Mass Spectrometry
DOI: 10.5702/massspectrometry.A0153

Technical Report

Rapid Analysis for α-Tocopherol and Its Oxidative Products in the *Pisum sativum* L. Leaf Using Supercritical Fluid Chromatography-Medium Vacuum Chemical Ionization Tandem Mass Spectrometry

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A method for the rapid determination of α-tocopherol (α-T) and its oxidative products in plant tissue has been developed using supercritical fluid extraction (SFE) coupled with supercritical fluid chromatography (SFC) and medium vacuum chemical ionization (MVCI) with tandem mass spectrometry. The method is designed to study changes in levels for α-T and its oxidative products in plant cells during photosynthesis, aiming to observe the light response curves. α-T oxidation is a non-enzymatic self-defense mechanism in plant cells. Unlike enzyme-involved reactions, it cannot be stopped, so the oxidation continues in crude extracts even after extraction. Therefore, a real-time *in-situ* method is essential for tracking the light response curves. To optimize the selective reaction monitoring method, the reaction mixture of α-T and singlet oxygen ($^1O_2$), generated by rose Bengal under light illumination, was used as the source of oxidative products. The relative abundance changes in α-tocopherylquinone and 8a-hydroperoxy tocopherone in *Pisum sativum* L. (Pea) leaves under excessive light illumination have been preliminarily analyzed as part of the light response curve study. The method archives a throughput of 10–15 minutes for analyzing duplicate leaf samples. This process includes cutting off the leaf, sectioning it, placing the sample in a frozen SFE vessel, and conducting SFE/SFC analysis. Consequently, the average throughput is approximately 5–7 minutes per sample.

Please cite this article as: Mass Spectrom (Tokyo) 2024; 13(1): A0153

Keywords: reactive oxygen species, α-tocopherol oxidation, light response curves

(Received July 22, 2024; Accepted September 17, 2024; advance publication released online October 2, 2024)

1. INTRODUCTION

Cyanobacteria utilize the abundant water supply on Earth as an electron donor and produce oxygen as a byproduct of photosynthesis. Since their revolutionary appearance, cyanobacteria have gradually increased oxygen concentration on our planet. This oxygen-rich environment enabled the evolution of aerobic bacteria, which harnessed oxygen for high-energy respiration. However, oxygen's role in energy exchange processes also forms reactive oxygen species (ROS), such as singlet oxygen, peroxides, and other oxidative compounds within the electron transport system. ROS play a crucial role in the plant energy exchange process, but they can also be harmful molecules.[1–4] Excessive light intensity produces ROS in chloroplasts, which can damage photosynthetic apparatuses such as the thylakoid membrane. Singlet oxygen add the double bond through the ene reaction that forms lipid hydroperoxide (LOOH), which can be further oxidized to form lipid peroxyl radicals (LOO•) in the presence of oxidized transition metals (*e.g.*, Fe_3^+).[5] To protect membrane lipids from photooxidation, higher plants have developed various self-defense systems.[6] They synthesize lipid- and water-soluble antioxidants, such as tocopherols and ascorbate. Tocopherol biosynthesis is initiated in the cytoplasm and continues in plastids, where plastoglobules are thought to be the site of tocopherol accumulation.[7] Plastoglobules in the chloroplasts are also involved in recycling α-tocopherol (α-T).[8] It indicates that enough amount of α-T is present in advance at the photosynthesis apparatus under a dark environment. Then, it will be oxidized or reduced depending on the light environment. Plants respond rapidly, as fast as tens

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of minutes, by changing the light environment.[9] Since α-T oxidation is a non-enzymatic self-defense mechanism in plant cells, and oxidation continues in crude extracts even after liquid extraction, a real-time *in-situ* method capable of tracking the light response curves of sample throughput is essential. Using a rapid method to determine α-T and its oxidative product in the cell may be applied to studying light response curves.

We have reported the determination of α-T oxidative products produced from the α-T by incubating in the laboratory ambient environment using supercritical fluid extraction (SFE) coupled with supercritical fluid chromatography (SFC) hyphenated to medium vacuum pressure chemical ionization (MVCI) mass spectrometry (MS).[10] The combination of SFE/SFC and MVCI is particularly advantageous due to the specific properties of both techniques. SFE rapidly extracts lipophilic analytes directly from tissues without co-extracting salts, which often interfere with analysis. This SFE from crude tissue can be introduced directly into SFC without the need for sample solvent adjustment, unlike what is typically required for high-performance liquid chromatography. This minimizes the use of laboratory ware, organic solvents, and preparation steps, reducing the risk of contamination and significantly speeding up the analytical process—crucial for analytes like α-T—which are prone to rapid oxidation in ambient conditions.

Furthermore, the SFC and MVCI interface is designed to be simple yet highly effective. Unlike other interfaces that require additional solvents to assist in the ionization process, this setup efficiently transfers the analytes into the ionization chamber, offering excellent sensitivity, particularly for negative ions. Given that α-T can be oxidized rapidly in ambient conditions, the determination of α-T and its oxidative products requires careful handling to prepend autoxidation during pre-analysis sample processing.[11]

The present study intends to determine α-T, α-tocopherylquinone (α-TQ), and α-T hydroperoxide in plant cells with minimal sample preparation steps using SFE/SFC combined with a tandem mass spectrometer. The reported liquid chromatography (LC)-tandem mass spectrometry (MS/MS) methods[11,12] were referenced as a starting point for the selected reaction monitoring (SRM) transitions of α-T and α-TQ.

2. EXPERIMENTAL

2.1. Materials

A cylinder of general-grade helium (99.995%) and carbon dioxide (99.5%, siphon type) (Iwatani Industrial Gases Corp., Osaka, Japan) was used. Acetonitrile, methanol (LC/MS grade), rose Bengal, polyethylene glycol (PEG) 200, 400, and 600, and DL-α-T (reagent grade) were purchased from FUJIFILM Wako Pure Chemicals Corporation (Osaka, Japan). A 13.6 mg of α-T was dissolved in 100 μL hexane (Reagent grade, FUJIFILM Wako), then diluted by methanol (LC/MS grade, FUJIFILM Wako) to 201 μmol · L^{-1}. This relatively high concentration was chosen to minimize the impact of trace oxygen in methanol, which can accelerate α-T autoxidation. Each 100 μL portion of a diluted α-T was stored in a 500 μL Eppendorf tube at −80°C until use. The sprout of *Pisum sativum* L. (Pea) was purchased from the local grocery store. Water was obtained from a Milli-Q Purification System (Merck, Germany).

