

Technical recommendations for liquid chromatography mass spectrometry analysis of oxylipins.

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1. Introduction.

As summarized in the accompanying focus article, research on eicosanoids such as prostaglandins and related mediators originated during the 1930s, with seminal work by Kurzrock and Lieb (1), and von Euler (2), which was then followed by the structural identification and characterization of prostaglandins (PG) and thromboxanes (TX) by Bergström, Samuelsson and Hamberg in the 1960s-1970s (3-5). During that time, related oxygenated structures were discovered, which originated from both longer and shorter chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic and linoleic acids (6-8). In the literature, the term “eicosanoids”, which chemically refers to species comprised of 20 carbons, was used generically to include related structures of fatty acids with different chain lengths (9, 10). In more recent years, the term “oxylipin” has become more generally used since it chemically includes all oxygenated metabolites of monounsaturated (MUFA) and PUFA regardless of chain length or synthetic origin (11). “Oxylipin” will be used in this document to cover relevant species being analyzed using targeted methods described herein.

The sensitive and selective analysis of oxylipins and their downstream metabolites requires the combination of chromatography and tandem mass spectrometry (MS/MS), with many studies describing targeted methods for their analysis in human plasma, serum, urine and other biological samples over the last 50 years. With advances in liquid chromatography (LC) and the availability of sensitive MS/MS systems originating in the early 2000s, comprehensive chromatographic approaches to separating and quantifying up to and beyond 100 eicosanoids using pure chemical standards and appropriate internal standards (IS) were developed (9, 12-16). This analysis later expanded to include a more comprehensive set of oxylipins when instruments with increased sensitivity and functionality became available (17-26). A selection of papers from the field are cited here, along with more recent reviews for background information (13-16, 27-36). Additional studies describe the overall fragmentation behavior of oxylipins in the gas phase following collision-induced dissociation (CID) (37, 38). Although gas chromatography is well suited for analysis of (derivatized) oxylipins, since the 2000s it has been superseded by LC combined with MS/MS, for which derivatization is not required. MS/MS interfaced with electrospray ionization (ESI), operating usually in negative ion mode, detects all species resulting from the deprotonation of the carboxyl group. Only a few oxylipins are sometimes monitored in positive ion mode, for example, leukotriene C₄ (LTC₄), where the glutamic acid amino group is ionized (38).

Not all isomers of oxylipins are well separated by reversed-phase (RP) LC (e.g. 12-hydroxy-eicosatetraenoic acid (HETE) and 8-HETE, 7,17-dihydroxydocosahexaenoic acid (DiHDoHE) and 7,14-DiHDoHE). Therefore, selective MS detection is undertaken following CID to form characteristic fragments. The quantification of oxylipins in biological samples generally requires high sensitivity assays due to their low abundance. However, some species have been reported at relatively high endogenous concentrations (e.g. 9-hydroxyoctadecadienoic acid (HODE)), and thus monitoring diverse species may require methods that have a wide dynamic quantification range. Triple quadrupole mass analyzers, operating in multiple-reaction monitoring (MRM) mode, also called selected reaction monitoring mode (SRM), are most often used for quantitative oxylipin analysis. These recommendations focus on analytical

(detection and quantitation of oxylipins by LC-MS/MS) and post-analytical (result reporting and nomenclature) aspects of the measurement process. Although the preanalytical pipeline is not covered in these recommendations, it may be useful to note that artefactual oxidation can occur during sample storage or preparation and this can lead to formation of significant amounts of several oxylipins (39, 40). Also, the generation of serum from whole blood results in activation of white cell and platelet enzymes that generate abundant oxylipins (41, 42).

Over the last 20 years, interest in lipid analysis using MS (lipidomics) increased substantially with numerous methods for analyzing large numbers of these molecules being developed and applied. This rapid development of the research field led to inconsistent data, uncertainty about data reporting, and hence reproducibility issues, particularly in relation to the use of untargeted methods (43-45). To address this, community initiatives were established, which included development of a “minimal reporting list” for the field of lipidomics as well as structured advice on how to annotate lipids based on the level of information provided by the analytical method, using shorthand nomenclature (46-49). Separately but related to these new recommendations, in the oxylipin field, it became apparent that detection of oxylipins called specialized pro-resolving mediators (50) may have, in some instances, not conformed to expected standards in the field, as discussed recently (51-55).

In the following paragraphs, methods and approaches to ensure robust and reproducible analysis of oxylipins using LC-MS/MS are presented, aiming to support the community with agreed principles to be applied in a discovery research setting. Measurements of specific oxylipins conducted as part of clinical studies (e.g. trials for drugs or other interventions in humans) or patient diagnostics, would need to meet criteria outlined in international bioanalytical guidelines, in addition to this document (56-58). Adopting analysis parameters described in the literature without method validation (parameters such as selectivity, sensitivity, calibration range, accuracy and precision; see below) is not recommended, since even on the same platform, instrument parameters can vary.

This recommendation is structured to provide guidance to researchers for both (i) initial method development and (ii) reporting data carried out using established methods. In the case of method development, all parameters described in boxes as “Method Development” can be reported, while if using an established routine assay, papers can instead report parameters listed under “Routine Analysis”, either in main text or a supplementary section. Any deviations from the reporting standards outlined here should be clearly reported. We also recommend that the Lipidomics Reporting Checklist is used as a template for reporting (46).

2. Chromatography.

Due to the structural complexity of oxylipins, numerous isomers of individual species with the same general structure can exist. For example, there are at least 10 different positional isomers for HDoHE, all forming a precursor $[M-H]^+$ ion with the same m/z value. While the overall pattern of fragment ions is distinct for each oxylipin structure, individual fragments are not always specific and furthermore, oxylipins with related structures can give rise to the same precursor-product ion m/z transition pairs, i.e. SRM/MRM transitions (Figure 1). This is of particular concern for isomers, such as enantiomers, diastereomers and geometric isomers.

Due to this issue, chromatographic separation is required for selective detection and quantification of individual oxylipins. This is most often undertaken using (ultra)high-performance reversed-phase columns filled with sub μm particle size or core-shell particles below 3 μm , applying a gradient of acidified water and organic solvent mixtures of methanol, acetonitrile and/or 2-propanol. Numerous methods are described that enable efficient separation of oxylipins into narrow chromatographic peaks that are well retained on the column and cleanly resolved from isobars (16, 20, 21, 23, 32). These methods can be used to guide chromatographic method development and may require adjustments depending on the specific instrumentation, and instrument configuration.

Besides RP separation, additional chromatographic systems including normal-phase/chiral (13, 59), supercritical fluid chromatography (24, 60, 61) and capillary electrophoresis (62, 63) have been used for oxylipin analysis. These show an orthogonal chromatographic selectivity, which can be helpful for the characterization of oxylipins in biological samples.

