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Development, validation, and application of an HPLC-MS/MS method for quantification of oxidized fatty acids in plants

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ABSTRACT

Oxylipins constitute a huge class of compounds produced by oxidation of long-chain unsaturated fatty acids either chemically (by radicals such as reactive oxygen species, ROS) or enzymatically (by lipoxygenases, LOX; cyclooxygenases, COX; or cytochrome P450 pathways). This process generates fatty acids peroxides, which can then be further modified in a broad range to epoxy, hydroxy, keto, ether fatty acids, and also hydrolyzed to generate small aldehydes and alcohols. In general, oxylipins are present in almost all living organisms and have a wide range of signaling, metabolic, physiological, and ecological roles depending on the particular organism and on their structure. In plants, oxylipins have been extensively studied over the past 35 years. However, these studies have focused mainly on the jasmonates and so-called green leaves volatiles. The function of early LOX products (like keto and hydroxy fatty acids) is yet not well understood in plants, where they are mainly analyzed by indirect methods or by GC–MS what requires a laborious sample preparation. Here, we developed and validated a straightforward, precise, accurate, and sensitive method for quantifying oxylipins in plant tissues using HPLC-MS/MS, with a one-step extraction procedure using low amount of plant tissues. We successfully applied this method to quantify the oxylipins in different plant species and *Arabidopsis thaliana* plants treated with various biotic and abiotic stress conditions.

1. Introduction

Oxylipins are compounds with an ancient evolutionary origin, produced by lipid oxidation. They are signaling molecules present mainly in all kingdoms of life and are often involved in ecological interaction between different organisms, playing different roles according to their molecular structure or the target organism [1–5]. The oxylipins can be generated chemically or enzymatically by the addition of one or several oxygen atoms into the carbon chain of polyunsaturated fatty acids (e.g., linoleic, linolenic, arachidonic acids). Much research has been done in mammalians regarding the biosynthetic enzymes and the role of oxidized fatty acids (hydroperoxides, hydroxy, and keto fatty acids) [6–8]. In plants, the first step of the biosynthesis leads to hydroperoxides fatty acids, which subsequently can be converted into a broad range of compounds such as epoxy-, hydroxy-, keto- or ether-fatty acids, among others [9]. Some of the fatty acid derivatives (13-hydroperoxide fatty acid) can be converted into one of the most studied plant hormones: jasmonic acid, which has been the main oxylipins studied in plants. The jasmonic acid biosynthetic pathway was elucidated in 1983 as a product of fatty acid oxidation with posterior cyclization, and all the genes and enzymes involved in the JA-biosynthesis were identified and characterized since [10]. These studies, together with numerous others on the function of jasmonates in plant physiology, metabolism, and defense, originated a whole research field. In the past years, plant lipoxygenases (LOX), as the first enzymes in oxylipin synthesis, have been shown to play a role in physiological functions (like seed germination, root morphology), plant defense against pathogens and herbivores, and response to abiotic stresses [11,12]. However, very little is still known about the role of the early LOX products, like keto- and hydroxyl-fatty acids in plants [13].

The quantification of oxidized fatty acids is challenging due to their chemical diversity, low concentration, and stability [14]. Several

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methods using GC–MS and LC-MS have been developed to quantify these compounds in biological samples. However, they focus mainly on mammalian tissues and fluids [15]. In plants and microorganisms, oxidized fatty acids have been quantified basically by indirect measurements of secondary species produced during lipid peroxidation, for instance, changing concentrations of malondialdehyde (a secondary product of lipid peroxidation) or hydroperoxides, and by GC–MS, respectively [16–20]. The main drawback of those methods is that indirect quantification is often unspecific and may provide misleading results, especially in plant extracts, where many other compounds can interfere in the measurements. In the GC–MS methods, the samples have to be derivatized by a time-consuming protocol, especially when analyzing a high number of samples.

There are only few studies published where the free oxidized fatty acids are directly measured [21–24]. In all these cases, the sample preparation still involves laborious/expensive protocols (using solid-phase extraction) and/or high amount of sample. Therefore, we developed and present here a straightforward, selective, sensitive, precise, and accurate method to quantify oxylipins in plant tissues. We employed this method to detect and analyze these oxylipins in different plant species and under different stress conditions.

2. Materials and methods

2.1. Reagents and standards

Solvents used during extractions were of HPLC grade. Chromatographic separation was carried out using methanol and acetonitrile HPLC grade purchased from J. T. Baker (Xalosto, Mexico) and formic acid from Sigma Aldrich (Seetze, Germany). All oxylipin standards were isolated as described for Trapp et al. [25]. The deuterated standards: (d3-13-KODE and d4-9-HODE) and the 9-HODE, 13-HODE, and 13-KODE (used in the validation with internal standards) were purchased from OlChemIm Ltd (Olomouc, Czech Republic). The plant or bacteria growth media were acquired from Sigma Aldrich Muhashige and Skoog Basal Medium (St Loius, USA) and King B agar (Steinheim, Switzerland).

2.2. Apparatus

A Waters Alliance®HPLC® (Waters Corporation, Milford, MA, USA) coupled to a Micromass Quattro LC mass spectrometer was used to develop and validate the analytical method for quantification of oxylipins. Chromatographic separation was carried out in a Luna Phenyl-Hexyl column (150 × 4.6 mm, 5 μm; Phenomenex, Aschaffenburg, Germany). Formic acid (0.001%, v/v) and acetonitrile with 0.001% (v/v) of formic acid were employed as mobile phases A and B, respectively. The elution profile was: 0–3 min, 50–80% B in A; 3–8 min, 80–100% B; 8–11 min 100% B; 11–11.1 min 100–50% B in A; and 11.1–16 min 50% B in A. The mobile phase flow rate was 1.1 mL/min (which was split in a rate of 1:6 before sending to ESI source). The injection volume was 25 μL. The mass spectrometer was equipped with an electrospray ionization source, operating in the negative mode. Measurements were carried out using the following ionization parameters: 25 V source voltage: 4.4 kV, capillary voltage: −48 V, tube lens −113 V, declustering potential 10 V, turbo gas temperature: 300 °C, auxiliary gas flow: 4.5 L/min, sheath gas flow: 9 L/min. Selected reaction monitoring (SRM) experiments were used to monitor specific precursor > product ion transitions for each oxylipin and for the internal standard. The collision energy was optimized for each compound separately.