High-power light-emitting diode (LED), XHP70.2 (CREE, Durham, NC, USA) was used to illuminate diluted α-T containing rose Bengal in the Eppendorf tube (3810X, Eppendorf AG, Hamburg, Germany) for α-T photooxidation. The ceramic metal halide lamp M360FCELSP-W/BUD (IWASAKI Electric, Hyogo, Japan) was used to illuminate excess light to Pea.

2.2. MVCI MS/MS instrumentation

The LCMS-8060 triple quadrupole mass spectrometer (LCMS-8060; Shimadzu, Kyoto, Japan) was used. The MVCI flow tube, which consists of a corona discharge electrode (quartz, stainless steel), a flow tube (stainless steel), and the mass analyzer interface, was built in-house.[13] The MVCI flow tube was attached to the LCMS-8060 by replacing the "ESI unit," which consists of a liquid chromatography inlet, an electrospray ionization (ESI) chamber, and a desolvation line (DL) with the in-house prepared flange. As illustrated in the right bottom of Fig. 1, the center of the flange consists of a 10 mm inner diameter with 10 mm depth, a 30° angle orifice with a 3 mm center hole, that is, an entrance from ions produced in the MVCI source into the ion guide (UF-Qarray).[14] The helium flow rate was set to 60 mL · min^{-1} ; with the 1 mL · min^{-1} flow rate of liquid CO_2; resulting pressures were 350 and 50 Pa for the MVCI source and ion guide, respectively. It achieves 100 μA of corona discharge current by applying −600 V.

The tuning and mass calibration of LCMS-8060 were carried out using an ESI source following a protocol provided by the manufacturer. The ion guide (UF-Qarray) voltages were then manually tuned using a mixture of PEG 200, 400, and 600 to maximize the ion intensities. For *m/z* values below 350, the ion guide voltages were adjusted to achieve reasonable ion intensities, while masses above 350 exhibited reasonable ion signals without the need for manual override.

Instrument control and data acquisition were carried out by LabSolutions Version 5.120 (Shimadzu). The acquired chromatograms were then exported in ANDI-MS[15] format by LabSolutions, and the chromatograms were manipulated by the open-source software "QtPlatz" (https://github.com/qtplatz/qtplatz). MarvinSketch was used for drawing, displaying, and characterizing chemical structures, substructures, and reactions, MarvinSketch version 24.1.3 (ChemAxon Ltd. https://www.chemaxon.com).

2.3. SFE/SFC apparatus

An SFC system consisting of the LC-30ADsf CO_2 pump, LC-40D modifier pump, and SFC-30A back pressure regulator (BPR; Shimadzu) was used. The methanol contents in the mobile phase were controlled by a control system for LC-30ADsf and LC-40D binary solvent delivery system via software (LabSolutions, Shimadzu). As shown in Fig. 1, a pressure restrictor made by a fused silica capillary is required behind the SFC-30A BPR to maintain a super to subcritical state to transport analyte molecules into the MVCI source. All the tubings after SFE extraction vessel and SFC injector sample loop (two-way valves: V1 and V2) to onward, where they may contact the analyte molecules, PEEKsil (SGE International Pty Ltd Corp., Victoria, Australia) tubings or inactivated fused silica capillary (GL Science, Tokyo, Japan), were used to avoid sample adsorption and carryover.

The MVCI prefers fewer modifier contents to achieve better detection sensitivity.[16] Using a BPR combined with a

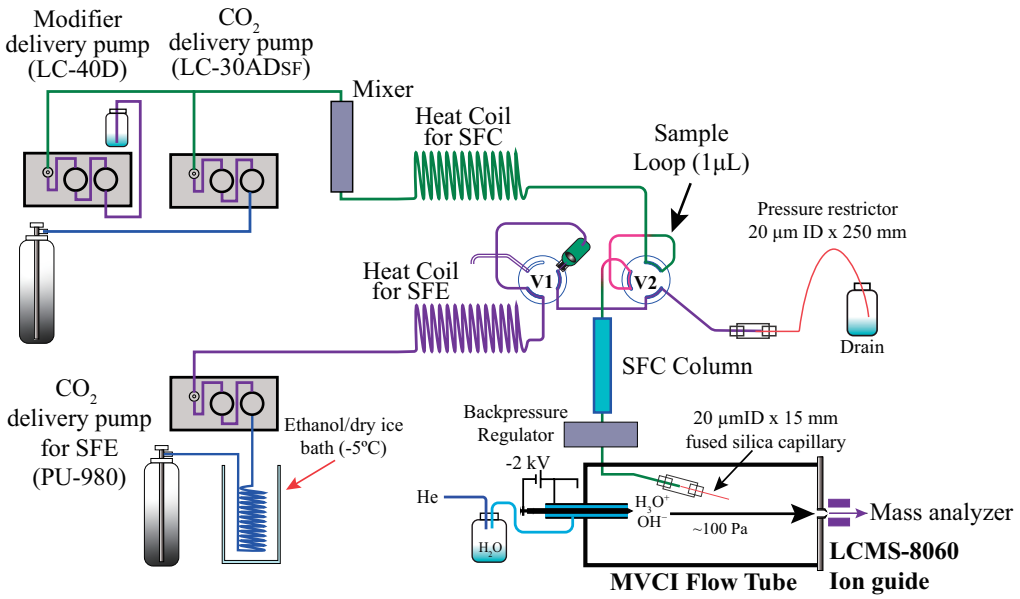

Fig. 1. Hydraulics of SFE/SFC system. The system comprises two independent mobile phase delivery hydraulics for SFE and SFC. Two hydraulics are connected via a column switching valve indicated as V2. The SFC mobile phase delivery system consists of a CO_2 delivery pump (LC-30ADSF, Shimadzu) and a mobile phase delivery pump (LC-40D, Shimadzu). Fluids delivered from two pumps are mixed using a mixer in a column oven (CTO-40C, Shimadzu). The other PU-980 (JASCO) was used as a CO_2 delivery pump for SFE. The SFC and SFE flow lines were drawn in green and purple, respectively. MVCI, medium vacuum chemical ionization; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction.