During method development, it is important to determine isomeric oxylipins which share both precursor and product ions and elute closely, leading to overlap in retention times in the same MRM detection channel. Examples include (i) PGE_2 , PGD_2 , and their 8-*iso* and 11- β isoforms and (ii) LTB_4 , 6-*trans*- LTB_4 and their C12 epimers, which elute within a narrow retention time window. Enzymatically formed *cis*-epoxy-PUFA elute close to, but slightly earlier than, *trans*-epoxy-PUFA formed by nonenzymatic oxidation using RP chromatography and generate identical MS/MS spectra (64). Furthermore, while epoxy- and hydroxy-PUFA are detected using identical MRM transitions, they are generally well separated by chromatography (Figure 1). Additional examples of critical separation pairs are listed in Table 1.

Uniquely among oxylipins, TXB_2 shows complex RP chromatographic behavior with a larger earlier eluting peak followed by a smaller later peak, with both connected by a sloped line (Figure 2). In short chromatographic runs, or when present at very low levels, it can appear as a broad tailing peak. This has been suggested to originate from tautomerism of the lactol ring (65), hence it is advised to integrate the entire peak to generate a single value.

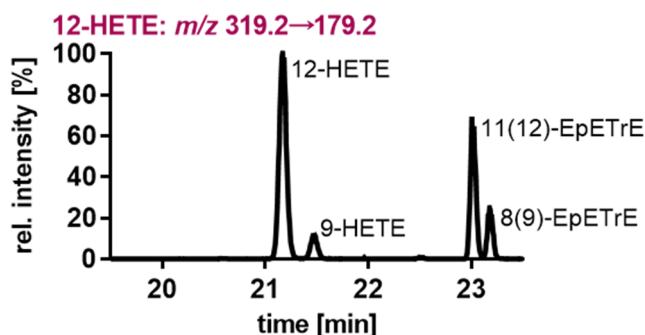


Figure 1. Different hydroxy- and epoxy-metabolites of arachidonic acid (AA) show signals in a transition channel typically used for 12-HETE in LC-MS/MS. A mixture of lipid standards (250 nM, 400 pg on column each) was separated using a 2.1 × 150 mm column with 1.8 µm fully porous RP18 particles with a gradient of water and acetonitrile/methanol and detected on a 5500 QTRAP following ESI(-) using the MRM transition m/z 319.2→179.2. Image reproduced from (39).

Table 1. Examples of critical separation pairs. In these examples, two or more lipids generate signals in the same MRM transition, requiring separation using chromatography to enable their quantification.

Critical chromatographic separation pairs	m/z of MRM transition
PGE ₂ and PGD ₂ and their 8- <i>iso</i> and 11-β isoforms	351.2→271.2
PGA ₂ , PGB ₂ and PGJ ₂	333.3→189.2
PGB ₂ , 5,15-DiHEPE	333.1→315.2
8-F ₁₁ -dihomo-phytoprostane (PhytoP)-DGLA and 15-F ₁₁ -dihomo-PhytoP-DGLA	355.2→281.1
PGF _{2α} and its 8- <i>iso</i> and 11-β isoforms	353.2→309.2
LTB ₄ , 6- <i>trans</i> -LTB ₄ and their C12 epimers	335.2→195.1
12,13-DiHODE and 15,16-DiHODE	311.2→223.2
ω and (ω-1) hydroxy fatty acids of AA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)	319.2→275.1, 317.2→273.2 and 343.2→299.1, respectively
8(9)-epoxy-eicosatetraenoic acid (EpETE) and 11(12)-EpETE	317.2→167.0
11(12)-EpETE and 14(15)-EpETE	317.2→207.2
8(9)-epoxy-eicosatrienoic acid (EpETrE) and 11(12)-EpETrE	319.2→167.2
15-oxo-ETE and 15-hydroperoxy eicosatetraenoic acid (-HpETE)*	317.2→113.0
12-oxo-ETE and 12-HpETE*	317.2→153.1
12-HODE and 10-HODE	295.2→183.1
7-HDoHE and 11-HDoHE	343.2→121.1

*[M-H-H₂O]⁻ ions of hydroperoxy-PUFA are isobaric with the corresponding [M-H]⁻ ions of oxo-PUFA (38)

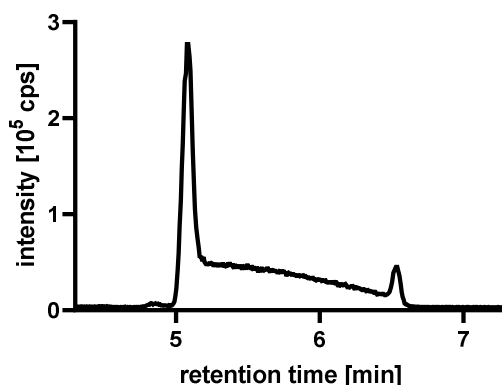


Figure 2. Typical peak shape of TXB₂ in RP-chromatography. Shown is the peak of the transition m/z 369.2→169.2 analyzed on a Sciex QTRAP 6500 of an injection of 520 pg of TXB₂ following separation on an Eclipse Plus C18 Column (Agilent).

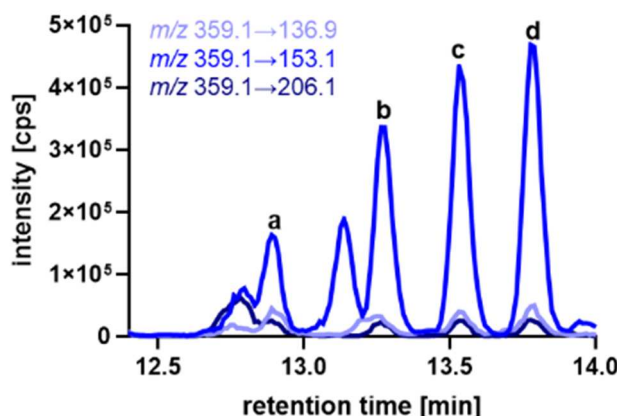


Figure 3. LC-MS/MS separation of 10,17-DiHDoHE isomers present in a commercially available pooled plasma. Oxylipins were extracted from commercially available plasma which contained high oxylipin levels, due to inappropriate storage. Oxylipins were isolated using a mixed mode (anion exchange/reversed-phase) solid phase extraction separated on a 2.1×150 mm column with 1.8 µm fully porous RP18 particles using a gradient of water and acetonitrile/methanol. Three MRM transitions of PDX were monitored following ESI in negative-ion mode. Several 10,17-DiHDoHE configurational isomers were detected (peaks a-d). Among them 10(*S*),17(*S*)-dihydroxy-4(*Z*),7(*Z*),11(*E*),13(*Z*),15(*E*),19(*Z*)-docosahexaenoic acid i.e. PDX and its enantiomer 10(*R*),17(*R*)-dihydroxy-4(*Z*),7(*Z*),11(*E*),13(*Z*),15(*E*),19(*Z*)-docosahexaenoic acid elute at the retention time of peak c, while 10(*S*),17(*R*)-dihydroxy-4(*Z*),7(*Z*),11(*E*),13(*Z*),15(*E*),19(*Z*)-docosahexaenoic acid and 10(*R*),17(*S*)-dihydroxy-4(*Z*),7(*Z*),11(*E*),13(*Z*),15(*E*),19(*Z*)-docosahexaenoic acid co-elute in peak d, while the rest are unassigned as yet. Image reproduced from (39).

