipases upon inflammatory stimuli or if it is bound in a triacylglyceride (fat) with unknown biological fate. One way of addressing this issue would be to separate the different lipid classes of a lipid extract prior hydrolysis, as commonly carried out for fatty acid analysis [35]. Another possibility is the direct detection of the esterified oxylipin, as recently successfully carried out, e.g. for C16:0/12-HETE-phosphatidylethanolamine [36] Combining all oxylipins and possible lipids, a fairly large number of analytes results. Thus, the integration of targeted oxylipin metabolomics in lipidomics is doubtlessly one of the biggest challenges for analytical chemistry of oxylipins. However, the low (total) concentration of the lipid mediators makes it questionable if 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, which is timely to be addressed, is the 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] (same SRM transition as the cis-isomer, eluting 1-3 min later). Though considerably large peaks of trans-epoxides are found in 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 role as lipid mediators. Particularly the timely question whether and to which extent trans-epoxy-FAs contribute to the biological effects associated 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 not only one, but in fact 4 compounds which are generated via CYP conversion as well as (aut)oxidation processes and can be hydrolyzed by sEH at isomer specific rates [37] (Fig. 1). Besides the epoxides, almost all oxylipins are chiral with more or less (understood) biological activity.

While enzymatic routes form products at a distinct enantiomer ratio, aut(oxidation) processes result in the formation of racemats. Therefore, the application of chiral separation can be very helpful to differentiate between the varying routes of formation. Moreover, in several cases, different enzymatic routes of formation can be distinguished based on the enantiomer ratio, e.g. (\pm)15-HETE (Fig. 1). While 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 demonstrated for this example, chiral separation would greatly assist to unravel 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, since current chiral-LC cannot keep up with the robustness and (overall) separation power of RP chromatography, they are comparably rarely used. Thus, targeted oxylipin metabolomics would massively 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. Taking all challenges for instrumental analytical chemistry mentioned in this article together, the integration of chiral separation in the routinely used targeted metabolomics platforms would have the largest impact on our understanding of the biology of oxylipins. With the data resulting from these methods at hand, 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 allow gaining an impressive understanding about the biological importance of the lipid mediators formed in the AA cascade. However, numerous questions remain to be answered, e.g. unraveling the mechanisms of the effects of dietary n3-PUFA intake on human health. The comprehensive investigation of hydroxy-n3-PUFAs, resolvins and n3-epoxides by methods summarized in this article, could be the key 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 entering the field may cause a revolution of targeted lipid mediator analysis. With the help of these instruments it is easy to generate peaks, areas and numbers. However, it will still be a challenge to get meaningful results, i.e. accurate concentrations in biological samples. Particularly, the optimization of sample collection, stabilization and preparation seem to be timely. With respect to the instrumental analysis the greatest challenges are the differential detection of stereoisomers and the analysis of esterified oxylipins. In order to foster this progress in the field of targeted oxylipin metabolomics, it is crucial that analytical chemistry is regarded as an integral part of the medical/biological research. Thoroughly developed methods and their continuous improvement require time and (grant) money. Though, not all method developments may address fundamental scientific questions (such as the detection of esterified oxylipins), in particular improvements on sample preparation, a fit-for-purpose validation and interlab comparison seem to be of high importance for the field.

Acknowledgements

Our work is supported by the Fonds der Chemischen Industrie, the German Research Foundation (DFG, Grant SCHE 1801) and the European Union (Grant CIG 293536).

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Figure Captions

Figure 1: Illustrative overview about the AA cascade, randomly highlighting few metabolites in order 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 [1,10].

Figure 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, based on specific SRM transitions. **(B)** Chromatographic separation of 9- and 12-HETE. Both show interfering SRM transitions because of almost identical collision induced dissociation MS/MS spectra of their $[M-H]^+$ ions **(C)**.

Figure 3: Concentrations of selected oxylipins in pooled human EDTA plasma 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 \pm SD (n=5). The following protocols were used:

SepPak – C18 phase, washing with water and n-hexane, elution with methyl formate [14]; **AnionEx** – C8 phase with anion exchange properties, washing with methanol/water (v:v, 1:1), elution with ethyl acetate/n-hexane (75:25) [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 carried out as described [11,4]. The complete evaluation of the performance of the procedures can be found in [12].

Figure 4: *Ex vivo* degradation/formation of oxylipins 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 g, 15 min, 4 °C), the plasma was immediately frozen (-80 °C) and the oxylipin concentration was analyzed within 5 days (Oasis SPE [11,12,4]). The resulting concentrations after different periods of time in sample preparation are compared to those with direct sample preparation ($t_{(5min, RT)}$). The results clearly show, that after 60 min storage of whole blood the levels of several oxylipins are massively reduced (e.g. 15-HETE and 14(15)-EpETrE) while other analytes are formed *ex vivo* (e.g. PGE₂). Shown are mean \pm SD (n=4). More results about the effect of storage during sample preparation of human blood can be found in the SI.

