

Comparison of sample preparation methods for the quantitative analysis of eicosanoids and other oxylipins in plasma by means of LC-MS/MS

Annika Irmgard Ostermann¹, Ina Willenberg¹ and Nils Helge Schebb^{1,2*}

¹ University 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, Wuppertal, Germany.

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

Oxylipins are potent lipid mediators. For the evaluation of their biological roles, several LC-MS based methods have been developed. While these methods are similar, the described sample preparation procedures for the extraction of oxylipins differ considerably. In order to deduce the most appropriate method for the analysis of non-esterified oxylipins in human plasma, we evaluated the performance of seven established sample preparation procedures. Six commonly used solid phase extraction (SPE) and one liquid-liquid extraction (LLE) protocol were compared based on the recovery of 13 added internal standards, extraction efficacy of oxylipins from plasma and reduction of ion-suppressing matrix. Dramatic differences in the performance in all three parameters were found. LLE with ethyl acetate was overall not a sufficient sample preparation strategy. The protocols using Oasis- and StrataX-material insufficiently removed interfering matrix compounds. Extraction efficacy of oxylipins on anion-exchanging BondElut cartridges was low, while removal of matrix was nearly perfect. None of the protocols led to a high extraction efficacy of analytes while removing all interfering matrix components. However, SPE on a C18-material with removal of matrix by water and n-hexane prior elution with methyl formate showed the best performance for the analysis of a broad spectrum of oxylipins in plasma.

Introduction

Oxidative metabolites of polyunsaturated fatty acids (PUFA) are an important class of lipid mediators. The conversion of arachidonic acid (20:4n6, AA) by cyclooxygenases (COXs) and lipoxygenases (LOXs) leads to highly potent eicosanoids controlling a multitude of biological functions [1]. A large number of eicosanoids and oxidation products of other PUFA, particularly docosahexaenoic acid (22:6n3, DHA) and eicosapentaenoic acid (20:5n3, EPA), generally referred to as oxylipins, have been described in recent years [2,3]. Their routes of formation are diverse, catalyzed by LOX, COX, Cytochrome P450 and soluble epoxide hydrolase among further enzymes and non-enzymatic (aut)oxidation [1,3]. For many of the formed oxylipins, the biological role has not been fully understood, however several are implicated to regulate physiological processes such as blood pressure, inflammation and pain, but also cellular functions, e.g. proliferation [1]. Quantitative analysis in biological samples is the key to a mechanistic understanding of the biological roles of oxylipins. Monitoring changes in a comprehensive pattern of oxylipins in different (patho)physiological conditions and in response to pharmacological treatment is one of the most promising strategies in the investigation of oxylipins. This led in the past, e.g. to the understanding of the remarkably different actions of n3- and n6-epoxides in angiogenesis and cancer growth [4,5] as well as their antiarrhythmic effects [6] or the discovery of inflammation resolving hydroxyl- [7] and multiple hydroxylated n3-PUFA, such as resolvins [2]. Several liquid chromatography (LC) mass spectrometry (MS) methods have been described allowing parallel quantification of a large number (~100) of oxylipins in biological matrices [8-11]. These approaches make targeted metabolomics analysis of the arachidonic acid cascade feasible because a comprehensive set of products is covered.

On the level of instrumental analysis, almost all current methods address the challenges of quantitative oxylipin analysis such as (i) very low concentrations (ii) huge concentration range of the different oxylipins in one sample (up to 10^4 fold) [12] and (iii) large number of structurally similar analytes with several (regio)isomers with a similar approach: Separation is carried out by modern reversed phase chromatography with sub-2- μm particles. Electrospray ionization is used in negative ion mode and the analytes are detected in selected reaction monitoring (SRM) mode on a highly sensitive triple quadrupol (qqq) MS [8-11,13]. In fact, even the same transitions are used for quantification, e.g. beta fragmentation of hydroxy-FA by collision-induced dissociation (CID) [11,13]. Overall, the differences in the well optimized methods are minor and mainly depend on analyte-coverage and sensitivity, most likely limited by standard availability and instrumental performance, respectively.

Regarding sample preparation the methods vary considerably. Mixing of the sample with organic solvents and direct injection is only possible for specific questions because of the low oxylipin concentrations in biological samples [14]. Thus sample preparation techniques are required allowing efficient extraction from the matrix and pre-concentration prior instrumental analysis. For this purpose, both liquid-liquid extraction (LLE) with ethyl acetate (EA) [15] and different solid phase extraction (SPE) protocols [16,11,10,17,8,13,6] have been described. For SPE, classical RP material [16,11] as well as modern polymeric stationary phases with embedded polar groups such as Oasis HLB (Waters, Eschborn, Germany) [10,17,8] or StrataX (Phenomenex, Torrance, CA, USA) [9] are employed. Moreover, materials with anion exchange properties are used [13,6] to extract the slightly acidic oxylipins from biological samples. Taking the different solvents used in the washing and eluting steps of SPE into account, the described protocols are even more diverse.

Isotope-labeled internal standards (IS) are only available for few oxylipins because of the large diversity of analytes. As a consequence a single heavy atom labeled IS is used for a whole group of oxylipins, e.g. $^2\text{H}_4$ -PGE₂ for all PGs and 5-HETE for all hydroxy-FA [10]. Even though structurally similar IS are used, their retention time differ. As a consequence, the IS cannot correct for all interferences caused by influence of matrix compounds on the ionization process. Ion suppression is in fact the Achilles heel for quantification using ESI-MS, since the signal is strongly affected by coeluting matrix compounds [18]. Thus, efficient removal of the matrix by sample preparation is the key for a successful quantification of oxylipins in complex biological samples.

However, neither the extraction efficacy of oxylipins from biological samples by the different sample preparation strategies nor their ability to remove ion suppressing matrix compounds has been investigated so far. In the present study, we therefore compared the performance of 7 commonly used sample preparation techniques for the analysis of free (i.e. non-esterified) oxylipins in human plasma. The methods were thoroughly evaluated according to three criteria: i) IS recovery ii) ion suppression and iii) extraction efficacy. The most efficient sample preparation protocols are identified and could be used as a basis for further optimization procedures.

Materials and methods

Chemicals and biological materials

LC-MS grade acetonitrile (ACN), acetic acid (HAc) and methanol (MeOH) were from Fisher Scientific (Nidderau, Germany). Oxylin standards and internal standards (Tab. 1) were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany). Further standards such as EpODEs and DiHODEs were a kind gift from the laboratory of Bruce Hammock, UC Davis, California, USA. 1-(1-(ethyl-sulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy)phenyl)urea synthesized as described [19] was used as internal standard 2 (IS 2). Sodium acetate was obtained from Merck (Darmstadt, Germany). n-Hexane (HPLC Grade), was purchased from Carl Roth (Karlsruhe, Germany) and formic acid (Acros Organics) was obtained from Fisher Scientific (Nidderau, Germany). All other chemicals were from Sigma Aldrich (Schnelldorf, Germany). Pooled human plasma was generated by mixing EDTA plasma obtained from healthy male volunteers. The pooled plasma was centrifuged (10 min, 4°C, 10 000 x g) and stored at -80°C in 1-2 mL aliquots.

Oxylin extraction

For each analysis a freshly thawed 500 µL aliquot of the same human plasma pool was used. In the first step, 10 µL of IS solution in MeOH (100 nM of ²H₄-6-keto-PGF_{1α}, ²H₄-PGE₂, ²H₄-PGD₂, ²H₄-TxB₂, ²H₄-LTB₄, ²H₄-9-HODE, ²H₈-5-HETE, ²H₈-12-HETE, ²H₆-20-HETE, ²H₁₁-14,15-DiHETrE, ²H₁₁-14(15)-EpETrE, ²H₄-9(10)-EpOME and ²H₄-9(10)-DiHOME) and 10 µL of antioxidant solution (0.2 mg/mL ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene and triphenylphosphine in MeOH/water (50/50, v/v)) were added to the plasma. Extraction was carried out according to 6 different established SPE protocols (a detailed description of all procedures can be found in the electronic supplementary material (Tab. S3) and one LLE protocol:

For **LLE** [15], 500 µL of 1 M sodium acetate (pH 6.0) was added to 500 µL of plasma. The sample was extracted twice with 750 µL EA. In each step, the sample was vortexed for 3 min and centrifuged for 5 min at 4°C at 20 000 x g.

For **Oasis-EA** SPE [10] the plasma sample was mixed with 500 µL MeOH/water (5/95, v/v) acidified with 0.1% HAc. After centrifugation (10 min, 4°C, 20 000 x g) the supernatant was loaded to a preconditioned Oasis HLB-SPE-column (3 mL, 60 mg, 30 µm particles; Waters, Eschborn, Germany). The column was washed with 6 mL MeOH/water (5/95, v/v) acidified with 0.1% HAc and the cartridge was dried by low vacuum (~200 mbar) for 20 min. Oxylin were eluted by gravity with 0.5 mL MeOH and 1.5 mL EA.

For the **Oasis-MeOH** SPE [17] the plasma sample was mixed with 500 µL MeOH/water (40/60, v/v) acidified with 0.1% formic acid. The sample was centrifuged (10 min, 4°C, 20 000 x g) and loaded to a preconditioned Oasis HLB-SPE-column (3 mL, 60 mg, 30 µm; Waters). The cartridges were washed with 3 mL of MeOH/water (20/80; v/v), acidified with 0.1% formic acid, dried with low vacuum (~200 mbar, 20 min) and eluted by gravity with 2 mL of MeOH.

For the extraction on the anion exchange column **BondElut** Certify II [6,13] the plasma was mixed with 500 µL of 1 M sodium acetate buffer (pH 6.0) /MeOH (95/5, v/v). The sample was centrifuged (10 min, 4°C, 20 000 x g) and the supernatant was loaded onto a preconditioned BondElut Certify II column (3 mL, 200 mg, 47-60 µm particles; Agilent). The cartridges were washed with 3 mL of MeOH/water (50/50, v/v) and dried by low vacuum (~200 mbar) for 20 min. Analytes were eluted with 2 mL of 75/25 (v/v) n-hexane/EA with 1% HAc (**AnionEx-Strong**) [6], or with 2 mL 25/75 (v/v) n-hexane/EA with 1% HAc (**AnionEx-Weak**) [13].

For **StrataX** SPE [9], 500 µL of plasma was mixed with 500 µL MeOH/water (20/80, v/v) centrifuged (10 min, 4°C, 20 000 x g) and loaded onto the preconditioned cartridge (3 mL, 100 mg, 33 µm; Phenomenex, Aschaffenburg, Germany). The columns were washed with 3.5 mL of 10% MeOH, dried (~200 mbar, 20 min) and eluted by gravity with 1.0 mL of MeOH.

For the **SepPak** tC18 SPE [16,11] 500 µL plasma were mixed with 1500 µL MeOH/water (20/80 v/v), centrifuged (10 min, 4°C, 20 000 x g) and acidified with 80 µL pure HAc to a pH of 3.0 directly before loading onto the preconditioned SPE column (6 mL, 500 mg, 37-55 µm particle, Waters). Samples were washed with 10 mL of water and 6 mL of n-hexane, dried and eluted by gravity with 8 mL of methyl formate.

All organic phases from the protocols were collected in sample tubes containing 6 μ L of 30% glycerol in MeOH. The extracts were then centrifuged (10 min, 4°C, 20 000 x g) and evaporated in a vacuum centrifuge (1 mbar, 30°C, 90-120 min; Christ, Osterode, Germany) until only the 2 μ L glycerol plug was left. The dried residues were immediately frozen at -80°C. Within 48 h the residues were reconstituted in 50 μ L of MeOH containing 40 nM of IS2, centrifuged (10 min, 4°C, 20 000 x g) and analyzed by LC-MS.

In a further set of samples, the IS was added after the LLE/SPE to the extracts to distinguish between a loss of IS during the extraction and a suppressed ESI signal. Samples for ion suppression analysis were prepared without addition of IS.

In order to investigate the effect of the cartridge size, the **SepPak** was carried out with a 3 mL (200 mg) cartridge and the **AnionEx-Weak** was additionally carried out on 6 mL cartridges (500 mg). The volumes for cleaning, equilibration, washing and elution were adjusted to the cartridge size. In brief, 3 mL SepPak cartridges were washed with 5 mL of water and 3 mL of n-hexane and eluted with 4 mL of methyl formate and samples loaded on the 6 mL AnionEx column were washed with one column volume of MeOH/water (50/50, v/v) and eluted with 5 mL of 25/75 (v/v) n-hexane/EA with 1% HAc.

LC-MS analysis

Quantification of oxylipins was carried out by LC-MS according to the method of Yang et al. [10,20] adapted to the instrument in our lab as described in detail in the electronic supplementary material.

In brief, separation was carried out utilizing an Agilent 1290 LC (Agilent, Waldbronn, Germany) on an Agilent Zorbax Eclipse Plus C-18 reversed phase column (2.1 x 150 mm, particle size 1.8 μ m) with a gradient of 0.1% aqueous HAc as solvent A and ACN/MeOH/HAc (800/150/1, v/v/v) as solvent B. Samples (5 μ L) were injected by an xt-PAL autosampler (CTC Analytics, Zwingen, Switzerland). The outlet of the analytical column was connected to a Valco six-port two-position valve implemented in the MS allowing a reduced contamination of the MS source by directing the void volume to waste. Mass spectrometric detection was carried out on an AB Sciex 6500 QTRAP instrument (AB Sciex, Darmstadt, Germany) in scheduled SRM mode following negative ion electrospray ionization (ESI). Instrument controlling was performed with Analyst 1.6.2. and data analysis was carried out with Multiquant 2.1.1. (AB Sciex).

Recovery of IS ($^2\text{H}_4$ -6-keto-PGF $_{1\alpha}$, $^2\text{H}_4$ -PGE $_2$, $^2\text{H}_4$ -PGD $_2$, $^2\text{H}_4$ -TxB $_2$, $^2\text{H}_4$ -LTB $_4$, $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE, $^2\text{H}_8$ -12-HETE, $^2\text{H}_6$ -20-HETE, $^2\text{H}_{11-14,15}$ -DiHETrE, $^2\text{H}_{11-14(15)}$ -EpETrE, $^2\text{H}_4$ -9(10)-EpOME and $^2\text{H}_4$ -9(10)-DiHOME) was calculated by an external calibration (2-40 nM) based on the peak areas. Quantification of oxylipins in plasma was performed by an external calibration using 13 deuterated internal standards (Tab. S1). For calibration, the analyte to IS area ratios were linearly fitted reciprocally weighted by concentration. Additionally the IS 2 peak area (added in the last step of sample preparation) was monitored as measure for precision of injection and detection signal over the analysis batch and was found to be within (100 \pm 10%). For ion suppression analysis a 30 nM solution of the IS ($^2\text{H}_4$ -PGE $_2$, $^2\text{H}_4$ -PGD $_2$, $^2\text{H}_4$ -TxB $_2$, $^2\text{H}_4$ -LTB $_4$, $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE, $^2\text{H}_8$ -12-HETE, $^2\text{H}_6$ -20-HETE, $^2\text{H}_{11-14,15}$ -DiHETrE, $^2\text{H}_{11-14(15)}$ -EpETrE, $^2\text{H}_4$ -9(10)-EpOME and $^2\text{H}_4$ -9(10)-DiHOME) in MeOH was postcolumnly added to the eluate of the LC column (flow 0.8 mL/h) and the transitions of the compounds (Tab. S2) were monitored with a dwell time of 20 ms.