During the inter-laboratory reproducibility, the analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Böblingen, Germany) connected to an LTQ Iontrap mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was carried out using the same conditions described above. The ionization parameters used during these analyses (ionization parameters, collision energy, precursor ion isolation width, and activation Q) were optimized for each compound separately

2.3. Plants and liverworts material

Arabidopsis thaliana Col-0 were obtained from publicly available collections. Surface sterilization of seeds was performed for 10 min with 0.05% SDS (sodium dodecyl sulfate) solution prepared in 70% ethanol. The sterilized seeds were plated on ½ MS (Murashige and Skoog medium) agar plates. Seeds were stratified at 4 °C for 2 d, and then plates were transferred in a growth chamber (Model CU36-L5, Percival Scientific, Perry, IA, USA) at 22 °C under long day (16 h photoperiod and 8 h dark) or short day (10 h photoperiod and 14 h dark) conditions for germination and seedling growth. Stem and leaves of *Alternanthera brasiliana* were collected at São Carlos area (Brazil) at the end of 2014. *Phaseolus lunatus'* Ferry Morse' var. Jackson Wonder Bush was grown in soil in a growth chamber at 23 °C (160 mE m^{22} s^{21} during a 14-h photoperiod; relative humidity 60%) for 2 weeks. *Marchantia polymorpha* ssp. *ruderalis*, eco-type BoGa, was kindly provided by Prof Dr. Sabine Sargo, and sterile plant cultivation was conducted according to Althoff [26]. *Ricidiocarpus natan* was obtained from a local shop and kept on the water under indirect sunlight until the analysis.

After collection, the tissues were immediately frozen in liquid nitrogen, ground, and dried in a SpeedVac under reduced pressure at −40 °C. After homogenization, 25 mg of plants were weighed into 1.5 mL tubes and stored at −20 °C until the measurements.

2.4. Oxylipins' extraction and analysis

The optimization of oxylipins extraction was performed in two steps. Initially, solvents with different polarities were evaluated, and in the next step, the water content was assessed to get a high oxylipin and low chlorophyll content.

2.4.1. Extraction using organic solvents

In the first step, tubes containing 25 mg of dried plant material were extracted with 250 μL of different solvents: ethyl acetate, acetonitrile, ethanol, and methanol. The samples were shaken for 20 min at 1100 rpm, centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was dried and resuspended in 80% methanol and analyzed by HPLC-MS/MS.

2.4.2. Aqueous solutions

In the second step, tubes containing 25 mg of dried plant material were extracted with 250 μL of different methanol: water ratios (90, 80, and 70% of methanol, respectively). Each extraction procedure was performed in triplicate. After extraction, all samples were centrifuged at 10,000 g for 5 min at 4 °C. The supernatant was directly analyzed by HPLC-MS/MS, as described above.

2.4.3. Radical scavenger and peroxide-reducing agents

Five biological replicates of *A. brasiliana* leaves were collected, immediately frozen in liquid nitrogen, ground using mortar and pestle, and lyophilized overnight. 25 mg of each biological replicate was aliquoted in 1.5 mL and extracted using 250 μL of either 80%. methanol or 80% methanol containing 0.5 mg/mL of butylated hydroxytoluene (BHT). The same procedure was repeated using 0.2 mg/mL of triphenylphosphine (TPP) instead of BHT.

2.4.4. Final method for extraction of oxylipins

During the validation of the analytical method, oxylipins were extracted in 1.5 mL tubes containing 25 mg of freeze-dried plant material. Samples were spiked with 10 μL of standard solutions (calibration curve or quality control levels, with the concentrations described in 2.5.2 and 2.5.3) and briefly shaken. The extraction was performed using 240 μL of 80% methanol, and samples were shaken for 20 min at 1100 rpm. Afterward, the samples were centrifuged for 5 min at 10,000 g and 4 °C and filtered using washed cotton. The supernatant was analyzed as

described above.

2.5. Method validation without internal standard

Two analytical methods for quantification of oxylipins were developed and validated during this work: one using deuterated oxylipins (13-KODE and 9-HODE) as internal standards and another without any internal standard. All validation parameters were evaluated for both methods to access the accuracy and precision according to the Guidelines on Bioanalytical Method Validation from European Medicines Agency (EMA), and FDA [27–30].

2.5.1. Limit of detection (LOD) and limit of quantification (LOQ)

LOD is usually established using matrix samples spiked with low amounts of standards. However, as no analyte-free matrix was available, the LODs and LOQs were determined by analyzing a serial dilution of each standard in solvent. The LOD was defined as the concentration where the signal was three noise level, while the LOQ was defined as the concentration where the signal was ten times the noise level. The basal levels were defined as the mean of the amount of each oxylipin present in ten independent blank samples, which were extracted as described in the *Final method for extraction of oxylipins*.

2.5.2. Calibration curve and linearity

The calibration curves were prepared in the matrix using a spiking solution containing pure compounds isolated from *A. brasiliana* (purity higher than 98%, defined by NMR as described by Trapp et. al) [24]. For each point of the calibration curve, 25 mg of dried plant material were spiked with 10 µL of spiking solution containing all the oxidized fatty acids in different concentrations (0.0625, 0.125, 0.250, 1.25, 2.50, 6.25, 12.5 µg/mL) and these samples were extracted as described in *Final method for extraction of oxylipins*. The final concentration of each calibration point (in ug/mL and ug/mg plant fresh weight FW) are presented in the Table S1.

2.5.3. Quality controls

Quality controls (QC) were used to assess the method's repeatability and reproducibility. Within-run repeatability was assessed by 5 replicates of each quality control level while 10 replicates were used to access repeatability and 20 replicates were used during the intra-lab repeatability. The quality controls were prepared spiking 25 mg of plant material with 10 µL of spiking solutions with different concentration of all oxylipins: high-quality control spiking solution (HQC) containing 11.25 µg/mL of each fatty acid; medium-quality control spiking solution (MQC) containing 7.5 µg/mL; and low-quality control spiking solution (LQC) containing 0.187 µg/mL. All these samples were also extracted as described above, given the following final concentration: 0.075 µg/g FW (LQC), 3.0 µg/g FW (MQC), 4.5 µg/g FW (HQC).