pressure restrictor enhances the reproducibility of retention times. However, the stainless-steel materials used in the BPR flow line can interact with molecules from biological samples, particularly under low modifier conditions, potentially causing artifacts such as peak tailing, carryover, and loss of sensitivity. In comparison, directly connecting the SFC separation column to the fused silica pressure restrictor via 50 μm inner diameter PEEKsil tubing without the BPR module minimizes peak dispersion and carryover. This approach improves overall sample throughput and sensitivity.

L-column3 C18 3 μm, 2.1 mm inner diameter ×100 mm length (CERi, Chemicals Evaluation and Research Institute, Saitama, Japan) (L-column3) was used for SFC. The SFE and SFC conditions used in this study were as follows: SFE was carried out with CO_2 at a flow rate of 0.2 mL · min^{-1}, 22 MPa, 40°C. SFC was performed using one percent methanol-modified CO_2 at 1 mL · min^{-1}, 24 MPa, 40°C.

An ACQUITY column in-line filter (Waters, Milford, MA, USA) was used as the SFE vessel for liquid sample analysis. For leaf section sample analysis, an in-line filter holder (P/N 160072; LC Packings, Thermo, Waltham, MA, USA) was utilized as the SFE vessel, with a copper washer (0.5 mm thickness, 3.3 mm inner diameter, and 8 mm outer diameter) inserted on the filter frit to create a 4.3 μL space for the tissue sample.

2.4. Accurate mass measurement

An ultra-performance liquid chromatography (UPLC; ACQUITY UPLC H-class plus, Waters) system coupled with an X500R QTOF quadrupole time-of-flight mass spectrometer (X500R; SCIEX, Framingham, MA, USA) was used for the determination of accurate masses for an α-T oxidative product. The tunings and mass calibration of X500R were carried out using a protocol provided by the manufacturer.

A 3 μL sample was applied to an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters) at a flow rate of 0.35 mL · min^{-1} with methanol in isocratic elution. The operating pressure was 3580 psi.

The X500R was operated in negative ESI mode with a capillary voltage of −4500 V. The source temperature was 350°C, and the declustering potential was −40 V. The precursor ion for tandem MS analysis was set to *m/z* 461.4, and the collision energy was 25 V.

2.5. Plant (Pea) sample preparation

Pea sprouts were prepared by cutting the root complex into 2 cm square sections and planting them into 6 cm diameter pots filled with vermiculite and water. Each pot contained approximately 10–12 seedlings. All pots were kept in the dark for at least 12 hours at 25°C. The experiments were conducted under two different light conditions:

1. Ceiling LED light with methanol/acetone extraction: Took two Pea pots, which were rested in the dark for 12 hours; one pot was placed under laboratory ceiling LED light (approximately 20 μmol · m^{-2} · s^{-1} photon flux) for 2 hours, while the other pot remained in the dark. Three leaves were collected from each pot, and the samples were extracted using methanol/acetone (50/50), as described in the next section.
2. Ceramic metal halide lamp with SFE/SFC analysis: A separate experiment was conducted using a single pot of seedlings. After 12 hours in the dark, the pot was exposed to light from a ceramic metal halide lamp positioned 1 m away from the Pea plant. Two leaves were collected at each time point for duplicate analysis, tracking the changes over the course of the experiment following the onset of illumination.

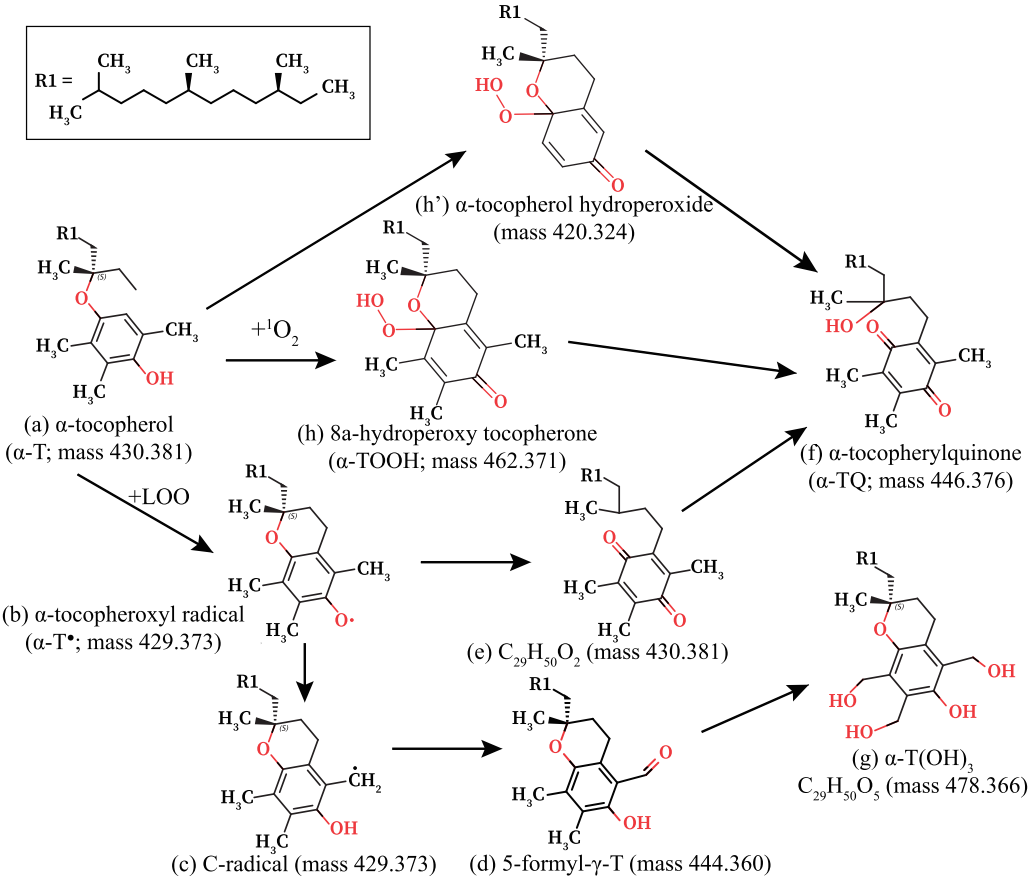

Fig. 2. A schematic of α-tocopherol (α-T) oxidative products described in the literature.