Results

Recoveries of internal standards

The % recoveries of the 13 deuterated IS spiked to 500 μ L human plasma using the SPE protocols are shown in Fig. 1 and recoveries with the LLE protocol are presented in Fig. S1. The results are also shown in more detail in the supplementary material (Fig. S1 and Tab. S4). With the LLE protocol recoveries of all 13 deuterated IS were below 40% (Tab. S4, Fig. S1), which is clearly below the performance of all SPE protocols (Fig. 1). Furthermore, the intersample (in batch) precision was low with a relative standard deviation (RSD) of the IS concentration of >34% for $^2\text{H}_4$ -6-keto-PGF $_{1\alpha}$, $^2\text{H}_4$ -TxB $_2$, $^2\text{H}_4$ -PGE $_2$, $^2\text{H}_4$ -PGD $_2$, $^2\text{H}_4$ -LTB $_4$ and $^2\text{H}_4$ -9,10-DiHOME and 15-26% for the other IS (Fig. S1, Tab. S4).

Using the Oasis-MeOH SPE protocol, recoveries of internal standards were below 62% (Fig. 1) except for $^2\text{H}_{11-14(15)}$ -EpETrE (68 \pm 2.1%). Compared to the other SPE protocols the recovery rates were generally low being the lowest among all protocols e.g. for $^2\text{H}_4$ -LTB $_4$, $^2\text{H}_4$ -9,10-DiHOME, $^2\text{H}_8$ -12-HETE and $^2\text{H}_{11-14(15)}$ -EpETrE (Fig. 1).

Overall good recovery rates ($\geq 65\%$) were obtained with the AnionEx-Weak protocol (Fig. 1). Following addition of the IS after the SPE-step the determined IS concentrations were slightly higher. This indicates, that small amounts of the analytes were lost during the SPE, probably due to incomplete elution from the sorbent bed. Using a less polar elution solvent (AnionEx-Strong), the polar IS $^2\text{H}_4$ -6-keto-PGF $_{1\alpha}$, $^2\text{H}_4$ -TxB $_2$, $^2\text{H}_4$ -PGE $_2$ and $^2\text{H}_4$ -PGD $_2$ were almost completely lost during SPE (recovery rates 0-54%) while recoveries were above 65% for all other IS. These losses are probably a result of insufficient elution during the SPE because recovery rates $\geq 55\%$ were found when adding the IS directly after the SPE-step (Fig. 1). The AnionEx-Strong protocol was designed for the analysis of medium to non-polar hydroxy-, dihydroxy- and epoxy-FA [6], thus low recovery rates of the polar prostanoids might be less important. However, it is interesting that even the recovery rates for the non-polar analytes were slightly better with a more polar elution solvent (AnionEx-Weak), though being acceptable ($>65\%$) for the AnionEx-Strong. Moreover, the precision of the AnionEx-Strong protocol was low (RSD $>17\%$ for medium to non-polar analytes) while RSD of the determined IS concentration in all other SPE protocols were $<10\%$ (Tab. S4).

Good overall recoveries ($\geq 68\%$) were observed for all IS with the SepPak protocol, except for $^2\text{H}_4$ -PGE $_2$ (recovery 41 \pm 2.4%). Losses during extraction can be ruled out, since there was no difference in the recovery rate of $^2\text{H}_4$ -PGE $_2$ between the addition of IS at the beginning of the sample preparation and addition of the IS directly after the SPE-step (Fig. 1, Tab. S4). This is consistent with the results from the ion suppression analysis for the $^2\text{H}_4$ -PGE $_2$ and $^2\text{H}_4$ -PGD $_2$ signal (Fig. 2A). Strong ion suppression of about 75% of the signal was observed exactly at the retention time of $^2\text{H}_4$ -PGE $_2$ ($t_R=4.56$ min) leading to the low recovery rate of $^2\text{H}_4$ -PGE $_2$ with this protocol.

The Oasis-EA protocol yielded recovery rates $\geq 64\%$ for all IS, except for $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE and $^2\text{H}_4$ -9(10)-EpOME. Nevertheless, when comparing recoveries with direct addition of IS and addition of IS after the SPE-step it becomes clear that significant amounts of almost all IS were lost during the SPE step (e.g. $^2\text{H}_6$ -20-HETE, $^2\text{H}_4$ -9(10)-EpOME and $^2\text{H}_{11-14(15)}$ -EpETrE). For $^2\text{H}_4$ -9-HODE no differences in the recoveries were observed (Fig. S2) when adding the IS before the SPE-step or after the SPE-step. As shown in Fig. 2B a significant ion suppression takes place in the $^2\text{H}_4$ -9-HODE signal at its retention time providing a mechanistic explanation for the poor recovery rate. Similarly, the recovery rate of $^2\text{H}_8$ -5-HETE with post-SPE addition of IS was with 69 \pm 4.1% significantly lower than for the other medium to non-polar IS (e.g. 89-97% for both epoxides). Ion suppression also occurred for $^2\text{H}_8$ -5-HETE (Fig. S3E), though not as pronounced as for $^2\text{H}_4$ -9-HODE. Slight ion suppression was also observable for $^2\text{H}_4$ -9(10)-EpOME (Fig. 2F) although the recovery rate with post-SPE addition of IS was 89%.

With the StrataX protocol, low recovery rates ($\leq 55\%$) were observed for $^2\text{H}_4$ -PGD₂, $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE and $^2\text{H}_4$ -9(10)-EpOME. Ion suppression accounted significantly for the loss of $^2\text{H}_4$ -9-HODE (Fig. S3D) and $^2\text{H}_4$ -9(10)-EpOME (Fig. 2G), although $^2\text{H}_4$ -9(10)-EpOME was also lost during the SPE-step (Fig. 1). The loss of $^2\text{H}_4$ -PGD₂ and $^2\text{H}_8$ -5-HETE can in part also be explained by slight ion suppression (Fig. S3A, Fig. S3E). The low recovery rate of $^2\text{H}_8$ -5-HETE could partly be explained by incomplete extraction (Fig. 1). Low extraction losses could also be observed for $^2\text{H}_4$ -9,10-DiHOME and $^2\text{H}_8$ -20-HETE (Fig. 1).

Overall, the ESI-signal in the ion suppression analysis was flatest with the Anion-Ex protocols followed by the SepPak protocol. Intense negative signals, particularly at late retention times occurred with the Oasis-EA, Oasis-MeOH and StrataX protocols (Fig. 2F-H, Fig. S3). A large number of oxylipins elutes at these retention times (Tab. S1). Thus, ion suppression strongly influenced the signal of these oxylipins.

Extraction efficacy of oxylipins from plasma

Aside from removing ion suppressing matrix compounds SPE should lead to an efficient extraction of oxylipins from plasma. Since all IS were more or less affected by the matrix, the absolute peak areas were used as measure for the extraction efficacy of oxylipins. Tab. 1 shows the absolute areas of a representative set of oxylipins following extraction of 500 μL of plasma with the different protocols. The areas of all other determined analytes can be found in the electronic supplementary material (Tab. S5). Consistent with the poor recovery of IS, the Oasis-MeOH protocol yielded low peak areas compared to the other protocols (e.g. prostanoides, diols, 20-HETE, 5-HETE). The extraction efficacy of the StrataX protocol was overall low too, yielding peak areas in the lower range for several epoxy-FA and hydroxy-FA, being lowest e.g. for 9(10)-EpOME, 19(20)-EpDPE, 9-HODE and 12-HETE. Furthermore, some epoxy-FA could not be detected at all, i.e. 11(12)-EpETE, 8(9)-EpETE and 13(14)-EpDPE (Tab. 1, Tab. S5). However, for several dihydroxy-FA the StrataX protocol yielded absolute areas in the higher range being highest, e.g. for 14,15-DiHETrE or 17,18-DiHETE (Tab. 1, Tab. S5).

Both Anion-Ex protocols led to absolute areas of epoxy-FA in the lower range compared to the other SPEs. Due to this low extraction efficacy several epoxy-FA could not be detected in the AnionEx extracts, e.g. 8(9)-EpETE and 13(14)-EpDPE (Tab. 1, Tab. S5). For various hydroxy-FA both protocols yielded areas in the higher range, e.g. 9-HODE and 12-HETE, while for dihydroxy-FA an overall low extraction efficacy was found, e.g. 14,15-DiHETrE (Tab. 1). The Oasis-EA protocol yielded intermediate results for the analytes. In a few cases it led to the highest or lowest peak areas, e.g. 5,6-DiHETrE and 15(16)-EpODE (Tab. S5) while extraction efficacy was in the upper range for epoxy-FA.

The SepPak protocol extracted oxylipins most efficiently from plasma and for many analytes this protocol yielded the highest peak areas (Tab. 1, S5). Only for a few polar analytes, e.g. TxB₂ as well as PGE₂, and for 15-oxo-ETE larger peaks were found with the other SPE protocols. A superior extraction efficacy of SepPak SPE was particularly found for the non-polar epoxides (Tab. 1 and S5): Compared to the AnionEx-Weak protocol up to 9-fold higher peak areas were found (16(17)-EpDPE, Tab. S5) and epoxides could be detected in human plasma which were below LOD using AnionEx and StrataX protocols, e.g. 13(14)-EpDPE (Tab. 1). Compared to the second most efficient protocol for the extraction of epoxides (Oasis-EA, Tab. 1) the area with the SepPak protocol was still up to 5-fold higher (16(17)-EpDPE, Tab. S5).

Cartridge Size

A possible reason for the differences in extraction efficacy is the varying cartridge size of the different SPE protocols (60-500 mg stationary phase) [1,9,11,21,2,8,18]. In order to assess the influence of the amount of stationary phase, the SepPak and AnionEx-Weak protocols were compared using 3 mL (200 mg) and 6 mL (500 mg) cartridges. The absolute areas of selected analytes are shown in Tab. 2 and all analytes are displayed in Tab. S5. Compared to the differences between the protocols, the cartridge size has only minor effects on the extraction

efficacy. Using the SepPak protocol with the smaller column the peak areas were for most analytes in the same range as with the original protocol ($\pm 20\%$), e.g. TxB_2 , 14,15-DiHETrE or 20-HETE. However, several analytes, especially epoxides, showed significantly higher absolute areas with the larger cartridge (120-290% of the area with the smaller cartridge), e.g. 9(10)-EpOME or 14(15)-EpETE.

A similar trend was observed for AnionEx-Weak SPE. With the larger column peak areas of most analytes were within a range of $\pm 20\%$ of the original protocol, while several analytes, mostly dihydroxy-FA, were in a range of 120-160% of the original protocol with extraction on the larger column, e.g. 13,14-DiHDPE or 13-HOTrE (Tab. S5).

Nevertheless, when comparing the SepPak and the AnionEx-Weak SPE (both 500 mg, 6 mL cartridge) the absolute areas of most epoxides were still up to 6-7 fold higher, e.g. 16(17)-EpDPE and 9(10)-EpODE, while extraction efficacy of dihydroxy-FA and hydroxy-FA was in the same range ($\pm 20\%$).

Calculated concentrations

Finally, all plasma concentrations of the oxylipins were calculated using the analyte/IS ratio (Tab. S1) and external calibration. Fig. 3 shows the concentrations of a representative set of eicosanoides determined with the different protocols. The concentration of all analytes can be found in supplement Tab. S6.

For the AA derived polar prostanoides TxB_2 , PGE_2 and PGD_2 , which were all quantified with an isotopically labeled surrogate as IS, concentrations were in the same range with the different protocols. However, for TxB_2 the Oasis-EA led to a higher concentration and for PGD_2 the StrataX protocol yielded a significantly lower concentration. These results were consistent with the extraction efficacy of the analytes which was highest for TxB_2 with the Oasis-EA and lowest for PGD_2 with the StrataX protocol (Tab. 1). Though group specific IS were used (Tab. S1), all protocols also led to overall similar concentrations for HETEs and DiHETrEs. However, the plasma concentration of 12-HETE was significantly higher for both AnionEx protocols, consistent with the high extraction efficacy for this analyte (Tab. 1). For 11,12-DiHETrE the Oasis EA and the StrataX protocol yielded lower concentrations compared to the other SPE protocols.

Dramatic differences were observed in the concentrations of epoxy-FA. Consistent with the extraction efficacy, the SepPak protocol clearly led to the highest concentrations followed by the Oasis-EA and the Oasis-MeOH protocol. Interestingly, the precision of the Oasis-EA protocol was low for some epoxides, e.g. 11(12)-EpETrE, 8(9)-EpETrE, 5(6)-EpETrE (RSD=43-53%). For the Anion-Ex SPE, 8(9)-EpETrE could even not be quantified and 11(12)-EpETrE as well as 14(15)-EpETrE for example were detected in a concentration ≤ 0.20 nM while the SepPak protocol yielded concentrations ≥ 1.1 nM.

Discussion

The aim of the present study was to identify the most appropriate protocol for the analysis of free (non-esterified) oxylipins in EDTA plasma. Aside from analysis of total oxylipins after liberation of esterified oxylipins by saponification, quantification of free oxylipins in plasma is the most commonly carried out analysis in studies on the biology of oxylipins (for discussion see [21,22]).

Taking the high costs for standards, cartridges and instrument time into account, we did not perform the common tiered step-by-step-optimization procedure to develop a sample preparation strategy. Instead, we compared the efficacy of well-established sample preparation protocols. All these protocols are "fit for purpose" as they have been successfully used to study oxylipin biology: The Oasis-EA method is from the Hammock lab (Davis, CA, USA) [10] and has also been successfully employed by other groups [8]. Modifications of this protocol, e.g. with different eluents, are also commonly used and elution with methanol (Oasis-MeOH) according to Balvers et al. [17] was included in the comparison. The AnionEx SPE protocols originate from the contract research organization (CRO)/laboratory Lipidomix, Berlin, Germany

and have been successfully employed by the groups of Schunck and Weylandt (Berlin, Germany) [6,13] among others. The StrataX and the SepPak SPE protocols are from the major labs in the field of oxylipin research, i.e. E. Dennis (San Diego, CA, USA) [9] and C. Serhan (Boston, MA, USA) [11]. Also the LLE protocol is commonly used, e.g. by the lab of Fleming (Frankfurt, Germany) [15]. All methods were carried out as described by these groups. However, sample preparation was in some cases adapted for plasma and the overall procedure (waiting times, used glassware, plastic tubes etc.) was kept the same in order to assure comparability (Tab. S3). The sample volume was set to 500 μ L, since lower volumes are, because of the low concentration of most oxylipins, doomed to failure in plasma despite highly sensitive LC-MS instruments [14].