For a given method, the retention time of analytes should be evaluated and documented. Relative retention time, specifically retention times in relation to the corresponding IS, is an important parameter for structural confirmation. Deuterated forms of oxylipins typically elute a little earlier (about 0.1-0.2 min) than their unlabeled analogs.

In contrast to the well-known oxylipins that are available as synthetic standards, many found in biological samples are not fully structurally characterized. Examples include the large number of isomers of 10,17-HDoHE formed by autoxidation. These need to be separated efficiently, in order to enable detection and quantification of single isomers. For example, 10(*S*),17(*S*)-dihydroxy-4(*Z*),7(*Z*),11(*E*),13(*Z*),15(*E*),19(*Z*)-

docosahexaenoic acid (PDX) and its enantiomer coelute and need to be separated from their related isomers for quantification (shown in Figures 3-4) (39). Where coelution is observed, but standards of the isomers are not available, different chromatographic conditions should be tested to aid identification. This can include testing different columns (e.g. polar embedded or chiral) solvents or applying ion mobility spectrometry (66-68) to verify the absence of coelution with related structures. If the full structure of the isomer is not confirmed, then only a partially annotated name (e.g. 10,17-DiHDoHE) should be reported. The appropriate naming of oxylipins, based on the assay used, is discussed in more detail below.

Report

Routine analysis:

Instruments used.

Column specifications including guard column.

Column oven temperature, solvents, flow rate, gradient, retention times of analytes.

Method development:

Retention time, peak width at 50% height (FWHM) for a defined injected amount of each oxylipin (e.g. 10 pg on column).

Procedures used to assess for potential coeluting compounds.

Definition of critical separation pairs as in Table 1, their chromatographic resolution as well as limitations (i.e. co-elution of oxylipins yielding the same ions in MRM).

Variation of the (relative) retention time (e.g. RSD) over at least 10 injections.

3. MS/MS Detection.

To correctly identify and accurately quantify oxylipins, structurally meaningful oxylipin-specific MRM transitions need to be carefully selected, specifically the precursor $[M-H]^-$ ion and a product ion that is at least in part specific for the molecule being analyzed.

In the case of oxylipins, product ions are typically formed through cleavage of a C-C-bond adjacent to an oxygenated functional group (α -cleavage) with or without further fragmentation, neutral loss, or rearrangement. Non-specific fragmentation resulting from loss of CO_2 /acetaldehyde $[M-H-44]^-$ or H_2O $[M-H-18]^-$ or multiples should be avoided when more specific fragment ions are available, since distinguishing between CO_2 and acetaldehyde requires high-resolution MS.

When establishing an assay, method parameters described in publications can act as a guide, however instrument-specific optimization will still be required. MS source parameters (including temperature, gas flows, ESI voltage) are first optimized for selected representative oxylipins. Parameters specific to each lipid, such as collision energy (CE) then need to be individually established for each oxylipin and its transitions. Depending on the instrument, other electronic parameters may then require optimization. Once MRM settings and chromatographic parameters are established, the simultaneous optimization of all targeted compound MS parameters can be performed (18).

When selecting product ions to monitor oxylipins, it is important to consider the potential for interference from co-eluting peaks in biological samples, and which MRM transitions give the highest signal-to-noise ratio (S/N) both using standards and in extracted matrix samples. A secondary MRM transition (qualifier) can be monitored in addition to the quantifier ion to support the identification of an oxylipin. This is especially useful in the case of co-eluting isobars (analytes yielding ions with the same m/z) when the particular oxylipin has not been previously shown to be present in the matrix being analyzed. A secondary MRM can also provide further evidence for functional group position (on which carbon the hydroxy, or epoxy group is located). If an oxylipin bears more than one oxygen-containing functional group (hydroxy, epoxy, or keto group), an α -cleavage at each site is expected. For example, 7,17-DiHDoHE gives rise to fragment ions at m/z 140 and m/z 261 due to fragmentation at bonds on C7 and C17, respectively (21). A published list of CID fragments from over 180 oxylipins and deuterated IS can aid selection of transitions for metabolites not separated by chromatography (19).

If two MRM transitions are monitored, the ratio of the quantifier to the qualifier (secondary) peak areas should be at a similar level (typically $\pm 30\%$) for the standard and the lipid in the sample, to evidence the structure. As an example, the MRM transition for 10,17-DiHDoHE shows several chromatographic peaks when analyzing biological samples (Figure 3). While the ratio of the two transitions provides evidence for the presence of PDX in serum (Figure 4), the peak at the retention time of PD1 results from an isomer, another compound, or mixtures thereof (21, 39, 69). Here the quantifier/qualifier ratio clearly shows that the compound(s) detected in the targeted analysis of patient serum are not the same as the standard.

