Chapter 2

Volatile Oxylipins and Related Compounds Formed Under Stress in Plants

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Summary

Plants form volatile oxylipins and related compounds under stress. Some of them are important flavor chemicals and give big impact on the flavor quality of food made from plant materials. They are also involved in defense responses of plants against pathogens and herbivores. Furthermore, in some instances, they cause harmful effects on plants themselves. Because of these significances of volatile oxylipins and related compounds, demands to perform comprehensive analyses of these compounds are increasing. In this chapter, we describe the simple but efficient procedures to reveal profiles of volatile oxylipins and related compounds by using HPLC and GC-MS. They are simple and can be performed in biochemical laboratories equipped with common facilities.

Key words: Volatile oxylipins, Reactive aldehydes, Plant stress responses, HPLC, GC-MS

1. Introduction

Oxylipin is a group name of compounds derived from fatty acids usually through at least one step of oxidation reaction. In mammals, C20 fatty acids such as arachidonic acid are the precursor of oxylipins. Prostaglandins and leukotrienes are the ones of most important mammalian oxylipins because of their diverse and prominent physiological functions. Therefore, various excellent protocols for analyses of mammalian oxylipins are available. Plants also form diverse arrays of oxylipins but usually from C18 and C16 fatty acids. Ones of most important plant oxylipins are jasmonic acid, its methyl ester, and its amino acid conjugates, which are involved in plant defense, growth, and development (1). Plants
have a unique oxylipin pathway forming short-chain, volatile oxylipins (2). Fatty acid hydroperoxide lyase (HPL) that cleaves 13- and/or 9-hydroperoxides of linolenic or linoleic acid is the key branching enzyme in the pathway. The first products formed from 13-hydroperoxides are C6-aldehydes, whereas C9-aldehydes are formed from 9-hydroperoxides. These aldehydes could be further reduced by alcohol dehydrogenase to form the corresponding short-chain alcohols, and in some cases, acetyl transferase converts them to their acetates.

These volatile oxylipins have unique, green leaf-like flavor properties; thus, they are important flavor compounds in food materials of plant origin. With soybean, n-hexanal is a cause of unpleasant, beany flavor, whereas in tomato fruits, \((Z)-3\)-hexenal and \((Z)-3\)-hexen-1-ol are important for the freshness of the fruits. In intact and healthy plant tissues, the amounts of volatile oxylipins are usually low; however, once the tissues suffer stresses, such as mechanical wounding, pathogen infection, herbivore attack, high temperature, or draught, they are immediately formed (2). From this, it has been assumed that volatile oxylipins are involved in stress responses of plants. For example, volatile oxylipins are involved in defense responses of Arabidopsis against necrotrophic fungal pathogens, such as \textit{Botrytis cinerea} (3). Also, their involvement in the tritrophic system consisting of plants, herbivores, and parasites to recruit parasitic wasps has been reported (4). Volatile oxylipins can also be airborne signal compounds to prime neighboring maize plants in order to defend efficiently against forthcoming herbivore attack (5).

Other than volatile oxylipins formed through HPL pathway, various reactive aldehydes are formed in plants especially when plants suffer stresses. Oxidation of polyunsaturated fatty acids with radicals and subsequent addition of dioxygen leads to the formation of peroxyl radicals (LOO•). LOO• may be reduced by other organic molecules to form a peroxide (LOOH). For example, lipoxygenase-independent formations of 12- and 16-hydroperoxides of linolenic acid are observed in leaves under oxidative stress (6). Alternatively, LOO• radicals are converted to monocyclic peroxides and bicyclic endoperoxides (7, 8). These peroxides decompose by nonenzymatic mechanisms to form a variety of molecules comprising carbonyl moiety. Typical aldehyde-producing reactions are \(\alpha\)- and \(\beta\)-scission of LOOH. Bicyclic endoperoxides decompose to form malondialdehyde (MDA) (8) or highly reactive \(\gamma\)-ketoaldehydes (9). Note that chemical property and biological activity of each carbonyl compound differ greatly, and the composition of them in plant cells changes depending on the physiological and stress status of the plant. Aldehyde profiles obtained by HPLC analysis, therefore, can provide more detailed information than does the conventional thiobarbituric acid assay, which detects aldehydes collectively.
In this chapter, three protocols to analyze volatile oxylipins and related compounds are described. First one is on HPLC analyses of short-chain aldehydes after derivatization with 2,4-dinitrophenylhydrazine (DNPH). This protocol can determine the amounts of even very short-chain (and therefore water-soluble) aldehydes in an accurate way. Although many noncarbonyl oxylipins escape from this, the DNP derivatization has an advantage to increase the sensitivity due to a strong light absorbance of the derivatives. In addition, focusing on carbonyl compounds has physiological significance because they generally have high biological activities. Second protocol uses solid phase microextraction (SPME) fiber to collect volatile compounds. Subsequent direct injection of the fiber to GC-MS gives detailed profiles of volatile compounds. SPME system is developed as a convenient and an efficient system to extract volatile organic chemicals on a fiber coated with absorbing matrix (10). For volatile oxylipins, SPME fiber coated with 50/30 μm DVB/Carboxen/PDMS is suitable (Note 1). If enough amounts (>10 g) of plant tissues are available, the closed loop stripping (CLS) system with a charcoal trap is suitable to analyze highly volatile compounds (11). Vaporization of the volatiles is facilitated by the airflow from a minipump, then the volatiles are splitted onto a charcoal trap. The trapped compounds are desorbed with dichloromethane for subsequent GC-MS analyses. Instead of the charcoal filter, other absorbing matrix, such as Porapak Q or Tenax, can be used. However, again, one must notice that the recovery of each compound significantly differs with each matrix (12).