Regarding the IS recovery rates (Fig. 1, Fig. S1, Fig. S2, Tab. S4), LLE and the Oasis-MeOH SPE are clearly outperformed by the other protocols. With the low IS recovery and the low precision, LLE is in our hands not an appropriate sample preparation for the analysis of oxylipins in plasma and thus excluded from further discussion. With acceptable recovery rates (>60%) for few oxylipins Oasis-MeOH SPE seems to be appropriate for specific questions. However, compared to the other protocols there is no reason to choose this protocol in targeted oxylipin metabolomics particularly because eluting with MeOH and EA (Oasis-EA) yielded much better results and acceptable IS recoveries (>60%) except for $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE and $^2\text{H}_4$ -9(10)-EpOME. The StrataX SPE showed a similar performance, leading to low recoveries (<60%) for four IS ($^2\text{H}_4$ -PGD₂, $^2\text{H}_4$ -9-HODE, $^2\text{H}_8$ -5-HETE and $^2\text{H}_4$ -9(10)-EpOME). Ion suppression analysis of the extracts unveiled that both StrataX and Oasis SPE insufficiently remove interfering matrix compounds (Fig. 2, Fig. S3). These compounds led to massive ion suppression and caused for example the poor recovery of $^2\text{H}_4$ -9-HODE (Fig. 2B, Fig. S3D) or $^2\text{H}_4$ -9(10)-EpOME (Fig. 2F-H). In comparison, the SepPak and AnionEx SPE led to a better removal of matrix and the ion suppression signals were smoother and more flat (Fig. 2, Fig. S3). For the SepPak SPE only a single, strong signal occurred in the ion suppression analysis at t_R =4.56 min. As a consequence the recovery of the coeluting $^2\text{H}_4$ -PGE₂ is poor while the recovery rate of all other IS is good (68-97%). Nevertheless, the ion suppression analysis showed several signals of potentially interfering matrix compounds in the SepPak extract. However, these seem not be prone to disturb the analysis since for example the small peak at 15.95 min does not cause a relevant suppression of the coeluting $^2\text{H}_4$ -9(10)-EpOME. The ion suppression analysis of the AnionEx extracts was even superior and showed only few small peaks. Consistently, the IS recovery for the AnionEx-Weak is nearly perfect (all analytes \geq 65%). The AnionEx-strong SPE led also to good – however not better – recoveries for IS eluting later than $^2\text{H}_4$ -LTB₄, while the polar oxylipins were lost.

The solvent used to elute the broad diversity of oxylipins from the SPE material has to be polar enough to elute the polar analytes (AnionEx-Strong vs. AnionEx-Weak), but must also possess a sufficiently strong elution power (see Oasis-EA vs. Oasis-MeOH) to elute all analytes quantitatively. While all protocols simply use polar eluents for equilibration, sample loading and washing followed by non-polar eluents for elution, the SepPak protocol utilizes the poor elution power of highly non-polar eluents for oxylipins: Here the polar washing step (with water) is followed by a washing step with pure n-hexane (eluting potentially interfering lipids [23]), before the oxylipins are eluted with the medium polar methyl formate. This results in an efficient reduction of ion suppressing matrix, particularly compounds of low polarity are removed. Thus at run times >10 min the ion suppression signal of the SepPak protocol is dramatically better than that of Oasis and StrataX protocols (Fig. 2, Fig. S3). However, it does not seem like the elution strategy for the SepPak SPE can easily be transferred to these polymeric stationary phases (Tab. S8). Regarding IS recovery and ion suppression only the AnionEx-Weak protocol, making use of the acidic properties of all oxylipins (oxidative fatty acid derivatives) can compete with the SepPak protocol. Because of the poor recovery of $^2\text{H}_4$ -PGE₂ in the SepPak SPE (Fig. 1, Fig. 2), the AnionEx-weak SPE is overall the best protocol in terms of IS recovery.

It is somewhat striking that our results clearly demonstrate that good IS recovery does not necessarily translate to a high extraction efficacy of oxylipins from the plasma matrix (Tab. 1,

Tab. S5). While several oxylipins were extracted in the same range by SepPak, StrataX, AnionEx-Weak and Oasis-EA, e.g. TxB₂, 9,10-DiHOME, 20-HETE, dramatic differences were found for extraction efficacy of analytes of low polarity, e.g. 9(10)-EpOME and 10(11)-EpDPE. The AnionEx-Weak protocol led even for the latest eluting epoxides to a better extraction efficacy than the AnionEx-Strong protocol (Tab. 1), and thus is for all oxylipins investigated clearly the more appropriate protocol compared to the AnionEx-Strong procedure. However, the peak areas of epoxides with the AnionEx-Weak were up to 7-fold lower compared to the SepPak SPE. Because of the flat ion suppression (Fig. 2E, Fig. S3), this effect seems to be caused by poor extraction efficacy rather than by ion suppression. Interestingly, the ratio between column dimension and sample volume (analyte and matrix amount) was found to be only of minor importance for the extraction efficacy. Even with a large 6 mL (500 mg) cartridge, the peak areas of epoxy-FA resulting from the AnionEx-Weak protocol were dramatically lower than those of the SepPak SPE (Tab. 2, Tab. S5). The resulting smaller peaks with the AnionEx-Weak protocol do not only influence the calculated concentrations (see below) but sacrifice sensitivity. For example 13(14)-EpDPE which can be well quantified with SepPak and Oasis SPE is below the detection limit with AnionEx-Weak. Thus, no information about this potentially biologically active lipid mediator [6,3] in human plasma can be obtained despite using a state of the art LC-qqq-MS instrument.

The central aim of the LC-MS analysis of oxylipins is to provide quantitative information about their concentration in biological matrices. Compared to other LC-MS based analyses, e.g. residues of pesticides where a heavy isotope surrogate is used as internal standard for each analyte, only few isotopically labeled oxylipins are available as IS. Thus, a single IS is used for the quantification of a group of oxylipins. Typically, ²H₄-PGE₂ is used for PGs, one or few deuterated hydroxy-FA are used as IS for the whole group of hydroxy-FA, one or few deuterated dihydroxy-FA for the class of dihydroxy-FA and so on. Though being structurally similar, the retention time of the analyte differs from that of the IS. Thus, the IS cannot compensate for ion suppressing effects from the matrix, which directly influence the calculated concentrations. As a result, the different SPE protocols lead to significantly different plasma concentrations of the oxylipins (Fig. 3, Tab. S6). While the obtained concentrations of several prostanoids, hydroxy-FA and dihydroxy-FA were in same range, the determined concentrations of epoxy-FA are dramatically different (Fig. 3). It therefore seems to be difficult to compare results from studies using different SPE methods. In order to investigate the role of oxylipins in biology, their levels always have to be compared within the same study using exactly the same analytical method.

So what is the most appropriate protocol for the analysis of free oxylipins in plasma?

The Oasis-EA protocol efficiently extracts oxylipins from plasma (Tab. 1, Tab. S5). However, IS recovery and analytes are strongly affected by severe ion suppression caused by matrix compounds not removed by the sample preparation procedure (Fig. 1, Fig. 2, Fig. S2, Fig. S3, Tab. S4). Particularly the concentration of analytes with a retention time >10 min could significantly be underestimated because the few IS used cannot compensate for the strong ion suppression in the analyte signals. Despite a near perfect removal of ion suppressing matrix, the use of the AnionEx-Weak protocol seems to be problematic since the concentration of epoxy-FA is massively underpredicted or these potent lipid mediators [6,3,20,4,5] are not detected at all. The only SPE method tested yielding good extraction efficacy and reduction of matrix compounds is the SepPak protocol. Only the ion suppression at 4.56 min directly affecting the quantification of PGE₂ is an obvious problem of the method. As shown in Fig. 3 the determined PGE₂ levels are consistent with the other SPE methods because of the use of ²H₄-PGE₂ as IS is correcting for the ion suppression. However, the use of ²H₄-PGE₂ (rec=41±2.4%) for other PGs, as carried out in our LC-MS method (Tab. S1), is problematic and another IS, e.g. ²H₄-PGD₂ should be used for quantification of PGE₃, TriHOMEs, PGF_{2α}, PGE₁ and LXA₄. With these adjustment about 50% lower concentrations for these analytes result (Tab. S7) which are consistent with the concentrations obtained with the other protocols (Tab. S6). However, in the long term, the underlying cause of this ion suppression should be identified and an effort should be undertaken to remove it, e.g. by an optimized SPE procedure

or improved LC separation. It should be noted, that the type of octadecyl modification (e.g. endcapping chemistry, carbon loading) of the SPE material has dramatic effects on the extraction efficacy of oxylipins from plasma (electronic supplementary material, Tab S8). Even comparable RP-18 materials from different companies lead to remarkably different results. It is striking that these differences affect mostly epoxy-FA, as already shown for the different SPE protocols above.

Conclusion

Overall, our data clearly demonstrates that the SepPak SPE is considerably advantageous for the analysis of free oxylipins in plasma compared to the other described sample preparation methods. Nevertheless, our results also show that there is still need for further improvements of the protocol.

It should be noted that the ion suppression effects presented here might affect other LC-MS methods in another way. Different gradients and RP columns will lead to slightly different elution profiles and different coelutions of oxylipins and matrix. Moreover, our conclusions for extraction efficacy and precision are only valid for analysis of free oxylipins in human plasma. Other sample matrices, e.g. saponified samples or tissue extracts, might lead to different results and conclusions.

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Figure Captions:

Fig. 1: Recoveries of internal standards (IS) for the tested SPE protocols. IS was added to the samples either at the beginning of the analysis (panel A) or after the SPE-step (panel B). Shown is the mean recovery rate \pm SD (n=5).

Fig. 2: Ion suppression analysis for (A) $^2\text{H}_4$ -PGE₂ with SepPak-SPE, (B) $^2\text{H}_4$ -9-HODE with the Oasis-EA-SPE and (C-E) for $^2\text{H}_4$ -9(10)-EpOME with all SPE protocols. The retention time window of each IS is highlighted in dark grey, the elution window of all analytes using this IS is depicted in light grey.

Fig. 3: Calculated oxylipin plasma concentrations with the different SPE protocols: (A) prostanoides (B) hydroxy-AA, (C) dihydroxy-AA and (D) epoxy-AA. Internal standards used for the quantification of the analytes are shown in Tab. S1. All results are shown as mean \pm SD (n=5). It should be noted that panel A does not include the AnionEx-Strong protocol because it is not suited for the analysis of polar oxylipins.

Tables

Tab. 1: Peak areas of selected oxylipins obtained after the extraction of 500 μL plasma. Shown is the mean \pm SD (n=5). The areas of all oxylipins detected are presented in the SI (Tab. S5).

[1000 counts]	TxB ₂	PGE ₂	PGD ₂	9,10-DiHOME	14,15-DiHETrE	20-HETE	9-HODE
SepPak	31 \pm 1.7	21 \pm 2.2	30 \pm 1.7	2200 \pm 140	220 \pm 16	29 \pm 5.2	6500 \pm 280
AnionEx-Strong	<LOQ*	15 \pm 2.0*	22 \pm 5.0*	1500 \pm 300	180 \pm 35	22 \pm 3.8	6000 \pm 1100
AnionEx-Weak	39 \pm 0.72	36 \pm 1.9	27 \pm 2.1	1700 \pm 53	190 \pm 4.2	27 \pm 2.1	6100 \pm 180
Oasis-EA	44 \pm 14	41 \pm 4.4	18 \pm 1.6	1500 \pm 35	220 \pm 7.4	24 \pm 1.7	2400 \pm 100
StrataX	29 \pm 1.2	62 \pm 6.7	4.9 \pm 0.95	1400 \pm 83	250 \pm 19	24 \pm 1.7	2200 \pm 97
Oasis-MeOH	26 \pm 2.0	26 \pm 1.9	16 \pm 2.2	1300 \pm 36	150 \pm 6.3	23 \pm 2.9	2500 \pm 110

	12-HETE	5-HETE	14(15)-EpETrE	9(10)-EpOME	13(14)-EpDPE	10(11)-EpDPE	8(9)-EpETrE
SepPak	580 \pm 21	340 \pm 21	48 \pm 9.3	1200 \pm 150	32 \pm 6.1	63 \pm 11	11.6 \pm 2.2
AnionEx-Strong	860 \pm 180	300 \pm 60	8.8 \pm 2.0	260 \pm 53	<LOQ	11 \pm 2.4	2.9 \pm 0.56
AnionEx-Weak	740 \pm 38	280 \pm 8.3	10 \pm 0.88	310 \pm 18	<LOQ	14 \pm 1.1	3.4 \pm 0.51
Oasis-EA	370 \pm 28	330 \pm 13	23 \pm 5.8	520 \pm 160	6.8 \pm 3.2	22 \pm 7.8	9.1 \pm 3.8
StrataX	320 \pm 18	260 \pm 11	11 \pm 0.56	180 \pm 16	<LOQ	13 \pm 1.2	3.2 \pm 0.44
Oasis-MeOH	330 \pm 19	200 \pm 26	15 \pm 1.5	450 \pm 29	7.0 \pm 0.75	20 \pm 1.2	4.4 \pm 0.65

*protocol not suited for the analysis of these oxlipins, IS rec <60%

Tab. 2: Effect of SPE-cartridge size on the extraction efficacy. Peak areas obtained after extraction of 500 μ L plasma on 6 mL cartridges (500 mg) were compared to 3 mL cartridges (200 mg). Shown is the mean \pm SD (n=5). The areas of all oxylipins detected are presented in the SI (Tab. S5).

[1000 counts]	TxB ₂	PGE ₂	PGD ₂	9,10-DiHOME	14,15-DiHETrE	20-HETE	9-HODE
SepPak 6 mL	31 \pm 1.7	21 \pm 2.2	30 \pm 1.7	2200 \pm 140	220 \pm 16	29 \pm 5.2	6500 \pm 280
SepPak 3 mL	36 \pm 3.2	10 \pm 0.88	23 \pm 5.7	2100 \pm 150	270 \pm 30	29 \pm 5.7	7400 \pm 320
AnionEx-Weak 3 mL	39 \pm 0.72	36 \pm 1.9	27 \pm 2.1	1700 \pm 53	190 \pm 4.2	27 \pm 2.1	6100 \pm 180
AnionEx-Weak 6 mL	40 \pm 6.9	24 \pm 4.3	24 \pm 4.7	2000 \pm 330	240 \pm 48	28 \pm 8.7	8200 \pm 830
	12-HETE	5-HETE	14(15)-EpETrE	9(10)-EpOME	13(14)-EpDPE	10(11)-EpDPE	8(9)-EpETrE
SepPak 6 mL	580 \pm 21	340 \pm 21	48 \pm 9.3	1200 \pm 150	32 \pm 6.1	63 \pm 11	11.6 \pm 2.2
SepPak 3 mL	660 \pm 47	340 \pm 41	17 \pm 1.7	680 \pm 46	20 \pm 2.5	50 \pm 5.1	7.2 \pm 0.71
AnionEx-Weak 3 mL	740 \pm 38	280 \pm 8.3	10 \pm 0.88	310 \pm 18	<LOQ	14 \pm 1.1	3.4 \pm 0.51
AnionEx-Weak 6 mL	680 \pm 92	300 \pm 32	12 \pm 2.3	280 \pm 39	<LOQ	16 \pm 3.3	3.9 \pm 0.85