2.6. Preparation of methanol/acetone extract of Pea leaf

The collected Pea leaf was applied to a 100 mL glass vial after being weighed, and then a 1 mL of chilled methanol/acetone (50/50) was added and sonicated for 3 minutes (US102, SND Co., Ltd. Suwa, Nagano, Japan). The liquid phase was collected and stored below −5°C in the chilled box until applied to the SFE/SFC analysis.

2.7. Leaf section analysis using SFE/SFC without solvent extraction

Using a sharply honed 3-mm diameter center punch, the collected Pea leaf was punched out, and the 3-mm diameter leaf section was immediately placed in the SFE vessel. The SFE vessel was chilled to dry ice temperature before applying a leaf section to minimize the cause of autoxidation of α-T.

3. RESULTS AND DISCUSSION

3.1. Preparation of a model α-T oxidative products

To obtain an oxidative product profile of α-T, a solution of 50 μmol · L^{-1} of α-T containing 1 μmol · L^{-1} rose Bengal in methanol was illuminated with high-power LED light at 3400 μmol·m^{-2} · s^{-1} for 30 s. The α-T oxidization reaction scheme from the literature[5,17] is summarized in Fig. 2, where the 8a-hydroperoxy tocopherone (α-TOOH) (h) was added by the authors as discussed below. Since α-T hydroperoxide (h′) is the first product from α-T oxidized by singlet oxygen ($^1O_2$), we focused on its level changes in response to light, alongside α-TQ. The molecular structure of α-T hydroxyperoxide is depicted as (h′)[5,18] in Fig. 2. We detected a few chromatographic peaks at *m/z* 419.3 in selected ion monitoring (SIM) from α-T with rose Bengal incubate (Fig. 3), which we initially assumed to be the deprotonated molecule of (h′). However, it is unlikely that three de-methylations occur on the chromanol ring in this reaction, and it is equally improbable that the three methyl groups would be re-added in the subsequent reaction to form α-TQ. Furthermore, all other photooxidation products showed product ions at *m/z* 163.1 and 135.1 on the chromatographic peaks corresponding to their precursors, which are the chromanol ring-originated product ions. However, none of those product ions were detected from the *m/z* 419.3 precursor ion, casting doubt on the identification of α-T hydroxyperoxide. The structure is shown as (h), 8a-hydroperoxy tocopherone (α-TOOH) is more reasonable. Both α-TOOH (h) and α-T hydroperoxide (h′) are suggested primary products by the $^1O_2$ and α-T reaction from the study using the density functional theory computation.[19]

Based on the above, we added the precursor ion of *m/z* 461.4 (deprotonated molecule of α-TOOH (h)) into the acquisition protocol (Table 1). As shown in Fig. 3, the SRM transitions from an *m/z* 461.4 precursor to 163.1 and 135.1 showed two major peaks at retention times 57.2 and 72.4 s, which is reasonable to consider the precursor ion contains a chromanol ring. The obtained chromatogram is very complex, with two significant peaks with an unresolved shoulder peak at 77.4 s. In addition, a chromatographic peak at the same retention time of 72.2 s appeared on the SRM transition of *m/z* 429.4 → 163.1, which appeared only in the rose Bengal incubate with high-power LED illumination. Fortunately, such