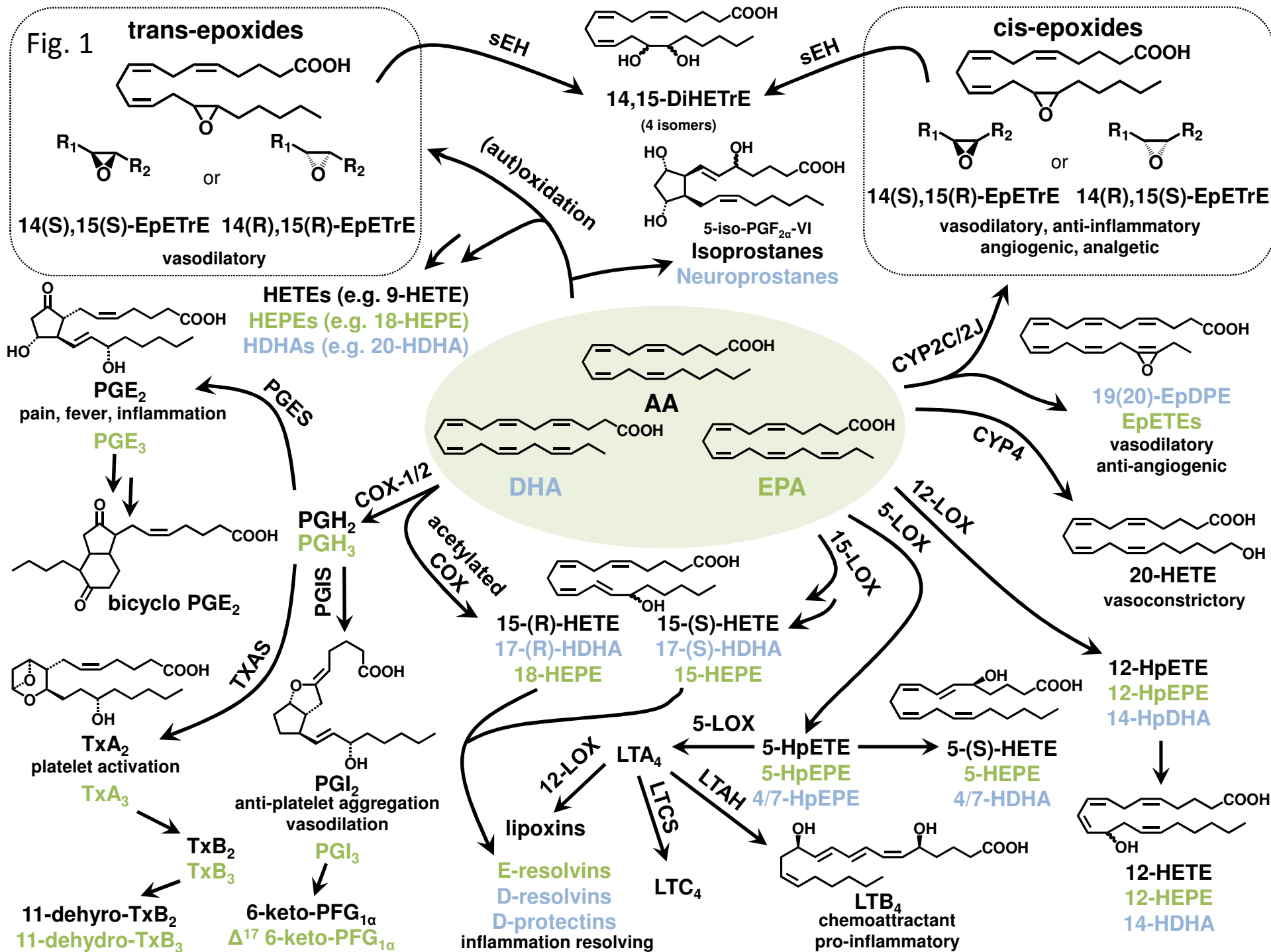


Fig. 2

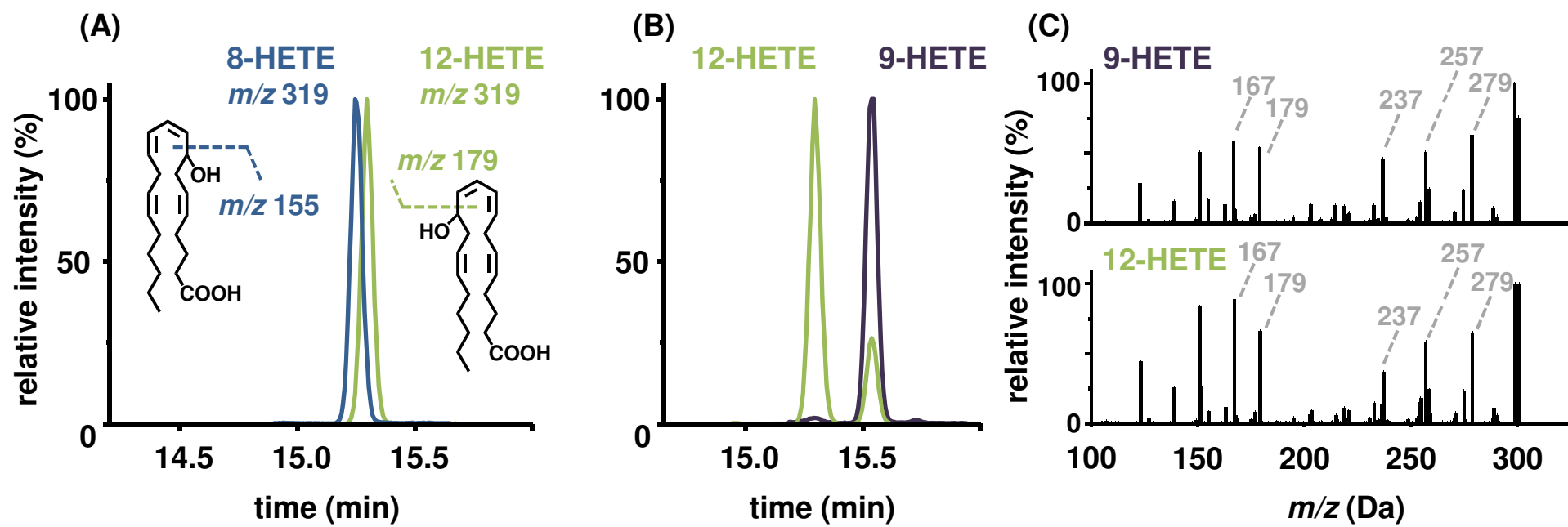