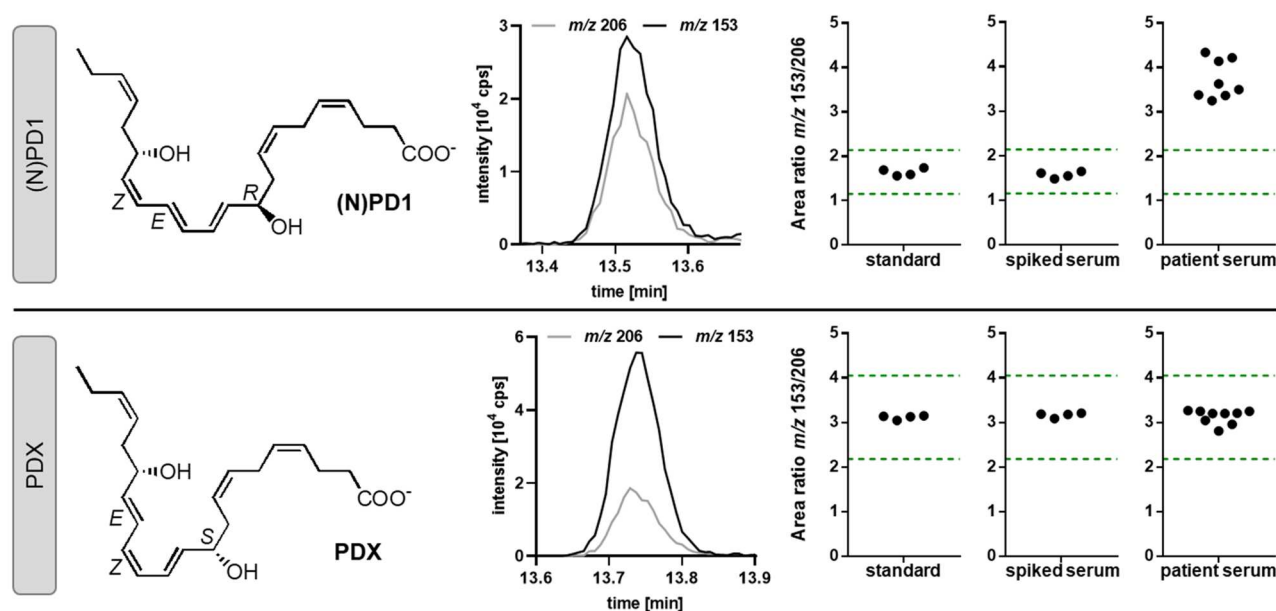


Figure 4. A secondary MRM transition demonstrates the absence of PD1 and presence of PDX in serum.

Oxylipins were extracted from healthy human serum spiked with PDX and PD1 (also known as NPD1) or from serum from patients with end-stage renal disease by solid-phase extraction. Chromatographic separation was performed on a 2.1×150 mm column with $1.8 \mu\text{m}$ fully porous RP18 particles using a gradient of water and acetonitrile/methanol. Oxylipins were detected on a 5500 QTRAP following ESI(-). Serum from healthy individuals did not yield signals of PDX or PD1. **Left:** Structures of PD1 and PDX as described (70). **Middle:** Representative peaks from standards using two MRM transitions, m/z 359.1 \rightarrow 153.1 and m/z 359.1 \rightarrow 206.1, corresponding to PDX and PD1 standards. **Right:** Area ratios of the oxylipin reference standards, extracts of human serum spiked with the reference standards (noting that serum from healthy subjects did not yield signals of either lipid), and extracts of serum from patients. While the ion ratio is not affected by matrix (standard vs. spiked healthy serum), differences between the patient's serum and the standard are observed for the PD1 signal, indicating that it arises from a different oxylipin, or a mixture of isomers that may or may not include an unknown amount of the lipid of interest. (21, 69).

Monitoring secondary MRMs and determining the ratio of qualifier and quantifier in a representative biological sample to demonstrate that it is consistent with that of the synthetic standard is important for low abundant lipids, such as multiply oxygenated forms where many closely eluting or co-eluting isomers exist. However, for simultaneous quantification of large numbers of more abundant oxylipins, such as mono-hydroxy fatty acids (e.g. 12-HETE) being measured in well-studied matrices, such as plasma, it is not routinely practical. This is due to limitations of the dwell and cycle times (see below).

An alternative approach for establishing the structure involves the collection of a product-ion spectrum of the oxylipin in the sample at or around the apex of the chromatographic peak and comparing it to the spectrum of the standard obtained using the same method. If sufficient lipid is present, then triple-quadrupole instruments can generate MS/MS spectra of sufficient quality for comparative purposes. QTRAP instruments, which can operate the third quadrupole as an ion trap, generate higher-quality spectra, particularly in cases where low amounts of the lipids are present. Here, the resulting spectra for the sample and standard need to be visually comparable, particularly for the more abundant structurally relevant ion m/z values, and their relative ratios (Figure 5). However, it is generally difficult to obtain spectra for comparison when analyzing very low amounts of lipids. In this situation, the use of qualifier

and quantifier transitions and comparing their ratios (see above) is more suitable for identity confirmation. Software tools for determining spectral fit are increasing in availability and popularity, particularly for untargeted and discovery studies (71). However, for confirmation of structure in targeted oxylipin assays, they should be used with caution and in general, regardless of the result from a computational comparison, a strong clear visual match between standard and proposed biological compound is expected. The same major ions should be seen clear of the baseline in both standard and sample, and with similar relative ratios (Figure 5).

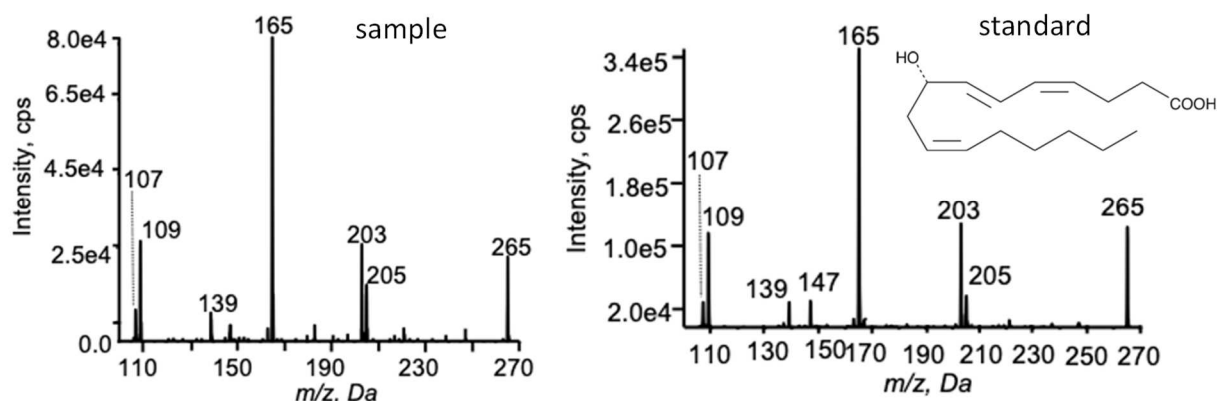


Figure 5. Example comparison of fragment spectra from a standard and a cell-derived oxylipin. Tetranor 12-HETE was analyzed either from macrophage extracts (left panel) or a commercial standard (Cayman Chemical, right panel) using LC-MS/MS. Images reproduced from (72).

Report

Routine analysis:

List of MS settings and transitions measured.

The identification criteria for the oxylipins should be stated, i.e. (relative) retention time, ion ratios of MRM transitions or fragmentation spectra.

The software and libraries used should be reported.

Method development:

All determined ratios of quantifier and qualifier and/or the fragmentation spectra of a peak in a sample and of a comparable amount of a standard.

4. Dwell time and cycle time.

For triple-quadrupole-based LC-MS/MS methods, the dwell time, which represents the duration of the detection of a single MRM transition, needs to be optimized in conjunction with the total cycle time for all transitions monitored. Short dwell times will reduce the total cycle time, and are useful when generating narrow chromatographic peaks, but they will also significantly reduce the sensitivity (based on S/N). On the other hand, if the cycle time is too long, S/N will be higher but narrow peaks will not be defined properly when quantifying a large number of analytes, since there will be insufficient data points per peak.