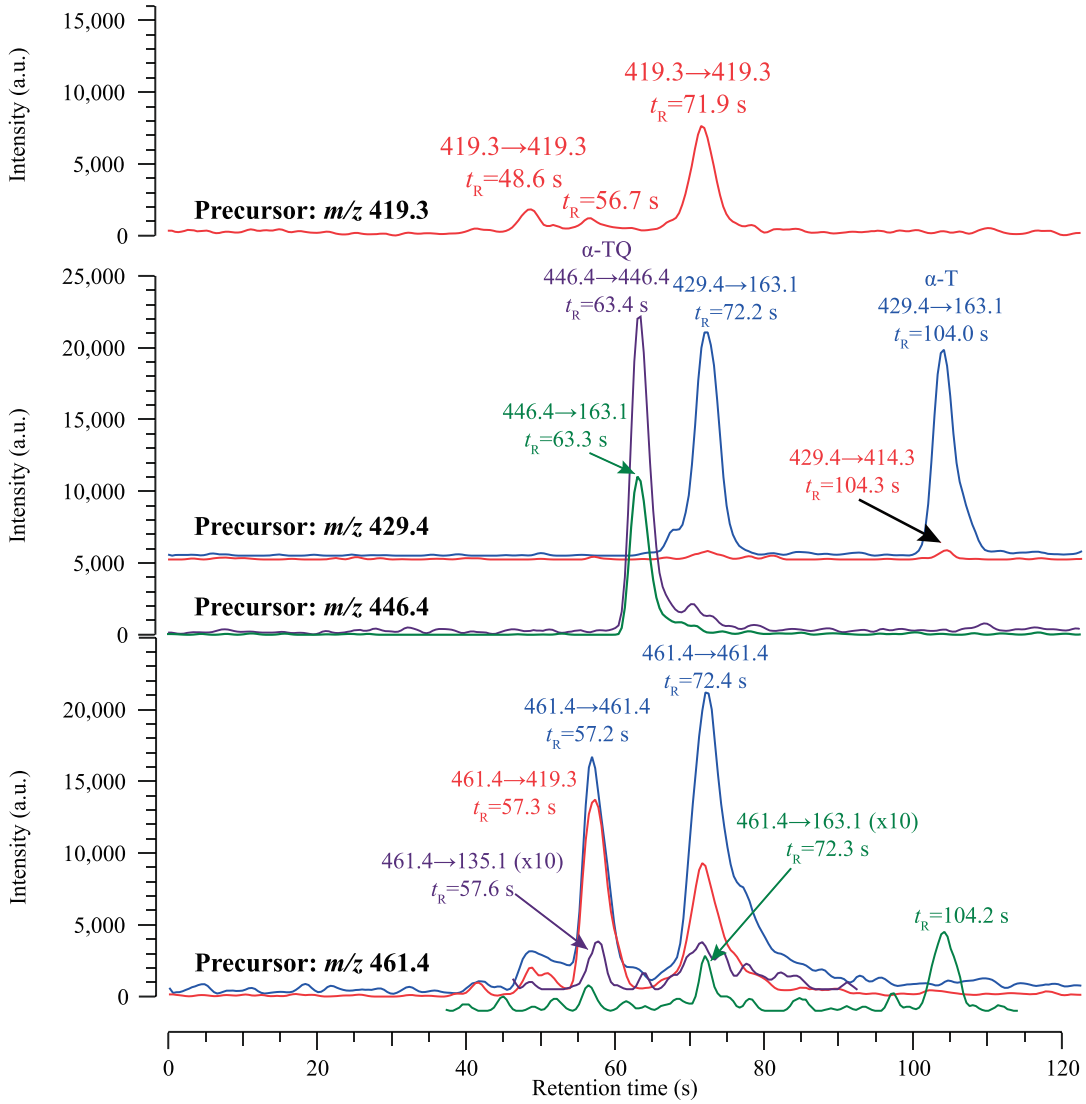

Fig. 3. Chromatograms of α-tocopherol (α-T) incubate using high power LED. A 50 μmol · L^{-1} α-T containing 1 μmol · L^{-1} rose bengal was illuminated with high power LED (3400 μmol · m^{-2} · s^{-1}) for 30 s. SRM transitions are indicated on each chromatogram; the collision energies are listed in Table 1. LED, light-emitting diode; α-T, α-tocopherol.

Table 1. The ion monitoring conditions for negative ions of α-T oxidative product.

Analyte	Q1	Q3	CE (V)	R.A.
α-T $[M-H]^-$	429.4	SIM	n/a	1.0
		163.1	30	0.83
		135.1	40	0.035
		414.4	28	0.024
		189.1	43	0.005
		161.1	52	0.001
		107.1	53	0.3×10^{-3}
α-TQ $[M]^-$	446.4	SIM	n/a	1.0
		163.1	42	0.19
		135.1	51	0.005
		177.1	40	0.006
		189.1	60	0.001
		175.1	37	0.8×10^{-3}
		149.1	55	0.6×10^{-3}
α-TOOH $[M-H]^-$	461.4	461.4	10	1.0
		419.4	24	0.44
		163.1	25	0.019
		135.1	49	0.015

CE, collision energy; R.A., relative abundance; SIM, selected ion monitoring.

complexity was eliminated from the chromatograms obtained from the Pea leaf sample, as described later. For this incubate, chromatograms for *m/z* 477.4 and 428.4 corresponding to (g) and (b), (c) in SIM did not show any peaks.

3.2. Accurate mass for α-TOOH using UPLC-X500R QTOF

The accurate masses for α-TOOH and its product ions were measured by UPLC-X500R. The obtained mass spectra are shown in Fig. 4. The deprotonated molecule of α-TOOH has appeared in *m/z* 461.3636 (off by −0.6 mDa) at a retention time of 65.4 s. The product ion spectrum for *m/z* 461.4 as precursor ion is shown in Fig. 4 bottom. The most abundant product ion was *m/z* 419.3531, corresponding to a chemical formula of $C_{27}H_{47}O_3$. We cannot find any peak corresponding to (h′) from the extracted ion chromatogram computed for *m/z* 419.3167 ± 0.0050.

3.3. SRM protocol for MVCI-LCMS-8060

According to the result from UPLC-X500R shown in the previous section, we have concluded that α-TOOH is detected