2.5.4. Matrix effect

To access the matrix effect on compound ionization, the angular coefficient of two calibration curves were compared: one prepared in the matrix (the plant extract) and another prepared in solvent. For the calibration curve prepared in the matrix, 250 mg of dry plant material was extracted with 2.5 mL of 80% methanol as described in the *Final method for extraction of oxylipins* and aliquoted in 9 epp tubes, each one containing 240 µL. These samples were spiked with 10 µL of each concentration of the spiking solution (see *Calibration curve and linearity)*. The calibration curve in solvent was prepared by adding 10 µL of each concentration of spiking solution in 240 µL of 80% methanol.

2.5.5. Selectivity

Since all the analyzed oxylipins are plant endogenous metabolites, the selectivity of each SRM transition could not be evaluated in matrix. Therefore, the selectivity was evaluated by analyzing each standard and internal standard at 100 ng/mL, individually and making sure that none of the other oxylipins would interfere in the chromatographic peak.

2.6. Method validation using internal standard

In order to evaluate whether the use of internal standards would add any improvement to the method, all parameters described above (linearity, selectivity, repeatability, and reproducibility) were also evaluated during the validation using internal standards. However, since some of the analyzed oxylipins are not commercially and we did not have enough isolated compounds, as proof of concept these parameters were assessed for 3 oxylipins: 9-HODE, 13-HODE, and 13-KODE, which are commercially available. Extractions for the calibration curve and QC samples were performed in the same way as described above. The only difference was that the extraction solution was spiked with 20 ng/mL of d3-13-KODE and d4-9-HODE (internal standards, IS)

2.7. Quantification of oxylipins in plants under biotic and abiotic stress conditions

To evaluate whether this method would be suitable to detect changes in the oxylipins' content in *Arabidopsis thaliana*, when it is exposed to biotic and abiotic stresses, we performed the following experiments.

2.7.1. Heat-shock experiment

Heat stress experiments were performed by modifying previous studies [31]. Five days seedling of almost equal length were transferred onto new ½ MS plates (Murashige and Skoog medium). For heat shock treatments, plants were divided into two sets: in set 1, plants were exposed to 44 °C heat-shock (HS) for 30 min on day 11, and plant material was harvested after 1 h of HS treatment. In set 2, plants were grown under normal conditions at 22 °C, and plant material was harvested as controls with HS-treated plants.

2.7.2. Bacteria inoculation experiment

Arabidopsis plants were grown under short-day conditions. *Pseudomonas syringae pv. tomato* DC3000 avrRpm1 (Pst avrRpm1) were grown overnight on King B agar plates at 28 °C. Bacteria were harvested and resuspended in 10 mM $MgCl_2$. Leaves of 4-week-old *A. thaliana* grown under short-day conditions were syringe infiltrated with 10^6 CFU of Pst avrRpm1 and harvested after 24 h. Non-infiltrated and mock (10 mM $MgCl_2$) infiltrated leaves served as control.

2.7.3. Wounding experiment

For the wounding experiments, leaves of 4-week-old *A. thaliana* grown under short-day conditions were wounded using a pattern wheel as described in Vadassary et. al. [32]. The wounded leaves were collected 1 h after the treatment.

2.7.4. Analyses of oxylipins

All samples were collected in 1.5 mL tube, immediately frozen in liquid nitrogen and freezer dried. Around 25 mg of freeze-dried plant material were extracted using 250 µL of 80% methanol spiked with 20 ng/mL of d3-13-KODE and d4-9-HODE. The samples were shaken for 20 min at 1100 rpm. Afterward, the samples were centrifuged for 5 min at 10,000 g and 4 °C and filtered using washed cotton. The supernatant was analyzed by using the developed method.

3. Results

The quantification of oxidized fatty acids was performed by HPLC MS/MS using Selected Reaction Monitoring (SRM), and the SRM transitions were selected based on the fragmentation pattern reported by Trapp et. al. as shown in Fig. 1, [25]. Chromatographic separation, ionization, and fragmentation parameters were optimized in order to achieve high intensity, good selectivity, and signal to noise ratio.