2. Materials

2.1. Equipment

1. Wakosil DNPH-II column (4.6 × 150 mm) for HPLC (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Equivalent ODS column should work; however, resolution of some aldehydes might be worse.

2. Common HPLC system with an absorption detector (Such as Shimadzu LC-10 system equipped with a photodiode array detector, SPD-M10A).

3. Common GC-MS apparatus (such as Shimadzu GC-MS QP-5050). If MS detector is not available, an FID detector can work. However, the retention time of each standard compound must be carefully determined for assignment of the peak).

4. SPME fiber assembly (50/30 mm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA) with a manual holder (also available from Supelco). Bake the fiber before use in the injection port of GC (set at 230°C) at least for 30 min just before use.
5. A glass vial (22 ml) with butyl stopper and crimp top seal (open top). Bake the vial at 180°C for 3 h before use.
6. ORBO-32 mini charcoal tubes (Supelco).
7. Glass apparatus for closed loop splitting system (Fig. 1).

2.2. Reagents

1. Acetonitrile, HPLC grade.
2. 2-Ethylhexanal as an internal standard (IS) for HPLC, 0.5 mM in HPLC grade acetonitrile.
3. Butylated hydroxytoluene as antioxidant.

Fig. 1. Closed loop splitting (CLS) system. Plant materials are put into the three-way round-shaped flask (500 ml) equipped with a condenser to remove moisture. With the hot plate/stirrer, the materials can be warmed up and stirred to facilitate vaporization of volatile compounds. The airflow is introduced from the pump through the materials to the charcoal cartridge.
4. DNPH. DNPH must be purified by recrystallization because commercial DNPH contains water in order to avoid explosion and usually contaminated with spontaneously formed hydrazones. Dissolve DNPH as much as you can into hot acetonitrile (60°C), then gradually cool the solution to room temperature. Recover the crystal by simple filtration through filter paper. Handle the pure DNPH gently with avoiding any shock. Immediately dissolve the crystal to pure acetonitrile to be 20 mM.

5. Formic acid, HPLC grade.


7. NaHCO₃.

8. Deionized, purified water (equivalent to Milli-Q water).

9. HPLC Mobile Phases, Mobile phase A: Wakosil DNPH-II Eluent A (Wako Pure Chemical, Osaka, Japan), Mobile phase B: Wakosil DNPH-II Eluent B (Wako Pure Chemical), Mobile phase C: Acetonitrile. Mobile phase C is used just to facilitate the elution of highly hydrophobic compounds, and hence, it can be replaced by B, although a longer time is required for chromatography.

10. IS solution for headspace SPME-GC analyses. Take 80 mg of \( n \)-heptanal and mix it well with 280 mg of Tween 20. Add 100 ml of water, then, thoroughly suspend the contents with a sonicator. Fill up to 700 ml to make 1.0 mM \( n \)-heptanal solution in 0.04% Tween 20. The solution can be kept at −20°C, but resuspend the contents with sonication every time before use.

11. Saturated solution of CaCl₂. Put 500 g of CaCl₂·2H₂O in a glass bottle, then add 300 ml of distilled water, and mix them. A part of the crystal remains. Take the solution and pass it through a column packed with charcoal (3 cm id x 10 cm) in order to remove contaminating organic compounds. Since saturated CaCl₂ solution is very viscous, vacuum filtration is better.