Fig. 3

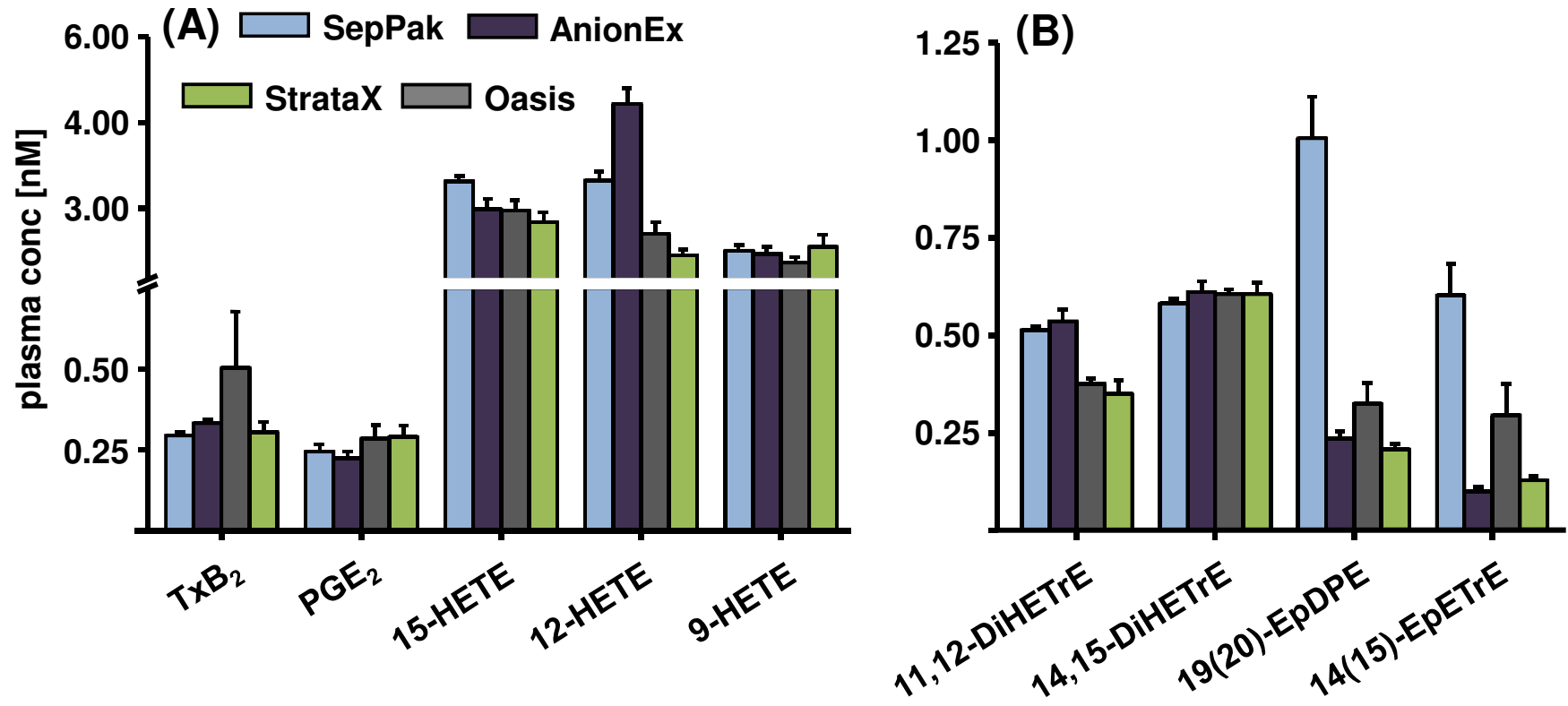
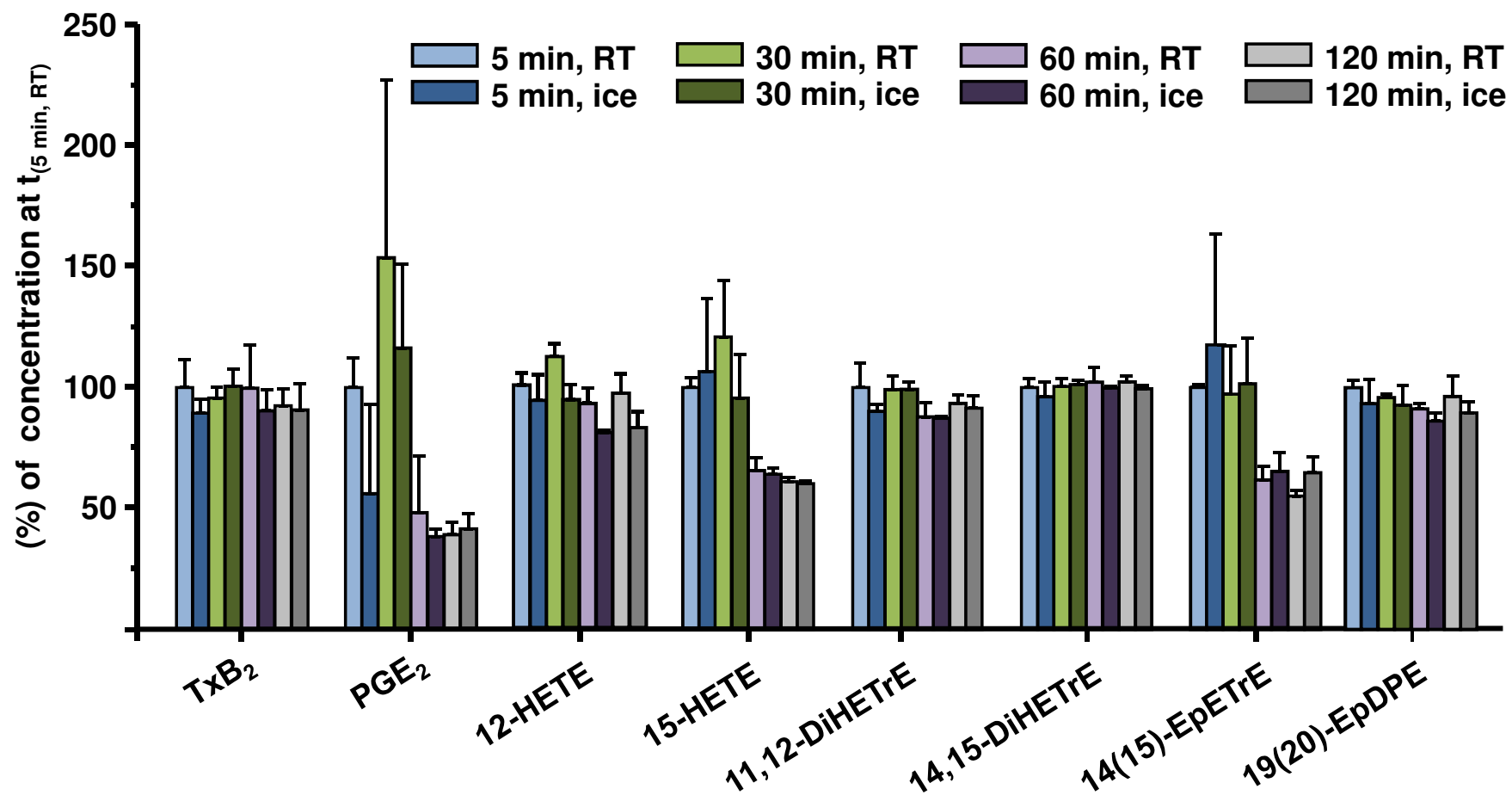