For quantification, a minimum of 7-10, but ideally 20, data points per peak are required to define a peak (73). As an example, a typical chromatographic peak with a full width of 10 s would allow for the measurement of only 50 transitions using a dwell time of 20 ms (cycle time 1 s; 1 Hz) while generating 10 points across the peak. If the detection of more transitions is desired, the MS method can be split into several periods as described (16), or if the instrument allows it, scheduled MRM can be used. This is particularly important when using modern columns with sub-2 μm particles.

With scheduling, the instrument only monitors each transition within a defined retention time window. This substantially reduces the number of MRM transitions being monitored at any particular time, enabling longer dwell times per analyte and thereby dramatically improving data quality and sensitivity. In scheduled mode, a fixed cycle time is defined, and dwell time is then dynamically adjusted based on the number of transitions being measured per individual cycle. The parameters for a scheduled MRM mode that need careful consideration, are as follows:

- i.) The cycle time should be sufficiently short to achieve the required number of data points per peak. A typical setting would be 0.5 s, which generates around 20 data points for a 10 s full width peak.
- ii.) The detection window should be sufficiently wide to allow robust detection even if the retention time shifts and to allow recording of a full baseline on either side of the peak enabling proper S/N calculations. Therefore, **2-4 full peak widths** should be monitored at both sides of the peak.

Report

Routine analysis:

The minimal number of data points used to define a peak (recommended 10-20).

Classical MRM: dwell time and number of transitions per period.

Scheduled MRM: cycle time and detection window used (may include the number of concurrent MRMs at each retention time)

5. Analytical and internal standards.

Targeted quantification of oxylipins requires an authentic analytical standard of the target molecule. The use of the enantiomer of an authentic analytical standard, which co-elutes in RP chromatography, is also acceptable. Reliable quantification in complex biological samples also requires stable isotope labeled IS, which are usually added prior to extraction and sample preparation (13, 19). Quantification is then performed by external calibration, based on the ratio of analyte to IS. Although not required for research assays, when the method is intended for clinical studies or patient diagnostics, external and IS should be certified using a guideline-conforming technique to determine absolute amounts (e.g. q-NMR) (74).

Ideally, an isotopically labeled IS would be included for each oxylipin. While these are not available for most, it is encouraged to use as many as possible, with at least one for each group of structurally-related analytes eluting at a similar retention time (e.g. PG, LT, hydroxy-PUFA, epoxy-PUFA and dihydroxy-PUFA or multiple hydroxy-PUFA). An isotopically labeled IS would ideally be included for a specific oxylipin when it is of greater interest to the study than others. This is because ion suppression caused by co-eluting molecules from the matrix can vary between the retention time of the IS and that of the analyte (see below), and this would not be accurately corrected by the (analyte/IS) area ratio. If ion suppression (see “sample preparation” section below for detailed description of ion suppression analysis) is thought to specifically impact a subset of analytical targets in a sample matrix, a comparison of peak quantitation against two independent IS (e.g. $^2\text{H}_4$ -5-HETE and $^2\text{H}_4$ -15-HETE) can be considered as a means of highlighting the issue. In addition, a representative extracted matrix pool can be used to generate a calibration curve dilution, and the slope of the analyte responses in the presence and absence of matrix can be compared. The stability and variation of the retention time of oxylipins in relation to their corresponding IS can also be evaluated to determine the relative retention time.

The amount of IS to add should be informed by the sensitivity of the analysis. Specifically, the amount/concentration should be within the lower range of the calibration standards (e.g. 10-50 times above the LLOQ). However, if the concentration of the IS is too low, accurate integration may be difficult particularly if losses or ion suppression occur. Conversely, if the IS amount is too high, a signal in the MRM trace of the unlabeled analyte can become visible, due to inevitable contamination with the unlabeled compound (generally < 1%), and/or it can cause ion suppression of the analyte.

If a commercial external standard is not available, and the oxylipin structure is not fully defined, then analysts can report fold changes relative to an IS, instead of quantified amounts. This allows generation of biological insight into the behavior of a given lipid, e.g. upon treatment with a test compound. However, for naming such lipids, shorthand nomenclature, as described here (47) is more scientifically correct than full stereochemical names. Furthermore, the data would be reported as relative, e.g. % of control, and not quantitative. In this way, an oxylipin is only fully annotated when a synthetic reference standard is available.

Report

Routine analysis:

Standards and IS used (and amounts added) and the assignment of each IS to each oxylipin.

Method Development:

Relative retention time and its variation between standards vs. matrix containing samples.

6. Sensitivity of the instrument.

The lowest amount on column at which each oxylipin can individually be detected, as well as the lowest quantifiable amount, is determined separately for each oxylipin. These values are compound specific due to differences in ionization efficiency and fragmentation behavior. For a standard, this amount (in pmol or pg) is calculated based on the concentration of the standard in the solution and the injected volume (usually 5-20 μL). Typically, a serial dilution of primary standards is performed and measured several times. Therefore, the amount of oxylipin generating a S/N of at least 3 (in at least 3 repeated injections of primary standards) is first determined and set as the limit of detection (LOD) of the instrument.

The amount of standard injected which generates a $\text{S/N} \geq 5$ is the lowest standard for which peaks are integrated and the starting point for the determination of the lower limit of quantification (LLOQ). The LLOQ of the instrument method is that (calibrator) level which also leads to an accuracy (calibrator recovery) of $100\% \pm 20\%$ (see below) and a variation (CV) of $\leq 20\%$ in repeated injections if tested.

Determination of the S/N

For the determination of the S/N, the following parameters are considered:

- Unsmoothed chromatograms should be used to determine S/N.
- Chromatographic peaks should demonstrate visible stable baseline noise, ideally on both sides.
- S represents the height of the peak above the mean of noise (N) of the baseline (e.g. if the peak height is 6250 cps and the mean level noise is 875 cps (maximum 1300, minimum 450), then the peak signal is 5375 cps (Figure 6).
- N represents the absolute height of the noise (peak to valley, i.e. the highest and the lowest point of the signal) determined across a representative period of the chromatogram, within the region of the scheduled MRM window usually covering **at least 1 but ideally 2 peak widths**.
- The noise in a blank injection (e.g. methanol) can also be evaluated.
- The noise in an extraction blank injection should be evaluated as well to ensure no interferences (e.g. linoleic acid derived HODEs are sometimes detected in extraction blanks).