12. Sodium phosphate buffer (50 mM, pH 6.3 containing 0.1-mM diethylenetriamine penta-acetic acid as a chelating agent). After preparing the buffer, remove organic chemicals with a charcoal column as described above for saturated CaCl₂ solution.

13. Freshly distilled dichloromethane.

14. IS solution for CLS system. \( n \)-Nonyl acetate dissolved in distilled dichloromethane to make final concentration of 2 μg/ml. Seal the container tightly in order to avoid evaporation of dichloromethane.
3. Methods

3.1. HPLC Analyses of Short-Chain Aldehydes After Derivatization with DNPH

1. Immerse leaf samples (0.20–0.30 g fresh weight) in acetonitrile (2.5 ml) containing 2-ethylhexanal (12.5 nmol) and butylated hydroxytoluene (0.005%) in a glass tube with a screw cap. Tighten the cap, and incubate them in a water-bath at 60°C for 30 min. (Methanol and ethanol should be avoided for extraction of the aldehydes from plant tissues because they contain significant amounts of carbonyl compounds that cannot be removed easily.

2. Collect the extract into another glass tube by decantation and add DNPH solution (62.5 μl; final concentration of 0.5 mM) and formic acid (48.4 μl; final concentration of 0.5 M; Note 2). Tighten the cap, mix well, and incubate the mixture at 25°C for 60 min.

3. Add 2.5 ml of saturated NaCl solution and 450-mg NaHCO₃ for neutralizing formic acid. Shake at intervals for 10 min. Small bubbles are formed, and cease when neutralization is accomplished.

4. After centrifugation to facilitate phase separation, collect the upper acetonitrile layer, and dry it up in vacuo.

5. Add 400 μl acetonitrile, vortex, and collect the solution.

6. To remove chlorophylls and other pigments, load the sample solution on a BondEluteC18 cartridge (sorbent mass 200 mg, Varian), which has been pre-washed with 2 ml acetonitrile, and collect the pass-through. Apply additional 350 μl acetonitrile and combine the eluted solution with the pass-through. This solution may be kept for a while at −20°C in a tightly capped vial. Avoid drying up the solution, else the hydrazones easily decompose spontaneously.

7. Subject the solution to the reversed phase HPLC system. The compounds can be separated with 100% A (0–5 min), a linear gradient from 100% A to 100% B (5–20 min), and subsequently, 100% B (20–25 min) with a flow rate of 1 ml/min. In order to clean the column, 100% C can be used. DNP-derivatives of n-alkanals have a characteristic absorption peak at around 360 nm, and those of 2-alkenals at around 380 nm (Fig. 2). The DNP-derivative of MDA, in contrast, has a peak at 307 nm because of its ring structure (13). For photometric detection of these DNP derivatives, a photodiode array detector is suitable. For single-wavelength detection, 340 nm is a compromise (Fig. 2). In order to assign each peak, corresponding standard aldehyde-DNP derivative must be prepared (Note 3).
1. Plant leaves (0.5 g fresh weight) are homogenized with a Polytron mixer (Kinematica) with 5 ml of 50-mM sodium phosphate buffer (pH 6.3) containing 0.1-mM diethylenetriamine pentaacetic acid in the 22-ml-glass vial.

2. The vial is capped with the butyl stopper, and then incubated for 5 min at 25°C in order to facilitate enzymatic reactions to form volatile oxylipins. The IS (10 µl of 1.0-mM n-heptanal in 0.04% Tween 20) is added.

3. Afterward, 5 ml of saturated solution of CaCl₂ is added to inactivate the enzymes, and the stopper is tightly fastened with the crimp top seal. The homogenate can be stored at −80°C for a week. If volatiles in intact plant leaves are analyzed, homogenize the leaves with 5 ml of saturated solution of CaCl₂ to avoid any enzymatic reactions during and after homogenization. Thereafter, add the buffer and IS as described before.

4. The homogenate is warmed to 40°C, then insert the SPME fiber through the butyl stopper. The matrix is exposed to the headspace for 30 min at 40°C. The fiber is inserted into the insertion port (set at 230°C) of the GC-MS (Shimadzu QP-5050) equipped with 0.25 µm × 30 m Stabilwax column (Restek, Bellefonte, PA). The column temperature is programmed as 40°C for 5 min to 180°C for 5 min at 10°C/min with a carrier gas (He) at 1 ml/min. The mass detector was operated in electron impact mode with ionization energy of 70 eV. The glass insert must be a special one for SPME.
analysis (SPME Sleeve, available from Supelco), otherwise highly volatile compounds appear as broad peaks. Splitless injection with a sampling time of 2 min is used. The fiber is kept inserted into the injection port until the end of analysis in order to bake out any compounds from the matrix.