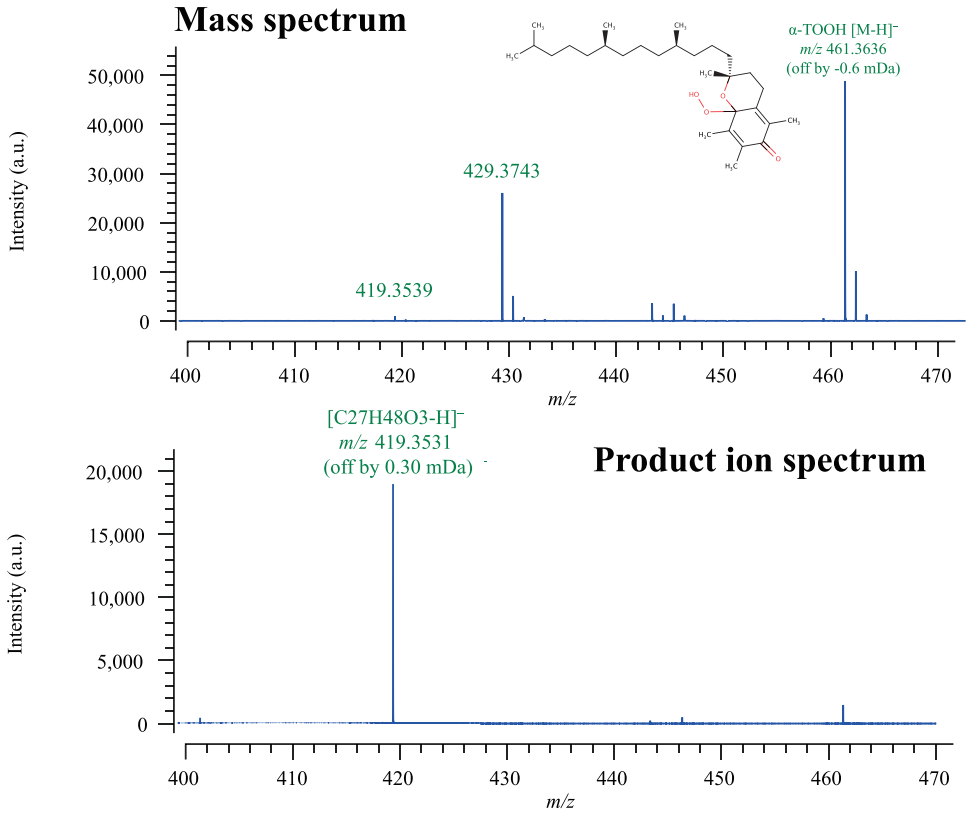

Fig. 4. Mass spectrum and product ion spectrum acquired by UPLC-X500R. α-Tocopherol incubate using high-power LED was used as a sample. Spectra are taken from a retention time of 65.4 s, an apex on the extracted ion chromatogram for *m/z* 461.364. LED, light-emitting diode. The spectrum data files are available in J-STAGE Data. https://doi.org/10.50893/data.massspectrometry.27133806

as $[M-H]^-$, which can be detected using the SRM transition *m/z* 461.4 → 419.4 and 135.1. The ion that appeared on SIM at *m/z* 419.3 might be a by-product of the MVCI since peak retention times are matched to the peaks of α-TOOH in SRM.

A linear response range of the developed method was evaluated using a dilution series of α-T in the range of 1–20 pmol, and a linear relationship was observed with χ^2 = 1.17 (standard error = 1.1 pmol; data not shown). α-T level in the acquired chromatograms from the Pea sample describing subsequent sections are within the linear response range.

3.4. α-T and its oxidative products in Pea leaf

Triplicate leaf samples were collected from two Pea pots: one that remained in the dark and another one that was exposed to laboratory ceiling light for 2 hours. Collected leaves were applied for methanol/acetone extraction described in the Experimental section. The obtained chromatograms from methanol/acetone extracts for dark and lighted Pea leaves are shown in Fig. 5.

An absolute amount of α-T in a leaf extract was varied in the range of 75 to 190 pmol · mg^{-1} fresh weight and the relative standard deviation of the method was 36.2%. An average peak area ratio for α-TQ and α-T was calculated within the same leaf sample as a relative abundance of α-TQ.

3.5. High sampling rate analysis using SFE/SFC-MVCI

The method described in the previous section uses solvent extraction and ultrasonication. The steps were ultimately shortened compared to the standard extraction method, which uses high-power ultrasonication, homogenization, liquid–liquid partition, *etc.* Even though steps still require 15–30 minutes of preparation time, which is longer than monitoring the light response curve. Therefore, directly applying a leaf section into the SFE vessel described in the Experimental section was preliminarily tested. Using a sharply honed 3-mm diameter center punch, the collected Pea leaf was punched out and immediately placed in the chilled SFE vessel. The SFE vessel and a leaf sample are shown in Fig. 6. The key to obtaining better reproducibility for α-T peak intensity using SFE/SFC was using a sharp-edged punch, a short manipulation time, and keeping the obtained section chilled. The sample preparation time from cutting a leaf from the plant to starting the SFE was approximately 3 minutes. Using two sets of SFE vessels, two leaves were taken at a time and placed in the SFE vessels simultaneously. During the SFE/SFC-MVCI analysis (2.5-minute run time), the other SFE vessel holding the sample was stored at dry ice temperature. Using this procedure, two leaf samples collected simultaneously can complete the SFE/SFC analysis within 10 minutes from when the leaves were collected.

Figure 7 presents SFE/SFC-MVCI MS/MS chromatograms for leaf samples collected at different time points: before illumination, 20, 35, and 50 minutes of exposure to excess light (3600 μmol · m^{-2} · s^{-1}) using a ceramic metal halide lamp, positioned 1 m away from the Pea plant in a pot. The relative abundance (peak area ratio) of α-TQ and α-TOOH over α-T is shown in Fig. 8. Due to the limitations of the SFE/SFC analysis method, it is not possible to weigh tissue sections or account for the leaf-to-leaf variance of analytes; therefore, only relative changes can be measured. The peak area obtained from SRM transition *m/z* 429.3 → 163.1 (α-T),