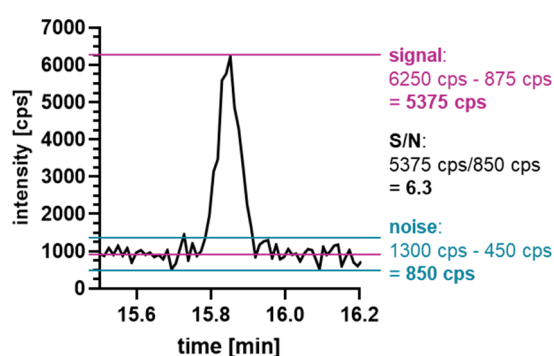


Figure 6. Representative illustration for the determination of S/N. The peak of the injection of a standard (0.25 nM, 0.4 pg on column) and signal-to-noise ratio is determined.

- Care should be taken when software is used to determine S/N. It is important to note that some S/N calculation tools from instrument manufacturers generate incorrectly high S/N values, most likely because smoothing is applied and other parameters such as SD of noise are used in place of noise. If software is used, users should first determine that the software returns values that are consistent with simple manual calculation as described above. If needed, it may be possible to improve software performance by adjusting/correcting parameters such as noise retention time window. The validation of any software used, and its settings should be reported in the manuscript.
- In addition to S/N, the coefficient of variation (% CV) or relative standard deviation (RSD) of repeated injections can be used to inform assay sensitivity, e.g. $\leq 20\%$ at LLOQ. However, if this criterion is also reported, only analyte peaks in samples that have an $S/N \geq 5$, should still be considered for integration.
- Alternative methods to determine S/N exist which are standard for clinical applications, including using SD of noise instead of noise (75), or multiplying the signal height by 2 before dividing by noise ($2H/h$, where H is height of peak, and h is height of noise (European Pharmacopoeia, Ph. Eur. (76)). This stringent level of validation requires the use of matrix-spiked standards (75) and is generally not feasible for the analysis of endogenous oxylipins in research settings due to sample number and volume limitations. If researchers decide to use these approaches, it is essential to follow them fully, and some pointers are provided below.
 1. Using the SD of baseline noise for h assumes the noise is normally distributed and would result in value for h that is approximately 1/5 of the peak-to-peak value (approx. 99% confidence interval). The minimum S/N required when using SD for noise must also be adjusted to be larger by a factor of 5.
 2. Similarly, if $2H/h$ is used, the resulting S/N values for the LOQ would need to be doubled to compensate. For example, the LOQ for the Ph. Eur. (76) would become $S/N \geq 10$, which is compatible with $S/N \geq 5$, as suggested in these recommendations. If this is not corrected, poor-quality peaks may be processed.
 3. The LOQ can also be established by performing precision and accuracy studies using repeated injections. Using this approach, the concentration that can be measured with a coefficient of variance (CV) less than 20% establishes the LOQ. For studies like this, the experimental design must include enough samples to compute the CV with an acceptable 95% confidence interval. When the CV of a low-concentration sample is used, the experimental design should be described, including sample count and other variance expected to be encountered (inter-assay, inter-day, inter-instrument).

As stated already, this level of validation is generally not feasible for the analysis of endogenous oxylipins in research settings due to the unavailability of analyte-free matrix, and low amounts of precious samples being available in many experiments. Other variance methods exist which use matrix-matched calibration curves, and these are also described for clinical studies and diagnostics, but they are also not usually feasible for research assays and are not covered in these recommendations.

Based on the criteria provided above, the LLOQ is defined for a given oxylipin and instrument as a unique amount ("pg or pmol on column" or concentration of the injected solution). For oxylipins, this is typically about 1 pg (Table 2 in (55)) on column but can be as low as 0.1 pg on column for some lipids using the newest instruments (21, 24). Quantification of oxylipins in biological extracts can only be performed if the compound is present at this or higher levels in the sample being extracted. The LLOQ can instead be reported as the minimal amount of lipid that can be quantified in a specific amount of biological sample (e.g. a concentration in ng/mL or pg/mg tissue, from a defined volume or wet weight), provided the corresponding signal in the instrument used fulfills the criteria described in this section.

It is important to note that the presence of matrix can increase the LLOQ of an assay due to the detection of chemical noise generated from unrelated components present in the sample and a reduced response to the analyte due to ion suppression (see above). Due to this, an essential requirement for reliable quantification in biological samples is that the analyte in samples demonstrates a clear chromatographic peak with a sufficient S/N of ≥ 5 for each reported value. Integrated area values alone, e.g. 2000 cps, can't be used to determine the presence of a peak in a sample, since these levels can also originate from background noise (51).

Report

Routine analysis:

Definition of LLOQ and LOD.

LLOQ and S/N for each oxylipin as determined by the method using analytical standards without matrix.

A representative unsmoothed chromatogram from a sample showing the typical peak for each oxylipin relevant for the study being reported in the batch of samples together with the amount which was calculated based on this peak.

Method development:

Representative unsmoothed chromatograms of standards used for LLOQ determination.

If software is used for S/N calculation: Settings of the software program used, and documentation that it leads to similar results to manual determination.

7. Calibration and calculation of concentration.

For the standard curve, starting from the LLOQ, a series of at least 6 concentrations for each oxylipin quantified is required for external calibration. At least 2 concentrations per order of magnitude (i.e. a log-linear distribution) are used. For example, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1000 nM corresponding to 0.16, 0.48, 1.6, 4.8, 16, 48, 160, 480, and 1600 pg on column, respectively, with 5 µL injected for an oxylipin with the molecular weight 320 g/mol. Detailed procedures for preparing and analyzing a calibration series have been published (18, 77-79). Standards of known purity such as those with a certificate are preferable, and laboratories are encouraged to confirm their purity and identity (80). Analysis of the calibration series should be performed in ascending order, starting with a solvent blank, and ending at the upper LOQ (ULOQ, top of linear range) or at the highest amount relevant for the samples being analyzed, followed by analysis of a solvent blank to evaluate injection carryover. As described above, it is advisable that the concentration of IS in the standard curve and prepared samples being analyzed by LC-MS/MS are equivalent.

For calibration and calculation of the amount of lipid in the samples, the area of the oxylipin peak is divided by the area of the IS used. To improve the accuracy and efficiency of integration and peak picking algorithms, peak smoothing algorithms can be applied, but this should only be done after raw data peaks have been verified to exceed the minimum S/N criteria for LLOQ (see above).

A linear calibration function is generated by linear regression of the ratio of oxylipin and IS peak areas as a function of the oxylipin amount/concentration. Alternatively, the ratio of oxylipin and IS amounts can be plotted on the x-axis. A reciprocal weighting of $1/x$ or $1/x^2$ of the analyte/IS concentration/amount is strongly advised (81) because it leads to a better accuracy for quantification of low-abundant oxylipins.