3.3. CLS System to Analyze Volatile Oxylinps and Related Compounds

1. Fresh mushrooms (50 g fresh weight) are diced and homogenized with 75 ml of 0.1 M sodium phosphate (pH 6.5) with a Polytron mixer (Kinematica).

2. Put the homogenate into the round-shaped flask of close loop splitting system (Fig. 1). For highly volatile compounds such as C6-volatiles, the temperature of the hot stirrer is set at 30°C. For low volatile compounds, the temperature can be increased, but one should be careful not to make the charcoal wet (because of the high humidity of the airflow), and not to heat-denature labile volatile compounds. In order to facilitate vaporization of volatiles, the airflow can be introduced into the homogenate with bubbling. In some cases, addition of antifoam is essential; however, avoid the one consisted of silicon polymer because it results in contamination of Si compounds. Antifoam consisted of monoglycerides and lecithin works well. If one would like to avoid using antifoam, flow the air on the surface of homogenate with stirring the homogenate vigorously with a magnetic stirring bar.

3. The volatiles from the sample are split on a charcoal trap (ORBO-32 Mini) for 3 h with a flow rate of 4 L/min. In order to clean the pump, connect the outlet and inlet directly with the ORBO cartridge, and keep flowing for 1 h before use.

4. After splitting, take off the cartridge, and remove the retaining plugs and backup adsorbent and the additional retaining plugs. Slowly and carefully add 250 μl of dichloromethane containing 2 μg/ml of nonyl acetate as an IS into the outlet of the cartridge to elute volatiles. Rapid addition of the solvent results in explosion of the charcoal from the end. Repeat the elution once again with the same volume of the dichloromethane solution. Now, the eluted solution is ready for GC-MS analysis. It can be kept at −20°C for at least a week.

5. GC-MS analysis is performed as described for SPME-GC-MS analyses, but the insert glass must be replaced with the normal one for splitless injection. One microliter of the eluted solution usually gives satisfactory result.

Table 1 is a list of typical DNP-aldehydes with their retention

3.4. Results

3.4.1. Peak Identification and Quantification of Aldehyde-DNP Derivatives

DNP-aldehydes can be identified by their retention time. For the plant sources that have not been analyzed before, however, it is better to confirm the identity of each peak with mass spectrometer.
time. In order to determine the amount of an aldehyde from the peak area at 340 nm, the peak area ratio of the aldehyde and the IS (2-ethylhexanal) is determined first. The content of the aldehyde can be obtained by multiplying this ratio by the conversion factor $k$ (Table 1). The $k$ value of each aldehyde, empirically determined, reflects both the derivatization/extraction efficiency and the absorption coefficient of its DNP-derivative.

A representative chromatogram is shown in Fig. 3. For quantification of each compound, an aqueous solution of the corresponding authentic compound should be prepared with Tween 20 as described for IS solution ($n$-heptanal). Organic solvents must

### Table 1
Retention time and conversion coefficient ($k$) when detection is performed at 340 nm for DNP derivatives of aldehydes

<table>
<thead>
<tr>
<th>Aldehyde or ketone</th>
<th>Retention time (min)</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>4.55</td>
<td>3.24</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>5.28</td>
<td>3.32</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>6.96</td>
<td>0.49</td>
</tr>
<tr>
<td>4-Hydroxy-(E)-2-hexenal</td>
<td>7.45</td>
<td>0.61</td>
</tr>
<tr>
<td>Acetone</td>
<td>9.37</td>
<td>3.66</td>
</tr>
<tr>
<td>Acrolein</td>
<td>10.09</td>
<td>1.36</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>11.22</td>
<td>0.56</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>13.78</td>
<td>2.89</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>15.63</td>
<td>0.54</td>
</tr>
<tr>
<td>Phenylacetaldehyde</td>
<td>17.95</td>
<td>0.59</td>
</tr>
<tr>
<td>(E)-2-Pentenal</td>
<td>18.30</td>
<td>2.71</td>
</tr>
<tr>
<td>$n$-Pentanal</td>
<td>19.69</td>
<td>0.62</td>
</tr>
<tr>
<td>(Z)-3-Hexenal</td>
<td>20.30</td>
<td>0.52</td>
</tr>
<tr>
<td>(E)-2-Hexenal</td>
<td>22.19</td>
<td>2.36</td>
</tr>
<tr>
<td>$n$-Hexanal</td>
<td>23.37</td>
<td>0.46</td>
</tr>
<tr>
<td>2-Ethylhexanal (IS)</td>
<td>29.00</td>
<td>1.0</td>
</tr>
<tr>
<td>(E)-2-Nonenal</td>
<td>34.33</td>
<td>2.72</td>
</tr>
</tbody>
</table>
Fig. 3. A representative total ion chromatogram of volatile oxylipins formed in disrupted *Arabidopsis* leaves (ecotype Nossen) analyzed with SPME GC-MS. The labels represent following: 1, acetone; 2, 1-penten-3-one; 3, *n*-hexanal; 4, (Z)-3-hexenal; 5, *n*-heptanal (IS); 6, (E)-2-hexenal; 7, (Z)-2-penten-1-ol; 8, 6-methyl-hepten-2-one; 9, *n*-hexan-1-ol; 10, (Z)-3-hexen-1-ol; 11, butyl 1-isothiocyanate; 12, 2,4-hexadienal (two peaks of geometrical isomers); 13, 3-methylbutyl 1-isothiocyanate; 14, 5-ethyl-2(5H)-furanone; 15, b-cyclocitral; *, contamination from a plastic ware.