Fig. 4



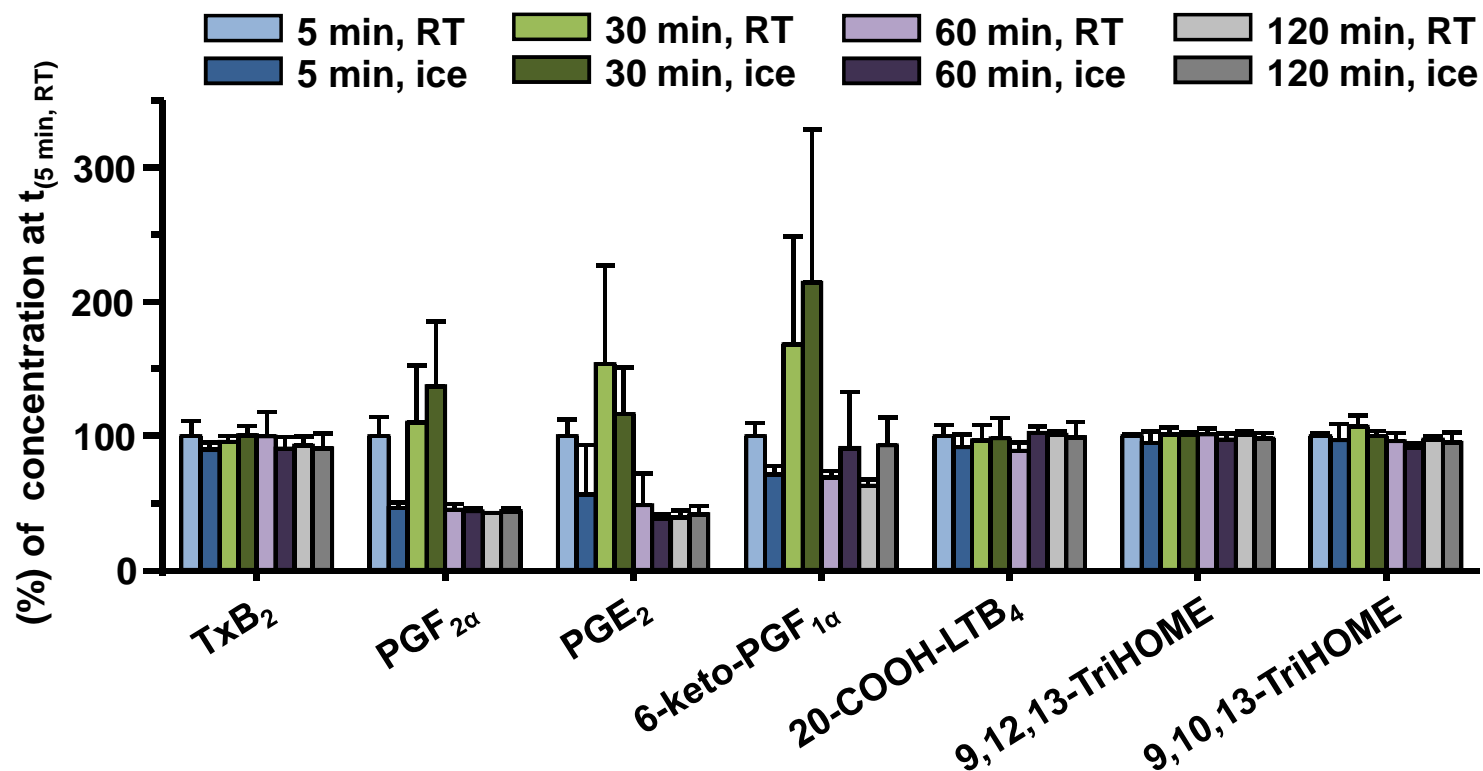


Fig. S1: *Ex vivo* degradation/formation of TxB_2 , prostaglandins (PGs) and trihomes in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (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 ($t_{(5 \text{ min, RT})}$). 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 \pm SD (n=4).