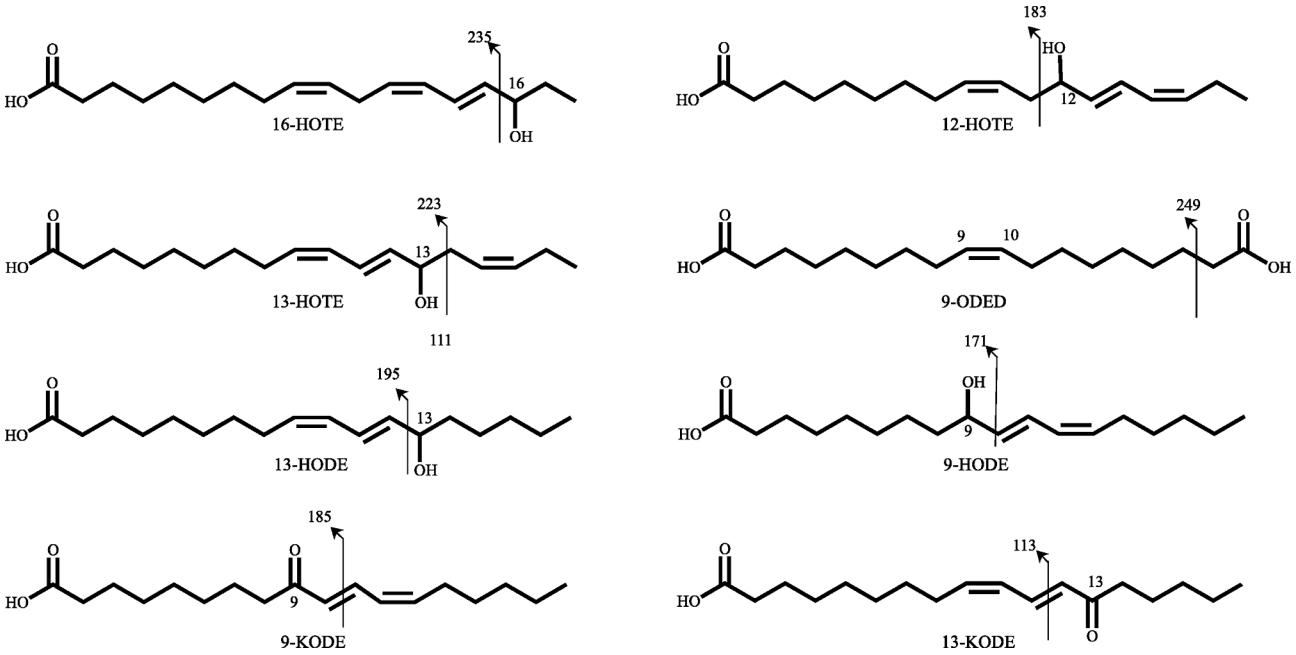

Fig. 1. Chemical structure of oxylipins quantified in the present study. The arrows represent the fragments used for quantification of each metabolite. **16-HOTE** (16-hydroxy-9,12–14-octadecatrienoic acid), **12-HOTE** (12-hydroxy-9,13,15-octadecatrienoic acid), **13-HOTE** (13-hydroxy-9,11,15-octadecatrienoic acid), **9-ODED** (9-octadecenedioic acid), **13-HODE** (13-hydroxy-9,11-octadecadienoic acid), **9-HODE** (9-hydroxy-10,12-octadecadienoic acid), **9-KODE** (9-keto-10,12-octadecadienoic acid), **13-KODE** (13-keto-9,11-octadecadienoic acid).

3.1. Development of extraction method

Several organic solvents were tested to extract the oxidized fatty acids from *A. brasiliana* tissues: ethyl acetate, acetonitrile, ethanol, and methanol. However, all the extraction solution presented high chlorophyll content, and a clean-up process was necessary to remove it from the samples. During the HPLC-PDA-MS analysis, the chlorophyll present in *A. brasiliana* tissues showed a non-polar nature (data not shown). So, instead of cleaning up the samples to remove the chlorophyll, we tested aqueous extraction solutions to extract a lower quantity of chlorophyll. After extraction, all samples were analyzed by HPLC-ESI-MS/MS using SRM. The areas present in the SRM chromatograms are summarized in Figure S1 for compounds 13-KOTE, 13-HODE, and 9-HODE, respectively.

These results show that independently of the oxidation nature (ketone or hydroxyl group) and position, the better extraction for these oxidized fatty acids was achieved using 80% methanol as extraction solvent. Therefore, 80% methanol was chosen for extractions of oxylipins to be applied in this method.

Many of the published methods for quantification of oxylipins employed either radical scavengers (e.g., butylated hydroxytoluene, BHT) or peroxide-reducing reactants (e.g., triphenylphosphine, TPP) during the extraction in order to avoid further oxidation of free fatty acids or to reduce the hydroperoxides into hydroxy fatty acids [23,24]. Thus, we also tested the influence of these compounds on the amount of extracted oxylipins from *A. brasiliana* tissues. As shown in Figure S2, neither BHT nor TPP had a significant impact on the concentration of the analyzed oxylipins. Therefore, no additive was used in the extraction solution during the method validation.

3.2. Validation parameters

The validation parameters (selectivity, linearity, reproducibility, repeatability, matrix effect, and robustness) were evaluated according to the EMA, FDA, and European Commission guidelines [27–30]. Since no blank matrix was available (all the oxylipins are endogenous in *A. brasiliana*), the quantification was performed using standard addition method, by preparing the calibration curve and quality controls in the plant extract (matrix), as described in 2.5.4 and 2.5.3.

3.2.1. Linearity

Linearity was measured using seven calibration curves prepared individually. Each calibration curve was composed by a blank sample and 7 points, as shown in Table S1. These values were chosen based on the basal levels present in *A. brasiliana* tissues and 120% of the maximum expected levels during biological experiments (i.e., exposure to biotic and abiotic stresses).

The SRM transitions, linear regression, weighting factor, $R^{2,}$ and the basal level of each compound in *A. brasiliana* are shown in Table 1. The SRM transitions were defined according to data published by Trapp et al. [25] and considering their selectivity since some compounds had a common fragment and retention time. (SRM chromatograms are shown on Figure S3)

For unweighted regression, the variance was not evenly distributed throughout the points of the calibration curve, and therefore 3 different weighting factors were evaluated: 1/x, $1/x^{2,}$ and 1/y. The optimal weighting factor was determined based on the % relative error (%RE, Supplementary Information, Figure S4), and F(exp) [33]. All the F(exp) values shown in Table 1 are lower than F(tab) = 10.967 ($F_{5,5}$, 99%), showing that the applied regression model and weighting factor were able to homogenize the variance of the residues providing a suitable model for quantification of the oxylipins in *A. brasiliana* tissues.

It is important to highlight that the best weighting factor and the regression model were determined evaluating not only the R^2 but also the residues for all calibration points (data not shown). In this case, all the residues must be below 20% for the lower calibration curve point and 15% for the others. When these parameters are not satisfied for one of the points of the calibration curve, this point is excluded from the calculations. According to the guidelines, neither the lowest point nor two consecutive points can be excluded. All these requirements were satisfied during the statistical processing, showing the method is linear for quantification of oxylipins in *A. brasiliana*. The limit of quantification in solvent was 0.1 ng/mL for the hydroxy fatty acids and 0.5 ng/mL for the keto fatty acids and 9-octadecedioic acid.

Table 1
SRM transitions, linear fit parameters for quantification of each oxylipin without internal standard.