The calibrator recovery ("accuracy" in several software programs, or "relative mean error" (RME)) should be $100\% \pm 20\%$ at LLOQ and $100\% \pm 15\%$ for all other calibration points. The accuracy here describes the ratio between the added (actual) concentration of the compound in the calibration solution and the concentration which is calculated using the regression function of the calibration series:

$$\text{calibrator recovery} = \frac{C_{\text{calculated}}}{C_{\text{added}}} \cdot 100 [\%]$$

Also, the precision of the calculated amount of repeated injections (e.g. $n = 5$ in three batches) can be evaluated and is expected to be in the range of $<20\%$ CV. The linear range of the method can also be defined, starting with the LLOQ, up to the upper limit of quantification (ULOQ), the highest standard level which results in a linear detector response with an accuracy of $100\% \pm 15\%$ for repeated injections.

In samples, amounts of oxylipins can only be determined if the level detected is within the linear range.

In research settings, limited amounts of matrix often constrain generation of matrix-matched calibrations. Thus, the above-described matrix-free external calibration with IS method is common practice. However, determining and reporting recovery (accuracy) and variation (precision) by standard additions (spiking to samples, see below) is recommended as part of initial development/establishment of a method.

Direct quantification based on IS can be conducted for individual oxylipins if their corresponding internal standard (IS) is available and the method is operating in the linear dynamic range (see above). For this approach, it is essential to determine the response factors of the analytes relative to the IS.

Report

Method development:

Table of a typical linear calibration, including amounts on column, the slope, the Y-intercept, the R^2 value, and the accuracy (calibration recovery (\pm SD)) and precision.

8. Sample preparation and ion suppression analysis.

Prior to LC-MS/MS, extraction of oxylipins from biological samples reduces levels of interfering molecules such as proteins and concentrates analytes of interest in an LC-MS-compatible solvent. This can be achieved by several methods including protein precipitation (23), liquid/liquid extraction, solid phase extraction (SPE), or a combination thereof (82). Most often, SPE procedures are used since they also (at least partially) remove other highly abundant lipids such as phospholipids and neutral lipids. It is important to note that some oxylipins are unstable during handling and extraction (for example prostaglandins show pH sensitivity and instability during base hydrolysis) with many being also present in complex lipid pools (19, 83). Thus, it is important to assess stability of oxylipins and IS during sample processing particularly for the determination of total (hydrolyzed) oxylipins. In addition, certain oxylipins can be formed during sample preparation (e.g. the clotting process used to produce serum) and extraction, if appropriate precautions are not taken (40, 77, 84).

Co-extracted compounds from matrix can cause significant ion suppression (or “ion enhancement”, both are referred to here as “ion suppression”) of oxylipins and their corresponding IS. To check this, during method development, the detection of IS in extracted samples can be monitored. Where apparent recovery of the IS appears low (< 50%), it can be determined whether this is caused by ion suppression, low extraction recovery, or degradation of the compound. This can be assessed by comparing data generated from a sample with IS added at the beginning of sample preparation with a sample where the IS was added at the end of sample preparation, directly to the extract (Figure 7). If ion suppression dampens IS detection, the signal will be reduced in both samples. In this case, sample preparation and/or chromatographic conditions may require further optimization (82, 85) and it should be evaluated whether a different IS should be used instead. It is important to note that ion suppression can dramatically vary across the chromatogram, and consequently, between an IS and its associated analyte(s). As an example, ion suppression for linoleic acid-derived HODE is different from AA-derived HETE in plasma lipid extracts, because the oxylipins elute at different retention times (82). Ion suppression analysis (Figure 7) can be applied to monitor these effects.

Alternatively, dilution integrity experiments can be performed. For this, the prepared solution ready for injection in the LC-MS is analyzed after a 2–10-fold dilution, and the concentrations calculated based on IS and external calibration are compared. After correcting for dilution, the same concentration is expected for each dilution. Significantly higher or lower levels of oxylipin after dilution indicate that the signals of the analyte and the allocated IS are not equally interfered by matrix effects. If this is observed, measures to reduce ion suppression should be considered, including modifying the choice of IS.

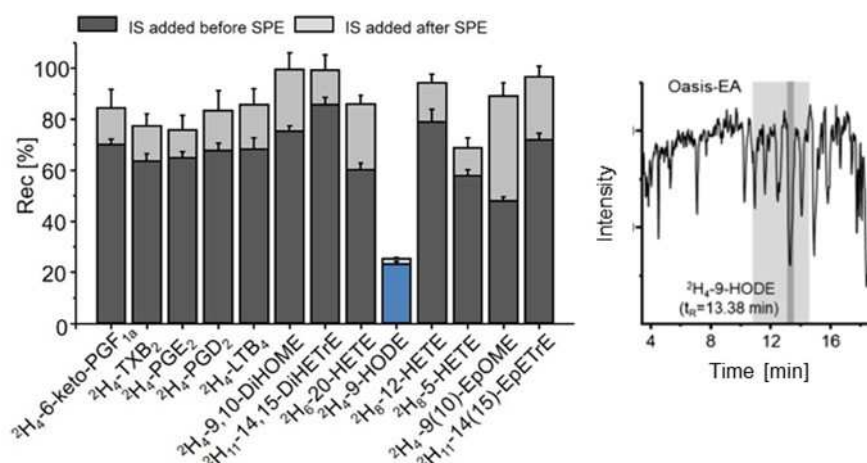


Figure 7. Analysis of recovery and ion suppression for different oxylipin IS extracted from plasma using an OasisHLB-based SPE. Left: The recoveries (Rec [%]) of IS added at the beginning of sample preparation (before SPE) and at the end of sample preparation (after SPE) are shown. Differences between the two originate from inevitable losses during SPE. If the apparent recovery rate is low in both, it indicates a reduction of the signal by ion suppression as shown for $^2\text{H}_4$ -9-HODE. **Right:** LC-MS/MS ion suppression analysis of an SPE extract of human plasma (82). The plasma extract without IS is injected onto an HPLC column. Post-column, the IS is added by flow injection using a syringe pump, and the IS signal is monitored. Drops in the signal intensity indicate ion suppression. In dark grey, the retention time of $^2\text{H}_4$ -9-HODE is indicated, and the light grey area highlights the retention time region of oxylipins to which $^2\text{H}_4$ -9-HODE is allocated as the IS. Images reproduced from (82).

Report

Routine analysis:

A detailed sample preparation procedure including solvents used for reconstitution.

Average IS recovery for each sample type (e.g. plasma, tissue, urine).

Method development:

Dilution integrity analysis, and a statement if and how the sample preparation technique was optimized to reduce matrix effects.

9. Accuracy, precision and system qualification.

During method development, accuracy and precision should ideally be determined for each sample matrix of interest (e.g. plasma or urine) to demonstrate the suitability of the method. However, it is recognized that in research settings, analytical laboratories routinely analyze many types of samples, and sufficient biological material is often not available for this purpose. Therefore, accuracy and precision could at least be provided for a relevant matrix as described below, when the method is being established.