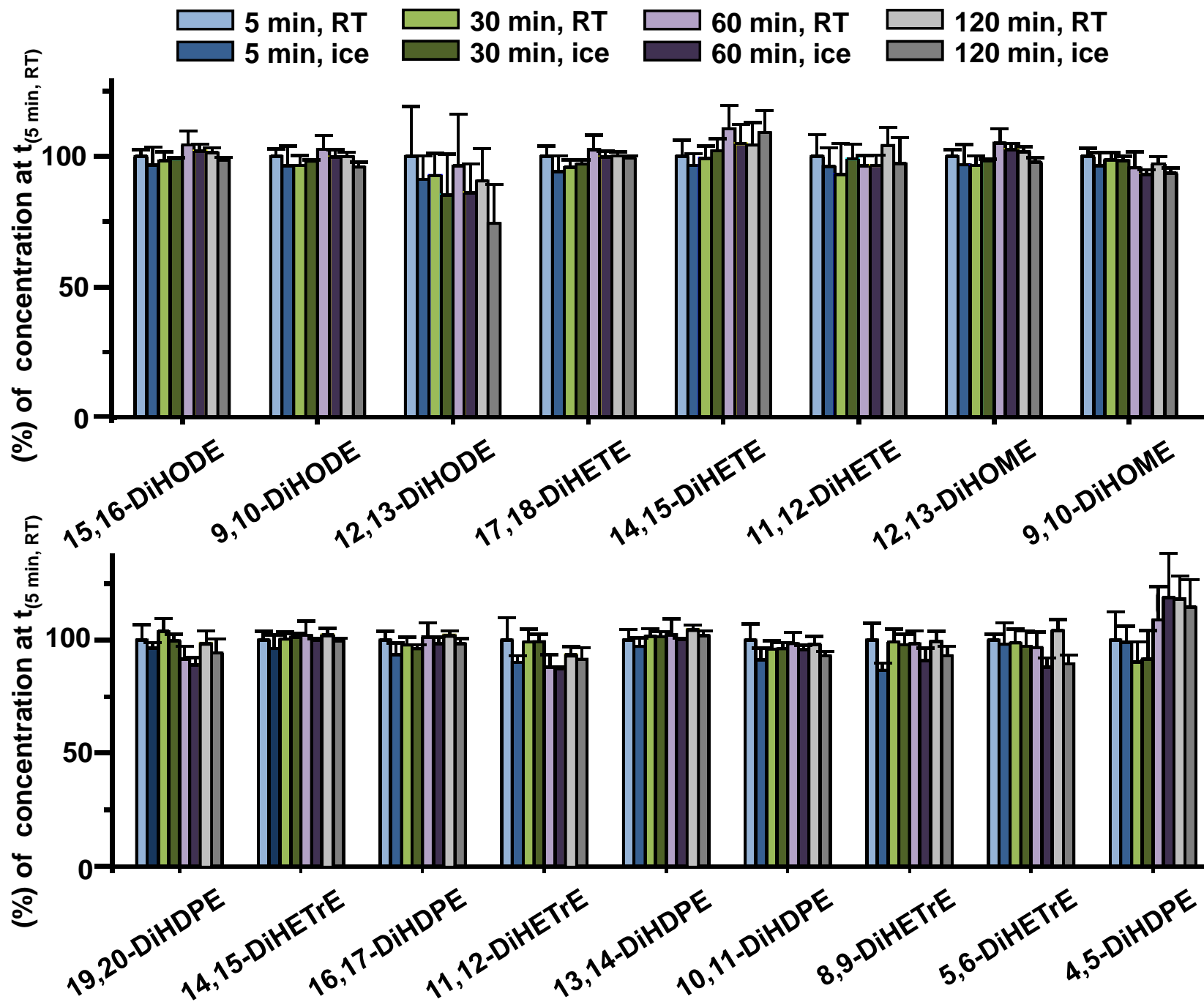


Fig. S2: *Ex vivo* degradation/formation of diols in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), 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 ($t_{(5 \text{ min, RT})}$). Shown are mean \pm SD (n=4).