Compound	SRM	Curve	Weighting factor	R^2	F (exp)	Amount in blank (µg /g DW)
16-HOTE	293 > 235	155389x + 1429,5	$1/x^2$	0,992	0.682	0.092
12-HOTE	293 > 183	305350x + 2753,7	$1/x^2$	0,999	0.175	0.090
13-HOTE	293 > 195	169905x + 2821,6	1/x	0,998	0.926	0.167
9-ODED	311 > 249	137555x + 5278,9	$1/x^2$	0,995	1.634	0.384
13-HODE	295 > 195	19817,6x + 2582,7	$1/x^2$	0,994	7.811	1.300
9-HODE	295 > 171	321671x + 19816,0	$1/x^2$	0,995	2.898	0.616
13-KODE	293 > 113	433527x + 14471,0	1/x	0,998	0.773	0.334
9-KODE	293 > 185	211053x + 9060,6	$1/x^2$	0,989	3.117	0.429

DW: Dry weight of *A. brasiliana*.

3.2.2. Matrix effect

It is well known that matrix, in this case, plant metabolites, can have a pronounced effect on the mass spectrometer response during the quantification of some compounds by HPLC-ESI-MS/MS. For this reason, the validation guidelines recommend that for quantifications using HPLC-ESI-MS/MS, all samples (calibration curve and quality controls) must be prepared in the matrix. Although some guidelines accept methods using samples prepared in solvent, it should only be done once it is proved that the matrix does not affect the response for different analyte concentrations.

Evaluation of the matrix effect can be done by comparison of the angular coefficient of both calibration curves: prepared in the matrix and in the solvent. If the difference is below 15%, the method can be validated in solvent; otherwise, the validation must be carried out in the matrix. Curves in solvent and in the matrix for all oxylipins are shown in Figure S5 (supplementary information). The angular coefficient (α) for calibration curves prepared in matrix and solvent, as well as the relative difference between these values, are summarized in Table S2. These results show that the matrix does affect the analyzer response for half of the oxylipins (samples highlighted in blue). So, for these compounds, the validation must be carried out in the matrix. Therefore, we decided to validate the method in the matrix for all oxylipins present in this study.

3.2.3. Repeatability and reproducibility

Accuracy and precision were assessed by the overall mean, relative standard deviation (RSD%), and relative error (Error%) of independent quality controls samples (QC). In this method, the quality control levels were defined as: low quality control (3 folds the concentration of lowest point of the calibration curve (CC)), medium quality control (75% of the concentration of the highest point of CC), and high (90% of the concentration of the highest point of CC).

Method repeatability was assessed by the accuracy and precision of two batches of samples prepared by the same analyst on two different days. The method reproducibility was calculated by accuracy and precision within four batches prepared in two different days by two analysts working in different laboratories: at Laboratório Micromolecular de Micro-organismos in São Carlos, Brazil, and at Max Planck Institute for Chemical Ecology in Jena, Germany. Each batch was composed by one calibration curve (CC) and 5 samples of each quality control level (LQC, MQC, and HQC). The data are shown in Table 2 and demonstrating that both relative standard deviation (RSD%) and relative error (Error%) are much lower than the limits described in the analytical guidelines: 20% for LQC and 15% for the other QCs.

3.2.4. Robustness

Robustness is the capacity of an analytical method to remain unaffected by small variations in the method parameters. It can be measured by comparing the concentration of quality control samples analyzed under two different conditions. Here, the method's robustness was evaluated by changing acetonitrile to methanol in the chromatographic method. So, a batch of samples (calibration curve and quality controls) was prepared and analyzed using both methods (standard and modified). After the quantification, the overall means of each quality control level obtained in each method were compared. The correlations between these means are given in Table S3 and showing that these solvents can be exchanged without impacting the method efficiency.

3.3. Validation using internal standards

All the recommendations from the EMA and FDA guidelines were fulfilled, and the method was considered precise, accurate, and robust for quantification of oxylipins in *A. brasiliana* tissues even without the addition of internal standards. However, we also evaluated whether or not a method using internal standards could contribute to decrease some of the method variability. The addition of internal standard can also be beneficial while analyzing real samples by correcting pipetting mistakes done during the sample preparation. Due to the lack of standards, the addition of internal standards was evaluated only for 13-HODE, 9-HODE, and 13-KODE. The linear fitting, $R^{2,}$ and weighting used during these evaluations are shown in Table 3 and were chosen as described before for the method without internal standard.

The contribution of internal standard was evaluated by accuracy and precision during repeatability and reproducibility studies, and the results are shown in Table 4, where all values of RSD and error are below 15%.

To further compare the accuracy and precision of the quantification performed with and without IS and evaluate whether or not internal standards contribute to reduce the number of outliers during the quantification of oxylipins, we analyzed the residues of quality control samples (for these three oxylipins) when the data was processed in two different ways:

A) **By area (external quantification)**: QC samples were quantified based on a calibration curve drawn by correlating the amount of oxylipins and the *response area*. This procedure is the same used in the quantification without internal standard.
B) **By ratio (internal quantification)**: QC samples were quantified based on a calibration curve that correlates the amount of oxylipins with the *ratio standard/internal* standard. In this case, *none weighting factor* was applied to the calibration curve

After quantification of quality controls samples (LQC, MQC, HQC) using these methods, the measured concentration of each QC sample was compared to the nominal values, and the residues were arranged graphically as shown in Fig. 2. The pink dots are outliers based on EMA and FDA rules. Although the number of outliers did not vary much for 13-HOTE and 9-HODE (changes from 0 up to 7.5% of the total number of samples), for 13-KODE, the addition of IS reduced the number of outliers from 20 to 7% (for LQC points). These results suggest that the IS helps to reduce the number of outliers, but the oxylipins can still be quantified with good precision and accuracy even without the addition of IS, as it was shown by the parameters evaluated during the validation of this

Table 2
Repeatability and reproducibility for quantification of oxylipins in *A. brasiliana* plant tissue.