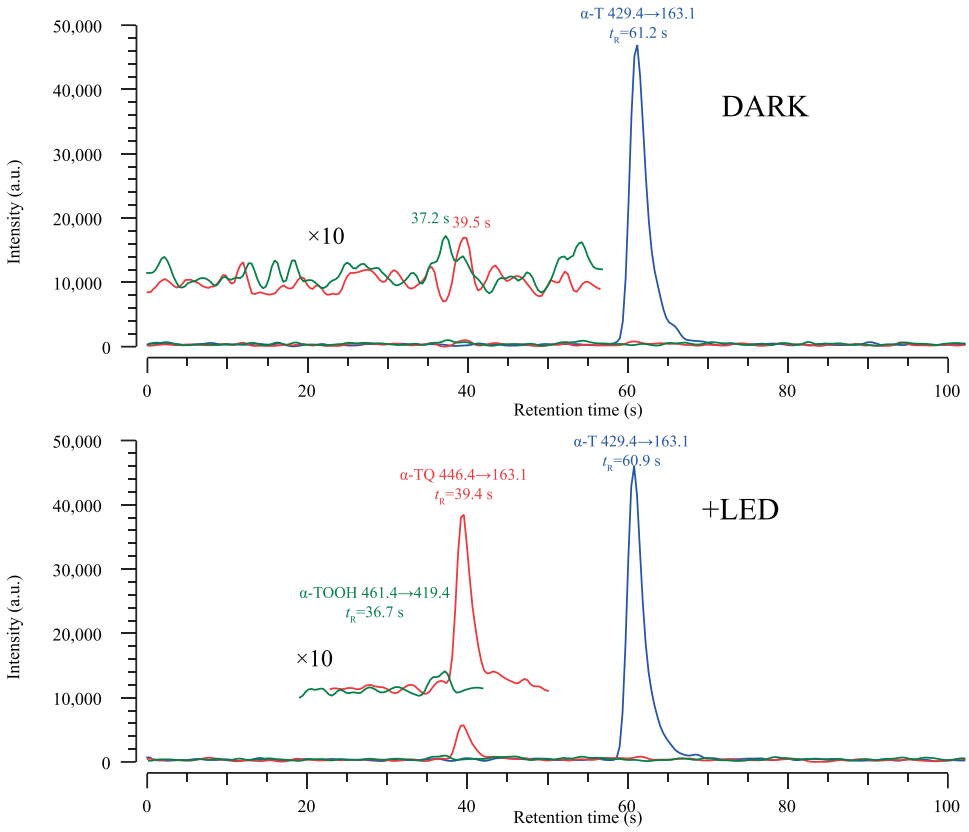

Fig. 5. α-Tocopherylquinone (α-TQ) formation in *Pisum sativum* L. (Pea) leaves under dark and ceiling-lighted conditions using methanol/acetone extract.

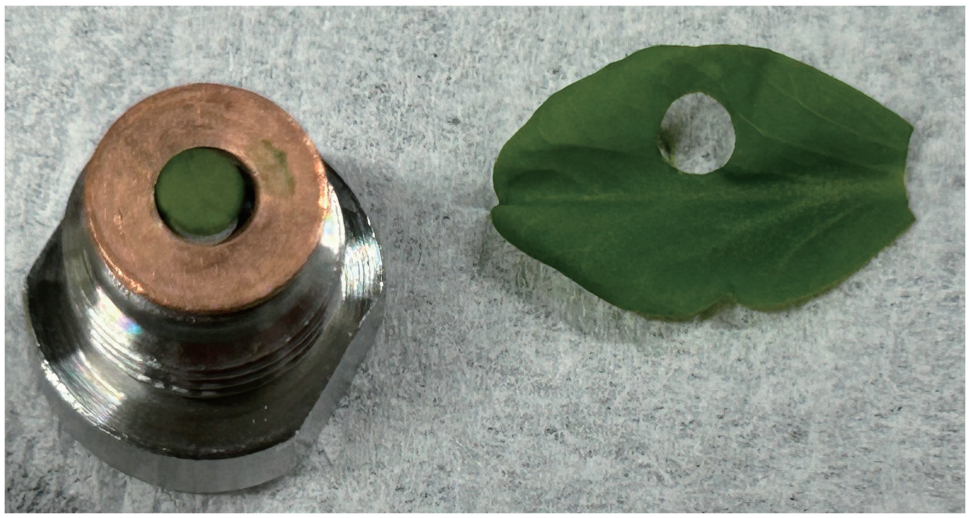

Fig. 6. A leaf sample on the bottom half piece of SFE vessel (inline filter). A sampled leaf from *Pisum sativum* L. (Pea) under excess light was taken from the plant and made a 3-mm diameter of disk shape using center punch. A 3-mm diameter leaf section was set to a chilled SFE vessel spaced by the copper washer. SFE, supercritical fluid extraction.

446.4 → 163.1 (α-TQ), and 461.4 → 419.4 (α-TOOH) were used for relative abundance calculation, with the peak area of α-TQ and/or α-TOOH divided by the peak area of α-T within each sample. The results indicate a drastic increase in α-TQ and α-TOOH at 35 minutes, potentially corresponding to increased photosynthesis activity and resulting release of singlet oxygen by the electron transport system. A significant standard deviation was observed at the 35-minute time point. Although the cause has not been fully addressed, it is possible that increased leaf-to-leaf variance at the onset of the photosynthetic reaction contributed to this error, as this reaction triggers rapid changes in the levels of α-T, α-TQ, and α-TOOH. The effect of excess light may also vary between individual leaves. This variance could potentially be reduced using a rosette leaf plant, such as *Arabidopsis thaliana*, which may have less position dependency relative to the light source. Due to the nature of the sampling method, the relative abundance results shown in Fig. 8 are based on duplicate