Fig. 1

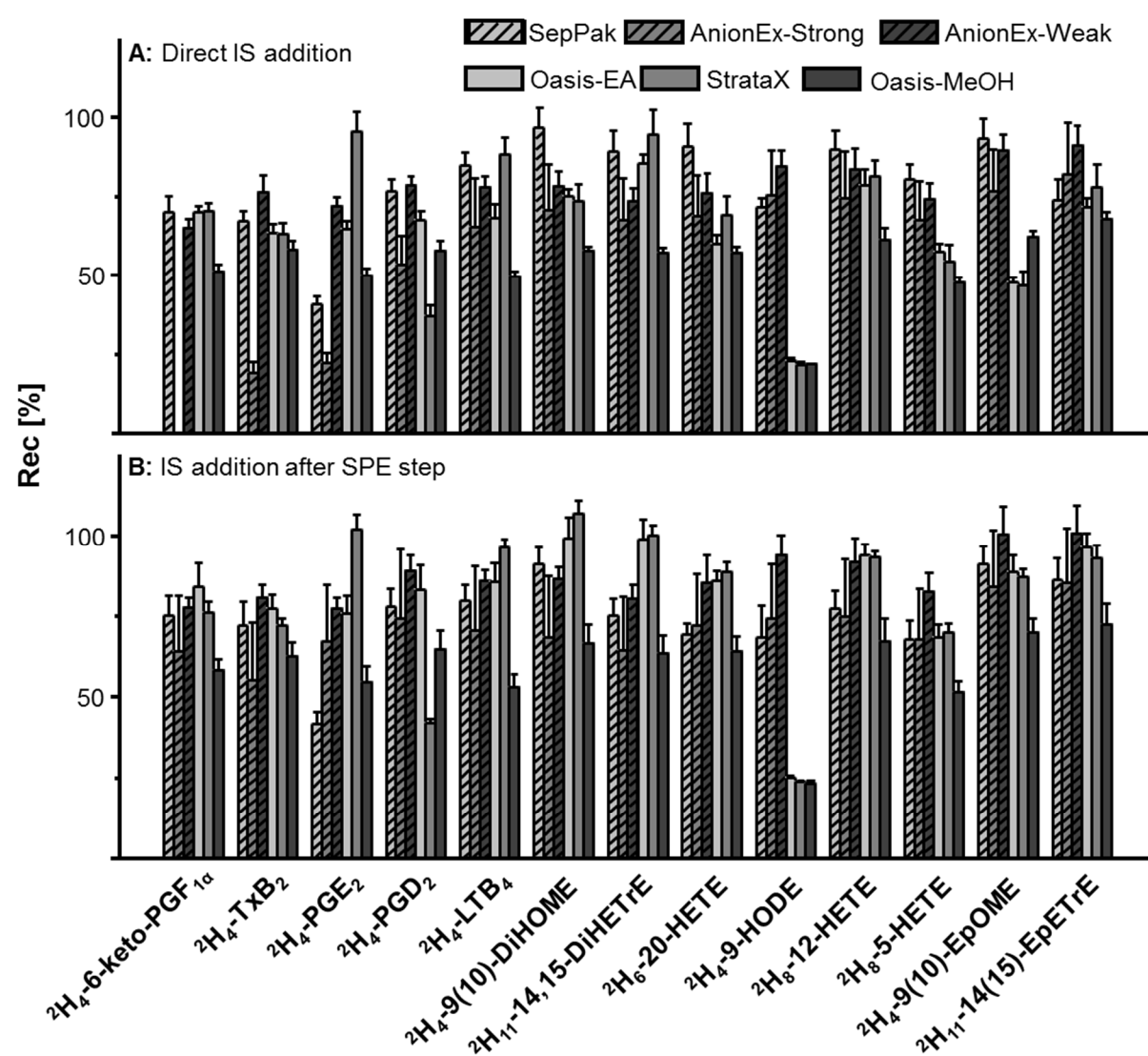


Fig. 2

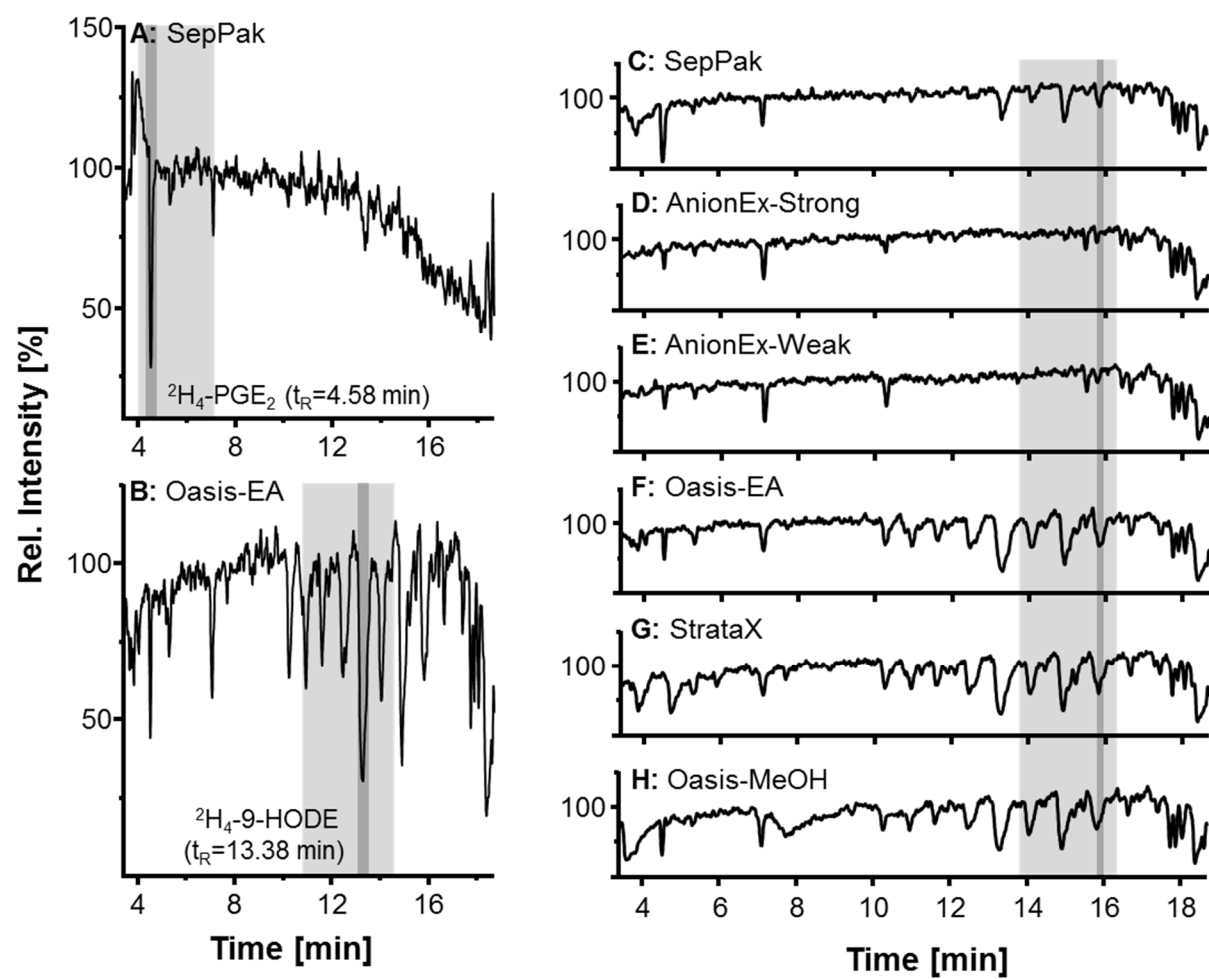
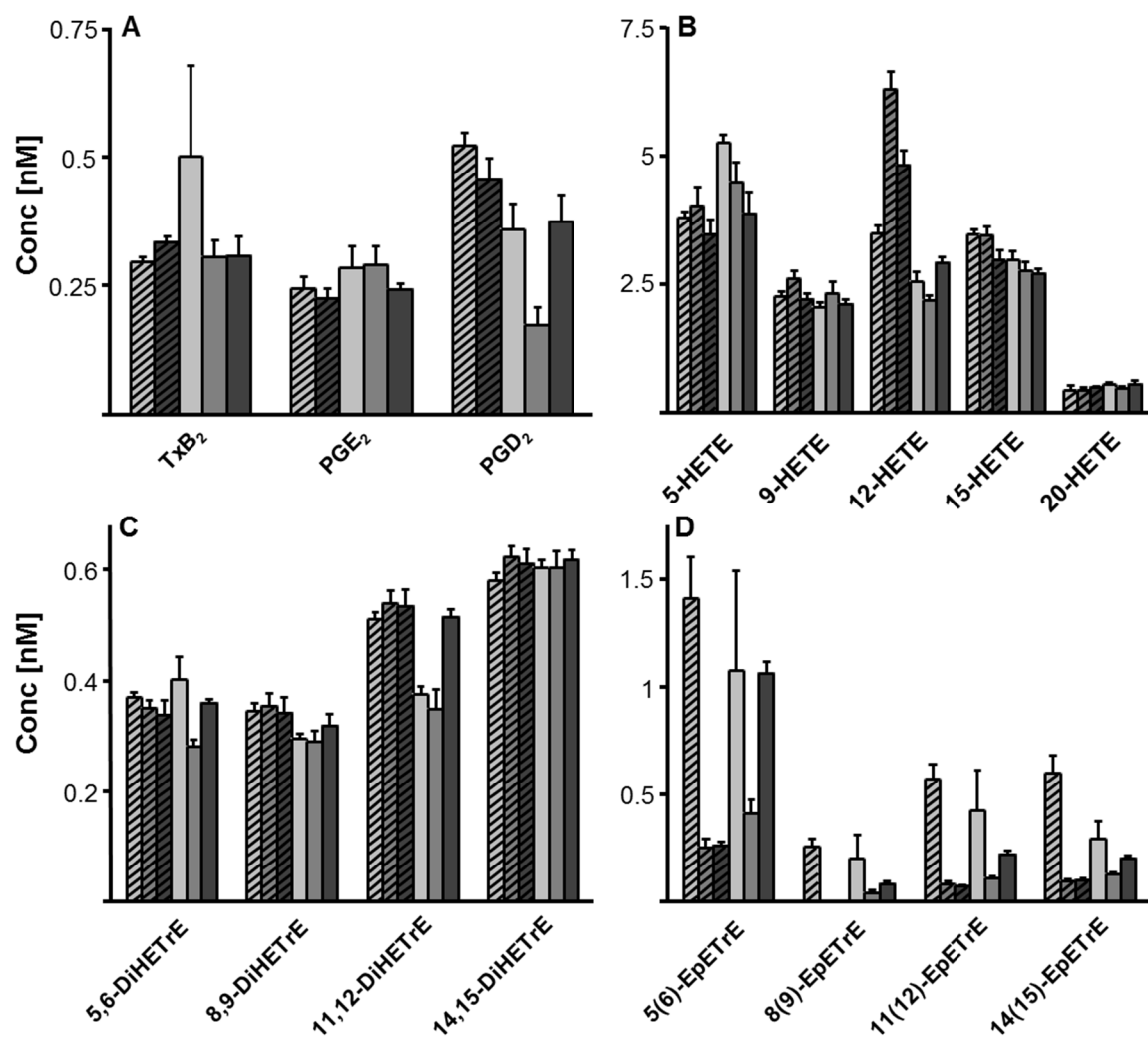


Fig. 3

 SepPak
  AnionEx-Strong
  AnionEx-Weak
  Oasis-EA
  StrataX
  Oasis-MeOH



LC-MS oxylipin analysis

Prior analysis, the samples were kept at 4°C in a HTS xt-PAL autosampler (CTC Analytics, Switzerland, local distributor: Axel Semrau, Sprockhövel, Germany) equipped with a 20 µL sample loop and 100 µL syringe.

A 5 µL aliquot of the sample solution is injected in the flow of an 1290 LC System (Agilent, Weilbronn, Germany), on an Agilent Zorbax Eclipse Plus C-18 reversed phase column (dimensions 2.1 x 150 mm, particle size 1.8 µm) with a Phenomenex C-18 SecurityGuard Ultra C18 cartridge as precolumn (cat. nr. AJ0-8782, Phenomenex, Torrance, CA, USA) kept in a column oven at 40 °C.

The oxylipins are separated by a binary solvent gradient with 0.1% acetic acid as solvent A and 800/150/1 (v/v) acetonitrile/methanol/acetic acid as solvent B at a flow rate of 0.3 mL/min: 0-0.25 min isocratic 35% B, 0.25-3.00 min linear from 35% B to 53% B, 3.00-12.50 min linear from 53% B to 68% B, 12.50-17.50 min linear from 68% B to 95% B, 17.50-19.00 min isocratic 95 % B, 19.00-19.10 linear from 95% B to 35% followed by reconditioning for 2.40 min. Utilizing the 2-position-6-port valve build in the MS the eluent was directed to waste during the first 2.5 min and the last 3.5 min of each run to reduce contamination of the MS source.

The detection was carried out using a 6500 QTRAP instrument (AB Sciex, Darmstadt, Germany) following negative electrospray ionization. The oxylipins were detected in scheduled selected reaction monitoring mode (Tab. S1). The detection window was set to ± 22.5 s around the expected retention time and a maximum cycle time of 0.5 s allowing the detection of at least 18 data points per compound.

The optimized source settings are: Ion-spray voltage of -4500 V, 35 psi curtain gas, 60 psi nebulizer gas (gas 1) and 60 psi drying gas (gas 2) at a temperature of 475 °C. The vertical axis offset of the sprayer was 0.528 cm and the horizontal 0.540 cm. Nitrogen was used as collision gas at 12 psi ("high") and all transitions were monitored in unit resolution with an entrance potential of -10 V. Analyst Software (version 1.6.2., AB Sciex) was used for controlling the LC-ESI-MS system and data acquisition. Multiquant (version 2.1.1, AB Sciex) was used for integration and quantification. The analyte concentrations of the samples were calculated directly by comparison of the analyte peak area detected with that of the IS (Tab. S1). For calibration, the analyte to IS ratios were fitted in a linear way reciprocally weighted by concentration. Only those oxylipins were included in data analysis exceeding LOQ in >60% (e.g. 3 out of 5) of the samples.

Table S1 Parameters of the LC-ESI(-)-MS/MS method for the determination of the concentration of oxylipins in biological samples. Shown are all analytes covered, the mass transition used for quantification in scheduled SRM mode, the electronical MS parameters (Declustering Potential (DP), Collision Energy (CE), Collision Cell Exit Potential (CXP)), the internal standard (IS), the retention time and its standard deviation (SD), peak width (full width and full width at half maximum height (FWHM)), limit of detection and the calibration range. The actual dynamic range for the quantification in the sample matrix depends on the dilution or concentration steps (10 fold for 500 μ L plasma analysis) during sample preparation.