Compound	Nominal conc. (µg/g DW)*	Within-run n = 5			Repeatability n = 10			Intra-lab Reproducibility n = 20		
		Mean ± SD	RSD (%)	Error (%)	Mean ± SD	RSD (%)	Error (%)	Mean ± SD	RSD (%)	Error (%)
16-HOTE	0.075	0.067 ± 0.004	6.08	−10.40	0.073 ± 0.010	12.8	−2.53	0.075 ± 0.009	12.5	−0.15
	3.000	2.963 ± 0.231	7.79	−1.23	2.880 ± 0.212	7.08	−4.01	2.858 ± 0.241	8.05	−4.73
	4.500	4.297 ± 0.121	2.88	−6.44	4.092 ± 0.210	4.68	−0.07	4.246 ± 0.398	8.85	−5.65
12-HODE	0.075	0.075 ± 0.008	10.32	0.27	0.078 ± 0.009	11.8	3.33	0.079 ± 0.009	11.5	4.96
	3.000	2.868 ± 0.171	5.95	−4.39	2.826 ± 0.157	5.25	−5.81	2.814 ± 0.147	4.89	−6.22
	4.500	4.105 ± 0.213	5.20	−8.77	4.153 ± 0.102	4.25	−7.71	4.120 ± 0.249	5.54	−8.43
13-HOTE	0.075	0.074 ± 0.008	10.29	−1.33	0.069 ± 0.004	5.93	−7.60	0.072 ± 0.006	8.50	−4.39
	3.000	3.162 ± 0.169	5.34	5.41	2.978 ± 0.249	8.29	−0.73	2.946 ± 0.249	8.32	−1.80
	4.500	4.781 ± 0.084	1.75	−6.24	4.425 ± 0.410	9.10	−1.66	4.430 ± 0.456	10.1	−1.56
9-ODED	0.075	0.075 ± 0.013	8.36	0.00	0.075 ± 0.006	7.73	0.27	0.077 ± 0.008	10.43	2.77
	3.000	2.759 ± 0.196	7.10	−8.02	2.812 ± 0.238	7.94	−6.25	2.872 ± 0.259	8.62	−4.28
	4.500	4.134 ± 0.356	8.62	−8.14	4.108 ± 0.468	10.4	−8.71	4.429 ± 0.511	11.3	−1.58
13-HODE	0.075	0.067 ± 0.005	7.00	−11.33	0.067 ± 0.004	5.43	−10.81	0.069 ± 0.007	9.77	−7.42
	3.000	2.989 ± 0.212	7.08	−0.37	2.969 ± 0.221	7.35	−1.05	2.880 ± 0.197	6.57	−4.02
	4.500	4.628 ± 0.145	3.13	−2.84	4.414 ± 0.253	5.63	−1.80	4.272 ± 0.273	6.07	−5.08
9-HODE	0.075	0.071 ± 0.005	6.40	−5.33	0.071 ± 0.005	6.56	−5.00	0.075 ± 0.007	9.31	−0.08
	3.000	2.717 ± 0.160	5.88	−9.43	2.75 ± 0.150	5.01	−8.33	2.732 ± 0.007	0.23	−8.94
	4.500	3.959 ± 0.112	2.84	−12.03	3.958 ± 0.152	3.37	−12.04	4.011 ± 0.185	4.10	−10.88
13-KODE	0.075	0.072 ± 0.006	8.06	−4.44	0.073 ± 0.004	13.44	−2.22	0.075 ± 0.010	13.2	−0.33
	3.000	2.800 ± 0.251	8.97	−6.65	2.732 ± 0.213	7.11	−8.92	2.826 ± 0.206	6.86	−5.81
	4.500	4.112 ± 0.153	3.71	−8.62	3.999 ± 0.228	5.06	−11.13	4.150 ± 0.233	5.17	−7.77
9-KODE	0.075	0.077 ± 0.006	8.36	2.40	0.075 ± 0.008	10.7	0.00	0.077 ± 0.008	10.6	2.42
	3.000	2.934 ± 0.291	9.93	−2.21	3.221 ± 0.284	9.47	7.37	2.813 ± 0.008	0.27	−6.22
	4.500	4.297 ± 0.285	6.62	−4.52	4.117 ± 0.294	6.53	−8.51	4.242 ± 0.388	8.62	−5.73

Table 3
Linear fitting, weighting, and correlation coefficient for oxylipins calibration curves using the standard internal method.

Compound	Calibration curve	Weighting	R^2
13-HODE	Y = 0.488678 + 0.00714018*X	1/X2	0.9869
9-HODE	Y = 0.59446 + 0.0188614*X	1/X2	0.9855
13-KODE	Y = 0.116593 + 0.00289745*X	1/X	0.9970

analytical method.

3.4. Oxylipin profile in plants and liverworts

To verify whether this method would be suitable for detecting and analyzing oxylipins in vegetal samples other than *A. brasiliana*, we also analyzed leaves of *Arabidopsis thaliana*, *Phaseolus lunatus*, and two liverworts (*Marchantia polymorpha* and *Riccidiocarpus natan*) and compared the oxylipins profile present in these samples. Since the analytical

Table 4
Validation parameters for quantification of oxylipins in *A. brasiliana* using IS method. (n = number of samples for each QC level)

Comp.	Quality control		Repeatability n = 10			Reproducibility n = 15		
	Level	Conc.	Mean	RSD %	Error%	Mean	RSD %	Error%
13-HODE	**LQC**	**0.075**	0.084	12.44	10.51	0.081	7.76	4.67
	MQC	**3.000**	3.060	1.99	2.72	3.027	0.89	1.55
	HQC	**4.500**	4.718	4.85	2.57	4.866	7.52	4.24
9-HODE	**LQC**	**0.075**	0.083	10.00	8.35	0.081	8.03	2.52
	MQC	**3.000**	2.774	−7.52	5.34	2.905	−3.25	6.60
	HQC	**4.500**	4.173	−7.28	0.88	4.639	2.99	14.29
13-KODE	**LQC**	**0.075**	0.078	4.44	13.4	0.077	2.36	2.19
	MQC	**3.000**	2.814	−6.19	3.64	2.975	−0.85	7.83
	HQC	**4.500**	4.153	−7.72	0.80	4.521	0.48	11.62