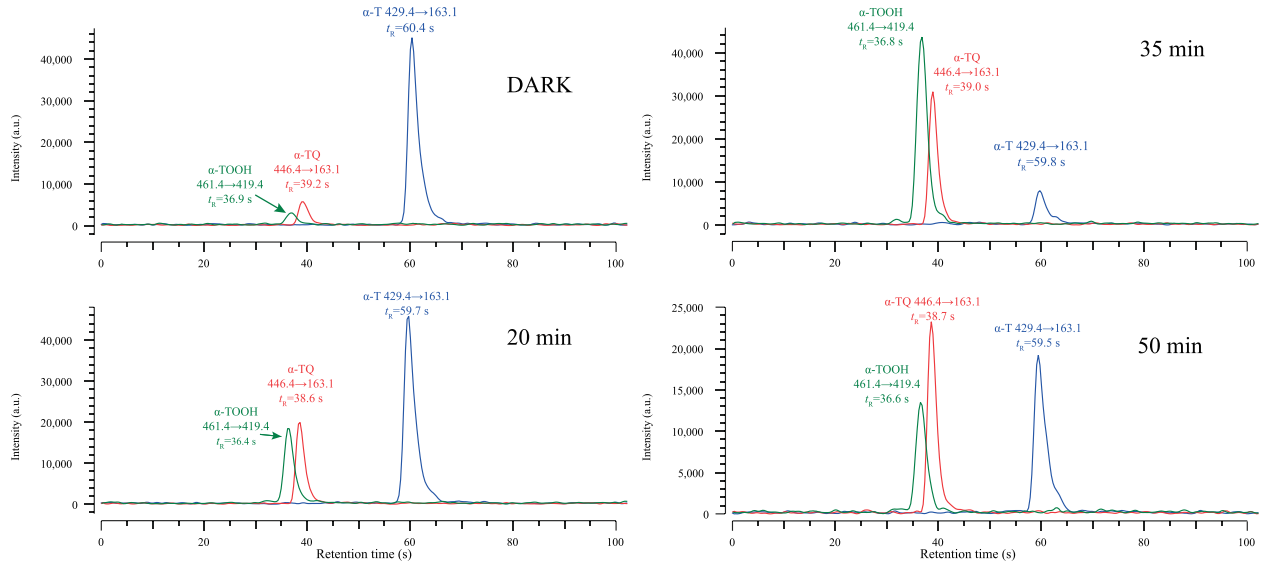

Fig. 7. α-TQ and α-TOOH formation in *Pisum sativum* L. (Pea) leaves by excess lights, applied 3-mm diameter of leaf-disk into SFE vessel. α-TOOH, 8-hydroperoxytocopherone; α-TQ, α-tocopherylquinone; SFE, supercritical fluid extraction.

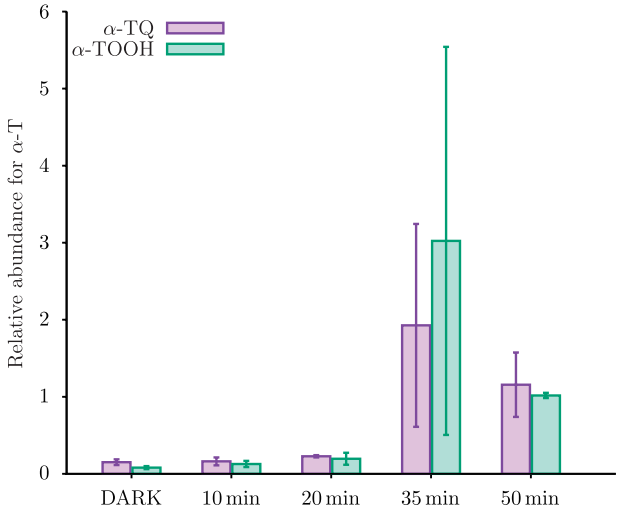

Fig. 8. Light response for α-TQ and α-TOOH formation in *Pisum sativum* L. by excess lights illumination. Error bars indicated in the figure are standard deviation. α-TOOH, 8a-hydroperoxy tocopherone; α-TQ, α-tocopherylquinone.

measurements ($N = 2$) for each time point. A significant challenge with this method is the rapid oxidation of α-T, which progresses during the experiment and cannot be fully halted, making it a race against time. This oxidation contributes to the observed variance, underscoring the need to find better reference materials for more consistent quantification. No peaks for (b), (c), (d), and (g) were detected from all of the acquired leaf samples.

The analytical method developed in this study offers significant potential for investigating lipid oxidation processes in small tissue regions. Since lipids can oxidize rapidly during pre-processing in a laboratory environment, it is crucial to minimize handling time and prepare samples quickly. Combining micro-scale SFE with subsequent SFC separation enables the analysis of small tissue regions, enabling the exploration of metabolic specificity at the structural level, such as differences in mesophyll, midrib, and vein tissues. The use of MVCI for ionization is particularly advantageous, as it allows the ionizing of a wide range of lipophilic molecules in tissues with intensive sensitivity. Notably, this method is not limited to plant tissue analysis; it is also applicable to mammalian tissues[16] and a cultured cell.[20]

However, identifying a suitable internal standard for absolute quantification remains a crucial challenge. In this study, α-T was used as a reference material, but its levels varied between 75 and 190 pmol per milligram of tissue. Despite this variability, the well-characterized α-T oxidation pathway, along with the low levels of α-TQ in leaves from dark-rested plants, provided sufficient insight into the photo-oxidation response. For broader application to other ROS-involved processes, it will be essential to establish a robust internal standard and a method for its consistent incorporation into native tissue samples.

4. CONCLUSION

The SFE/SFC-MVCI-MS system previously reported was reconfigured and optimized for mounting up to an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu). We initially utilized a standard ESI source for instrument tuning and calibration. Subsequently, the ESI source was replaced with our in-house designed MVCI source. Although the manufacturer provided auto-tuning for the ESI source, which was largely compatible with the MVCI source, manual overriding of the ion guide tuning parameters was necessary to enhance ion intensities below mass 350.

The linear response for the α-T was exhibited in the range of 1–20 pmol. The SRM transitions and collision energy protocol were determined using α-T incubate with rose Bengal and light illumination. The oxidative product induced by singlet oxygen, α-TOOH and α-TQ was effectively produced using high photon flux LED.

The light response curve of *Pisum sativum* L. (Pea) under excess light (3600 μmol · m^{-2} · s^{-1}) was preliminarily tested. Two Pea leaf samples were processed every 10–15 minutes. This process included cutting off the leaf, sectioning it using a center punch, placing the sample in a frozen SFE vessel, and conducting SFE/SFC analysis. This method allows for

sampling two leaves at a time, each cycle taking 10–15 minutes to perform a duplicate sample analysis. It is about the accepted throughput for observing light response curves.

ACKNOWLEDGMENTS

We thank Shimadzu Corporation for loaning us the LCMS-8060 and Dr. Daisuke Okumura and Dr. Masaru Nishiguchi for their technical assistance and optimization of the LCMS-8060 for MVCI. The authors thank Dr. Shunji Kato of Tohoku University for the valuable discussion concerning the possible reactions for α-T oxidation, particularly for α-TOOH. The authors thank Mr. Toshio Ichihara of Osaka University for building an SFC to MVCI interface, flange for adapting LCMS-8060, and corona discharge electrode assemblies. This work was supported by JSPS KAKENHI Grant number JP23H01985. LC-MS/MS analysis was performed using research equipment shared in the MEXT Project for Promoting Public Utilization of Advanced Research Infrastructure (Program for Supporting the Construction of Core Facilities) [JPMXS0441200024].

DATA AVAILABILITY STATEMENT

The spectrum data files of Fig. 4 are available in J-STAGE Data. https://doi.org/10.50893/data.massspectrometry.27133806

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