Analyte	Mass transition		MS Parameters			Internal Standard (IS)	Retention time		Peak width		LOD	Calibration range	
	m/z (MS1)	m/z (MS3)	DP (V)	CE (V)	CXP (V)		(min)	SD ¹ (sec)	Full (s)	FWHM (s)	LOD (nM)	lower conc ² (nM)	upper conc ³ (nM)
6-keto-PGF _{1α}	369.3	163.2	-70	-36	-6	² H ₄ -6-keto-PGF _{1α}	3.18	0.69	34	18	< 0.90	0.90	361
20-COOH-LTB ₄	365.2	347.2	-80	-25	-8	² H ₄ -TxB ₂	3.22	1.50	17	3	0.50	1.00	200
Resolvin _{E1}	349.3	195.0	-65	-22	-10	² H ₄ -TxB ₂	3.25	0.34	13	3	0.60	1.20	480
20-OH-LTB ₄	351.2	195.2	-80	-25	-8	² H ₄ -TxB ₂	3.37	0.30	14	3	0.10	0.25	200
TxB ₂	369.2	169.1	-60	-25	-7	² H ₄ -TxB ₂	3.69	0.19	22	3	0.25	0.63	500
PGE ₃	349.3	269.2	-60	-22	-6	² H ₄ -PGE ₂	4.01	0.20	7	3	0.15	0.30	120
PGD ₃	349.3	269.2	-60	-22	-6	² H ₄ -PGD ₂	4.22	0.26	9	3	0.50	1.00	200
9,12,13-TriHOME	329.2	211.1	-80	-32	-10	² H ₄ -PGE ₂	4.31	0.16	22	3	0.50	1.25	1000
9,10,13-TriHOME	329.2	171.1	-80	-32	-8	² H ₄ -PGE ₂	4.38	0.17	16	2	0.20	0.50	400
PGF _{2α}	353.2	309.2	-80	-26	-7	² H ₄ -PGE ₂	4.39	0.34	13	3	0.35	0.70	281
PGE ₂	351.2	271.3	-60	-24	-6	² H ₄ -PGE ₂	4.58	0.15	11	3	< 0.10	0.10	200
PGE ₁	353.3	317.2	-60	-20	-6	² H ₄ -PGE ₂	4.71	0.17	9	2	0.13	0.33	260
PGD ₁	353.3	317.2	-60	-20	-6	² H ₄ -PGD ₂	4.82	0.17	13	3	0.25	0.50	200
PGD ₂	351.2	271.3	-60	-24	-6	² H ₄ -PGD ₂	4.85	0.29	12	3	0.50	1.00	200
LXA ₄	351.2	115.2	-60	-21	-8	² H ₄ -PGE ₂	5.25	0.18	13	3	0.09	0.18	70
11,12,15-TriHETrE	353.2	167.1	-80	-28	-10	² H ₄ -PGE ₂	5.23	0.10	10	3	0.50	1.00	100
LTB ₅	333.3	195.2	-65	-22	-8	² H ₄ -LTB ₄	6.58	0.16	10	3	0.10	0.25	200
PGJ ₂	333.3	189.2	-60	-25	-8	² H ₄ -PGE ₂	6.56	0.16	10	3	0.80	1.60	160

PGB ₂	333.3	175.1	-60	-28	-8	² H ₄ -PGE ₂	6.67	0.11	16	3	<0.40	0.40	800
THF diol	353.2	127.1	-80	-32	-8	² H ₄ -LTB ₄	6.77	0.19	15	3	0.13	0.25	100
15,16-DiHODE	311.2	223.2	-80	-29	-10	² H ₄ -9,10-DiHOME	7.36	0.16	12	3	0.20	0.50	400
8,15-DiHETE	335.2	235.2	-65	-22	-4	² H ₁₁ -14,15-DiHETrE	7.39	0.14	13	3	0.40	0.80	80
9,10-DiHODE	311.2	201.2	-65	-27	-10	² H ₄ -9,10-DiHOME	7.39	0.13	20	3	<0.20	0.20	400
12,13-DiHODE	311.2	183.1	-80	-30	-8	² H ₄ -9,10-DiHOME	7.47	0.16	14	4	1.00	2.00	400
6-trans-LTB ₄	335.2	195.1	-65	-23	-9	² H ₄ -LTB ₄	7.75	0.15	15	4	0.25	0.50	200
5,15-DiHETE	335.3	173.2	-60	-21	-8	² H ₁₁ -14,15-DiHETrE	7.77	0.14	17	4	0.13	0.25	100
17,18-DiHETE	335.3	247.2	-65	-24	-8	² H ₁₁ -14,15-DiHETrE	7.91	0.16	18	4	0.13	0.25	100
LTB ₄	335.2	195.1	-65	-23	-9	² H ₄ -LTB ₄	8.18	0.16	18	4	0.25	0.50	200
14,15-DiHETE	335.3	207.2	-65	-25	-10	² H ₁₁ -14,15-DiHETrE	8.43	0.17	14	4	0.13	0.25	100
11,12-DiHETE	335.2	167.1	-65	-26	-5	² H ₁₁ -14,15-DiHETrE	8.62	0.16	16	4	0.13	0.25	100
12,13-DiHOME	313.2	183.2	-80	-30	-8	² H ₄ -9,10-DiHOME	8.82	0.20	28	4	0.50	1.25	1000
8,9-DiHETE	335.2	127.1	-65	-26	-5	² H ₄ -9,10-DiHOME	8.98	0.19	15	4	0.25	0.50	100
9,10-DiHOME	313.2	201.2	-80	-29	-8	² H ₄ -9,10-DiHOME	9.23	0.19	27	4	<0.50	0.50	1000
19,20-DiHDPE	361.2	273.2	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	9.89	0.21	17	4	0.50	1.00	100
14,15-DiHETrE	337.2	207.1	-65	-25	-10	² H ₁₁ -14,15-DiHETrE	9.86	0.23	18	4	0.10	0.25	200
LTB ₃	337.2	195.2	-65	-22	-8	² H ₄ -LTB ₄	10.09	0.19	26	4	0.25	0.50	200
16,17-DiHDPE	361.2	233.2	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	10.45	0.19	16	4	0.25	0.50	100
11,12-DiHETrE	337.2	167.1	-65	-26	-8	² H ₁₁ -14,15-DiHETrE	10.60	0.22	21	4	<0.25	0.25	200
13,14-DiHDPE	361.2	193.2	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	10.71	0.23	19	4	0.13	0.25	100
9-HOTrE	293.2	171.2	-65	-22	-8	² H ₄ -9-HODE	10.95	0.18	18	4	0.25	0.50	100
10,11-DiHDPE	361.2	153.2	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	11.07	0.25	18	4	0.25	0.50	100
8,9-DiHETrE	337.2	127.1	-70	-30	-8	² H ₁₁ -14,15-DiHETrE	11.22	0.23	19	4	0.25	0.50	200
EKODE	309.2	291.1	-65	-20	-6	² H ₄ -9-HODE	11.26	0.23	16	4	0.25	0.50	100
13-HOTrE	293.2	195.1	-70	-24	-8	² H ₄ -9-HODE	11.30	0.32	15	4	0.30	0.60	60
5,6-DiHETE	335.2	115.2	-60	-21	-8	² H ₁₁ -14,15-DiHETrE	11.71	0.24	18	5	0.13	0.25	100
15-deoxy-PGJ ₂	315.2	271.2	-65	-20	-6	² H ₁₁ -14,15-DiHETrE	11.76	0.24	25	5	0.50	1.00	400
7,8-DiHDPE	361.2	113.1	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	11.86	0.31	16	4	0.50	1.00	100

20-HETE	319.2	275.1	-80	-23	-6	² H ₆ -20-HETE	12.04	0.25	18	5	1.30	2.60	260
15-HEPE	317.2	219.2	-60	-20	-10	² H ₈ -12-HETE	12.04	0.25	17	5	0.63	1.25	500
5,6-DiHETrE	337.2	145.1	-70	-26	-10	² H ₁₁ -14,15-DiHETrE	12.11	0.34	22	5	0.25	0.50	200
8-HEPE	317.2	155.2	-60	-20	-8	² H ₈ -12-HETE	12.36	0.26	20	5	0.25	0.63	500
12-HEPE	317.2	179.2	-65	-20	-8	² H ₈ -12-HETE	12.54	0.26	19	4	0.25	0.63	500
5-HEPE	317.2	115.1	-60	-20	-6	² H ₈ -12-HETE	13.06	0.22	20	5	0.20	0.50	400
4,5-DiHDPE	361.2	229.3	-65	-24	-6	² H ₁₁ -14,15-DiHETrE	13.09	0.29	16	5	1.00	2.00	100
13-HODE	295.2	195.2	-80	-26	-9	² H ₄ -9-HODE	13.27	0.26	27	5	<1.00	1.00	2000
9-HODE	295.2	171.1	-80	-26	-7	² H ₄ -9-HODE	13.38	0.27	27	5	<1.00	1.00	2000
15(16)-EpODE	293.3	235.2	-65	-20	-4	² H ₄ -9(10)-EpOME	13.89	0.29	17	4	0.13	0.25	100
15-HETE	319.2	219.2	-60	-20	-8	² H ₈ -12-HETE	13.97	0.26	22	4	0.50	1.25	1000
9(10)-EpODE	293.3	171.2	-65	-20	-8	² H ₄ -9(10)-EpOME	14.04	0.19	18	4	0.10	0.20	80
17(18)-EpETE	317.2	215.2	-65	-20	-6	² H ₁₁ -14(15)-EpETrE	14.11	0.28	16	4	0.25	0.50	100
11-HETE	319.2	167.2	-60	-23	-7	² H ₈ -12-HETE	14.47	0.20	23	4	<0.50	0.50	1000
12(13)-EpODE	293.2	183.1	-65	-24	-8	² H ₄ -9(10)-EpOME	14.44	0.24	16	4	0.13	0.25	100
13-oxo-ODE	293.2	195.1	-75	-20	-8	² H ₄ -9-HODE	14.43	0.24	15	4	0.50	1.00	100
15-oxo-ETE	317.2	113.1	-65	-25	-8	² H ₈ -5-HETE	14.66	0.24	11	4	0.25	0.50	100
9-oxo-ODE	293.2	185.1	-90	-28	-8	² H ₄ -9-HODE	14.66	0.18	15	4	0.50	1.00	100
14(15)-EpETE	317.2	207.2	-65	-20	-6	² H ₁₁ -14(15)-EpETrE	14.73	0.22	11	4	0.13	0.25	100
8-HETE	319.2	155.2	-60	-22	-6	² H ₈ -12-HETE	14.81	0.17	16	4	1.25	2.50	1000
12-HETE	319.2	179.2	-60	-20	-8	² H ₈ -12-HETE	14.83	0.17	16	4	<0.50	0.50	1000
11(12)-EpETE	317.2	167.2	-65	-20	-6	² H ₁₁ -14(15)-EpETrE	14.87	0.15	10	4	0.25	0.50	100
8(9)-EpETE	317.2	127.2	-65	-20	-6	² H ₁₁ -14(15)-EpETrE	15.01	0.23	13	3	0.50	1.00	100
9-HETE	319.2	167.2	-60	-23	-7	² H ₈ -5-HETE	15.10	0.14	15	3	1.25	2.50	1000
15(S)-HETrE	321.2	221.2	-70	-23	-10	² H ₈ -5-HETE	15.15	0.12	16	3	0.25	0.50	200
5-HETE	319.2	115.2	-60	-21	-7	² H ₈ -5-HETE	15.33	0.11	17	3	0.50	1.25	1000
19(20)-EpDPE	343.2	241.2	-65	-20	-7	² H ₁₁ -14(15)-EpETrE	15.80	0.11	10	3	0.13	0.25	100
12(13)-EpOME	295.3	195.2	-80	-23	-8	² H ₄ -9(10)-EpOME	15.86	0.16	13	3	0.10	0.25	200
14(15)-EpETrE	319.2	219.3	-65	-20	-4	² H ₁₁ -14(15)-EpETrE	15.99	0.14	15	3	0.25	0.50	100

9(10)-EpOME	295.3	171.1	-80	-23	-8	² H ₄ -9(10)-EpOME	16.03	0.17	14	3	0.10	0.25	200
16(17)-EpDPE	343.2	233.2	-65	-20	-7	² H ₁₁ -14(15)-EpETrE	16.18	0.15	19	3	0.13	0.25	100
13(14)-EpDPE	343.2	193.2	-65	-20	-7	² H ₁₁ -14(15)-EpETrE	16.25	0.19	19	3	0.25	0.50	100
5-oxo-ETE	317.2	273.2	-65	-22	-6	² H ₄ -9(10)-EpOME	16.30	0.10	10	3	1.00	2.00	100
10(11)-EpDPE	343.2	153.2	-65	-20	-7	² H ₁₁ -14(15)-EpETrE	16.34	0.15	17	3	0.13	0.25	100
11(12)-EpETrE	319.3	167.2	-60	-20	-7	² H ₁₁ -14(15)-EpETrE	16.44	0.10	12	3	0.25	0.50	200
8(9)-EpETrE	319.3	167.2	-60	-20	-7	² H ₁₁ -14(15)-EpETrE	16.58	0.13	10	3	0.13	0.25	100
8(9)-EpETrE 2	319.2	155.2	-65	-20	-6	² H ₁₁ -14(15)-EpETrE	16.58	0.20	10	3	1.00	2.00	100
5(6)-EpETrE	319.2	191.1	-60	-20	-7	² H ₁₁ -14(15)-EpETrE	16.71	0.12	10	3	0.50	1.00	100

¹ Reported retention time variance within a batch of 20 injections. Following analysis on the machine with different mobile phases and another column a larger retention time drift (≤ 0.2 min) was observed and the retention times in the method were adapted accordingly. ² LLOQ was set to the lowest calibration standard injected within the sample set yielding a signal to noise ratio ≤ 9 and an accuracy in the calibration within $\pm 20\%$. ULOQ does not reflect the end of the linear range but the concentration of the highest calibrator.

Table S2 List of internal standards used and parameters of ESI(-)-MS/MS detection. Shown are the mass transition used for quantification in scheduled SRM mode and the electronical MS parameters (Declustering Potential (DP), Collision Energy (CE) and Collision Cell Exit Potential (CXP)).

Analyte	Retention time (min)	Mass transition		MS Parameters		
		m/z (MS1)	m/z (MS3)	DP (V)	CE (V)	CXP (V)
² H ₄ -6-keto-PGF _{1α}	3.18	373.3	167.1	-80	-36	-8
² H ₄ -TxB ₂	3.95	373.3	173.2	-65	-24	-8
² H ₄ -PGE ₂	4.56	355.2	275.3	-60	-25	-6
² H ₄ -PGD ₂	4.84	355.2	275.3	-60	-25	-6
² H ₄ -LTB ₄	8.12	339.2	197.2	-65	-23	-9
² H ₄ -9(10)-DiHOME	9.15	317.2	203.4	-80	-29	-8
² H ₁₁ -14,15-DiHETrE	9.72	348.2	207.1	-65	-25	-10
² H ₆ -20-HETE	11.97	325.2	281.2	-70	-23	-6
² H ₄ -9-HODE	13.27	299.2	172.3	-80	-26	-6
² H ₈ -12-HETE	14.68	327.2	184.2	-65	-22	-8
² H ₈ -5-HETE	15.21	327.2	116.1	-60	-21	-8
² H ₄ -9(10)-EpOME	15.95	299.2	172.2	-80	-23	-8
² H ₁₁ -14(15)-EpETrE	15.88	330.2	219.3	-65	-20	-4

Tab. S3: Overview of the sample preparation steps for the SPE protocols

	Column Preparation	Sample Preparation (500 µL plasma)	Sample Loading	Sample Wash	Elution
Oasis-EA (Oasis HLB, 3 mL, 60 mg, 30 µm)	1 x EA 1 x MeOH 2 x 5% MeOH, 0.1% HAc	1:1 dilution with 5% MeOH, 0.1% HAc Centrifugation		2 x 5% MeOH, 0.1% HAc	0.5 mL MeOH 1.5 mL EA
SepPak (SepPak tC18, 6 mL, 500mg, 37-55 µm)	3 x MeOH 3 x H ₂ O	+ 1.5 mL 20% MeOH Centrifugation + 80 µL conc HAc (sample: pH 3)		10 mL H ₂ O 6 mL Hex	8 mL Methyl Formate
BondElut (Bond Elut Certify II, 3 mL, 200 mg, 47-60 µm)	1 x MeOH 1 x 0.1 mol/L NaAc, 5% MeOH	1:1 dilution with 1 mol/L NaAc, 5% MeOH (pH 6.0) Centrifugation		1 x MeOH/H ₂ O (50/50, v/v)	AnionEx-weak 2.0 mL n-Hex/EA (25/75, v/v) ----- AnionEx-strong 2.0 mL n-Hex/EA (75/25, v/v)
StrataX (StrataX, 3 mL, 100 mg, 33 µm)	3.5 mL MeOH 3.5 mL H ₂ O	1:1 dilution with 20% MeOH Centrifugation		3.5 mL 10% MeOH	1.0 mL MeOH
Oasis-MeOH (Oasis HLB, 3 mL, 60 mg, 30 µm)	1 x EA 1 x MeOH 1 x 20% MeOH, 0.1% FA	1:1 dilution with 40% MeOH, 0.1% FA Centrifugation		1 x 20% MeOH, 0.1% FA	2.0 mL MeOH

Tab. S4: Recovery rates of internal standards (IS) for the SPE protocols with addition of IS before (A) the SPE-step and after (B) the SPE-step. Shown are the mean+/-SD (n=5).