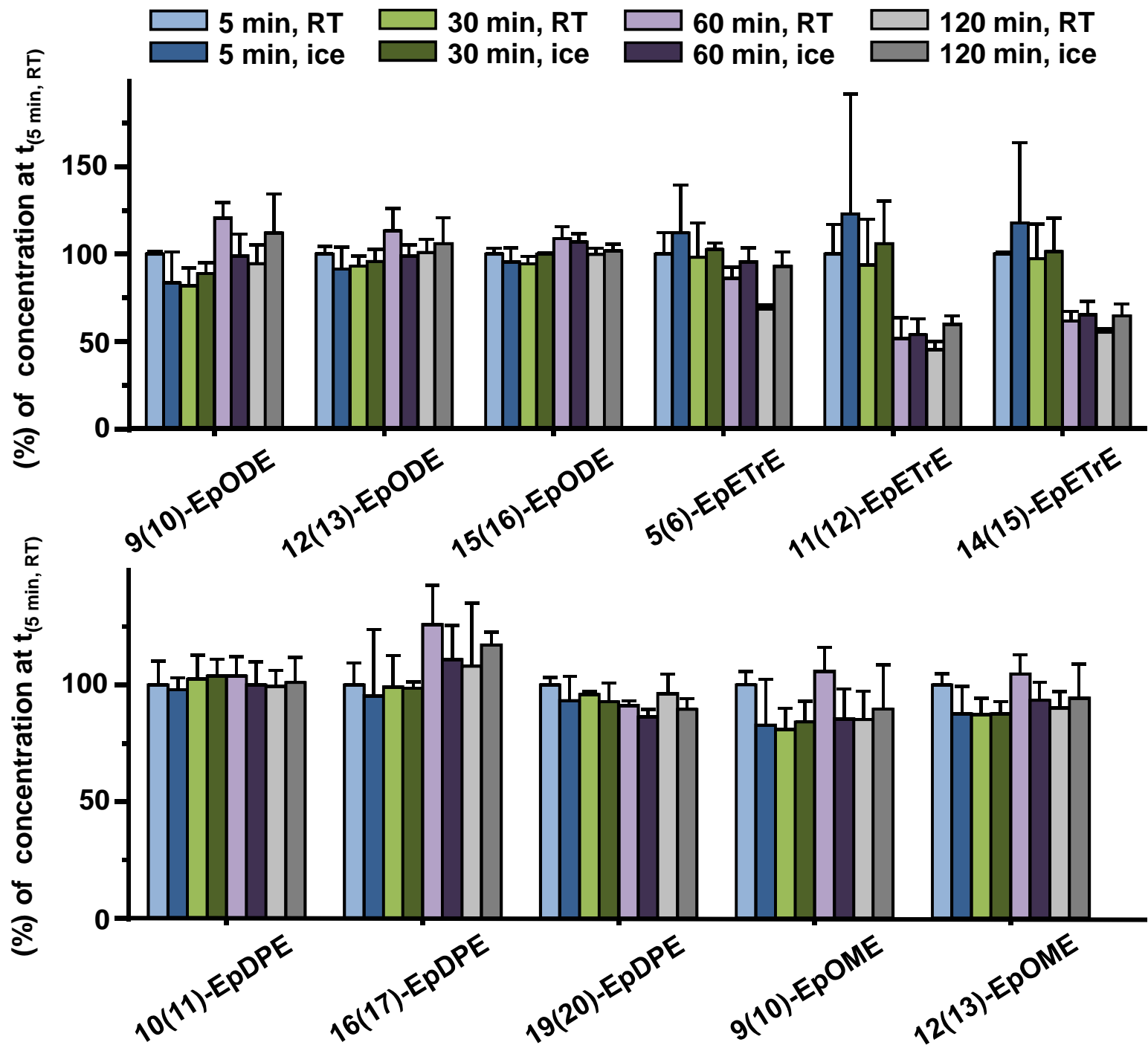


Fig. S3: *Ex vivo* degradation/ formation of epoxides in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °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 ($t_{(5 \text{ min, RT})}$). 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 \pm SD (n=4).

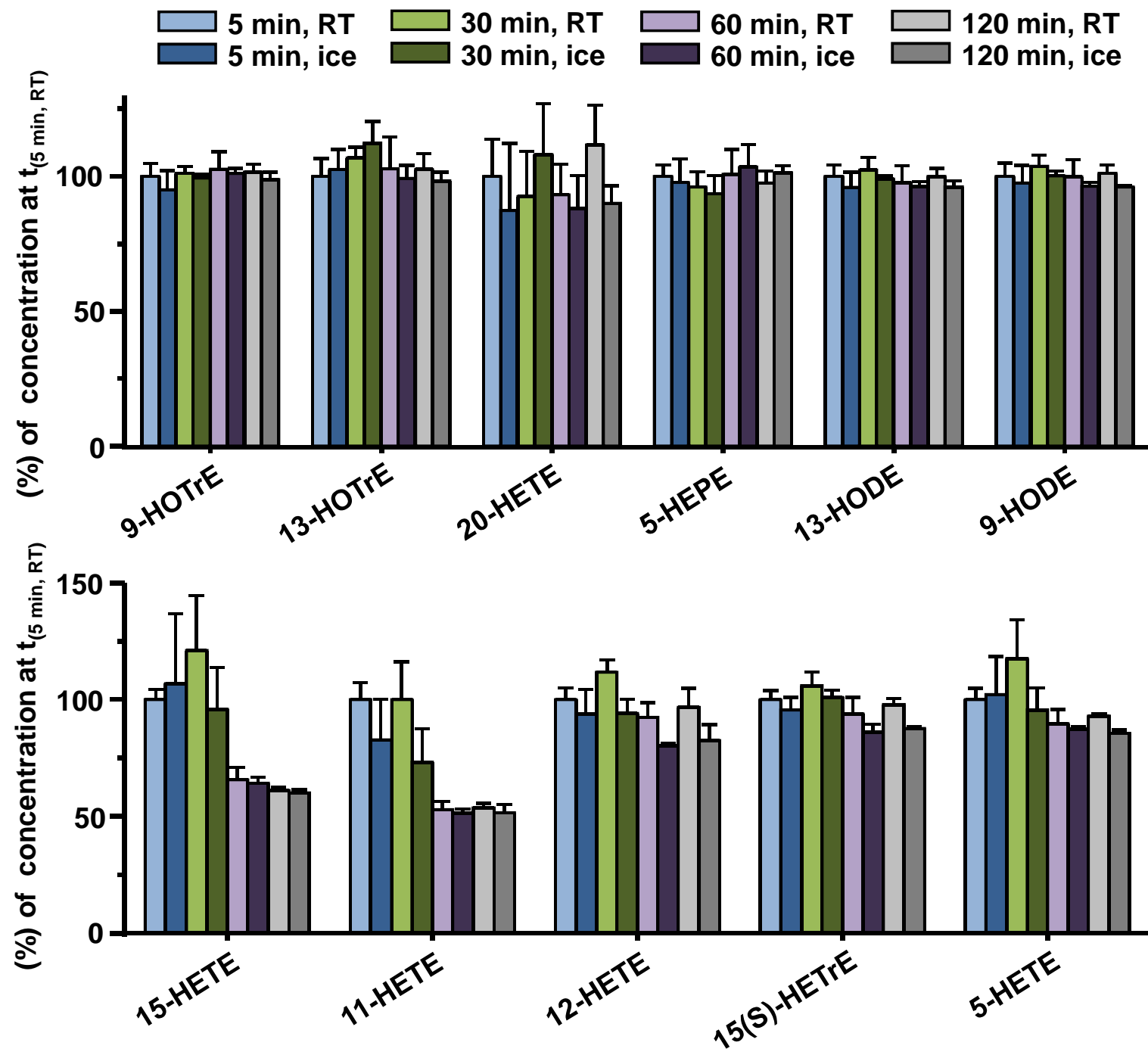


Fig. S4: *Ex vivo* degradation/formation of alcohols in human whole blood after blood withdrawal. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and left for 5 min, 30 min, 60 min or 120 min either at room temperature (RT) or on ice. After centrifugation (1200 x g, 15 min, 4 °C), 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 ($t_{(5 \text{ min, RT})}$). 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 \pm SD (n=4).