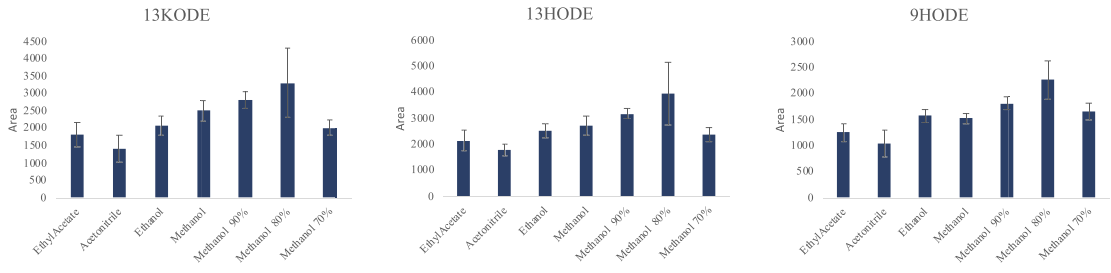

Fig. 2. Residual plot of quality control samples using distinct mathematical processing.

parameters (i.e., matrix effect, recovery, precision, and accuracy) were not evaluated for all the different tissues, we used the Comp/IS ratio to compare the profile of oxylipins in these tissues instead of absolute quantification. As can be seen in Fig. 3, we were able to detect all the oxylipins in vegetal samples of different species. Moreover, the basal oxylipin profiles vary considerably among the samples, both in quality and quantity.

3.5. Oxylipin composition upon biotic and abiotic stresses

In the past years, 9- and 13-LOX genes have been shown to be involved in plant development and responses to biotic and abiotic stress. Therefore, we applied the method for the quantification of the oxylipins upon infiltration with the bacterial pathogen *Pseudomonas syringae pv. tomato avrRpm1*, and upon different abiotic stresses (wounding, salt stress, and heat stress). We also use the same samples to measure jasmonates: 12-oxo-phytodienoic acid, (OPDA) jasmonic acid (JA), and jasmonic acid-isoleucine conjugated (JA-Ile) using the method published by Almeida-Trapp et. al. [34].

The oxidized fatty acids and the jasmonates present a very diverse profile depending on the stress the plants are exposed to. The jasmonates are well-known as signaling molecules produced upon mechanical stress, and the concentration of jasmonic acid and JA-Ile increased 500 and 100 folds, respectively, upon wounding. Other oxylipins quantified here did not respond at all to wounding (Fig. 4).

Upon salt stress, both jasmonates and oxidized fatty acids showed only minor changes in their concentration when compared to non-stressed plants (around 2–5 folds). On the other hand, the concentration of some oxidized fatty (i.e., 12-HODE, 9-HODED, 9-KODE) and jasmonates (JA-and JA-Ile) presented a considerable increase in response to heat stress. Moreover, there is no straight correlation between the position (C9, C12, C13, or C16) nor type of oxidation (hydroxy, or keto groups) with each stress.

The *Pst avrRpm1* caused a strong boost in the biosynthesis of both jasmonates and oxidized fatty acids, increasing the concentration of many oxidized fatty acids (9, 12, and 13 derivatives) up to 600 folds, and jasmonates up to 8000 folds.

4. Discussions

Oxylipins constitute a complex and important class of compounds found in a wide range of organisms, such as microbes, plants, insects, and animals. In plants, the best known and well-studied oxylipins are C6-volatiles and jasmonates (i.e., oxo-12-phytodienoic acid, jasmonic acid, jasmonic acid isoleucine conjugate), which over the past decades have been shown to play a role in a variety of developmental process, regulation of secondary metabolites, and abiotic/biotic stress responses [7–9,35–37]. In spite of that, little is known about the role of oxylipins other than the jasmonates, such as 9- and 13-oxidized fatty acids in plants. However, in the past ten years, some studies have shown that the 9-LOX derivatives might also play a role in physiological processes (such as root waving, loss of apical dominance in the root, decrease of root elongation), defense against pathogens, and stomata closure [35–37]. Although it is known that in *A. thaliana,* for instance, 6 lipoxygenases (LOX1-LOX6) are involved in the biosynthesis of oxidized fatty acids, gene regulation studies can only provide an incomplete view of the processes because two of the LOX are able to oxidize carbon 9 of C18 fatty acids (LOX1 and LOX5), while the other four (LOX2, LOX3, LOX4, and LOX6) are responsible for the oxidation of carbon 13. Sometimes LOXs with dual oxidation sites (9/13 carbon) were also identified [37]. Therefore, it is important to be able to detect and quantify the real metabolites in order to evaluate their function, in addition to the molecular analyses, including gene regulation strategies.

Although some methods have been used for quantification of these compounds in plant materials, they are mainly based on gas chromatography-mass spectrometry, involving extensive extraction and derivatization procedures. Some interesting methods using direct quantification of oxidized fatty acids by HPLC-MS/MS have been reported, but they applied to liquid fluids [10,38–39], which constitute a matrix less complex than plant tissues. These methods still employ solid-phase extraction (SPE), making the sample processing expensive and laborious. Since plant material constitute a much more complex matrix if compared to biological fluids, we present the development and validation of an HPLC-ESI-MS/MS-based method for direct quantification of oxidized fatty acids (members of oxylipins) (Fig. 1) in plant samples using a low amount of fresh plant tissue (100 mg fresh weight or 25 mg of dry weight) and a very straightforward extraction method, which comprises a direct extraction of plant tissues with 80% methanol followed by direct HPLC-MS/MS analysis. This method can also be adapted for quantification of jasmonates as described in Almeida-Trapp et al. [29].

According to the international guidelines for validation of bioanalytical methods, this is a reliable, reproducible, precise, accurate, and robust method for quantification of oxylipins in plant tissues, since all RSD and errors were bellow 15% for all quality controls in the repeatability and reproducibility tests (Table 2). It is also a robust method when the mobile phase is changed between methanol and acetonitrile since deviations in the quality controls are below 15 % as recommended by the international guidelines.