		² H ₄ -6-keto-PGF _{1α}	² H ₄ -TxB ₂	² H ₄ -PGE ₂	² H ₄ -PGD ₂	² H ₄ -LTB ₄	² H ₄ -9,10-DiHOME	² H ₁₁ -14,15-DiHETrE	² H ₆ -20-HETE	² H ₄ -9-HODE	² H ₈ -12-HETE	² H ₈ -5-HETE	² H ₄ -9(10)-EpOME	² H ₁₁ -14(15)-EpETrE
SepPak	A	70 ± 4.8	68 ± 3.1	41 ± 2.4	77 ± 3.8	85 ± 4.0	97 ± 6.2	89 ± 6.3	91 ± 7.4	72 ± 2.8	90 ± 6.2	80 ± 4.8	93 ± 6.3	74 ± 6.6
	B	75 ± 6.1	72 ± 7.2	42 ± 3.6	78 ± 5.5	80 ± 4.7	91 ± 5.3	75 ± 5.2	70 ± 3.3	69 ± 9.8	77 ± 5.6	68 ± 5.8	91 ± 5.8	86 ± 6.7
AnionEx-Strong	A		19 ± 3.4	22 ± 3.1	53 ± 9.3	66 ± 15	71 ± 15	68 ± 14	69 ± 13	75 ± 14	75 ± 15	68 ± 12	77 ± 13	82 ± 16
	B	64 ± 17	55 ± 18	67 ± 18	75 ± 22	71 ± 20	69 ± 19	64 ± 17	72 ± 16	75 ± 17	75 ± 18	68 ± 16	84 ± 18	85 ± 17
AnionEx-Weak	A	65 ± 2.8	76 ± 5.4	72 ± 2.7	79 ± 2.9	78 ± 3.7	78 ± 4.8	74 ± 4.2	76 ± 6.1	85 ± 5.3	84 ± 6.5	74 ± 5.3	90 ± 5.0	91 ± 6.5
	B	78 ± 3.1	81 ± 3.9	78 ± 3.3	89 ± 5.0	86 ± 3.3	87 ± 3.5	81 ± 4.2	86 ± 8.5	94 ± 6.2	92 ± 7.2	83 ± 6.0	100 ± 8.4	100 ± 8.7
Oasis-EA	A	70 ± 1.9	64 ± 3.0	65 ± 2.3	68 ± 2.9	68 ± 4.3	75 ± 2.1	86 ± 2.9	60 ± 2.7	23 ± 1.0	79 ± 4.9	58 ± 2.4	48 ± 1.7	72 ± 2.6
	B	84 ± 7.3	77 ± 4.5	76 ± 5.7	83 ± 7.7	86 ± 6.0	99 ± 6.5	99 ± 6.1	86 ± 3.2	25 ± 0.58	94 ± 3.4	69 ± 4.1	89 ± 5.1	97 ± 4.1
StrataX	A	70 ± 2.6	63 ± 3.2	96 ± 6.4	37 ± 3.3	88 ± 5.5	74 ± 5.6	95 ± 7.7	69 ± 6.2	22 ± 0.88	81 ± 5.2	55 ± 5.2	47 ± 4.5	78 ± 7.2
	B	76 ± 3.6	72 ± 2.2	100 ± 4.6	42 ± 1.2	97 ± 2.6	110 ± 3.7	100 ± 3.1	89 ± 2.8	24 ± 0.42	93 ± 2.1	70 ± 2.8	87 ± 2.6	93 ± 4.0
Oasis-MeOH	A	51 ± 2.1	58 ± 3.0	50 ± 1.9	58 ± 3.3	50 ± 1.8	58 ± 1.3	57 ± 1.6	57 ± 2.2	22 ± 0.21	62 ± 3.7	48 ± 1.7	62 ± 1.8	68 ± 2.1
	B	58 ± 3.7	63 ± 4.4	55 ± 4.9	65 ± 6.1	53 ± 3.9	67 ± 5.8	64 ± 5.7	64 ± 4.4	23 ± 0.79	67 ± 6.9	52 ± 3.1	70 ± 4.2	73 ± 6.5
LLE	A	23 ± 8.7	27 ± 11	30 ± 11	33 ± 13	36 ± 15	31 ± 11	28 ± 7.3	29 ± 4.8	27 ± 5.9	28 ± 4.7	32 ± 9.4	32 ± 5.4	32 ± 4.7
	B	30 ± 14	29 ± 14	28 ± 11	30 ± 15	29 ± 11	29 ± 7.3	27 ± 4.3	31 ± 3.2	28 ± 2.9	27 ± 2.7	28 ± 6.1	35 ± 3.6	36 ± 2.4

A: Direct IS addition

B: IS addition after SPE-step

Tab. S5: Peak areas following extraction of 500 µL plasma of all oxylipins included in the LC-MS method with the different SPE-protocols tested. Shown is the mean \pm SD (n=5).

Tab. S6: Calculated concentrations for human plasma of all oxylipins included in the LC-MS method determined with the different SPE-protocols tested. Internal standards used for quantification can be found in Tab. S1. Shown is the mean \pm SD (n=5).

Tab. S8: Peak areas following extraction of 500 µL plasma of all oxylipins included in the LC-MS method with the SepPak-protocol utilizing different SPE columns. Shown is the mean \pm SD (n=5).

→ See Excel Sheet

Tab. S7: Modified LC-MS quantification method using the SepPak protocol. Because of strong ion suppression occurring for $^2\text{H}_4$ -PGE₂ in this protocol, the quantification method (Tab S1) is modified and $^2\text{H}_4$ -PGD₂ is used for several analytes. The calculated concentrations of PGE₃, PGD₃, 9,12,13-TriHOME, 9,10,13-TriHOME, PGF_{2α}, PGE₂, PGE₁, PGD₁, PGD₂, LXA₄, 11,12-,15-TriHETrE, PGJ₂, PGB₂ with different internal standards is shown for the original (left) and modified method (right). Shown is the mean ± SD (n=5).

	Original Quantification Method		Modified Method	
	IS	Mean [pM]	IS	Mean [pM]
PGE ₃	$^2\text{H}_4$ -PGE ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ
PGD ₃	$^2\text{H}_4$ -PGD ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ
9,12,13-TriHOME	$^2\text{H}_4$ -PGE ₂	8300 ± 670	$^2\text{H}_4$ -PGD ₂	4700 ± 360
9,10,13-TriHOME	$^2\text{H}_4$ -PGE ₂	1600 ± 130	$^2\text{H}_4$ -PGD ₂	890 ± 42
PGF _{2α}	$^2\text{H}_4$ -PGE ₂	190 ± 20	$^2\text{H}_4$ -PGD ₂	100 ± 11
PGE ₂	$^2\text{H}_4$ -PGE ₂	250 ± 22	$^2\text{H}_4$ -PGE ₂	250 ± 22
PGE ₁	$^2\text{H}_4$ -PGE ₂	200 ± 28	$^2\text{H}_4$ -PGD ₂	110 ± 12
PGD ₁	$^2\text{H}_4$ -PGD ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ
PGD ₂	$^2\text{H}_4$ -PGD ₂	523 ± 25	$^2\text{H}_4$ -PGD ₂	520 ± 25
LXA ₄	$^2\text{H}_4$ -PGE ₂	440 ± 35	$^2\text{H}_4$ -PGD ₂	250 ± 20
11,12-,15-TriHETrE	$^2\text{H}_4$ -PGE ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ
PGJ ₂	$^2\text{H}_4$ -PGE ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ
PGB ₂	$^2\text{H}_4$ -PGE ₂	<LOQ	$^2\text{H}_4$ -PGD ₂	<LOQ

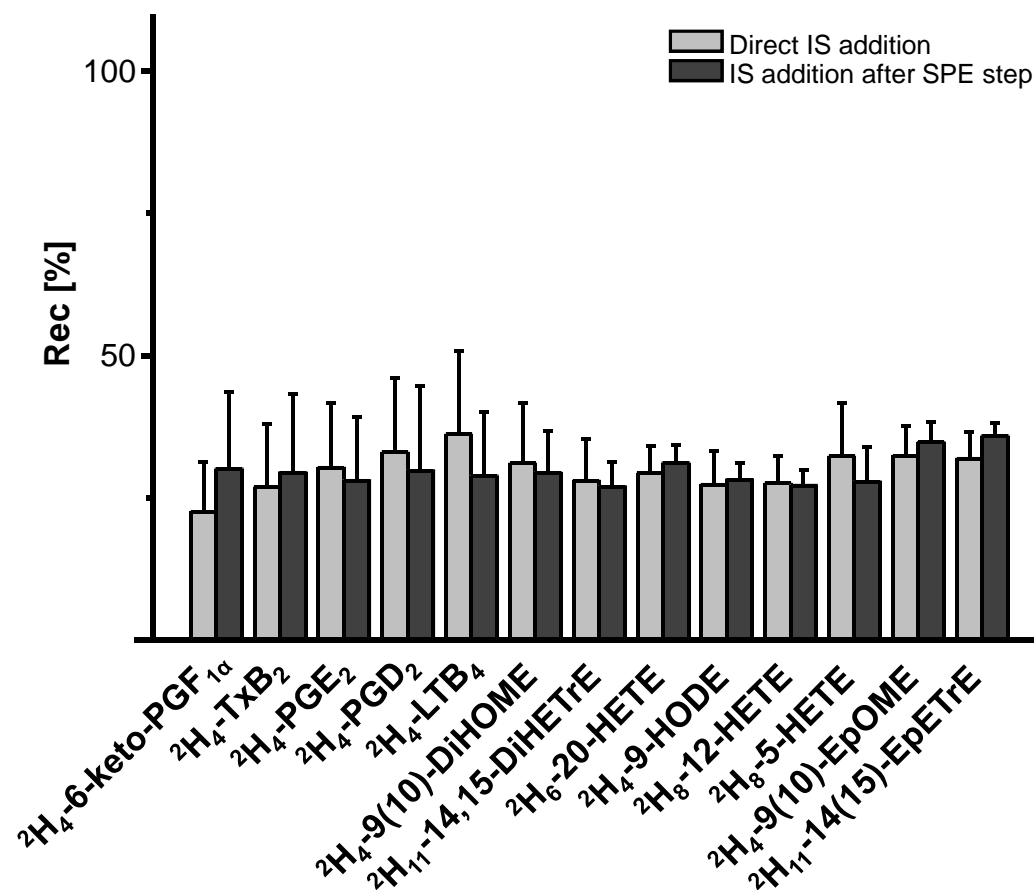


Fig. S1: Recoveries of internal standards (IS) for the LLE protocol with addition of IS before the SPE-step and after the SPE-step. Shown is the mean \pm SD (n=5).

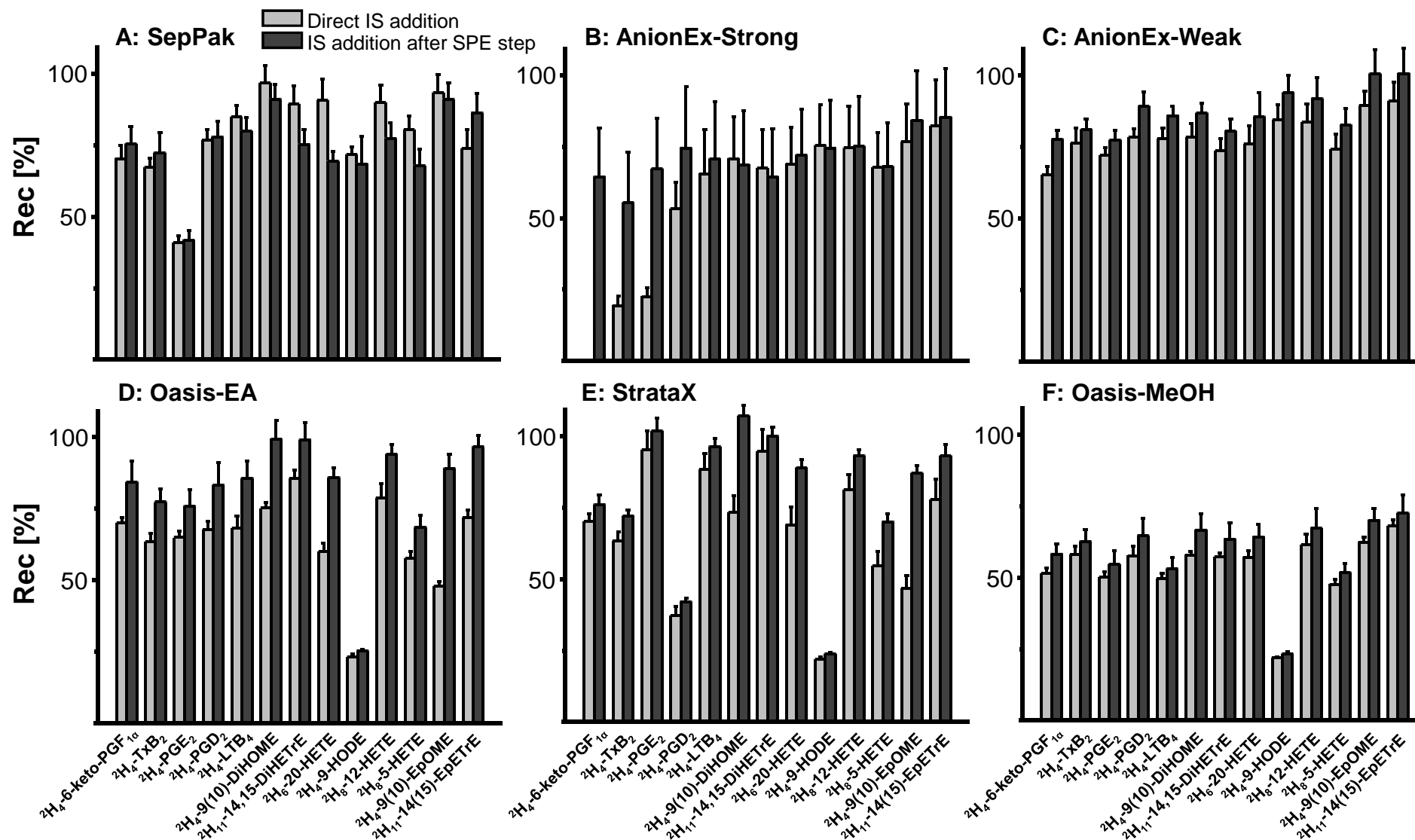


Fig. S2: Direct comparison of recoveries of internal standards (IS) with addition of IS at the beginning of the sample preparation (light grey bar) and after the SPE-step (dark grey bar) for (A) SepPak, (B) AnionEx-strong, (C) AnionEx-weak, (D) Oasis-EA, (E) StrataX and (F) Oasis-MeOH protocol. Shown is the mean \pm SD (n=5). The same data is presented in condensed fashion in Fig. 1.