Fig. 4. A representative total ion chromatogram of volatiles formed in disrupted mushrooms (*Astraeus hygrometricus*) extracted with CLS system. The labels represent following: 1, 1-butanol; 2, pyridine; 3, 1-pentanol; 4, 2-n-pentylfuran; 5, 3-octanone; 6, 3-hydroxy-2-butanone; 7, unknown (M⁺ = 111, C₇H₁₁O); 8, 6-methyl-5-hepten-2-one; 9, 4-hydroxy-4-methyl-2-pentanone; 10, *n*-nonanol; 11, 3-octanol; 12, tetradecane; 13, 2-cyclohexen-1-one; 14, 1-octen-3-one; 15, 1-octen-3-ol; 16, furfural; 17, 2-ethyl-1-hexanol; 18, benzaldehyde; 19, 1-octanol; 20, (E)-2-octen-1-ol; 21, 4-(5-methyl-2-furanyl)-2-butanone; 22, (E,E)-2,4-decadienal; 23, 4-(2-furanyl)-(E)-3-buten-2-one.

be avoided because they extensively affect adsorption kinetics of compounds on the matrix. With the aqueous solution, prepare serial dilution of it with the buffer and saturated CaCl₂, and calibration
curves must be constructed by using peak area ratio between the compound and the IS.

A representative chromatogram is shown in Fig. 4. The compounds are identified with their GC retention times and mass spectra by comparing them against those obtained with authentic standards. The retention index of each compound is calculated using \( n \)-alkanes (C\(_{7-30}\), Sigma) as external references. This procedure can also be used to analyze volatiles emitted from living plant parts without cutting even under their growing conditions. In that case, Tedlar bag is usually used to cover the plant organs. Because the air is circulated, humidity increases and CO\(_2\)/O\(_2\) balance changes during extraction, which might cause artificial effect on the physiology of plants. In order to avoid this problem, the fresh air is flowed into the Tedlar bag (14), but a special care must be paid to avoid contaminations.

4. Notes

1. For selection of the matrix of SPME fiber, one must notice that the recovery of a compound differs significantly depending on the nature of the matrix. Detailed information on SPME is available on the web site of Sigma-Aldrich (http://www.sigmaaldrich.com).

2. Formic acid can be substituted with 0.5-M acetic acid, but the former results in a more efficient derivatization of MDA (15). Harder acid such as phosphoric acid and HCl will facilitate the isomerization of \( \gamma, \delta \)-unsaturated carbonyl to \( \beta, \gamma \)-unsaturated carbonyl [e.g., (Z)-3-hexenal to (E)-2-hexenal], and hence should be avoided.

3. In order to prepare standard aldehyde-DNP derivatives, add 1 mmol of aldehyde (some aldehydes are available only as their acetals; see below) or ketone and a few drops of formic acid to the DNPH solution (ca. 0.3 g of DNPH in 20-ml ethanol warmed to 50°C), and incubate it at 50°C with gentle stirring until the color changes. Keep the solution at ambient temperature overnight to form the crystals of the DNP-derivative, and collect them on a glass filter. When an aldehyde has to be prepared from its acetal, first dissolve the acetal at 1–10 mM in 0.1-M HCl, and incubate at 40°C for 1 h. Then add 0.035% DNPH (in 1-M HCl) to an equimolar amount, and incubate it at ambient temperature for 1 h. Extract the formed DNP-derivative with hexane, chill the hexane solution to −20°C to facilitate crystallization, and collect the crystals.
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