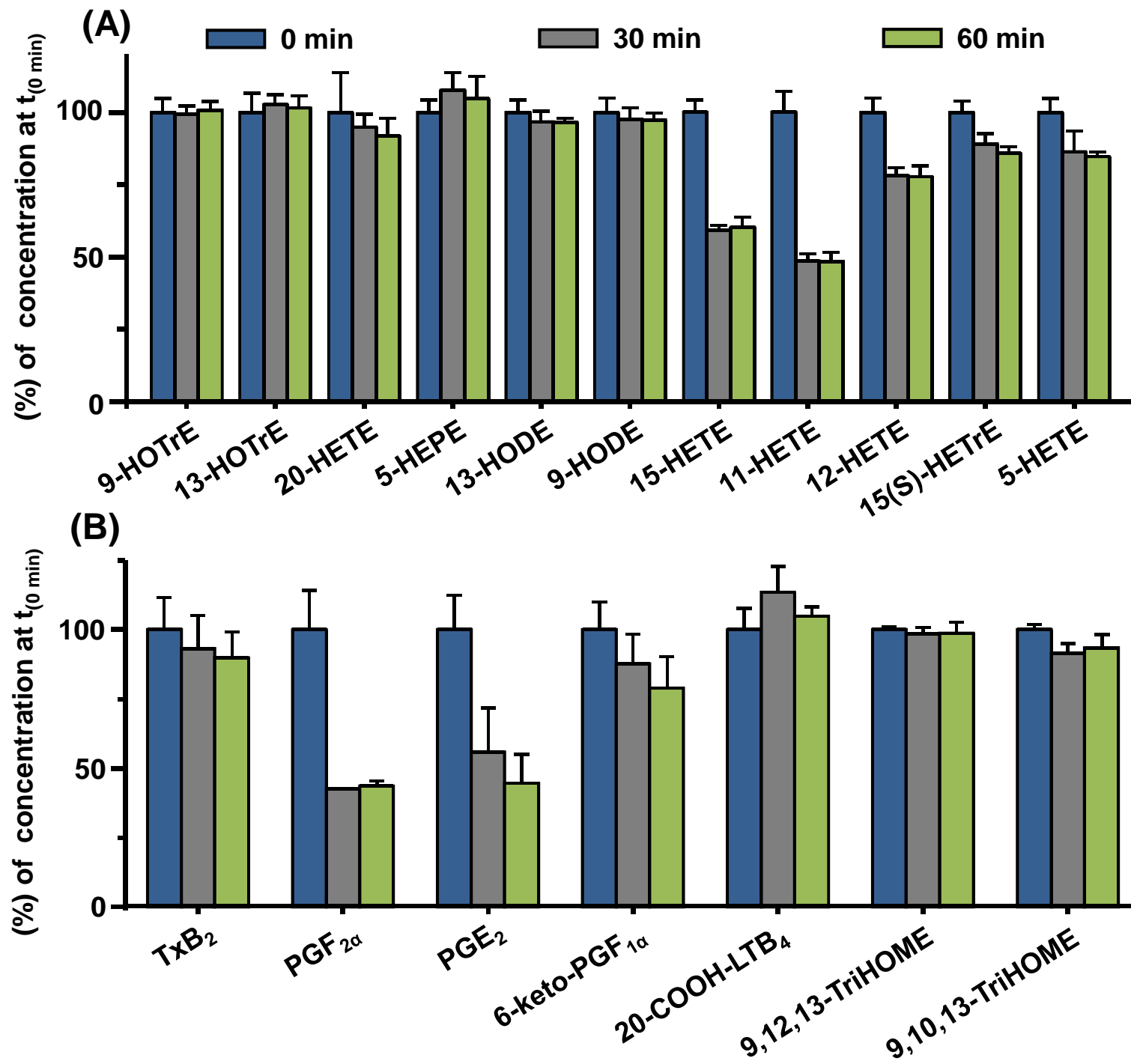


Fig. S5: *Ex vivo* degradation/formation of alcohols **(A)** as well as TxB₂, prostaglandins and trihomes **(B)** in freshly centrifuged human plasma. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and centrifuged (1200 x g, 15 min, 4 °C) after 5 min at room temperature (RT). 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. Oxylin 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 ($t_{(0 \text{ min})}$). 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 PGE₂ and PGF_{2α} significantly decreased in the first 30 min of storage while TxB₂, 20-COOH-LTB₄, 6-keto-PGF_{1α} and the trihomes showed no changes up to 60 min **(B)**. Shown are mean ± SD (n=4).