$^2\text{H}_4\text{-PGD}_2/{}^2\text{H}_4\text{-PGE}_2$ (A)

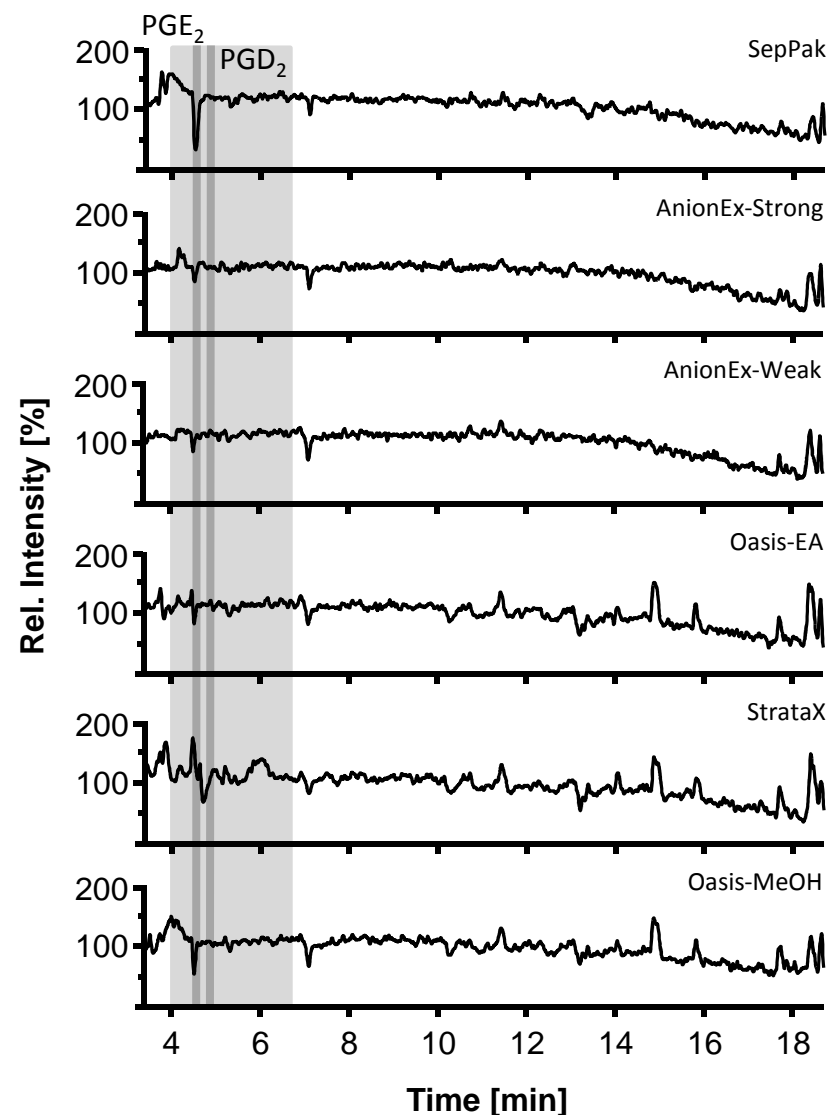


Fig. S3: Ion suppression analysis of the internal standards for the different SPE protocols tested: (A) $^2\text{H}_4\text{-PGE}_2/{}^2\text{H}_4\text{-PGE}_2$, (B) $^2\text{H}_4\text{-TxB}_2$, (C) $^2\text{H}_4\text{-LTB}_4$, (D) $^2\text{H}_4\text{-9-HODE}$, (E) $^2\text{H}_8\text{-5-HETE}$, (F) $^2\text{H}_8\text{-12-HETE}$, (G) $^2\text{H}_6\text{-20-HETE}$, (H) $^2\text{H}_{11}\text{-14,15-DiHETrE}$, (I) $^2\text{H}_{11}\text{14(15)-EpETrE}$, (J) $^2\text{H}_4\text{-9(10)-DiHOME}$. The dark grey bar indicates the retention time of the IS and the light grey bar indicates the retention time range of all analytes quantified with the IS. Mean intensity in the ion suppression (5-10 min) chromatogram was set to 100% relative intensity.

$^2\text{H}_4\text{-TxB}_2$ (B)