Since the number, nature, and expression of LOXs varies greatly among different plants [8,37], it is also important having a method able to detect LOX products in different plants. Therefore, we showed that although this method was developed and validated in *Alternanthera brasiliana* tissues, it was suitable for the detection of oxylipins in many other plants (from liverworts to dicots), as demonstrated in Fig. 3. It is important to highlight that when used for absolute quantification in plants other than *A. brasiliana*, the calibration curve must be prepared by standard addition in order to overcome the matrix effect. We also used

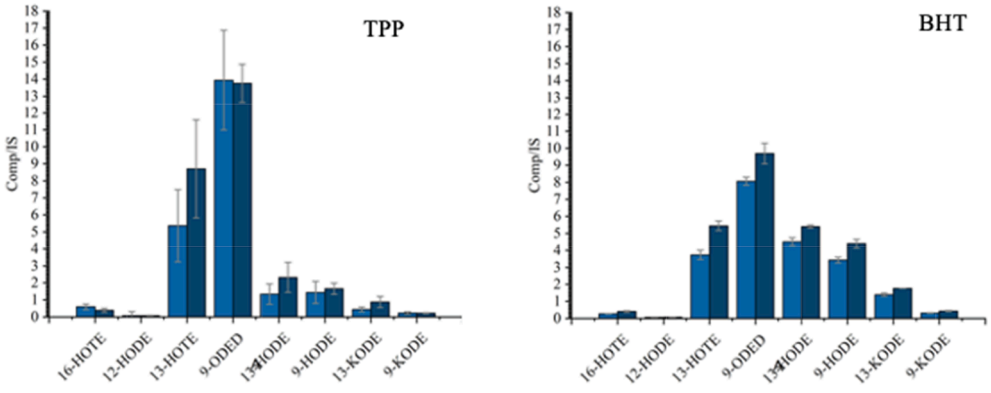

Fig. 3. Profile of oxylipins in vegetal tissues of various plant species.

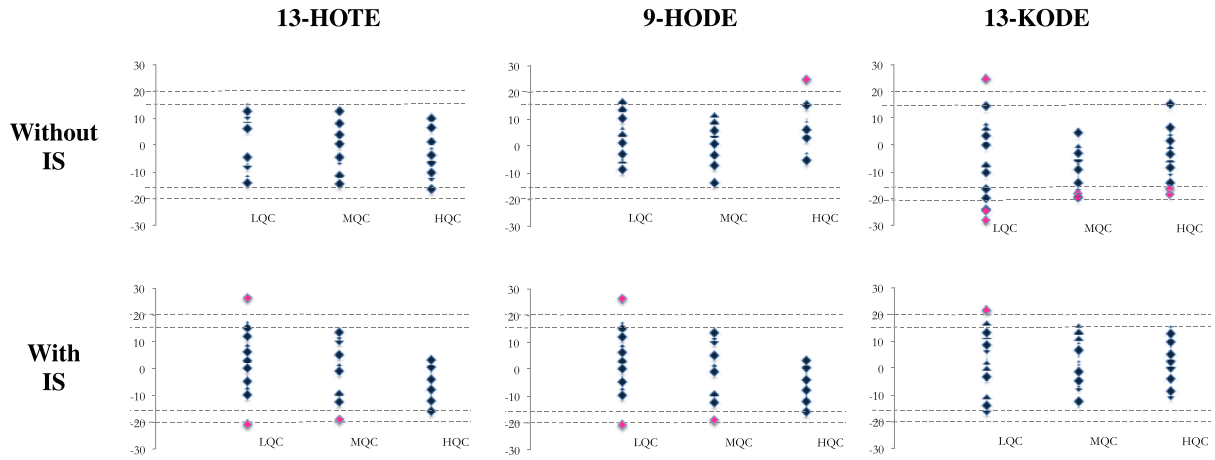

Fig. 4. Relative amount of oxidized fatty acids and jasmonates upon wounding in *A. thaliana*. The fold change is related to the amount of oxylipins in treated plants in comparison to control cultured under the same conditions as the treated plant ones, but not exposed to the stress. The pink axis refers to fold change for the oxidized fatty acids, and the blue (right) axis refers to the fold change for jasmonates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this method to analyze the relative amount of oxylipins in *A. thaliana* upon different biotic and abiotic stresses.

When *Arabidopsis thaliana* was exposed to different biotic and abiotic stresses, the profile of these oxidized fatty acids differed considerably depending on the stress, and it is also different from jasmonates, which are usually a model for oxylipins. The jasmonates, for example, strongly responded to wounding, heat, and *Pseudomonas syringae* avrRpm1 infection, and only slightly upon salt stress. On the other hand, the amount of oxidized fatty acids did not change upon wounding, although gene expression related to 9 and 13 LOX have been reported to increase upon mechanical stress in many plants [40]. In this case, the products of 9- and 13-LOX might be converted into jasmonates and green leaf volatiles [8,41]. Moreover, the amount of oxidized fatty acids changed considerably upon heat and *Pst avrRpm1* with a different profile, and only slightly upon salt stress. Showing that not all oxylipins respond in the same manner to different stresses.

5. Conclusions

The exact role of different oxylipins in plant development and stress tolerance has not been understood yet, and the function of specific compounds requires more attention. However, the methods used so far for quantification of those compounds in plants involve an extensive sample preparation and/or a high amount of plant tissues. Here, we developed and validated an accurate, sensitive and precise HPLC-MS/MS method for quantification of oxidized fatty acids in plant tissues, using a straightforward one-step extraction procedure, which allows the quantification of oxidized fatty acids in different plant tissues. This method has also been used for quantification of oxylipins in plants exposed to biotic and abiotic stresses, showing that the response of those oxidized fatty acids (member of the so called oxylipins) is quite different from the jasmonates (the most well-known plant oxylipins). Therefore, having a reliable, sensitive, accurate and straightforward method for quantification of oxidize fatty acids in plants will contribute to the understanding of the role of these compounds in plant physiology, defense and interactions with other organisms and stresses.

CRediT authorship contribution statement

Marília Almeida-Trapp: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Gezimar Donizetti de Souza:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Kirti Shekhawat:** Investigation, Writing – review & editing. **Arsheed H. Sheikh:** Investigation, Writing – review & editing. **Axel Mithöfer:** Resources, Writing – review & editing. **Heribert Hirt:** Resources, Writing – review & editing. **Edson Rodrigues-Filho:** Funding acquisition, Project administration, Resources, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.123006.

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