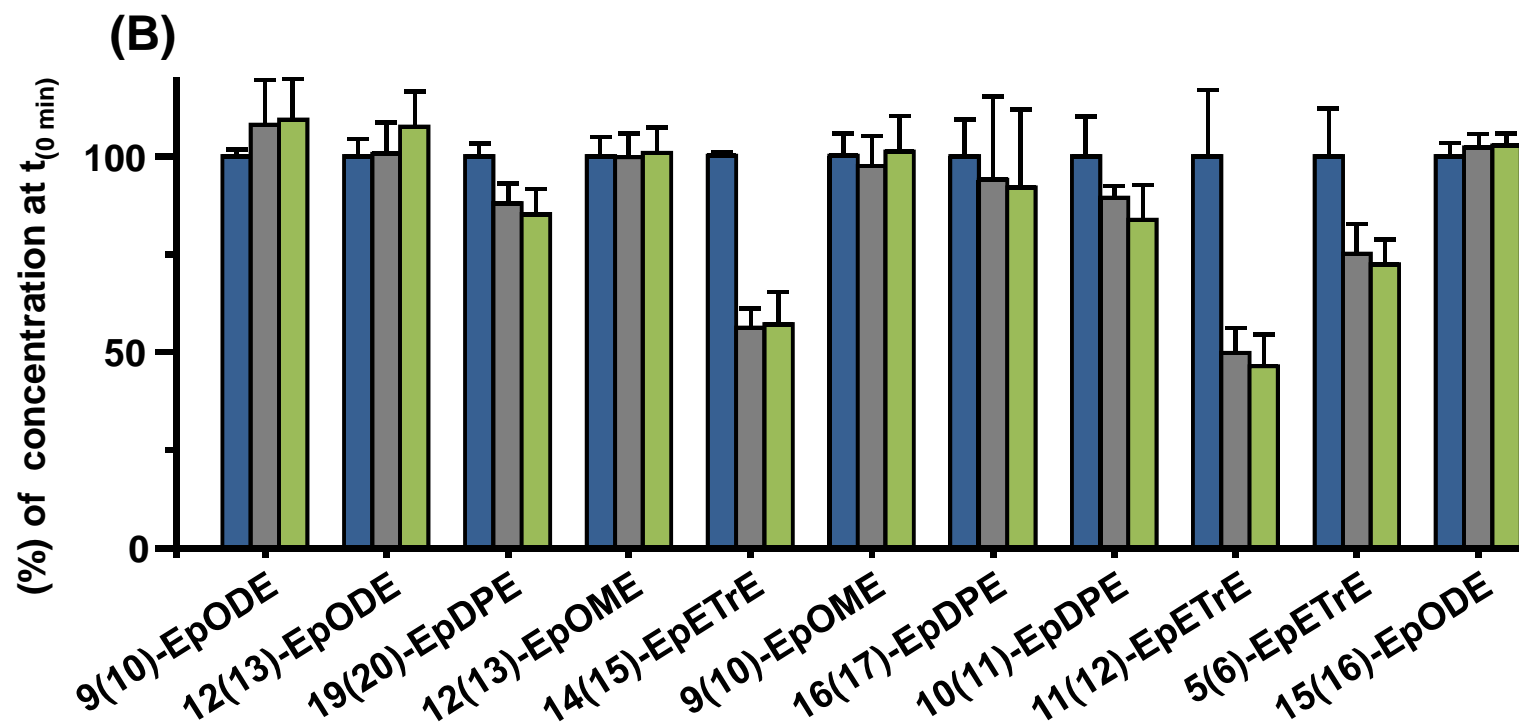
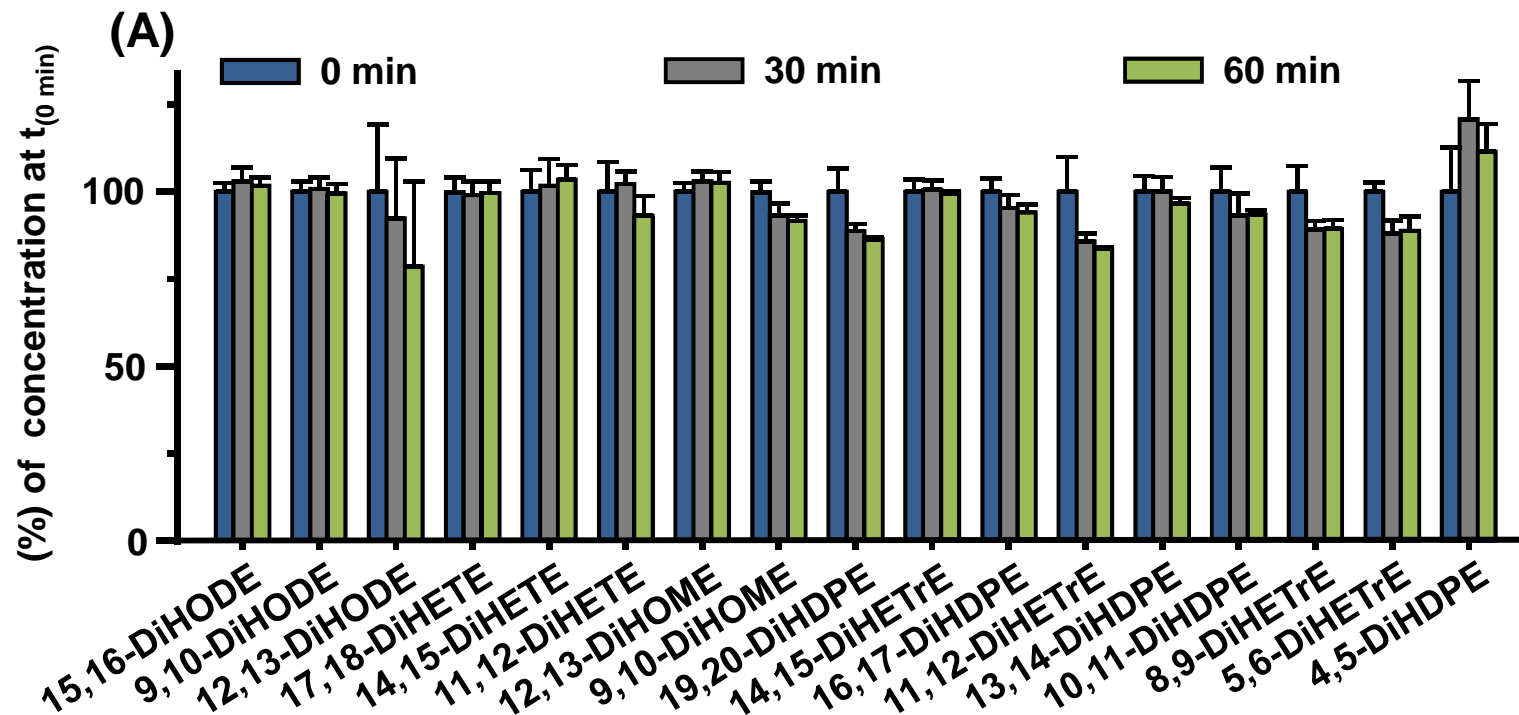


Fig. S6: *Ex vivo* degradation/ formation of diols **(A)** and epoxides **(B)** in freshly centrifuged human plasma. Blood of a healthy human volunteer was collected in EDTA tubes, pooled and centrifuged (1200 x g, 15 min, 4 °C) after 5 min at room temperature (RT). The resulting plasma was left with the cell pellet for 0 min, 30 min or 60 min on ice before freezing at -80 °C till analysis. Oxylin 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 ($t_{(0 \text{ min})}$). 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 \pm 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 _{2α}	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 _{1α}	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 _{2α}	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 _{1α}	206.3 ±	20.7	180.7 ±	22.4	163.0 ±	23.4
20-COOH-LTB ₄	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