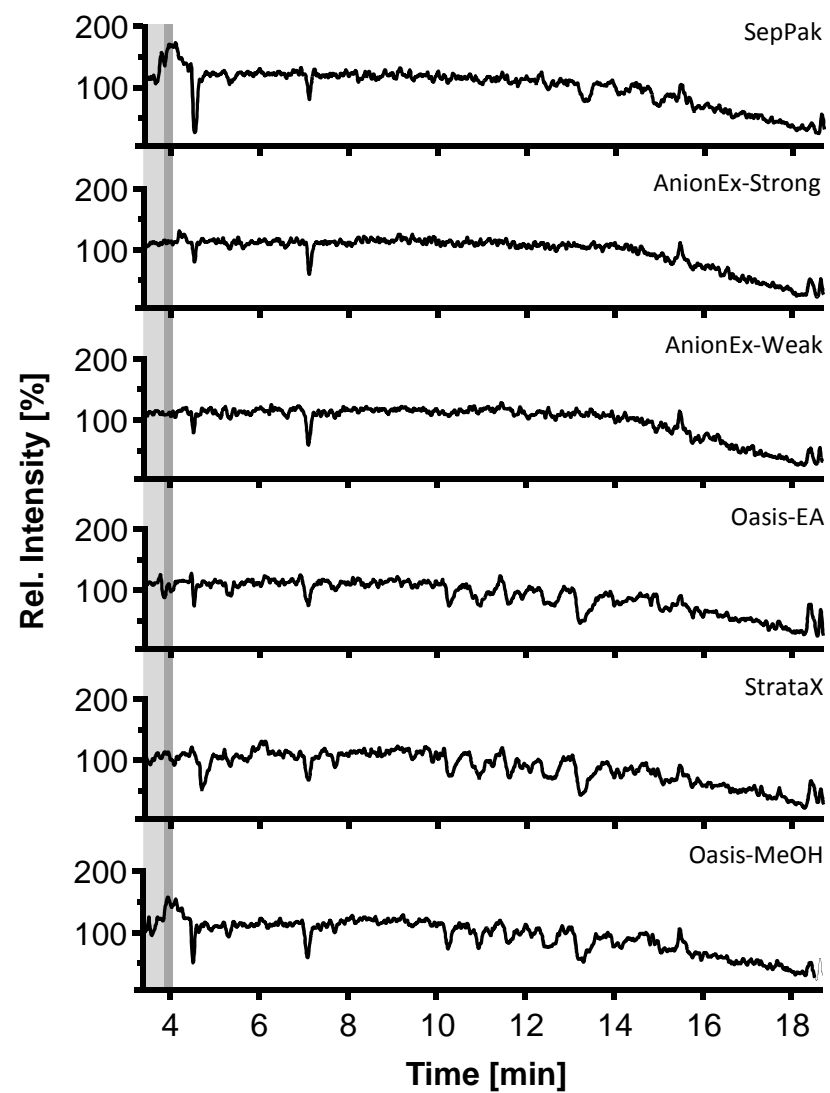


Fig. S3 continued

$^2\text{H}_4\text{-LTB4 (C)}$

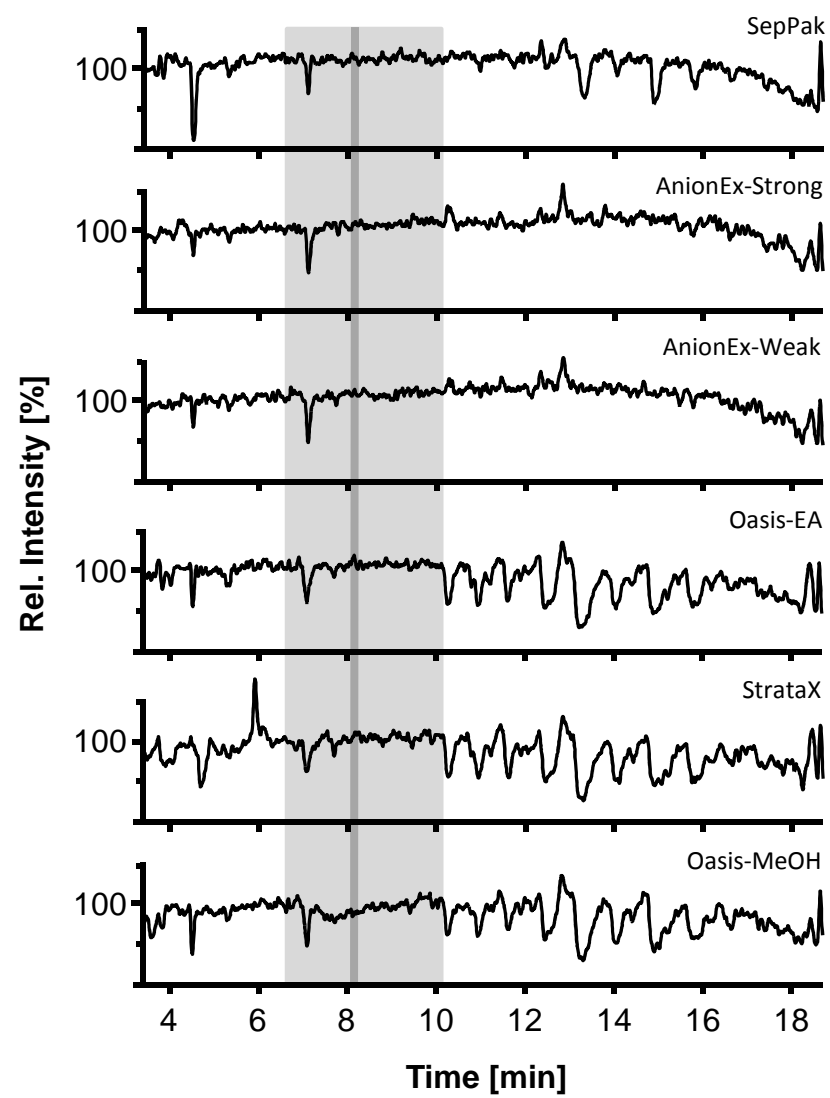


Fig. S3 continued

$^2\text{H}_4\text{-9-HODE (D)}$

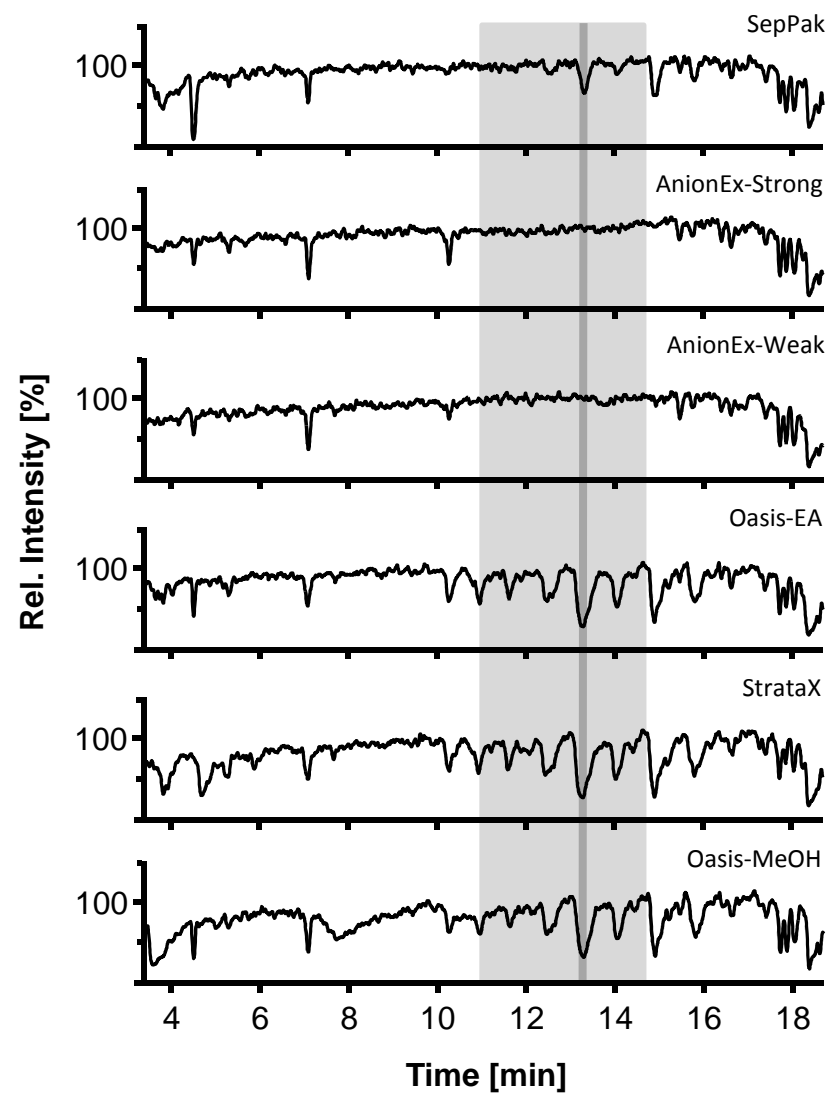


Fig. S3 continued

$^2\text{H}_8\text{-5-HETE (E)}$

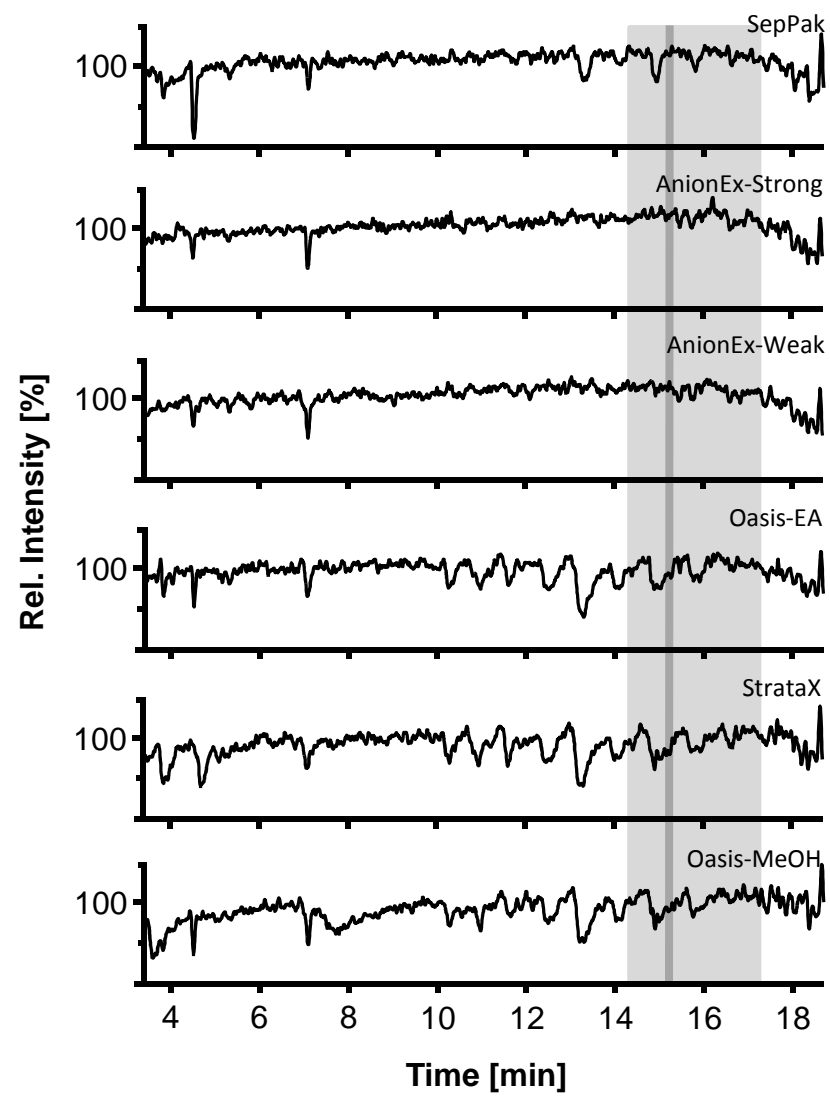


Fig. S3 continued

$^2\text{H}_8\text{-12-HETE (F)}$

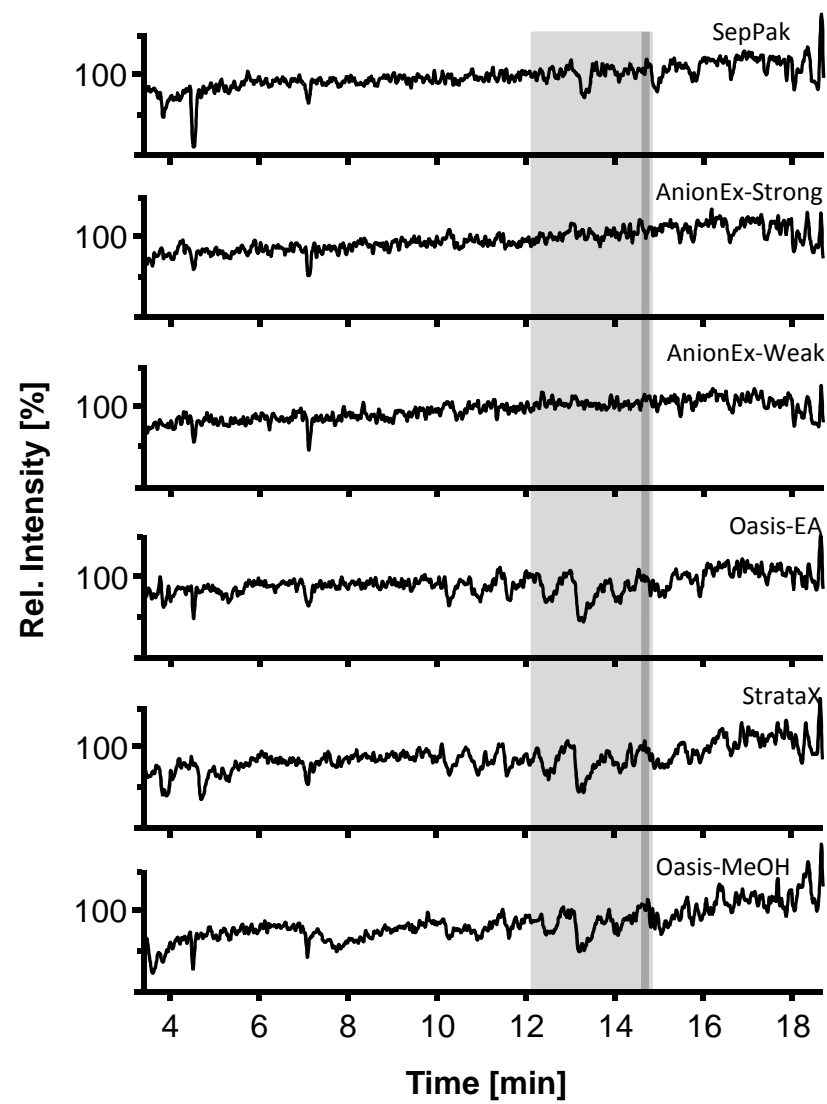


Fig. S3 continued

$^2\text{H}_6$ -20-HETE (G)

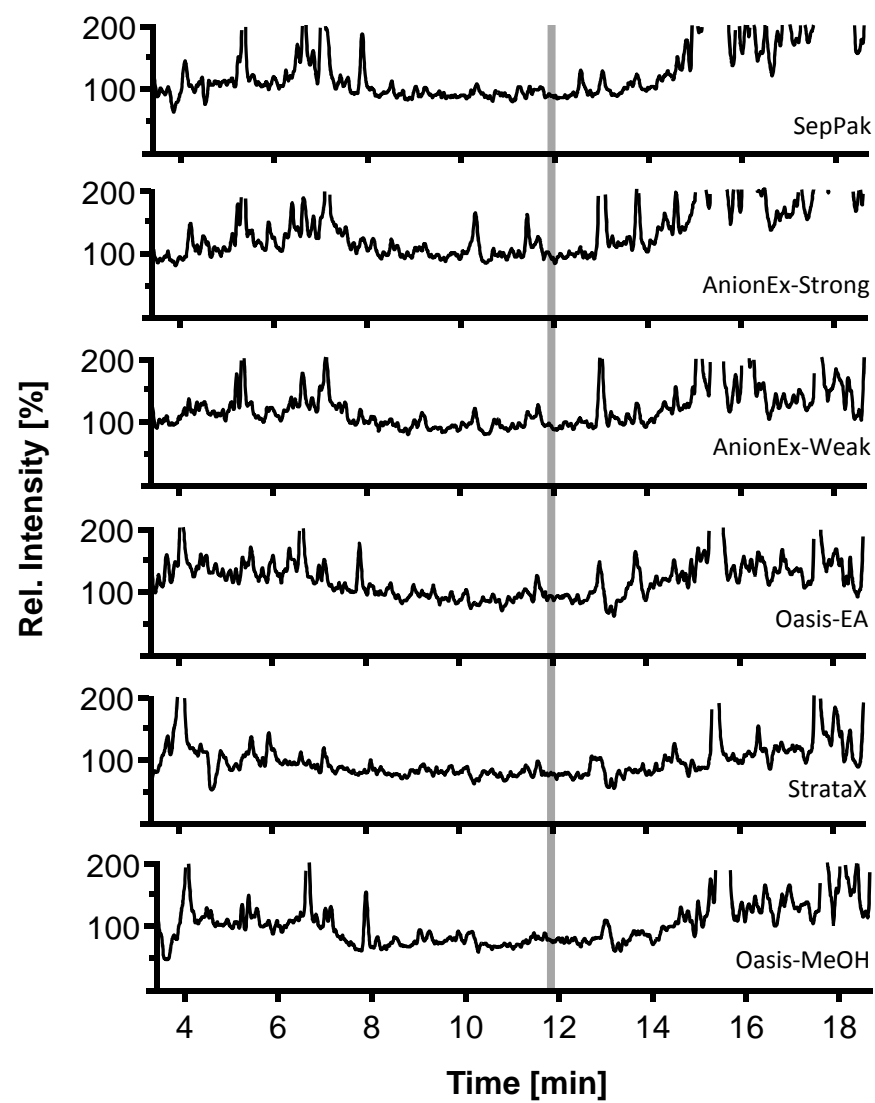


Fig. S3 continued

$^2\text{H}_{11}$ -14,15-DiHETrE (H)

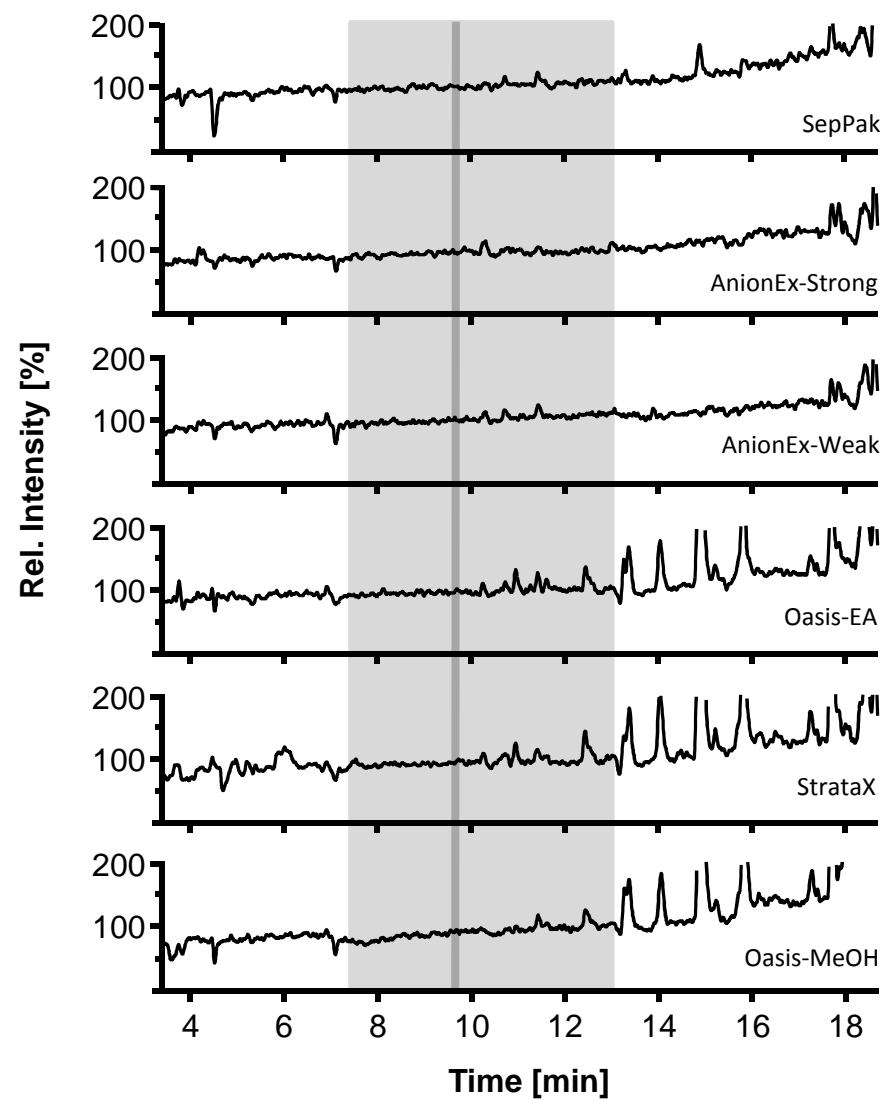


Fig. S3 continued

$^2\text{H}_{11}14(15)\text{-EpETrE (I)}$

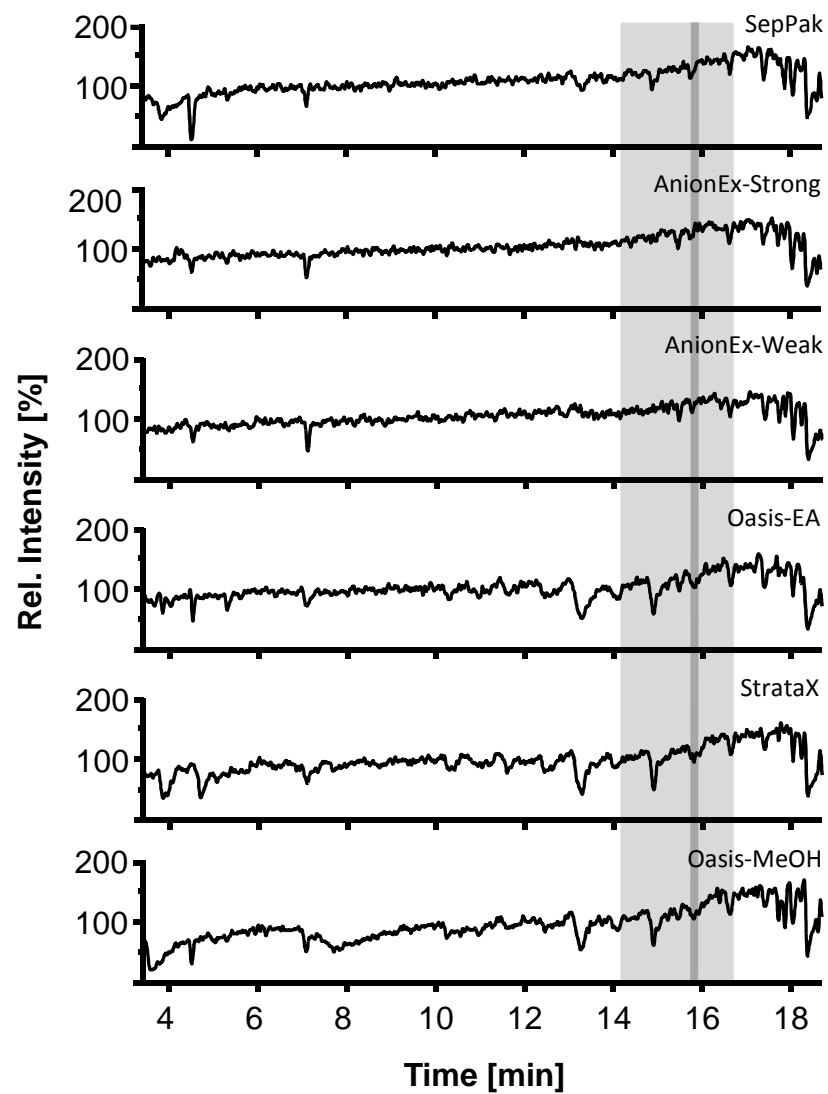


Fig. S3 continued

$^2\text{H}_4$ -9(10)-DiHOME (J)

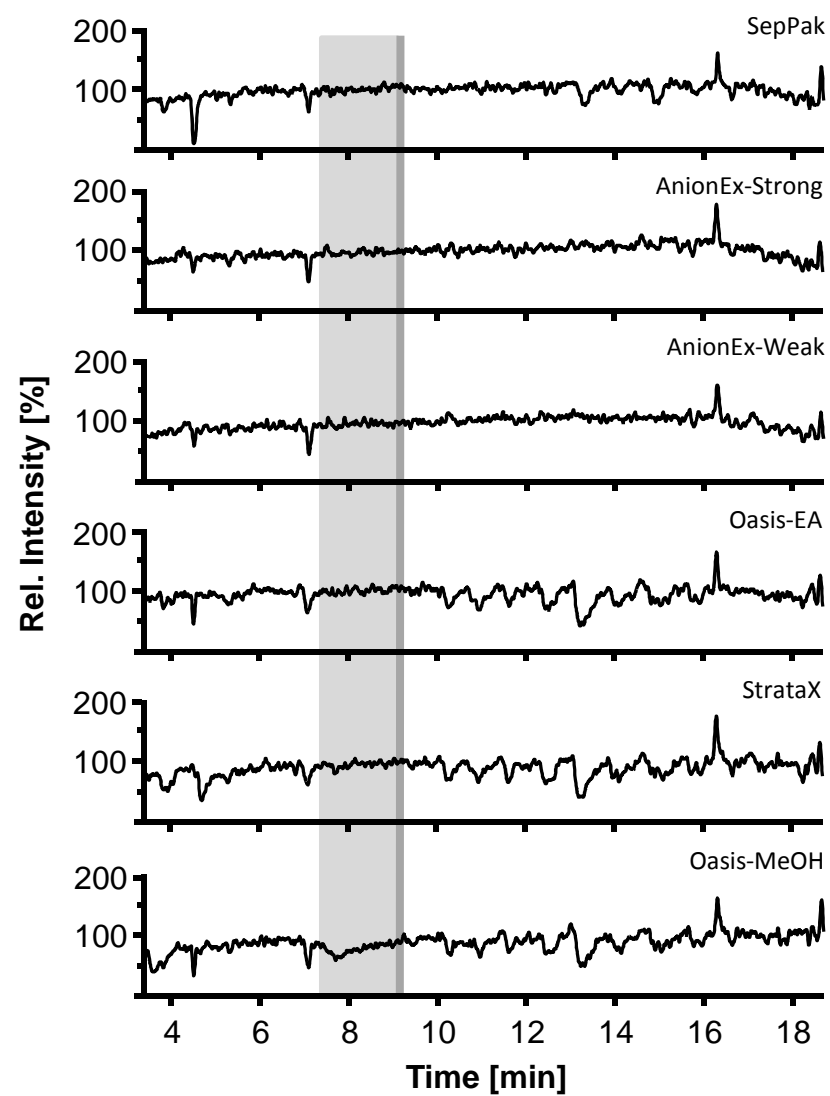


Fig. S3 continued