The performance of the method for quantification of (representative) oxylipins can be evaluated by repeatedly analyzing spiked samples. If a matrix without the oxylipin of interest is available (which is rarely the case), then two relevant amounts can be added (one close to the LLOQ) to determine accuracy and precision. Alternatively, an extracted matrix pool can be used to generate a calibration curve dilution, and the slope of the analyte responses in the presence and absence of matrix can be compared. An example of the determination of accuracy and precision of multiple hydroxy-PUFA in plasma and serum is shown in Table 2.

Table 2. Determination of accuracy and precision in spiked samples. Example of a data set for the analysis of samples spiked with oxylipins as part of method development. The oxylipins were spiked to plasma at the indicated concentration (0.1 nM to 1 nM) and the concentration was determined using LC-MS/MS (n = 4). Accuracy was calculated as: $accuracy = \frac{c_{calculated}}{c_{added}} * 100 [\%]$ and precision is expressed as RSD. No endogenous levels of RVD5 and PDX were detected. Data reproduced from (21).

Oxylipin	mass transition		0.1 nM in plasma		0.3 nM in plasma		1.0 nM in plasma	
			1.8 pg on column		5.4 pg on column		18 pg on column	
	Q1	Q3	acc. [%]	prec. [%]	acc. [%]	prec. [%]	acc. [%]	prec. [%]
7,17-DiHDoHE (RvD5)	359.1	199.1	109	3	111	4	109	10
10,17-DiHDoHE (PDX)	359.1	153.1	96	5	98	2	94	6

A challenge with oxylipin quantification is that most biological matrices contain oxylipins. To overcome this, a heavy isotope labeled oxylipin may be used, or a non-labeled oxylipin standard can be added at different amounts. When the added amount is plotted against the analyte-to-IS ratio, the linear regression function is expected to be parallel to that of the calibration. For the latter, spiking levels should be adjusted to be close to the amounts identified in the actual sample (e.g. equal and double of the endogenous present amount).

By preparing and analyzing a set (n=3-6) of non-spiked and spiked samples over three days, intra-day and inter-day precision can be determined. When it comes to precision over multiple days using the same sample, it is recommended to prepare different aliquots rather than thawing the same sample multiple times.

The stability of extracted samples and maximum storage times between preparation and analysis in the freezer and/or in the autosampler can be monitored and reported, since extracted oxylipins can degrade rapidly, or alternatively form *ex vivo* (39, 40, 77, 84, 86, 87). Related to this, oxylipin levels in fluids and tissues can change during storage. This is especially important to consider where samples have been stored for several years. Numerous studies have investigated the changes in the pattern of oxylipins after storage, and the impact of pre-analytical procedures such as plasma generation from whole blood (30, 40, 77, 84, 86-88). A simple strategy to test for stability involves the addition of ^{13}C -labeled IS at the time of collection and monitoring its losses alongside endogenous oxylipins in the sample.

It is recommended to include at least one quality control (QC) sample, containing, if possible, all analytes in a batch, to monitor the performance and stability of the analytical workflow. This can be generated from a pooled, spiked and aliquoted biofluid sample (as relevant), or a larger pool of homogenized and aliquoted tissue or cell pellet samples. QC samples can be included in each batch at defined intervals and the analyst is encouraged to define pass/fail criteria, e.g. a deviation from expected concentration < 30%.

Since solid tissues can be highly heterogeneous, it is important to use optimized homogenization and extraction methods that yield reproducible recoveries of IS and analytes. Due to this, attention should be paid to potential sources of variability in IS response in complex tissue samples. This also applies to more conventional fluids such as plasma and serum, which can vary significantly due to underlying disease. Maintaining a record of peak intensity of IS in each run can help provide an early indicator of unanticipated interferences, as well as alerting the analyst to variations in instrument performance. It is also important to monitor method performance over time. To this end, basic QC procedures should be established in each laboratory performing quantitative oxylipin analysis. By the analysis of control charts, using e.g. Westgard rules (89, 90) analytical accuracy and precision can be monitored and warning and action limits defined.

Before commencing a sample analysis, acceptable instrument performance should be demonstrated, both for chromatographic separation and MS sensitivity. It is suggested that a low-level or a medium-level standard (system suitability control, SSC) is injected for this purpose. If the complete calibration series is not run alongside each batch, selected calibration standards should be analyzed to validate that the entire calibration series is still valid. Injection of the full calibration series should be carried out regularly, or with every batch, and always after instrument maintenance or repair.

Report

Routine analysis:

Type of QC samples used and how these were evaluated.

Method development:

Recovery of spiked standards for a representative sample matrix, inter- and intraday precision and accuracy of different spiking levels

10. Reporting and naming.

The levels of all quantified oxylipins in all samples should be reported, either as amount (mass or molar units) per weight (tissues), cell number, or per volume (concentration; fluids) in a table. All oxylipins measured in the method should be listed, including those that were not detected or quantified, due to reasons that could include their signals being below the LOD or LLOQ, or above the ULOQ, or where signals were of poor quality due to interfering chemical noise.

Oxylipin nomenclature should be informed by the limitations of the chosen approach. Systematic names are impractical in many cases. However, as a general rule, the name of the reported oxylipin should only contain information that can be derived from the LC-MS/MS analysis, with exceptions as listed below. If no chiral analysis has been performed, then stereoisomer designation is not applicable. For example, 5-HETE should be used as name, in place of 5(*S*)- or 5(*R*)-HETE.

In situations where an oxylipin shares retention time with a defined stereoisomer, then that name could be also included within parentheses, for example: “5,15-DiHEPE (Resolvin E4)”, to indicate co-elution. Other examples where there is insufficient separation of diastereomers in RP chromatography include 5,15-DiHETE, 7,17-DiHDoHE and 10,17-DiHDoHE, which can coelute with standards of Resolvin D5, PD1 and PDX, respectively (39).

For well-known oxylipins such as prostaglandins, where stereochemistry in biological samples has been historically assigned, the common names are generally used. If biological information is used to inform naming, for example the detection of 12-HETE in serum, which is well known to be mostly 12(*S*)-HETE, then this could be reported in the discussion. Analogous information can be included when analyzing prostaglandin *versus* isoprostane (IsoP) isomers.

Minimal reporting

As stated at the start of this document, for manuscripts reporting the establishment of an oxylipin analytical method in a laboratory (even if adapting the method from a publication), all parameters listed in boxes above under “Method Development” are recommended to be reported, enabling other researchers to evaluate the performance of the described procedures.

Where an established method is being routinely used in a laboratory, the first published paper from the laboratory should report parameters listed under “Method Development”, while subsequent papers using that method to quantify the same set of oxylipins can instead report parameters listed under “Routine Analysis”, either in main text or a supplementary section. For transparency, the Lipidomics Reporting Checklist can be used for documentation and reporting (46).

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